Journal of Immunology Research

Recent Advances in the Immunopathogenesis and Immunotherapy of Autoimmune Diseases

Lead Guest Editor: Qingdong Guan
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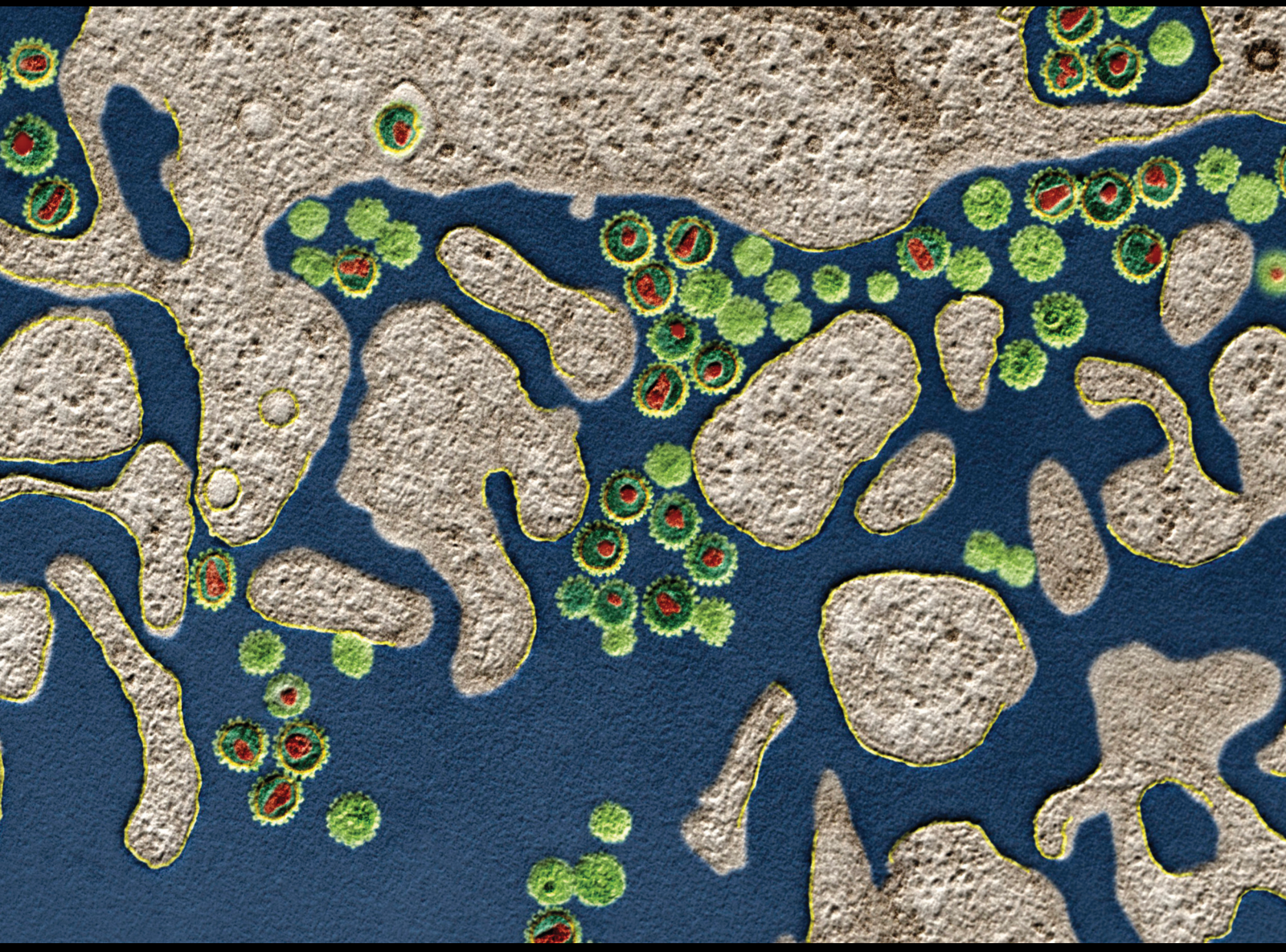

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Review Article

Overview of Strategies to Improve Therapy against Tumors Using Natural Killer Cell

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Guest Editor: Cheng Xiao

NK cells are lymphocytes with antitumor properties and can directly lyse tumor cells in a non-MHC-restricted manner. However, the tumor microenvironment affects the immune function of NK cells, which leads to immune evasion. This may be related to the pathogenesis of some diseases. Therefore, great efforts have been made to improve the immunotherapy effect of natural killer cells. NK cells from different sources can meet different clinical needs, in order to minimize the inhibition of NK cells and maximize the response potential of NK cells, for example, modification of NK cells can increase the number of NK cells in tumor target area, change the direction of NK cells, and improve their targeting ability to malignant cells. Checkpoint blocking is also a promising strategy for NK cells to kill tumor cells. Combination therapy is another strategy for improving antitumor ability, especially in combination with oncolytic viruses and nanomaterials. In this paper, the mechanisms affecting the activity of NK cells were reviewed, and the therapeutic potential of different basic NK cell strategies in tumor therapy was focused on. The main strategies for improving the immune function of NK cells were described, and some new strategies were proposed.

1. Introduction

Natural killer (NK) cells are the first line of antitumor lymphocyte cells [1]. They can directly lyse tumor cells in a non-MHC-restricted manner without prior activation or regulate the adaptive immune response with secreting immune regulatory cytokines [2–5]. There are many different factors influencing the NK cell functions. Firstly, it is the source of NK cells. For example, the NK cell line is an "off the shelf" cellular therapeutic, induced pluripotent stem cell-derived natural killer cells (iPSC-NK cells) have the advantages of homogenous and low immunogenicity, and peripheral blood stem cell- (PBSC-) derived NK cells can be gained from patients directly [6–8]. The function of NK cells is regulated by the interactions between receptors on NK cells and ligands on tumor cells, for instance, the activating receptors NK group 2D (NKG2D) receptor can recognize ligands displayed on the surface of tumor cells and improve its cytotoxicity [9]. But the tumor cells also evoluted various ways to escape the immune surveillance. One effective strategy to prevent immune escape is to modify the surface marker of NK cells, such as CAR-NK [10, 11]; the other strategy is to use monoclonal antibodies to block the inhibitory receptor, a promising treatment strategy called checkpoint blockade [12, 13]. The infiltration number of NK cells in tumor site is also a key factor that influences the treatment effect of NK cells. Many strategies were explored to improve the NK cell number in target sites, for instance, genetic modification of NK cells with chemokine receptor targeting tumor cells could improve the tendency to tumor site [14]. The physical methods such as ultrasound-mediated delivery were also involved to improve the NK cell infiltration in tumor site [15, 16]. To fulfil the ability of NK cell-based therapy, oncolytic virus, nanomaterials, and other physical methods were also involved to improve the NK cell therapy [17, 18].

In this paper, the mechanism affecting NK cells' activity was reviewed, and recent advances of innovative approaches based on NK cell therapy were also discussed. Particularly,

we focused on studies indicating the therapeutic potential of different NK cell-based strategies for the management of tumor and try to indicate new breakthroughs and trends in the area of NK cell-based therapy.

2. The Key Factors in NK Cell Education

The NK cells' function was regulated by the interactions between receptors on NK cells and ligands on tumor cells. The most important receptors on NK cells are major histocompatibility complex, also known as human leukocyte antigens (HLA) in human or Ly49 in mice. In this way, NK cells can sense the downregulation of MHC molecule to mount an effector response to damaged or infected cells in an "altered self" way. Based on whether the NK cell receptors (NKRs) can identify HLA-I or not, there are two predominant superfamilies of NKRs that have been identified.

2.1. HLA-I-Reliant Receptors

2.1.1. Killer Immunoglobulin-Like Receptors (KIRs). The activating and inhibitory KIR receptors control the development and function of NK cells adjusting to the tumor microenvironment immunity [19]. The interactions between KIRs and their HLA class I ligands in humans (Ly49 in mice) mediate NK cell self-tolerance or facilitating cytotoxicity against transformed cells. KIRs can bind to HLA-A, HLA-B, and HLA-C molecules and signal through long intraplasmatic tails with two immunoreceptor tyrosine-based inhibition motifs (ITIMs). In the absence of infection, inhibitory HLA-KIR signals dominate and protect cells from NK cell-mediated lysis [20], while KIR2DL and KIR3DL, binding ITIMs in intracytoplasmic, can inhibit the NK cells from lysis. For KIR2DS and KIR3DS, the DAP-12 can bind to immunoreceptor tyrosine-based activation motif (ITAM), which can activate signal and boost the NK cells to recognize tumor cells [21] (Figure 1(a)).

2.1.2. Killer Lectin-Like Receptors (KLRs). Separate from KIRs, other conserved MHC-binding receptors including killer lectin-like receptors confer additional diversity in NK education and protection from autoreactivity. HLA-E can be recognized by CD94/NKG2A and CD94/NKG2C for inhibition or activation, respectively. CD94/NKG2A is an example of KLR and has a similar structure that contains a ligand-binding domain resembling that of a C-type lectin. The inhibitory CD94/NKG2A NK cell repertoires have been classified based on their reliance on KIR or CD94/NKG2A for recognition of self-HLA [22]. CD94/NKG2C functions as an activating receptor by associating with the DAP12 signaling adapter [23]. C-type lectin-activating receptors CD94/NKG2C receptors of NK and their activated counterparts have short cytoplasmic tails with ITAMs. The other one is C-type lectin receptors that bind to nonclassical class I MHC molecules or "class I-like" molecules.

2.2. The Activating Receptor Does Not Rely on HLA-I

2.2.1. Natural Killer Group 2D (NKG2D). There are some receptors on the surface of NK cells which do not rely on HLA-I, such as the activating receptors NKG2D, a C-type lectin receptor, which can bind stress-inducible ligands including the MHC class I chain-related (MIC) peptides (MICA and MICB), and the human cytomegalovirus UL16 binding proteins (ULBPs) [24, 25]. The NK cells with NKG2D expression can detect and eliminate cells that have undergone "stress", showing its promising application in the treatment of infectious disease, cancer, and autoimmune disease [26]. However, advanced cancers frequently lost cell surface-bound MICA and MICB by proteolytic shedding and escape NK cell immune mechanism. When researchers specifically adopt small molecule inhibitors to block MICA and MICB, it can reactivate NK cells' antitumor immunity [27]. Some researchers also demonstrated that the circulating adoptive NK cells in patients with metastatic melanoma or renal cell carcinoma did not mediate tumor regression because of its significant low levels of NKG2D expression. When reacted with IL-2 in vitro, the NK cells with improved NKG2D expression could lyse tumor cells in vitro again [28]. Recent research also showed that CD4+CD25+ regulatory T cells (Treg) inhibited NKG2D-mediated NK cell cytotoxicity in vitro [29]. Therefore, it is important to figure out the exact mechanism of NKG2D signal pathway in NK cells.

The most well-characterized activating NKRs are currently not well defined. NKG2D encoded by a highly conserved gene (KLRK1) with limited polymorphism is an activating receptor expressed on the surface of NK cells, which can recognize an extensive repertoire of ligands, encoded by at least 8 genes in humans (MICA, MICB, RAET1E, RAET1G, RAET1H, RAET1I, RAET1L, and RAET1N) (Figure 2(b)). The NKG2D pathway serves a mechanism for the immune system to detect and eliminate cells that have undergone "stress." NKG2D provides an attractive target for therapeutics in the treatment of infectious disease, cancer, and autoimmune disease. But a more recent investigation observed that Treg inhibited NKG2D-mediated NK cell cytotoxicity in vitro.

2.2.2. Natural Cytotoxicity Receptors (NCRs) NKp46, NKp30, and NKp44. The NK cells secrete cytokines such as IFN-γ and TNFα via activating the natural cytotoxicity receptors which consisted of three receptors (NKp46, NKp30, and NKp44) [30]. NKp46 and NKp30 are expressed on the cell membrane of human peripheral blood NK cells, most of them are CD56 dim NK cells, while NKp44 is expressed on IL-2-activated NK cells and CD56 bright NK cells [31]. And ligands are systemically summarized in Kruse study [32].

2.3. Interleukin Improves NK Cell Response. Interleukin is an important immunostimulatory cytokine for immune cells including NK cells. IL-2 can enhance NK cell antitumor effects and systemic use along with NK cell-based treatment. It is reported that CD25 on NK cells, a component of the high-affinity IL-2R, can promote NK cell activation in response to low doses of IL-2. Some studies demonstrated that the use of both IgG and IL-12 can improve CD25 expression and further promote NK cell antitumor activity in

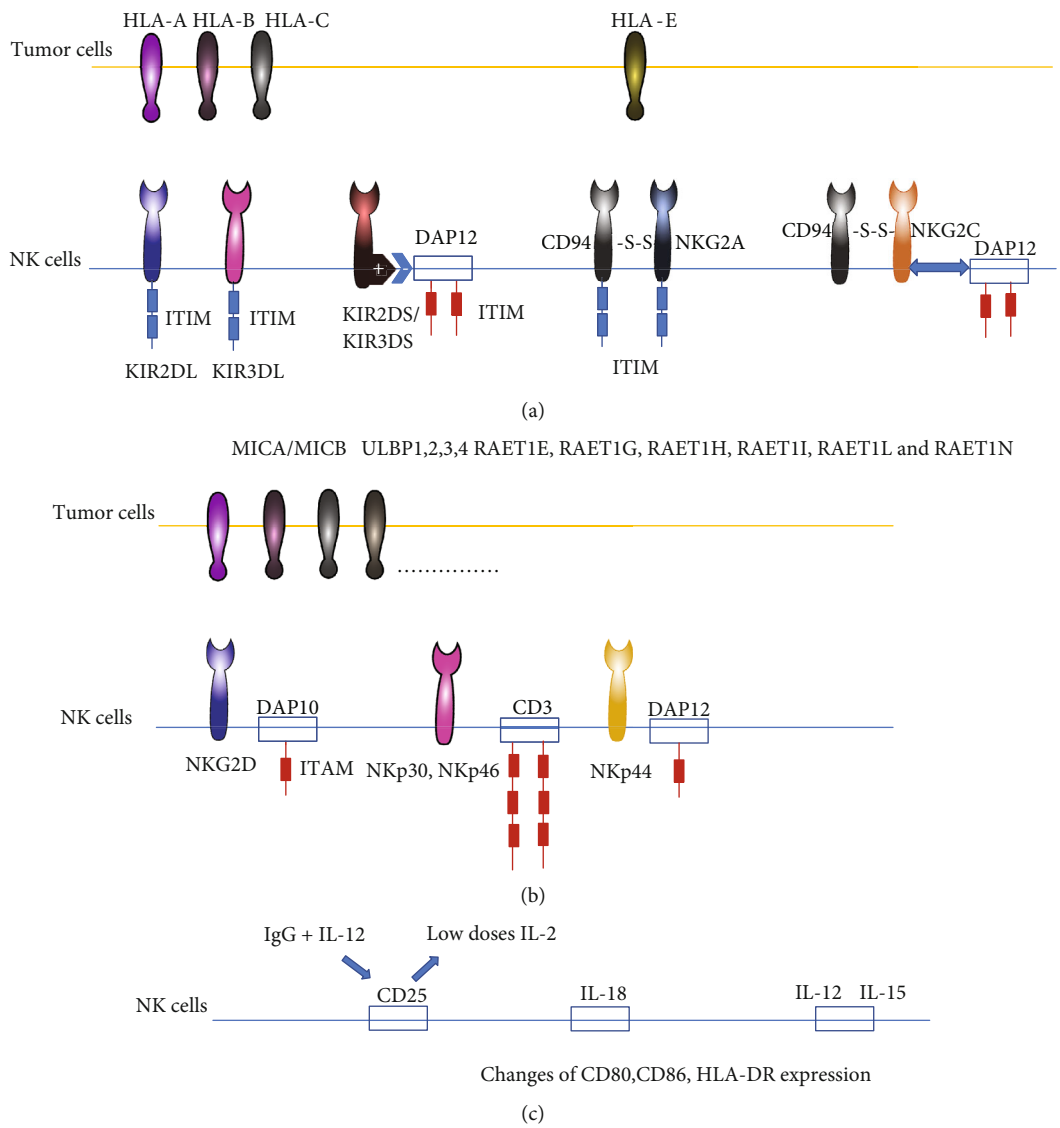

Figure 1: The key factors in NK cell education. (a) HLA-reliant receptors. (b) The activating receptor does not rely on HLA-1. (c) Interleukin improves NK cell response. HLA-1: human leukocyte antigen 1; ITIM: immunoreceptor tyrosine-based inhibitory motif; ITAM: immunoreceptor tyrosine-based activation motif.

response to low-dose IL-2 (10 pg/ml) [33]. For NK92 cells, tethering IL-2 to its receptor IL2Rβ can enhance antitumor activity and expansion [34]. Some other studies also demonstrated that when adding both IL-2 and IL-18, the NK cell clusters were observed earlier and NK cells promote the expansion of 56 folds on average on day 10 compared with stimulation with IL-2 or IL-8 alone. The potential mechanism is that IL-18 not only promoted the expansion of NK cells but also changed the phenotype of NK cells. It was observed that IL-18 enhanced the expression of CD80, CD86, and HLA-DR on NK cells, suggesting that IL-18 conferred NK cells an APC-like phenotype [35]. The NK cell-based immunity can also be augmented with other cytokines, such as IL12 and IL15 which can prompt peripheral NK cell tissue homing [36]. Some researchers demonstrated that ALT-803, an IL-15 superagonist complex alone, could also enhance the function of both normal and ovarian cancer patient-derived NK cells by increasing cytotoxicity and cytokine production [37] (Figure 1(c)).

3. Sources of NK Cells

There are various sources which could be used for NK cell immunotherapy, such as peripheral blood (PB), umbilical cord blood (UCB), bone marrow (BM), human embryonic stem cells (hESCs), mononuclear cells, induced pluripotent stem cell-derived NK cells (iPSC-NK cells), cord blood-derived NK cells, and NK cell lines [38, 39] (Table 1). Among them, PBNK cells have characteristics of safety, convenience, and strong killer ability against tumor cells [40]. However, NK cells from tumor patients may not expand sufficiently or NK cells with antitumor ability were rarely found. And a suitable donor providing allogeneic NK cells is evaluated strictly and not easy to find. Furthermore, it is time-

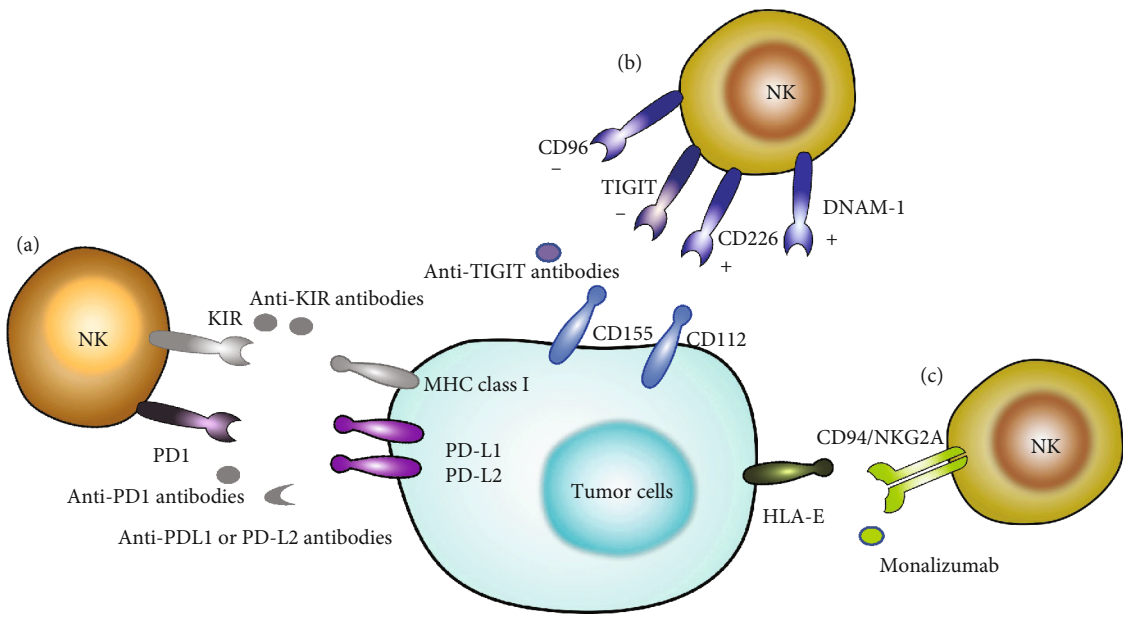

Figure 2: Checkpoint blockade therapy based on NK cells. (a) Using anti-PD1, anti-PDL1, anti-PDL2, or anti-KIR antibodies can release the inhibitory signal for NK cells. (b) TIGIT competes with immune activator receptor CD226 or DNAM-1 for the same set of ligands: CD155 and CD112; blocking CD96 is a promising therapeutic role combined with TIGIT. (c) CD94/NKG2A is an inhibitory receptor expressed by NK cells and T cells that recognizes HLA-E.

Table 1: The advantages and disadvantages of different sources of NK cells.

Sources of NK cells	Advantages	Disadvantages
Peripheral blood	Safe; conveniently collected; strong ability to kill tumor cells	Low numbers in patients; time-consuming and costly
Umbilical cord blood	Available; off-the-shelf; UCB-derived CD34+ cells have translated to the clinic; frozen for a long time	Only one time to get access to the umbilical cord blood
Human embryonic stem cells or induced pluripotent cell	Homogenous NK cell product; easy to amplify large numbers of NK cells	Need to induce iPSC into NK cells
Bone marrow	From patients	Invasive operation
NK cell line (i.e., NK-92 and NK-92MI)	Off-the-shelf; easy to amplify; lack most inhibitory receptors compared to naïve NK cells	Potential tumorigenicity

consuming and costly during NK cell isolation from peripheral blood, as it is always mixed with monocytes and other blood cells. Both autologous and allogeneic NK cells have been proven to be safe and tolerable in adoptive cell therapy [41, 42]. And some studies demonstrated that allogeneic NK cells exhibit better antitumor efficacy than autologous NK cells [43] because the autologous NK cells are potentially inhibited by self MHC class-I molecules on cancer cells. Umbilical cord blood (UCB) is a promising source of allogeneic NK cells which is an off-the-shelf frozen product to generate large numbers of highly functional NK cells from frozen UCB unites ex vivo [44]. iPSC-NK cells also have the characteristics to be banked and stored. And it has been established successfully to obtain large numbers of homogenous NK cells [45]. For acquiring NK cells with better antitumor ability, some researchers compared artificial antigen-presenting cells (aAPCs) expanded PB-NK and iPSC-NK cells with overnight-activated PB-NK cells. The researches demonstrated that aAPCs expanded PB-NK and iPSC-NK cells have better antitumor effect in vivo [46]. Compared with other sources of NK cells, NK cell lines such as NK-92 cells have the obvious advantage of being "off-the-shelf." The NK-92 cells have also entered clinical trials successfully [47]. And high intravenous doses of up to 10^{10} cells with some beneficial effect reported in melanoma and lung cancer patients have shown to be safe.

4. Modification of NK Cells

4.1. Chimeric Antigen Receptors. The tumor cells can escape immune surveillance by NK cells. Thus, it is necessary to modify NK cells with chimeric antigen receptors (CARs) to redirect their antitumor ability against resistant tumor cells [48]. To date, NK cells derived from different sources are modified with various CARs to redirect against different cancer antigens such as CD19, CD20, CD244, and HER2. For

example, NK-92 cells were engineered to express a variety of CARs specific against tumor-associated proteins (e.g., CD38, CD19, CD20, epithelial cell adhesion molecule (EPCAM), disialoganglioside (GD2), epidermal growth factor receptor variant III (EGFRvIII), and ErbB2 (HER2)), which rendered NK-92 cells cytotoxic against otherwise resistant hematologic and solid malignancies [49, 50].

To avoid side effects of on targets/off tumor side because some normal tissue also express cancer antigens, some efforts insert suicide gene into CAR in order to control this potential cytotoxicity. Liu et al. transduced cord blood-derived NK cells with a retroviral vector incorporating the genes for CAR-CD19, IL-15, and inducible caspase-9-based suicide gene (Ic9), and it demonstrated efficient killing of CD19-expressing cell lines and primary leukemia cells in vitro, with dramatic prolongation of survival in a xenograft Raji lymphoma murine model [51]. Clinical studies have indicated that CD19, CD7, and CD33 CAR-modified NK cells may be a good treatment option for lymphoma and leukemia [52, 53]. Just not long ago, Fate Therapeutics announced that the Food and Drug Administration has approved its research-based new drug (IND) application for FT596. FT596 is derived from the cloned primary induced pluripotent stem cell (iPSC) line iPSC-derived CAR-NK cells, which exhibit similar activity to CAR-T cells but are less toxic. CAR-modified natural killer (NK) cells derived from human induced pluripotent stem cells (iPSCs) (NK-CAR-iPSC-NK cells) in mouse ovaries enhanced antitumor activity in cancer models [54]. And this iPSC-derived CAR-NK therapy is approved for clinical use to address CAR-T recurrence and cost challenges.

Most CARs are derived from CAR-T and directly used to modify NK cell. These strategies omit the difference of intracellular mechanism between NK cells and T cells. Li optimized the CAR specially for NK cell, utilizing NKG2D, a type 2 transmembrane-anchored C-type lectin-like protein; the 2B4 costimulatory domain and the CD3z signaling domain have a better result compared with the specifically target cancer cells in an antigen-specific manner to improve survival in an ovarian cancer xenograft model [54].

4.2. Chemokine Receptor. Adoptive NK cell transfer is a promising method for cancer treatment, but only works in a few patients with hematological malignancies. One reason of the poor effect against solid tumor is that circulating NK cells are difficult to enter into tumor. Chemokine plays a significant role in regulation of the migration of leukocytes and it is feasible to improve NK cells homing through genetic expression of corresponding chemokine receptors. Some researchers modified human primary NK cells with CXCR2 to improve their ability to specifically migrate to renal cell carcinoma tumor which readily secreted CCR2 ligands. And CXCR2-transduced NK cells have increased adhesion with renal cells and prompt enhanced killing ability [55–57]. Some researchers also modified NK cells with chemokine receptor CCR7 via trogocytosis, a strategy to maintain the K562-based "donor" cell line expressing CCR7 and NK cells together. Through this process, the NK cells which can acquire CCR7 receptors via trogocytosis will move toward CCL19 and CCL21, then will be homing to the lymph node [58].

4.3. Checkpoint Blockade. Recent advances in immune checkpoint blockade (ICB) have revolutionized cancer treatment [59, 60]. The 2018 Nobel Prize in Physiology and Medicine was also awarded to the discoverers of cytotoxic T lymphocyte-associated protein 4 (CTLA4) and programmed cell death 1 (PD1) [61, 62]. Until now, six antibodies targeting immune checkpoint pathways have been approved for clinical use: ipilimumab (anti-CTLA-4), nivolumab (anti-PD-1), pembrolizumab (anti-PD-1), atezolizumab (anti-PD-L1), durvalumab (anti-PD-L1), and avelumab (anti-PD-L1). The mechanism of checkpoint blockade therapy was using monoclonal antibodies (mAbs) to activate immune cells through blocking the inhibitory receptors with ligands expressed on tumor cells. However, much attention is focusing on T cell immunotherapy. Considering NK cells have an important role in innate immunity, the roles of traditional and novel checkpoint blockades in NK cell-based immunotherapy are needed to be identified (Figure 2) [63].

4.3.1. PD-1. Blocking programmed death 1 (PD-1) showed its promising prospect to improve T cell functions and prevent tumor from immune responses. The expression/function of PD-1 on human NK cells was also explored recently. It was reported that CD16-KHYG-1 overexpress PD-1. And PD-1 blockade can block lytic granule polarization in NK cells, accomplished with function impairment of integrin outside-in signal pathway, which indicated that a novel mechanism of how PD-1 inhibition will disrupt NK cell function [64]. Some researchers also identify an increased number of PD-1 (bright) NK cells in the ascites of a cohort of patients with ovarian carcinoma. And PD-1 blockade strategy can restore part of PD-1 (bright) NK cell function [65]. Some studies support the use of cetuximab, which mediated antibody-dependent cytotoxicity (ADCC) against epidermal growth factor receptor- (EGFR-) overexpressing tumor cells, and PD-1 blockade can reverse NK cell dysfunction in head and neck cancer [66]. Furthermore, the safety of PD-1 blockade immunotherapy was confirmed in patients with different cancers.

4.3.2. KIRs. The KIRs presented on NK cell surface recognize MHC class I on tumor cells to maintain a "self-tolerance" condition. The anti-KIR antibodies can reactivate NK cells to kill tumor cells again. Lirilumab, an anti-KIR antibody, has shown a therapeutic effect in mice models of acute myeloid leukemia (AML) and B cell lymphoma. And anti-KIR monotherapy in patients with different cancers has been proven to be safe [67]. And combination of anti-KIR antibody IPH2101 and lenalidomide in phase I trial was also evaluated. But some reports showed the failure of lirilumab in treatment of multiple myeloma in phase II clinical trials [68].

4.3.3. TIGIT. T cell immunoglobulin and immunoreceptor tyrosine (TIGIT), an immunoglobulin superfamily receptor, presented on both NK and T cells and can be characterized to recognize CD155 [69, 70]. Many researches showed that

inhibitory receptors TIGIT, CD96, or activating receptors CD226 (also called DNAM-1) compete with the same receptors CD155 (poliovirus receptor) or CD112 (Nectin-2 or PVRL2), such competence regulate the NK cell functions [71–74].

A research reported that blocking CD96 directly or affecting CD96 through blocking TGF-β1 can break the balance and reverse NK cell exhaustion of liver patients, CD96 may be a promising inhibitory receptor which could combine with TIGIT in the future [75]. Recent studies also demonstrated that TIGIT blockade not only prevents NK cell exhaustion but also promotes T cell immunotherapy effects when NK cells existed. And the combination of TIGIT and PD-L1 blockade therapy has a better treatment effect in B16 pulmonary metastasis model [76]. The TIGIT blockade combined with anti-PD-1/PD-L1 antibodies has been proved to have synergy in preclinical models. And ongoing TIGIT blockade in phase I trials will answer whether TIGIT will work as well as PD-1/PD-L1 blockade. TIGIT is an immune checkpoint that inhibits activation of T cells and NK cells. A recent study showed that blockade of the checkpoint receptor TIGIT prevents NK cell exhaustion and elicits potent tumor-specific T cell immunity in an NK cell-dependent manner. TIGIT competes with immune activator receptor CD226 or DNAX accessory molecule-1 (DNAM-1) for the same set of ligands: CD155 (PVR or poliovirus receptor) and CD112 (Nectin-2 or poliovirus receptor-related 2 (PVRL2)) [71].

Some other studies demonstrated that CD16+ NK cells highly express TIGIT. When using anti-TIGIT or anti-CD112R, respectively, or together, human NK cells will trigger antibody-dependent cellular cytotoxicity (ADCC) via trastuzumab stimulation. Furthermore, this study indicates that poliovirus-like molecules are promising target in regulating NK cell function [77]. CD96, CD226 (DNAM-1), and TIGIT belong to an emerging family of receptors that interact with nectin and nectin-like proteins. CD226 activates NK cell-mediated cytotoxicity, whereas TIGIT reportedly counterbalances CD226. In contrast, the role of CD96, which shares the ligand CD155 with CD226 and TIGIT, has remained unclear. Some researchers found that CD96 competed with CD226 for CD155 binding and limited NK cell function by direct inhibition. Blocking CD96 may have applications in pathologies in which NK cells are important [74].

4.3.4. NKG2A. Despite the interactions between KIR and HLA-I control NK cell activation or inhibition, the activating receptors NKG2C and inhibitory NKG2A were also interacted with HLA-E on tumor cells [78]. Some researchers found NK cells expressing high NKG2A expression were exhausted and indicated a poor prognosis [79]. Using monalizumab, a monoclonal antibody which can block NKG2A, can improve NK cell cytotoxicity and ADCC [80, 81], especially in preclinical models [82], and some were under clinical trial [83]. Some researchers reported that IL-10 can enhance NKG2A expression. When blocking IL-10, the NKG2A expression on NK cells will decrease. It is reasonable that a higher HLA-E expression on tumor cells will destroy NK cell function. But some researchers reported that HLA-E was lowly expressed on U266 cells but upregulated *in vivo* in RAG-2(-/-) γc (-/-) mice. These researches indicated that HLA-E levels in vitro cannot not be an indicator *in vivo*. And further study demonstrated that both NKG2A-negative and KIR-ligand-mismatched NK cells are much powerful application [84].

4.3.5. CIS. CIS protein is a class of intracellular regulatory proteins known for its inhibition of cytokine signaling pathways. CIS protein has a central SH2 domain that binds to phosphorylated tyrosine motifs. In addition, the c-terminal of CIS protein has a domain called SOCS, which binds to the E3 ubiquitin ligase complex to facilitate ubiquitination and proteasome-mediated protein degradation. Delconte et al. found that the mutant NK cells were significantly more responsive to proliferation, killing, and activation after IL-15 stimulation than the control group. Transcriptome level detection found that the absence of CIS led to changes in the expression levels of many genes, including some serine proteases or their inhibitors, apoptosis regulators, transcription factors, and deubiquitinating enzymes. Through *in vitro* detection of NK cells, the author found that the absence of CIS could improve the signal strength of IL-15, in which JAK-STAT played an important role. Finally, the study demonstrated that blocking CIS can effectively reduce tumor progression by using lung and breast cancer models in mice [85].

4.3.6. IL-1R8. Interleukin-1 receptor 8 (IL-1R8, also known as SIGIRR or TIR8) is a member of the IL-1 receptor (ILR) family and has different structural and functional characteristics from other ILRs. Its primary role is the downstream signaling pathways and inflammatory responses of ILR, and Toll-like receptors (TLRs) play a negative regulatory role [86]. IL-1R8 is an important checkpoint for NK cell maturation and killing functions and plays a negative regulatory role in the antitumor and antiviral effects of NK cells. Recently, Molgora et al. have conducted in-depth research on the regulation mechanism of NK cells in antitumor and antivirus. The results showed that the interleukin receptor molecule IL-1R8 on the surface of NK cells has a negative regulatory effect on the antitumor and antiviral effects of NK cells [87]. The method of using the genetic engineering method to silence the expression of IL-1R8 molecules can effectively inhibit liver cancer, metastatic liver cancer, and cytomegalovirus infection.

5. How to Improve NK Cell Infiltration in Target Sites

One challenge for NK cell-based immunotherapy is the inadequate homing of adoptive transfer cell therapy [88–90]. Thus, it is urgent to explore novel strategies to improve NK cell infiltration in target sites (Figure 3). Ultrasound-mediated delivery has showed its promising prospect for enhancing the NK cell homing. Blood-brain barrier has been regarded as the main barrier that affected the efficiency of systemic injection of NK cells for brain tumor treatment. Alkins et al. demonstrated that the combination of focused

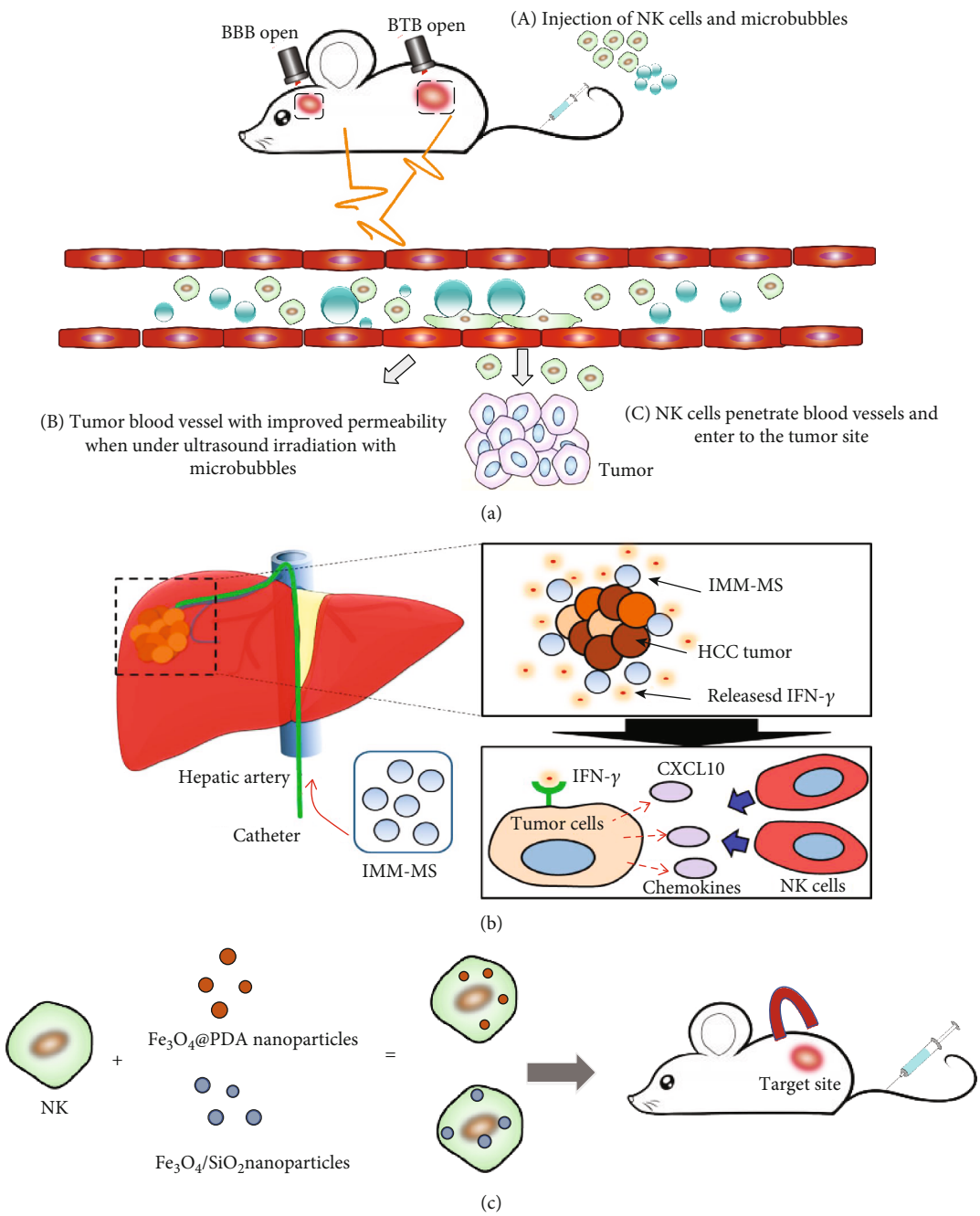

Figure 3: Strategies to improve NK cell infiltration in target sites. (a) Focused ultrasound improved NK cell infiltration in tumor sites. (b) Transcatheter intra-arterial local delivery of chemoattractant encapsulated in polymeric microspheres (reproduced from Ref. [93]). (c) Using magnets can attract NK cells which has uptake Fe_3O_4@PDA nanoparticles.

ultrasound (0.33 MPa average peak rarefaction pressure) and intravenous Definity ultrasound contrast can improve NK cell crossing through the BBB. With the interactions between focused ultrasound (FUS) and ultrasound contrast, the number of NK cells in metastatic breast tumor in the brain has a significant increase compared with delivery without BBB disruption. However, Alkins et al. also demonstrated that biweekly treatments with this method have no significant differences compared with the group of NK cells alone. Only adopting an early intensive treatment with targeted NK-92 cells (five times in the first five days and FUS), the tumor will be reduced statistically and further resulted in long-term survival in 50% of subjects [91].

Magnetic field is another useful strategy to increase NK cell recruitment and infiltration in the tumor sites. The key for this strategy is that NK cells have the ability to uptake nanoparticles containing Fe without affecting NK cell viability and cytotoxicity. Some researchers demonstrated that NK-92MI cells uptaking Cy5.5-conjugated Fe_3O_4/SiO_2 core/-shell nanoparticles can have a 17-fold enhancement into

tumor compared with NK-92MI cells alone via fluorescence microscopy. Wu et al. used polydopamine (PDA) to adhere Fe_3O_4 to improve biocompatibility and biodegradability. When putting a tiny magnetic device inside animals, the tumor will grow slowly compared with Fe_3O_4@polydopamine nanoparticle group and PBS group and without systemic toxicity in mice [92].

Considering IFN-γ can induce CXCL-10 expression and further result in improved NK cell infiltration, Park fabricated MRI visible immunomodulatory microspheres (IMM-MS) composed of IFN-γ, iron oxide nanocubes, and PLGA and demonstrated that such IMM-MS can attract NK cells into orthotopic liver tumor xenograft VX2 rabbit model. Transcatheter intra-arterial (IA) local delivery of chemoattractant encapsulated in polymeric microspheres is another strategy to induce efficient NK cell infiltration specifically for liver cancer. An immunomodulatory microsphere (IMM-MS) composed of biodegradable poly (lactide-co-glycolide) (PLGA) as a biocompatible polymer, IONC as an MR contrast agent, and IFN-γ as an immunomodulatory protein agent. The deposition of IMM-MS significantly increased NK cell infiltration into the liver tumor site [93, 94].

6. Treatment Strategies Combining NK Cells

6.1. Drug Delivery. Human immune cell can be used as vehicles for drug delivery or targeting agents because immune cells have the ability to traffic to the tumor or inflammatory sites [95–97]. NK cells with genetic modification have the ability to traffic to different solid tumors with associated ligand expression. NK92 cell with anti-CD19 to target to modification could serve as paclitaxel (PTX) nanoparticle drug delivery to target SKOV3.CD19 tumor subcutaneously in NSG mice. And the combination therapy through NK92 modified with PTX drug nanoparticles has showed better treatment results in both in vitro and in vivo studies [98]. The core technique of using immune cells in drug delivery is knowing how to attach the drug and leukocytes efficiently without affecting NK cells' naïve ability. And it has also been systematically explored, ranging from cell surface functionalization to internalization within leukocytes, which depends on the types of therapeutic delivered, leukocyte targeted, and the site of delivery [99].

What is more, some novel strategies of using NK cell membranes were also explored. Pitchaimani et al. developed "NKsome" composed of doxorubicin- (DOX-) loaded cationic liposomes and lysis NK-92 cells, which can recognize tumor cells through NK cell markers and further release chemotherapeutic drug DOX [100]. Deng et al. use NK cell membrane-decorated photosensitizer TCPP nanoparticles, which can induce proinflammatory M1 macrophage polarization and further activate effector T cells (CD4+ T cells and CD8+ T cells). A membrane camouflage fusogenic liposomal delivery system called "NKsome" is used for targeted tumor therapy. Activated NK cell membrane with receptor proteins was isolated from the NK-92 cells and extruded with the fusogenic liposome to form NKsomes. And chemotherapeutic drug doxorubicin (DOX) was further encapsulated into the aqueous core of NKsome and has improved its tumor homing ability and antitumor efficacy against MCF-7-induced solid tumor model [101] (Figure 4(a)).

6.2. Combination of Oncolytic Virus and NK Cells. Oncolytic virus (OV) therapy is a promising biological therapy, and FDA approved the first oncolytic HSV (oHSV) (T-Vec) used in advanced melanoma in 2015. However, the efficiency of OV therapy was not satisfying. The combined therapy with oncolytic viruses and NK cells was explored. Chen et al. demonstrated that group in EGFR-CAR NK-92 cells for 4 h followed by Ohsv-1 of breast cancer brain metastases mice will have a longer survival compared to monotherapies [102]. Suryadevara et al. combined OVs, proteasome inhibitors, and NK cell immunotherapy, which prolonged survival against glioblastoma (GBM). The mechanism of this triple combined therapy was the proteasome inhibitors bortezomib can increase heat shock protein 90 expression and resulted in OV replication [103]. Furthermore, oHSV and bortezomib will induce necroptosis and stimulate NK cell immunotherapy (Figure 4(b)).

6.3. Nanotechnology-Assisted NK Cell Therapy. Biomimetic nanotechnology is promising because it can imitate the physiological process and modulate immune cell functions and stimulate immunotherapy. Kang et al. fabricated a nanosized artificial necroptotic cancer cell (α-HSP70p-CM-CaP) vaccine composed of cancer membrane proteins (CM), α-helix HSP70 functional peptide (α-hsp70p), and CpG to stimulate both natural killer (NK) cells and APC. The tumor-associated antigens (TAA) on cancer cell membrane can trigger adaptive immune system. α-HSP70p, a danger-associated molecular pattern (DAMP), can activate both NK cells and DCs via binding with receptor CD49. CpG, an oligodeoxynucleotide, was also used as adjuvant which can stimulate strong host response. This nanosized vaccine (αHSP70p-CM-CaP) combined strategies of different antigens were used to acquire maximum antitumor effect. The αHSP70p-CM-CaP vaccine combined with anti-PD-1 antibody treatment therapy has provided tumor regression on mice B16OVA melanoma models [104].

To activate NK cells, nanoscale graphene oxide (NGO) with α-Hcd16 was synthesized; the cytolytic granule degranulation and IFN-γ secretion ability of NK cells were enhanced compared to α-Hcd16 alone [105]. The NK cell surface is an important bridge to deliver signals into intracellular level to active NK cells. Thus, the nanomaterials should mimic the biological conditions to interact with NK cell functions [106]. Delivering small interfering RNA (siRNA) into NK cells with high efficiency and low cytotoxicity is a difficult task. Nakamura et al. introduced siRNA core with protamine to decrease the total amount of lipid and further reduce the cytotoxicity. This study demonstrated that the ratio of YSK12-C4/siRNA at 5 has the similar gene silencing efficiency and lower cytotoxicity compared to a multifunctional envelope-type nanodevice (MEND) containing YSK12-C4 [107] (Figure 4(c)).

6.4. Bi- and Trispecific Killer Engagers. Recently, bispecific and trispecific antibodies known as killer cell adapters (BiKEs

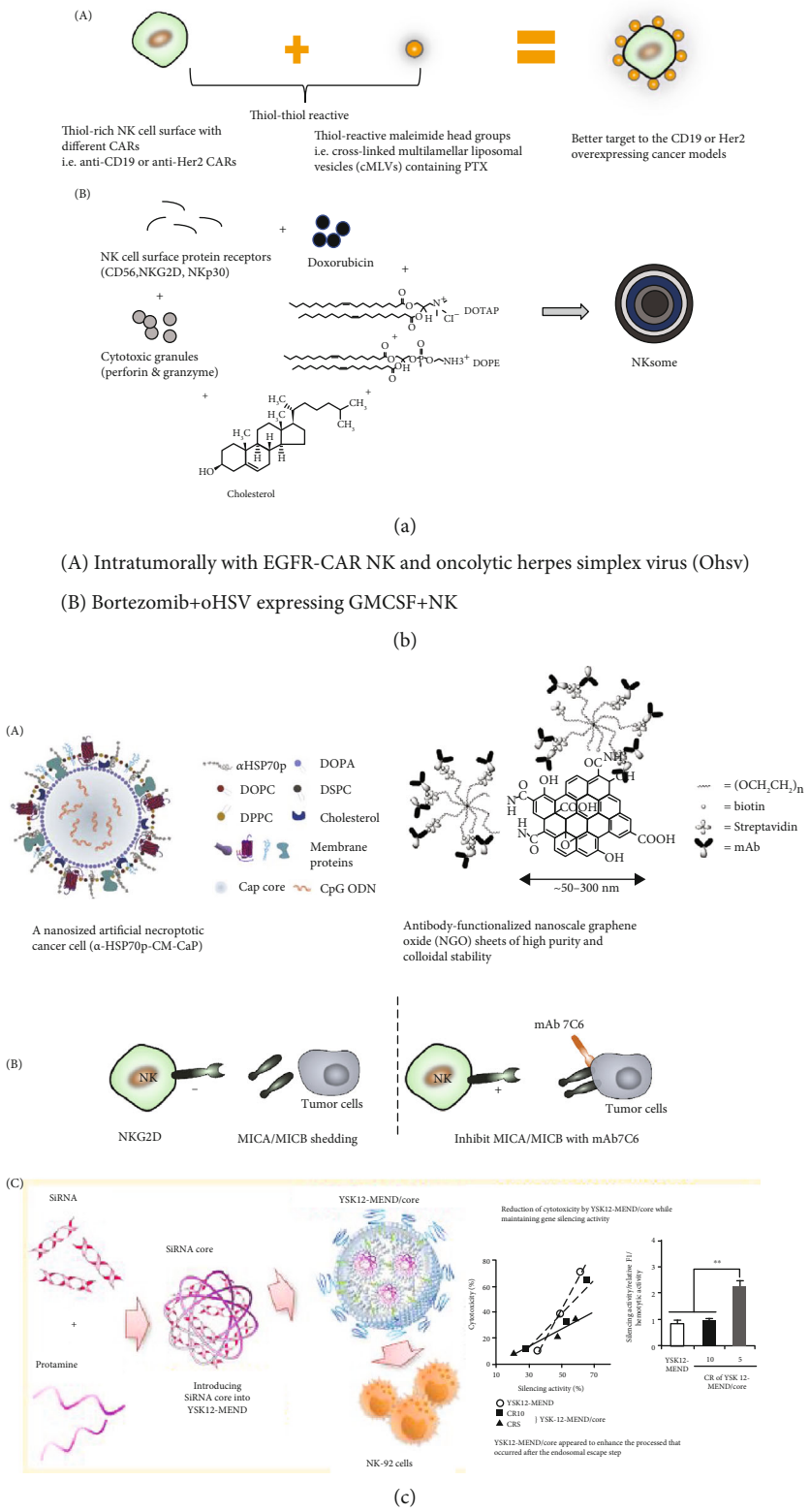

Figure 4: Different combined therapy improved therapeutic effect based on NK cells. (a) The NK cells of genetic modification and the membrane of NK cells can be used for drug delivery. (b) The combination of oncolytic viruses (OVs) and NK cells can improve the antitumor effects. Different combined therapy improved therapeutic effect based on NK cells. (c) The nanoparticles can assist NK cell-based therapy in different aspects. (Photo A of (c) was reproduced from Ref. [104]; photo C of (c) was reproduced from Ref. [107]).

Table 2: NK cell trafficking methods.

Imaging mode	Probes	NK cell types	Time in which NK cells retain the label	Advantages	Disadvantages
Optical imaging	DiR	Primary NK cells, activated/expanded NK cells, or NK cell lines	The DiR NK cells can be imaged for 8 days.	Little florescence interference; high tissue penetrance; using intravital microscopy	The DiR dye would persist in the membranes even in dead cells.
	Near-infrared dye DiD	NK-92 scFv (MOCC31)-zeta cells targeted to EpCAM antigen in tumors	Long-lasting at 72 h	Easily applicable; fast; inexpensive; and provides highly sensitive noninvasive imaging in preclinical and clinical settings	The absorption and emission wavelength of the probe is less than 65 nm. Many biological tissues are capable of autofluorescence under excitation, interfering with fluorescence analysis and biological imaging of biological samples.
	ESNF	Ex vivo-expanded NK cells	Long-lasting at 72 h using concentrations as low as 0.04 μM	Do not affect NK cell purity, expression levels of surface receptors, or cytotoxic functions	—
	Quantum dots (QD705)	NK-92MI	Up to 12 days post intratumoral injection in tumors	Low toxicity; high quantum yield; color availability; good photostability; large surface-to-volume ratio; surface functionality; and small size	Weakening of nonspecific background
	Ag_2 Se quantum dots (QDs)	NK-92	—	The emission is 1350 nm, in the second near-infrared window.	—
PET/SPECT	C-methyl iodide	—	Half-life = 20 min for PET as well as with gamma emitting radioisotope ^{111}in-oxine	High sensitivity for detecting labeled NK cells; high specificity	Poor spatial resolution (~1-5 mm), limited anatomic information, ionizing radiation, limited duration and number of scanning sessions
	^{18}FDG	NK-92-scFv (FRP5)-zeta cells	^{18}FDG 2-4 hours to days (^{111}In 4-7 days)	Clinically applicable; high specificity and sensitivity	Limited tracer uptake into the cells and loss of ^{18}FDG from labeled cells
Magnetic resonance imaging	USPIO	NK-92	2-4 weeks	Without significant adverse effect on the viability of cells; FDA approved	Hardly detecting small cell populations, relative high cost, long scan times, and low specificity; limited sensitivity, limited efficient labeling efficiency
Optical imaging/magnetic resonance imaging	Fluorescence organic dye (Cy5.5) with magnetic nanoparticles (Fe_3O_4/SiO_2)	NK-92MI	—	High specificity	Poor spatial resolution (~1-5 mm); limited anatomic information, ionizing radiation, limited duration and number of scanning sessions

and TriKEs) have been designed to efficiently participate in CD16 and induce antibody-dependent cell-mediated cytotoxicity (ADCC) response. They are designed by recognizing the fusion of the Fv domain of tumor cell antigens with the Fv domain that binds to CD16, with the ultimate goal of linking NK cells to tumor cells. Bispecific monoclonal antibodies have been successfully tested in primary lymphoma and Hodgkin lymphoma [108]. Although many strategies have shown promising success in *in vitro* and *in vivo* preclinical models, some of them are currently being evaluated in clinical trials. BiKEs contain two antibody fragments, one recognizes tumor antigens and the other one targets CD16 on NK cells, which together trigger ADCC. IL-15 is further integrated to create TriKE to drive strength of NK cells [109]. By

comparing with BiKEs, TriKEs produced superior NK cytotoxicity and NK cell persistence in *in vivo* xenograft tumor models and were recommended as an effective complement to existing NK delivery protocols. Importantly, TriKEs provide a versatile and cost-effective platform on which to incorporate novel targeting ligands with the potential to stimulate endogenous NK cells, thereby completely avoiding the need for cell transfer, predicting a new generation of immunity therapy.

7. NK Cell Tracking

How NK cells function its antitumor response in vivo is still not clear, and it is urgent to develop noninvasive imaging methods to track NK cells in vivo to explore the distribution of adoptive transfer and elucidate mechanism of NK cell-based immunotherapy. Three methods including optical microscopy, PET/SPET, and magnetic resonance imaging (MRI) were used to trace NK cells through labeling NK cells with fluorophores, radiotracers, or paramagnetic nanoparticles, respectively (Table 2).

Tavri et al. reported that a near-infrared dye DiD was employed to track the NK-92 scFv (MOC31)-zeta cells [110]. Lim et al. used anti-CD56 antibody conjugated with quantum dots (QD705) to label NK-92MI cells, which can be traced up to 12 days after intratumoral injection in human melanoma xenograft models [111]. Radiotracers including ^{18}FDG and C-methyl iodide were used for PET [110]. SPECT was used to label CAR-NK cells, which proved that targeting NK cells can accumulate more in tumor site compared to nontargeting NK cells [112]. For MRI, superparamagnetic iron oxide (SPIO) and ultrasmall superparamagnetic iron oxide (USPIO) nanoparticles were the most used probe to label NK cells; it is reported that NK cells can retain iron oxide up to 4 days [92, 113].

Conjugation of fluorescence organic dye (Cy5.5) with magnetic nanoparticles (Fe_3O_4/SiO_2) has also been used to visualize the migration of NK-92MI cells to the tumor site controlled by an external magnetic field [114]. PET and SPECT provide high sensitivity for detecting labeled NK cells (in the nanomolar concentration range), high specificity (target to background contrast), quantification of labeled cells and have immediate clinical translation through the use of FDA-approved labeled agents.

8. Conclusion

The key strategy for improving NK cell-based immunotherapy is to maximize NK cell reactive potential while minimizing NK cell inhibition. This review tried to give a whole picture of how NK cells were activated or inhibited and showed different strategies to improve the NK cell therapy. Various factors influence the functions of NK cells, the sources, the receptors on NK cell surfaces, the infiltration numbers in tumor sites, and so on. Nowadays, the clinical result of immune cell therapy indicated that adoptively transfer cell therapy and checkpoint blockade are the most promising strategies [115–118].

However, compared to T cell therapy, the mechanisms of NK cells are still needed to be furtherly explored. Luckily, some researches have been proceeded, such as developing the specific CAR for NK cells (NKG2D) rather than using the CAR which is designed for T cells originally. The TIGTI checkpoint blockade was also found to enhance NK cells' killing ability. These results indicated one promising direction is to develop strategies that can precisely regulate the NK cells' function. But the premise of this strategy is based on the full understanding of NK cells' activated and inhibitory functions. Activated NK cells were considered undergoing glycolysis and mitochondrial oxidative phosphorylation (OXPHOS), which is necessary for IFN-γ or granzyme B secretion of NK cells. Srebp-dependent regulation and amino acid-controlled c-Myc have been proved necessary for NK cell metabolic and functional responses. But NK cells in solid tumor are always dysfunctional which may be due to the tumor environments [119, 120]. These results indicated novel strategies of stabilizing c-Myc expression in NK cells which may be useful for normal functions of NK cells [121].

With the assistance of oncolytic virus and nanomaterials, the NK cell-based immunotherapy is promising. The oncolytic virus or nanomaterials can be designed to interfere with NK cell inhibition or change the tumor environment favouring NK cells' function. The NK cell in vitro culture is not to maintain the similar ability of NK cells in vivo. Some studies demonstrated that as the culture days prolong, some chemokine receptors of NK cells will be lost. Therefore, when administrating adoptive NK cell therapy, the surface marker expression of NK cells should be paid enough attention to ensure the NK cell ability [122, 123].

Finally, safety problems were important factors that should be considered when utilizing it in clinical studies. For example, targeted / non-targeted tumor toxicity of CAR-engineered NK cells needs to be considered, as for some cancer antigens are also expressed in healthy tissues. Insertion of suicide genes into CAR-modified effectors is one of the strategies to avoid these side effects [124]. However, such genetic modification needs to be sophistically designed and is laborious. Thus, the safety use of NK cells still needs to be further explored.

Abbreviations

NK:	Natural killer
MHC:	Major histocompatibility complex
HLA:	Human leukocyte antigens
NKRs:	NK cell receptors
KIRs:	Killer immunoglobulin-like receptors
ITIMs:	Immunoreceptor tyrosine-based inhibition motifs
ITAM:	Immunoreceptor tyrosine-based activation motif
KLRs:	Killer lectin-like receptors
NKG2D:	Natural killer group 2D
NCRs:	Natural cytotoxicity receptors
PB:	Peripheral blood
BM:	Bone marrow

hESCs: Human embryonic stem cells
iPSC-NK cells: Induced pluripotent stem cell-derived NK cells
aAPCs: Artificial antigen presenting cells
CAR: Chimeric antigen receptors
ICB: Immune checkpoint blockade
PD1: Programmed cell death 1
mAbs: Monoclonal antibodies
ADCC: Antibody-dependent cytotoxicity
EGFR: Epidermal growth factor receptor
AML: Acute myeloid leukemia
TIGIT: T cell immunoglobulin and immunoreceptor tyrosine
PVRL2: Poliovirus receptor-related 2
DAMPs: Danger-associated molecular patterns
iPSC: Induced pluripotent stem cell
FUS: Focused ultrasound.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Plasma MicroRNA Expression Profiles in Psoriasis

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Background. Psoriasis is an immune-mediated inflammatory chronic skin disease characterized by chronic inflammation in the dermis, parakeratosis, and excessive epidermal growth. MicroRNAs (miRNAs) are key regulators of immune responses. Although differential expression of miRNAs has been reported in certain inflammatory autoimmune diseases, their role in psoriasis has not been fully illuminated. Our aims were to confirm plasma miRNA expression signatures in psoriasis and to examine their potential influence on psoriasis pathogenesis. *Methods.* A miRNome PCR array was used to analyse the plasma of psoriasis patients and healthy donors. We performed miRNA pathway enrichment and target gene network analyses on psoriasis plasma samples. *Results.* We found several specific plasma miRNA signatures relevant to psoriasis. The miRNAs targeted pathways associated with psoriasis, such as the VEGF, MAPK, and WNT signaling pathways. Network analysis revealed pivotal deregulated plasma miRNAs and their relevant target genes and pathways regulating psoriasis pathogenesis. *Conclusions.* This study analysed the expression of plasma miRNAs and their target pathways, elucidating the pathogenesis of psoriasis; these results may be used to design novel therapeutic strategies and to identify diagnostic biomarkers for psoriasis.

1. Introduction

Psoriasis is an immune-mediated inflammatory chronic skin disease characterized by chronic inflammation in the dermis, parakeratosis, and excessive epidermal growth [1]. Skin lesions of psoriasis are characterized by infiltration of inflammatory cells and abnormal differentiation and hyperproliferation of keratinocytes [1]. Psoriasis affects 2-3% of the global population and seriously affects the quality of life of patients [2, 3]. There are 4 types of psoriasis, including psoriasis vulgaris, pustular psoriasis, psoriatic arthritis, and erythrodermic psoriasis [4]. However, the pathogenesis of psoriasis is still poorly understood, as psoriasis is a disease influenced by many different factors [4]. It is widely accepted that genetic susceptibility, cell cycle, immunity, inflammation, and neurotransmitters are involved [5–7]. Recently, abnormal genetic and environmental elements, particularly deregulated microRNAs (miRNAs) and their associated genes, have been indicated to be causative factors of psoriasis [8]. However, only a limited number of genes have been detected [9]. Currently, psoriasis is mainly diagnosed by clinical features (morphological evaluation of skin lesions). On occasion, a dermatopathologic evaluation may be helpful to confirm the diagnosis of psoriasis. However, unlike other autoimmune diseases, a histopathological examination and blood tests are generally not valuable tools to diagnose psoriasis, so diagnostic tests are almost unavailable [10].

The psoriatic lesion is characterized by T cell infiltration, increased chemokines, and angiogenesis, which may boost skin inflammation [11]. Increased Th17, $CD4^+$, and $CD8^+$ T cells and the interleukin- (IL-) 17 and IL-23 cytokines have been found in psoriatic lesions and peripheral blood, suggesting the involvement of innate and adaptive immunity in the pathogenesis of psoriasis [12, 13].

Currently, no specific markers that can help diagnose psoriasis and predict disease progression and remedial effects are found. Thus, a biomarker that can distinguish clinical types of psoriasis or can be used as a predictive biomarker for psoriasis progression is needed.

MicroRNAs (miRNAs) are small noncoding RNAs, approximately 22 to 25 nucleotides in length on average, with important roles in posttranscriptional gene expression. Deregulation of miRNAs and the corresponding target gene expression have been shown to be involved in psoriasis pathogenesis [14, 15]. miRNAs play a critical role in various autoimmune diseases, including psoriasis [16–18]. Recently, the number of miRNAs involved in immune system function and development has increased remarkably, and there has been a wide-ranging discussion of their possible use in therapies for immunological diseases [19]. miRNAs can regulate the proliferation, differentiation, and cytokine response of keratinocytes, the activation and survival of T cells, and the crosstalk between immunocytes and keratinocytes through the regulation of chemokine production in psoriasis. Recently, it has become evident that genetic polymorphisms in miRNA genes and/or in miRNA-binding sites of target genes can affect miRNA activity and contribute to disease susceptibility [20]. The concept that miRNAs take part in the pathogenesis of diseases, especially refractory diseases with unknown mechanisms, might lead to a new efficacious treatment. These studies emphasized the profound implication of miRNAs as regulatory molecules in autoimmunity and the intriguing possibility of using miRNAs as disease biomarkers in immunological diseases.

Studies have examined the role of miRNAs identified from human psoriatic skin, blood, and hair samples in relation to psoriasis pathogenesis, diagnosis, and treatment [4, 21]. Genetic polymorphisms related to specific miRNAs, such as miR-146a, are associated with psoriasis susceptibility [4]. Key roles of several unique miRNAs, such as miR-203 and miR-125b, in inflammatory responses and immune dysfunction, as well as hyperproliferative disorders of psoriatic lesions, have been revealed [22–24]. Moreover, circulating miRNAs detected from blood samples have the potential to be used in clinical applications as biomarkers of diagnosis, prognosis, and treatment responses [4]. These works underscored the potential importance of miRNAs in the diagnosis, prognosis, and treatment of psoriasis. However, further study in this field is needed, as the exact roles of miRNAs in psoriasis have not been fully elucidated. High-throughput miRNA expression studies have been performed to confirm plasma miRNAs specifically related to psoriasis [11, 24–26], but few studies have conducted a network analysis of plasma microRNAs and their target genes in psoriasis patients.

We performed a miRNome PCR array analysis on the plasma of psoriasis patients. Distinct plasma miRNA signatures relevant to psoriasis compared to healthy controls were confirmed. Pathway enrichment analysis of target genes of deregulated miRNAs revealed signaling pathways closely related to psoriasis pathogenesis, such as the VEGF signaling cascades, mitogen-activated protein (MAP) kinase pathway, and wingless-related integration site (WNT) pathway.

The network analysis of the interactions between plasma miRNAs and target genes/pathways in psoriasis illustrated psoriasis pathogenesis. This study illuminated some potential mechanisms of psoriasis pathogenesis by analysing deregulated plasma miRNAs, which may be used for the design of novel therapeutic strategies and diagnostic biomarkers of psoriasis.

2. Materials and Methods

2.1. Patients and Controls. Fifteen clinically diagnosed psoriasis patients (9 males and 6 females) and four healthy control subjects were included in our research. The psoriasis patients had not received systemic immunosuppressive medications for at least 1 month prior to study participation. The study was approved by the Ethical Committee of the Beijing Hospital of Traditional Chinese Medicine. Written informed consent was obtained from all the participants in the study. This research was performed in adherence to the principles expressed in the Helsinki Declaration.

2.2. Sample Preparation and RNA Extraction. Whole blood samples of healthy controls and psoriasis patients were collected using ethylenediaminetetraacetic acid- (EDTA-) containing tubes. All blood samples were centrifuged at 3,000 g for 20 min at 4°C. We collected the supernatants and then centrifuged again (13,000 g for 15 min at 4°C) to remove the cellular components. Finally, the purified plasma samples were obtained and stored at -70°C until further processing. One millilitre of each sample was subjected to RNA extraction.

Total RNA from each plasma sample was extracted using TRIzol-LS Reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. The concentration and purity of RNA was determined with NanoDrop ND-1000.

2.3. miRNA Profiling by Exiqon miRNA qPCR Panel. According to the RT^2 RNA QC PCR Array Protocol, RNA isolation (including homogenization, phase separation, RNA precipitation, RNA washing, RNA elution, and isolation of small-quantity RNA), RNA yield and quality assessment (including UV absorbance and denaturing agarose gel electrophoresis), cDNA synthesis (including diluting template RNA, preparing reagents, combining reagents, vortexing and centrifuging reagents, and incubating and heat inactivating reagents), and real-time PCR (including preparing reagents, diluting cDNA template 80x in nuclease-free water, combining reagents, vortexing and centrifuging reagents, and real-time PCR amplification) were performed by Exiqon miRCURY-Ready-to-Use PCR-Human-panel-I +II-V1.M (Exiqon miRNA qPCR panel, Vedbaek, Denmark), which could detect 764 miRNAs in plasma to identify differentially expressed miRNAs on the ABI PRISM 7900 system (Applied Biosystems) [27, 28]. Real-time PCR amplification was followed by melting curve analysis. Melting curve analysis of the PCR product(s) was performed to verify their specificity and identity. The relative expression between psoriasis patients and normal controls was estimated by the $2^{-\Delta\Delta Ct}$ method. We calculated the $\Delta\Delta$Ct for

each gene across two groups using the following equations, where group 1 is the control group and group 2 is the experimental group. Finally, we calculated the fold change for each gene from group 1 to group 2 by $2^{-\Delta\Delta Ct}$.

$$\begin{aligned}\Delta Ct\,(\text{group } 1) &= \text{average Ct} - \text{average of housekeeping genes' Ct for group 1 array},\\ \Delta Ct\,(\text{group } 2) &= \text{average Ct} - \text{average of housekeeping genes' Ct for group 2 array},\\ \Delta\Delta Ct &= \Delta Ct(\text{group } 2) - \Delta Ct\,(\text{group } 1).\end{aligned} \tag{1}$$

2.4. miRNA Target Gene Prediction. Screening of differential plasma miRNA was performed by the TwoClassDif database, and hierarchical clustering was performed by EPCLUST [29, 30]. The RVM t-test was applied to filter the differentially expressed plasma miRNAs for the control and experimental groups because the RVM t-test can raise degrees of freedom effectively in the cases of small samples. After the significant analysis and FDR analysis, we selected the differentially expressed plasma miRNAs according to the P value threshold. A P value < 0.05 was considered to have a significant difference.

MicroRNA target genes were predicted using the following miRNA target prediction tools: miRanda (August 2010 release) [31] and TargetScan (http://www.targetscan.org/vert_71/) [32]. Only genes predicted by both databases were chosen as conjectural miRNA targets for pathway analysis.

2.5. Pathway Enrichment and Network Analysis of MicroRNA Targets. Gene Ontology (GO) (http://www.geneontology.org) is a widely used database for functional enrichment analysis, which is helpful for understanding the functions of genes and proteins. GO analysis was applied to analyse the main function of the differentially expressed genes according to the GO database, which is the key functional classification system of NCBI and can organize genes into hierarchical categories and uncover the gene regulatory network on the basis of biological processes and molecular functions [33, 34]. Enrichment provides a measure of the significance of the function: as the enrichment increases, the corresponding function is more specific; this correlation helps us find those GO terms with more concrete functional descriptions in the experiment.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis is an enrichment and network analysis of a gene function database, which helps researchers find deregulated biological signaling pathways [35]. Pathway analysis was used to determine the significant pathway of the differential genes according to KEGG, Biocarta, and Reactome. We turned to Fisher's exact test and the χ^2 test to select the significant pathway, and the threshold of significance was defined by P value and FDR [35, 36].

In addition, the PathNet analysis is conducted, indicating the interacting relation between enriched pathways. The PathNet was the interaction net of the significant pathways of the differentially expressed genes and was built according to the interaction among pathways of the KEGG database to find the interaction among the significant pathways directly and systemically. It could summarize the pathway interaction of differentially expressed genes in diseases and determine the reason why certain pathways were activated [36, 37].

Moreover, intersecting genes of GO and KEGG were detected, and a miRNA-gene network was constructed. The relationship between plasma miRNAs and genes was counted by their differential expression values according to their interactions in the Sanger miRNA database, indicating the pivotal plasma miRNAs and genes in the pathogenesis of psoriasis [38]. A P value threshold of 0.05 and FDR correction were applied to the analysis.

2.6. Statistical Analysis. The relative quantification of plasma miRNAs was calculated by the equation $2^{-\Delta\Delta Ct}$. Data were expressed as the mean ± standard deviation (SD). A P value ≤ 0.05 was considered statistically significant. All statistical calculations were performed using SAS 9.4 software (Beijing Hospital of TCM Version, Order Number 9C1XJD).

3. Results

3.1. Global miRNA Expression Analysis of Plasma from Psoriasis Patients and Controls. To identify plasma miRNA expression signatures associated with psoriasis, we analysed global miRNA expression profiles by the miRNome PCR array in plasma derived from 15 psoriasis patients and 4 healthy volunteers. The clinical characteristics of patients included in the miRNome PCR array study are reported in Table 1. In the pool of plasma miRNAs, we identified that 15 plasma miRNAs were upregulated and 15 plasma miRNAs were downregulated ($P \leq 0.05$, fold change $\geq |2|$) in the plasma of psoriasis patients compared to healthy volunteers, showing a statistically significant difference between psoriasis patients and volunteers (Figure 1 and Table 2). Figure 1 shows that the colour of the patient group was different from that of the control group, which indicated the difference in plasma miRNA expression between the two groups.

3.2. Pathway Enrichment Analysis of Plasma miRNAs Deregulated in Psoriasis. To identify all the biological pathways targeted by deregulated plasma miRNAs in psoriasis, a pathway enrichment analysis based on annotated gene targets in GO was performed. This database can be used to evaluate miRNA regulatory action and to identify molecular pathways regulated by miRNAs. A functional pathway analysis using the KEGG pathway database was also performed. Genes targeted by deregulated miRNAs may be significant to psoriasis pathogenesis. Of note, a distinct enrichment of certain pathways was found; for example, pathways relevant to the immune system and proteoglycan metabolism and signaling pathways regulating apoptosis and/or the cell cycle were found [39].

In addition, many enriched pathways were found to be relevant to the multisystemic features of psoriasis. Figure 2 graphically represents enriched pathways in psoriasis. The

Table 1: Clinical characteristics of psoriasis patients.

Patients utilized for a gene array study	15 (100%)
Sex	
Male	9
Female	6
Age at diagnosis	36.80 ± 10.16
Types of patients	
Plaque psoriasis	15 (100%)
Psoriatic arthritis	0
Pustulosis of the palms and soles	0
Erythrodermic psoriasis	0
Stage	
Active stage	11 (73%)
Stable period	4 (27%)

enriched pathways were associated with angiogenesis (i.e., angiogenesis, positive regulation of angiogenesis, blood vessel remodelling, blood circulation, VEGF signaling pathway, and PI3K-Akt signaling pathway), cell cycle (positive regulation of the cell cycle, cell death, and negative/positive regulation of cell proliferation), and apoptosis (apoptotic process, intrinsic apoptotic signaling pathway, and PI3K-Akt signaling pathways). Moreover, other associated enriched pathways in GO and KEGG databases were implicated in the immune response (i.e., T cell receptor signaling pathway, T cell activation, B cell receptor signaling pathway, leukocyte transendothelial migration, and natural killer cell-mediated cytotoxicity) and the inflammatory response (i.e., chemokine signaling pathway, negative chemotaxis, NF-kappa B signaling pathway, and JAK/STAT signaling pathway).

Moreover, the MAPK and WNT pathways were found to be significantly enriched in psoriasis patients (Figure 2). Psoriasis-associated plasma miRNAs target MAP kinases; it has been reported that miR-148a-3p targets MAPK1, MAP2K3, MAP3K4, and MAP4K3 [40–43]. Some miRNA target genes associated with the MAPK pathway were also found in our analysis: miR-320c and miR-320d were found to target MAPK1.

Interestingly, the pathways related to stress and nerves (central and peripheral) were found to be distinctly enriched in their responses, such as in their response to stress, neurotrophin signaling pathway, long-term depression, glioma, Alzheimer's disease, nerve development, dopaminergic synapse, and serotonergic synapse.

The "proteoglycans in cancer" pathway was overrepresented in the plasma miRNAs of the patients studied. These target genes include FGF1, WNT1, WNT10B, WNT11, WNT10A, and WNT4.

3.3. Network Analysis of Pathways Targeted by Deregulated Plasma miRNAs in Psoriasis. The interactions of the pathways regulated by deregulated plasma miRNA target genes were determined by a network analysis including 49 pathway interactions constructed by KEGG (Figure 3).

Densely interconnected nodes expected to be associated with important biological processes of psoriasis pathogenesis were represented. The pathway network is graphically represented in Figure 3. The most enriched pathways, such as the MAPK, WNT, VEGF, and JAK/STAT signaling pathways; apoptosis; cell cycle; and pathways in cancer, were detected. Interestingly, the T cell receptor signaling pathway, B cell receptor signaling pathway, and natural killer cell-mediated cytotoxicity were significantly enriched. Moreover, the pathways related to long-term depression, Alzheimer's disease, and glioma were enriched.

3.4. Network Analysis of Deregulated Plasma miRNAs and Target Genes. The relationship between the deregulated plasma miRNAs and the target genes in psoriasis was determined by network analysis. A microRNA-target gene network comprising 28 microRNAs and 144 target genes was constructed (Figure 4). The deregulated plasma miRNAs and targeted genes were relevant to biological processes confirmed in psoriasis pathogenesis, such as immune response, inflammation, angiogenesis, and apoptosis. Therefore, plasma microRNAs regulating genes relevant to psoriasis were identified.

Interestingly, some of the targeted genes were relevant to immune and inflammatory responses, such as IL4R. Moreover, upregulated plasma miRNA target genes associated with B cell receptor (BCR) (EGR3) and T cell receptor (TCR) (MLLT3, DUSP3, DUSP5, DUSP7, DUSP8, DUSP16, NR4A3, CD34, CD58, CD8A, CD247, and CD276) adaptive immune responses implicated in psoriasis were identified.

Moreover, target genes regulated by deregulated plasma miRNAs were also associated with the innate immune response, such as STAT2 (miR-214-3p and miR-665), STAT3 (miR-665), and genes belonging to the NF-kappa B pathway, including PLCG1 (miR-218-5p), PRKCB (miR-7-5p), CSNK2A1 (miR-760), and XIAP (miR-7-5p, miR-320c, and miR-320d). Notably, several miRNA-targeted genes were involved in the JAK/STAT signaling pathway that regulated PIK3R3, STAT3, JAK1, SOCS5, IL4R, IL11, JAK3, SOS1, and SOS2. The JAK/STAT signaling pathway is relevant to autoimmune diseases, which indicates that psoriasis is an autoimmune disease [44]. In addition, JAK/STAT signaling pathway inhibition is used for treating psoriasis [45].

Moreover, target genes of deregulated plasma miRNAs participated in angiogenesis; these target genes include PIK3CD, PIK3C2B, MAPK1, AKT2, and PIK3R3.

In addition, genes relevant to apoptosis were also targeted and include BCL2L11, PIK3CD, PIK3R3, PIK3C2B, MAP3K5, MAP3K11, and MAP4K4.

To detect the relationship between gene expression and deregulated plasma miRNAs, a network analysis was performed. The most upregulated miRNAs, such as miR-214-3p, miR-7-5p, miR-761, and miR-665, targeted significant pathways in psoriasis pathogenesis. The most targeted gene was DAGLA.

4. Discussion

Currently, systematic analyses of plasma miRNA expression profiles in psoriasis are being performed [4, 11, 22–26]. However, the potential roles of identified miRNAs in psoriasis

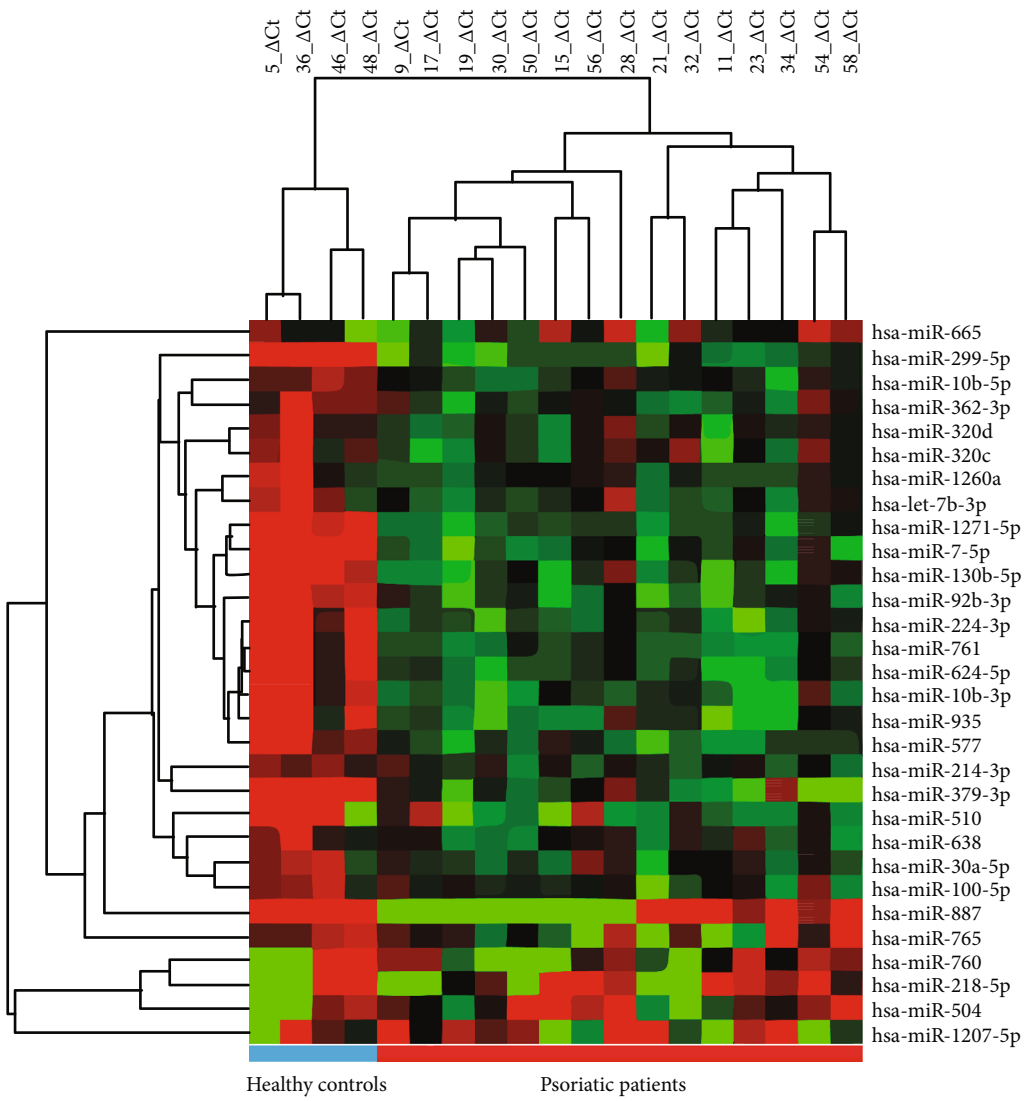

Figure 1: Hierarchical clustering of plasma samples. Heatmap showing expression of candidate miRNAs that are deregulated in the plasma of psoriasis patients compared with the plasma of normal controls (comparatively upregulated and downregulated miRNAs are indicated by red and green, respectively). The first 4 columns showed plasma from healthy individuals. The last 15 columns showed plasma from psoriasis patients. The lines indicate deregulated plasma miRNAs.

have not been predicted with bioinformatics analysis, so further study is necessary in this field. Thus, a global miRNA expression profile in the plasma of psoriasis patients was determined to identify miRNAs associated with psoriasis, assisting in the analysis of potential pathways involved in psoriasis pathogenesis. Deregulated miRNA-targeted pathways, such as the inflammatory response, angiogenesis, apoptosis, and/or cell cycle pathways, indicated a corresponding impact on psoriasis.

The target genes were completely associated with representative characteristics of psoriasis. Some genes targeted by deregulated plasma miRNAs were associated with angiogenesis (i.e., VEGF pathway) and adaptive and innate immune responses, and proinflammatory genes, such as IL4R, were also identified. In addition, the JAK/STAT pathway, which is related to autoimmune diseases [44, 46], such as psoriasis [45, 47, 48], was also observed. Plasma miRNA target genes play a role in apoptosis, which is associated with psoriasis pathogenesis. AKT2 participates in the PI3K/AKT/mTOR signaling pathway as a critical mediator in psoriasis [49]. SDC2, a target of miR-665 and miR-1207-5p, was found to be upregulated in the blood and lesions of psoriasis patients and to be involved in angiogenesis and the migration and retention of leukocytes [39, 50].

Notably, the VEGF, MAPK, and WNT signaling pathways, which are involved in psoriasis pathogenesis [50], were targeted by plasma miRNAs deregulated in psoriasis patients. The VEGF signaling pathway plays an important role in psoriasis [51]. Moreover, molecules associated with MAPK have been reported to be upregulated in the blood of psoriasis patients [52], and the p38 MAPK pathway is involved in psoriasis [53]. In addition, the WNT pathway is involved in skin inflammation in psoriasis patients [54].

Table 2

MicroRNA	Sequence	*P* value	Fold change (patients/control)	Regulation
(a) Plasma miRNAs upregulated in psoriasis patients versus healthy subjects				
hsa-miR-1271-5p	CUUGGCACCUAGCAAGCACUCA	0.0005695	3.31	Up
hsa-miR-299-5p	UGGUUUACCGUCCCACAUACAU	0.0006351	12.54	Up
hsa-miR-760	CGGCUCUGGGUCUGUGGGGA	0.001499	5.21	Up
hsa-miR-7-5p	UGGAAGACUAGUGAUUUUGUUGU	0.0022822	2.68	Up
hsa-miR-92b-3p	UAUUGCACUCGUCCCGGCCUCC	0.0045117	3.17	Up
hsa-miR-130b-5p	ACUCUUUCCCUGUUGCACUAC	0.0054175	2.43	Up
hsa-miR-510-5p	UACUCAGGAGAGUGGCAAUCAC	0.0155792	9.3	Up
hsa-miR-224-3p	AAAAUGGUGCCCUAGUGACUACA	0.015879	3.68	Up
hsa-miR-218-5p	UUGUGCUUGAUCUAACCAUGU	0.0167446	5.52	Up
hsa-miR-379-3p	UAUGUAACAUGGUCCACUAACU	0.0267967	3.13	Up
hsa-miR-761	GCAGCAGGGUGAAACUGACACA	0.033785	2.41	Up
hsa-miR-10b-3p	ACAGAUUCGAUUCUAGGGGAAU	0.0344881	4.04	Up
hsa-miR-624-5p	UAGUACCAGUACCUUGUGUUCA	0.0364547	2.74	Up
hsa-miR-577	UAGAUAAAAUAUUGGUACCUG	0.044155	3.05	Up
hsa-miR-935	CCAGUUACCGCUUCCGCUACCGC	0.0455152	4.76	Up
(b) Plasma miRNAs downregulated in psoriasis patients versus healthy subjects				
hsa-miR-665	ACCAGGAGGCUGAGGCCCCU	0.0003116	0.065	Down
hsa-miR-320d	AAAAGCUGGGUUGAGAGGA	0.0023854	0.42	Down
hsa-miR-10b-5p	UACCCUGUAGAACCGAAUUUGUG	0.0109296	0.43	Down
hsa-miR-1260a	AUCCCACCUCUGCCACCA	0.0121104	0.42	Down
hsa-miR-362-3p	AACACACCUAUUCAAGGAUUCA	0.0130663	0.49	Down
hsa-miR-214-3p	ACAGCAGGCACAGACAGGCAGU	0.0169923	0.37	Down
hsa-miR-30a-5p	UGUAAACAUCCUCGACUGGAAG	0.0170545	0.37	Down
hsa-let-7b-3p	CUAUACAACCUACUGCCUUCCC	0.0235985	0.45	Down
hsa-miR-638	AGGGAUCGCGGGCGGGUGGCGGCCU	0.0240685	0.36	Down
hsa-miR-320c	AAAAGCUGGGUUGAGAGGGU	0.0241893	0.39	Down
hsa-miR-504-5p	AGACCCUGGUCUGCACUCUAUC	0.0248942	0.21	Down
hsa-miR-100-5p	AACCCGUAGAUCCGAACUUGUG	0.0254685	0.36	Down
hsa-miR-765	UGGAGGAGAAGGAAGGUGAUG	0.0363717	0.24	Down
hsa-miR-887-3p	GUGAACGGGCGCCAUCCCGAGG	0.038367	0.23	Down
hsa-miR-1207-5p	UGGCAGGGAGGCUGGGAGGGG	0.0478966	0.13	Down

The "proteoglycans in cancer" pathway was prominently targeted by deregulated plasma miRNAs of psoriasis patients. Although the pathway is typically associated with many types of cancers, proteoglycans are important in angiogenesis and in the migration and retention of leukocytes [39]. Target genes such as AKT2, WNT1, WNT4, WNT10, WNT11, FGF1, FGF7, FGF9, FGF11, and FGF14 are involved in inflammation, which is a typical feature of psoriasis. Thus, proteoglycan metabolism was significantly affected by deregulated plasma miRNAs in psoriasis.

To prioritize pathways that may be important for psoriasis pathogenesis, a pathway network analysis was performed. The network analysis indicated significant pathways in psoriasis pathogenesis, such as apoptosis, cell cycle, angiogenesis, inflammatory response, T cell immune response, VEGF, MAPK, WNT, JAK/STAT, NF-kappa B, and B cell response. Interestingly, pathways in the network were relevant to psoriasis pathogenesis. In addition, enriched pathways related to long-term depression, Alzheimer's disease, and glioma were detected.

Our data were associated with pathogenesis in psoriasis patients. We found that miR-214-3p, miR-7-5p, miR-761, miR-665, and miR-1207-5p showed the highest degree in the network analysis of deregulated plasma miRNAs and their target genes. Interestingly, some of the pathways were targeted by upregulated miRNAs, such as miR-7-5p and miR-761, whereas some of the pathways were targeted by downregulated miRNAs, such as miR-214-3p, miR-665, and miR-1207-5p.

It has been reported that psoriasis, a psychosomatic disease, is closely related to chronic stress (i.e., depression and anxiety) via the hypothalamic-pituitary-adrenal (HPA) axis, which secretes neuroendocrine mediators and triggers skin inflammation in psoriasis [55–57]. In addition, stress can induce the interaction between mast cells and microglia and lead to inflammation [58], and inflammation of the

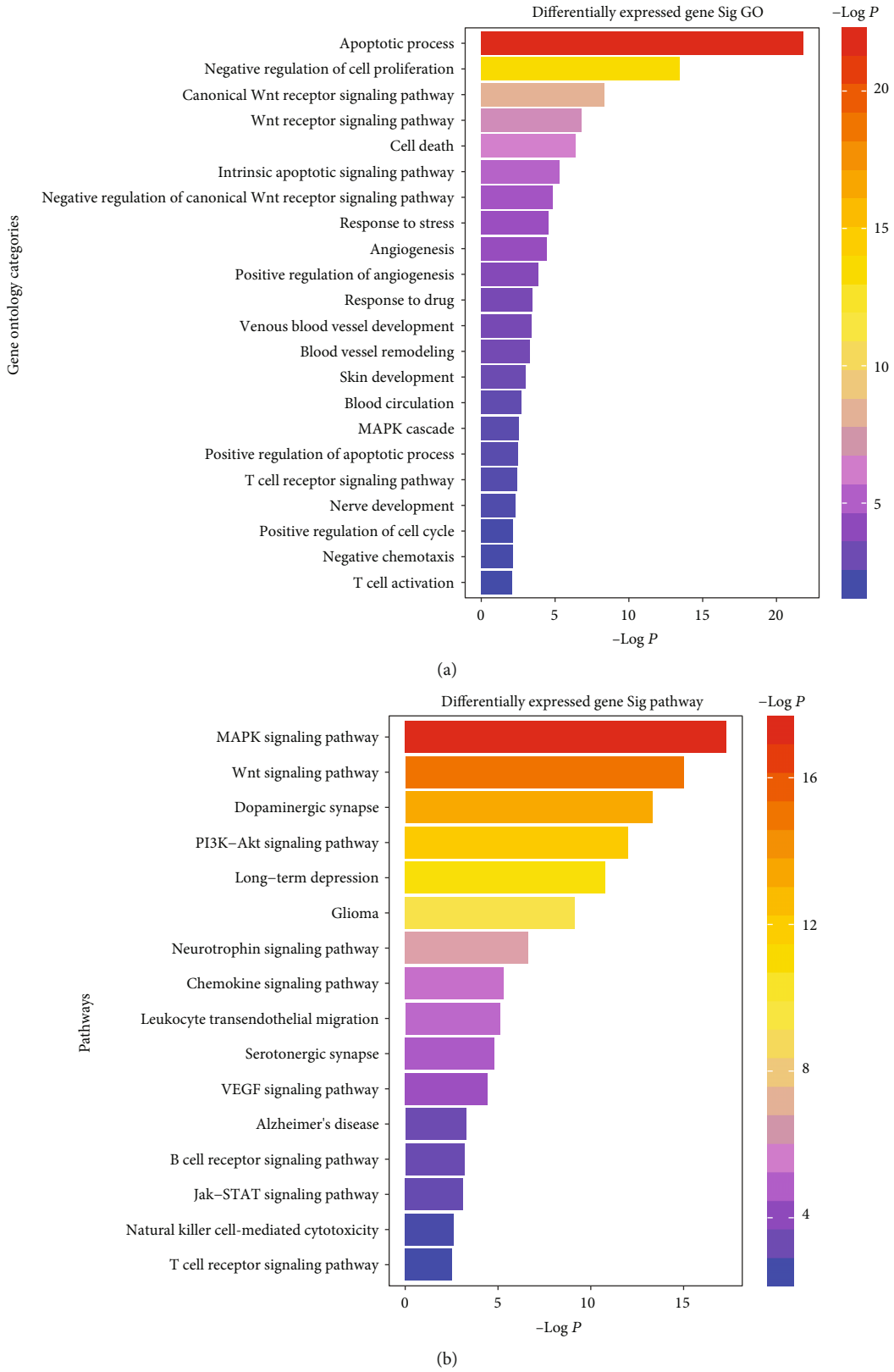

Figure 2: (a) GO-enriched biological process terms for the differentially expressed plasma miRNAs. (b) KEGG pathways enriched for differentially expressed plasma miRNAs.

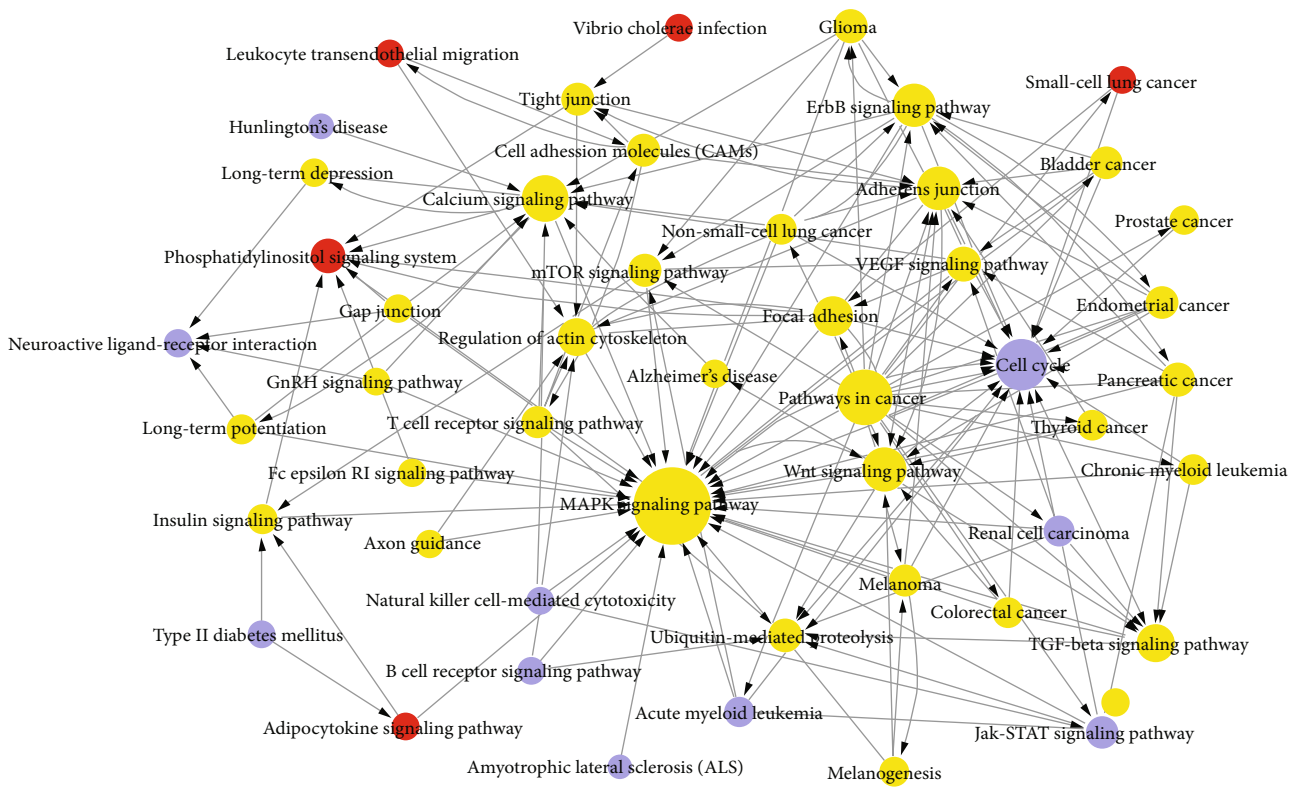

Figure 3: Network analysis of pathways targeted by deregulated plasma miRNAs in psoriasis (the red dots indicate the upregulated pathways, the blue dots indicate the downregulated pathways, and the yellow dots indicate the up/downregulated pathways).

hypothalamus can also induce the secretion of neuroendocrine mediators and skin inflammation [59]. miR-214-3p plays an important role in depressive-like behaviours, cognition defects, Alzheimer's disease, and endothelial cell dysfunction and mediates neural apoptosis, neuropathic pain, and the growth, migration, and invasion of glioma cells [60–65]. miR-7-5p is relevant to vascular endothelial cell proliferation, glioma, brain damage, and cognitive dysfunction [66–69]. Moreover, it has been reported that miR-7-5p boosts apoptosis but inhibits cell proliferation and apoptosis of T lymphocytes, explaining the function of miR-214-3p and miR-7-5p in psoriasis [70, 71]. It was reported that miR-1207-5p may exert an inhibitory effect on VEGF [72]. We indicated that miR-214-3p and miR-7-5p can regulate the VEGF pathway by deregulating NFATC4, PIK3R3, PRKCB, and PIK3CD, and angiogenesis typically occurs in psoriatic lesions [1]. Upregulated miR-761 has been found to be involved in modulating the proliferation and migration of glioma cells and in inducing skin inflammation by the neuroendocrine system and HPA axis [73]. Downregulated miR-665 has been found to be involved in cognitive dysfunction and astrocyte and neuroblastoma cell growth [74–76].

The differentially expressed miRNAs we identified do not correspond to those of other studies in psoriasis, and a possible explanation of the discrepancy is that most of the previous studies used samples such as psoriatic lesion tissues, keratinocytes, hair roots/shaft, PBMCs, psoriasis epidermal cells, and dermal T cells, but not plasma [4, 21–24]. miRNA profiling in plasma samples may be quite different from miRNA profiling in local skin cells such as epidermal keratinocytes, Th17 cells, and PBMCs. miRNAs have been identified to be deregulated in (1) psoriatic lesion tissues (miR-203, miR-21, miR-146a, and miR-423), dermal inflammatory infiltrates of psoriatic skin (miR-142-3p, miR-193b, and miR-223), the stratified epidermis of psoriatic lesions (miR-135b and miR-150), the upper part of the epidermis (miR-99a and miR-150), and psoriatic skin of animal models of psoriasis (miR-424); (2) keratinocytes (miR-99a, miR-150, miR-423, and miR-197), keratinocytes in psoriatic lesions (miR-31, miR-203, and miR-125b), normal human keratinocytes stimulated by combinations of proinflammatory cytokines (miR-203), and the reconstituted human epidermis and human keratinocyte cell line stimulated by IL-22 (miR-184); (3) psoriasis epidermal cells and dermal T cells (miR-21), Th17 cells (miR-193b and miR-223), and CD4(+) T cells (miR-210); (4) psoriasis hair roots (miR-19a) and psoriasis hair shafts (miR-424); and (5) PBMCs (miR-193b, miR-223, miR-143, and miR-146a).

miR-1266 and miR-33 have been detected to be deregulated in blood samples of patients with psoriasis. miR-1266 is overexpressed in psoriasis serum samples [77], which may be differentially expressed in plasma. In addition, since miR-33 is involved in the posttranscriptional regulation of genes associated with lipid metabolism, one study reported that the plasma levels of lipid and glucose metabolism-related miR-33 are increased and correlated with insulin in psoriasis patients [78].

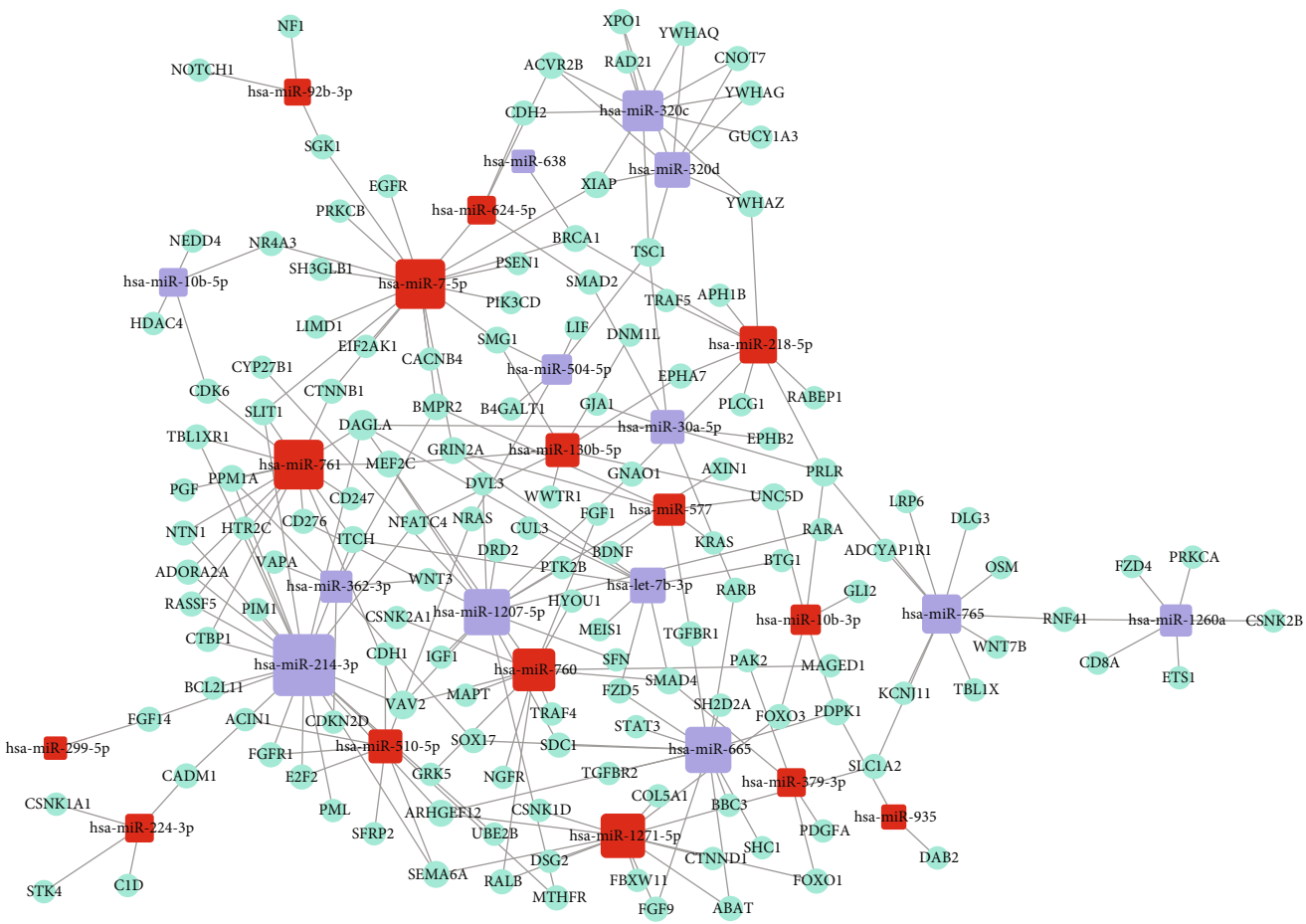

Figure 4: Network analysis of deregulated plasma miRNAs and target genes (the red squares indicate upregulated miRNAs, whereas the blue squares indicate downregulated miRNAs).

The potential limitations of the study are as follows: (1) We analysed deregulated miRNAs in the plasma samples from psoriasis patients, and the plasma miRNA profiles may be different than the miRNA profiles in skin tissue. (2) We identified the upregulation or downregulation of plasma miRNAs, and the changes in mRNA and gene expression were not confirmed. (3) Therapeutic interventions were not included in our study, so we cannot associate the expression of plasma miRNAs with the treatment responses; these experiments can be done in the future.

5. Conclusion

In summary, we studied the global plasma miRNA expression profile in psoriasis. Using this approach, we clarified specific plasma miRNA expression profiles that characterize psoriasis. The study analysed the expression of plasma miRNAs and the target pathways, elucidating the pathogenesis of psoriasis. This study illuminated some mechanisms of psoriasis pathogenesis by analysing deregulated plasma miRNAs, which may be used for designing novel therapeutic strategies and identifying diagnostic biomarkers for psoriasis.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this article.

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Review Article

Emerging Prospects for Nanoparticle-Enabled Cancer Immunotherapy

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One of the standards for cancer treatment is cancer immunotherapy which treats both primary and metastasized tumors. Although cancer immunotherapeutics show better outcomes as compared with conventional approaches of cancer treatment, the currently used cancer immunotherapeutics have limited application in delivering cancer antigens to immune cells. Conversely, in solid tumors, tumor microenvironment suppresses the immune system leading to the evasion of anticancer immunity. Some promising attempts have been made to overcome these drawbacks by using different approaches, for instance, the use of biomaterial-based nanoparticles. Accordingly, various studies involving the application of nanoparticles in cancer immunotherapy have been discussed in this review article. This review not only describes the modes of cancer immunotherapy to reveal the importance of nanoparticles in this modality but also narrates nanoparticle-mediated delivery of cancer antigens and therapeutic supplements. Moreover, the impact of nanoparticles on the immunosuppressive behavior of tumor environment has been discussed. The last part of this review deals with cancer immunotherapy using a combination of traditional interventional oncology approach and image-guided local immunotherapy against cancer. According to recent studies, cancer therapy can potentially be improved through nanoparticle-based immunotherapy. In addition, drawbacks associated with the currently used cancer immunotherapeutics can be fixed by using nanoparticles.

1. Introduction

Cancer is one of the most lethal diseases and is causing thousands of deaths annually throughout the world [1]. It is traditionally treated by using anticancer medicines and radiations [2]. However, these modalities are associated with certain drawbacks such as the high possibility of recurrence, limited therapeutic effectiveness, and distressing undesired effects. In recent years, clinicians have promisingly treated cancer by using immunotherapeutic moieties [3]. This approach has several advantages such as its effectiveness against metastasized cancer also as well as low risk of recurrence [4, 5]. Owing to these features, clinicians are interested in opting immunotherapy as a standard treatment option against cancer [6]. Thus, the researchers are actively developing different immunotherapeutic antibodies [6] and cell therapeutics [7]. Particularly, antibodies have been used in the development of immune checkpoint inhibitors against various regulatory molecules/receptors (Figure 1). Nonetheless, some undesired effects are also associated with cancer immunotherapeutics such as autoimmune disease [3]. In addition, immunotherapeutics are more effective against lymphoma than solid tumors [8, 9] likely due to difficult penetration of immunotherapeutic agents through their abnormal ECM (extracellular matrix) [10, 11]. Moreover, immune-suppressive tumor microenvironment (ISTM) is also responsible for the reduced efficacy of immunotherapeutics against solid tumors [12, 13].

Current research work is focused on the management of cancer immunotherapeutics' shortcomings, for instance, by using nanoparticles [14]. Nanoparticles are the biomaterial-

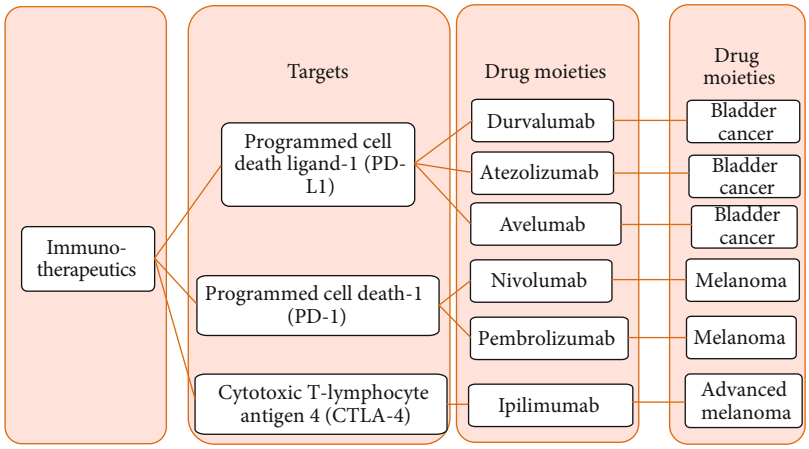

Figure 1: Examples of immunotherapeutics (mainly monoclonal antibodies) approved by the FDA for cancer treatment.

based nanosized vehicles [15, 16] which are extensively used in delivering drug molecules in a controlled fashion as well as to the target site [17].

Cancer treatment using immunotherapeutics depends on three important factors. The first factor deals with an effective transfer of cancer antigens to immune cells, particularly APCs (antigen-presenting cells), such as dendritic cells. The induction of anticancer immune response after delivery of adjuvant and cancer antigen to immune cells is the second requirement for this treatment. The third factor involves the modulation of the IDTM to induce a response to the anticancer immunotherapeutics. These aims can be achieved by using nanoparticulate systems, which can be potentially utilized for the induction of immune response against cancer. This review article describes the current trends in cancer therapy using nanoparticles as immune-modifying systems.

2. Mode of Action of Immunotherapeutics in Cancer

For the application of nanoparticles in the treatment of cancer, it is a prerequisite to comprehend the mechanistic aspects of cancer immunotherapy. The framework of cancer immunotherapy research depends on a cancer-immunity cycle (Figure 2) which involves the removal of tumor cells. Necrosis- or apoptosis-mediated death of cancer cells produces tumor antigens. APCs capture these antigens and present on major histocompatibility complex (MHC). The complexity of dendritic cells and cancer antigens induces the priming of immature T cells in the lymph nodes, followed by the infiltration of the activated TCLs (tumor-specific cytotoxic T lymphocytes) into the tumor site. TCLs interact with T cell receptors and MHC to recognize tumor cells. Then, effector T cell-mediated apoptosis of cancer cells releases additional cancer antigens which strengthen the immune response. These events lead to the induction of effective immunity against cancer, which is, however, interrupted by several barriers.

Proinflammatory cells, for instance, M1-polarized macrophages possess the capability of killing tumor cells. The deceased cells produce various immunosuppressive factors such as IL-10 (interleukin-10) inducing repolarization of macrophages from M1 to M2 [18–20]. In addition, these dead cells release the characteristic substances (for instance, monocyte chemoattractant protein-1 or MCP-1) which attract various cells (for example, leukocytes) towards them [19, 20], leading to the transfer of monocytes and MDSCs (myeloid-derived suppressor cells) into the tumor microenvironment [21–23]. Here, the differentiation of these monocytes into TAMs (tumor-associated macrophages) takes place. TAMs accelerate the growth of the tumor and camouflage it from immune attack [19–23]. On the other hand, the infiltrated MDSCs play a role in the inhibition of immune response against cancer through the secretion of anti-inflammatory cytokines, leading to Treg cell activation. Treg (regulatory T) cells have an immunosuppressive function and inhibit the maturation process of dendrites, resulting in the remission of the tumor [24–26]. The situation becomes more problematic when tumor evasion from an anticancer immune effect occurs due to the inhibition of TCLs by immune-suppressive entities present on PD-1, PD-L1, PD-L2, and CTLA-4 cells. Eventually, these phenomena limit the immunotherapeutic efficacy [27–30], revealing the significance of solving the issues of current immunotherapies against cancer. Immunotherapy can be intervened by nanomaterials to enhance immunity against cancer.

3. Types of Nanoparticle Systems

During current years, several nanoparticle systems (Figure 3) have been studied for cancer immunotherapy. Among a wide array of the currently studied nanoparticles for cancer immunotherapy, polymer-based nanoparticles are the most popular systems [31]. The Food and Drug Administration (FDA) has approved a variety of polymers, such as polyethylene glycol, poly (lactide-o-glycolic) acid, and chitosan owing to their biodegradable, biocompatible, and nontoxic features, for the synthesis of nanoparticle systems for cancer immunotherapy [32]. Other commonly used nanoparticulate systems include the inorganic (such as gold nanoparticles) and the lipid-based nanoparticles (such as liposomes) [33], as mentioned in Figure 3. All of these nanoparticles can be

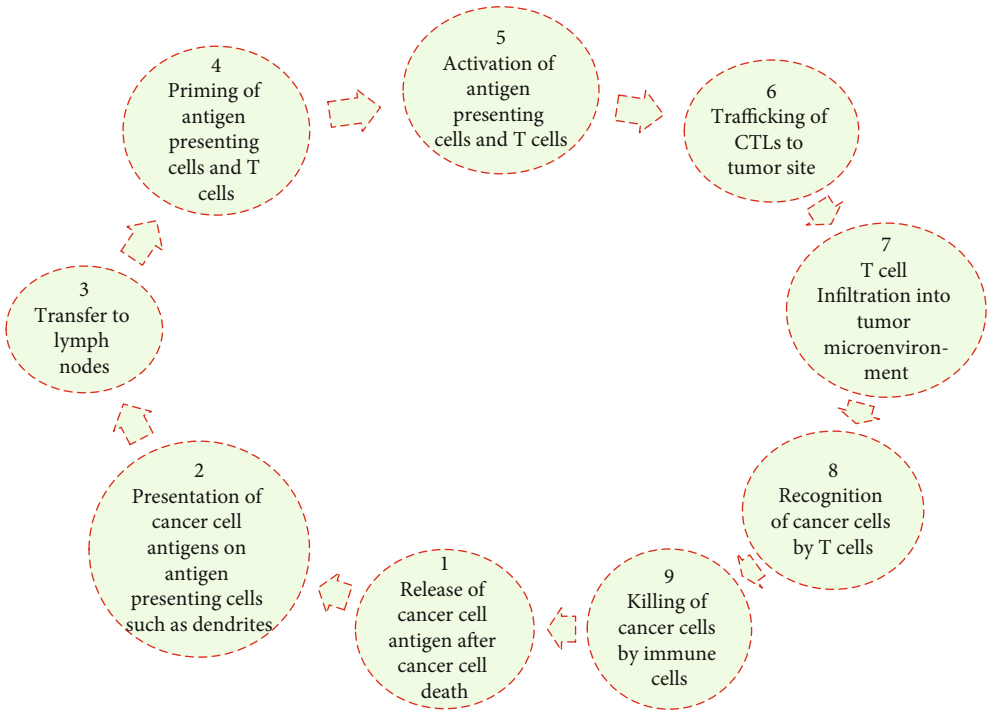

Figure 2: Cancer-immunity cycle showing its main stages such as release, presentation, transfer, priming, activation, trafficking, infiltration, recognition, and killing.

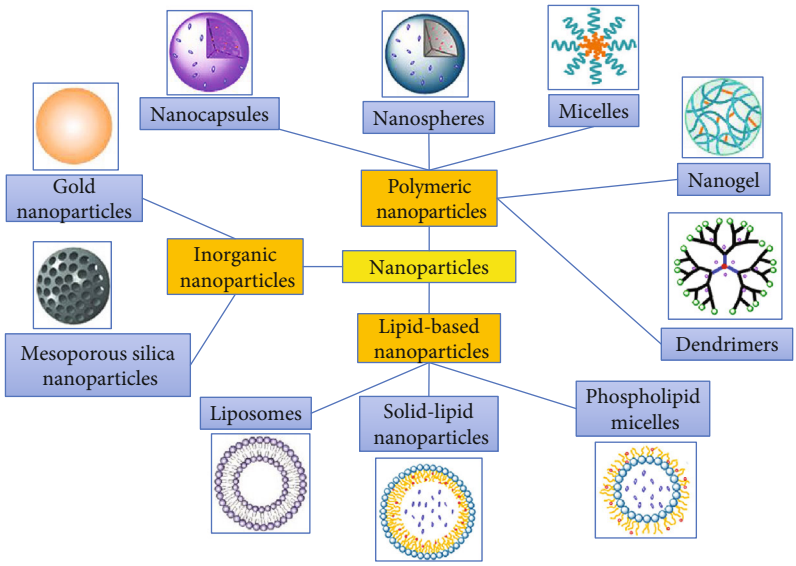

Figure 3: The representative examples of currently studied nanoparticles (polymeric, lipidic, and inorganic) for cancer immunotherapy.

promisingly used for targeting cancer and delivering antigens and supplements to the target site with a good accuracy and precision for the activation of the immune system.

4. Current Strategies for the Preparation of Nanoparticle Systems

Nanoparticles are produced through physical, biological, and chemical methods. Biological methods are mainly used for microorganism-assisted biogenesis of metallic nanoparticles such as gold nanoparticles [34]. Several approaches including emulsification, sol-gel synthesis, precipitation, spray drying, and salting out. Nanoemulsification is the generally adopted technique for the fabrication of polymer nanoparticles. This process involves the removal of organic solvents by the process of evaporation or extraction, leaving polymer nanoparticles in the pot [35]. However, it is crucial to remember that the selected approach affects the properties of the acquired nanoparticles, including size, shape, and charges [35].

5. Optimum Features of Nanoparticles for Efficient Immunotherapy

Nanoparticles possess distinguished physicochemical properties including size, shape, and charge, which can be customized to achieve various therapeutic goals such as cancer immunotherapy [36]. For example, the size of nanoparticles affects the cellular uptake and endocytosis. As compared with the larger nanoparticles (>100 nm), smaller ones (25-40 nm) have a greater potential of immune response activation, since smaller nanoparticles are allowed to move to lymph nodes via dendritic cells, while the larger ones are retained at the target site. Very large nanoparticles (>500 nm) are engulfed in the macrophages through phagocytosis [37]. In addition, the nanoparticle's shape also influences its uptake and distribution. Nonspherical nanoparticles experience prolonged systemic circulation, because of their potential to avoid nonspecific cellular phagocytosis. On the other hand, nonspherical nanoparticles are more readily engulfed by dendrites, in comparison with spherical nanoparticles [38]. Furthermore, the surface charge of nanoparticles also influences the mechanism of their internalization. For instance, cationic nanoparticles are quickly engulfed by macrophages or dendrites, resulting in a significant lysosomal escape. Conversely, there is stronger affinity between cationic nanoparticles and serum proteins, which provokes the reaction of cationic nanoparticles with anionic components such as hyaluronic acid and other moieties in the tumor microenvironment, resulting in the reduced leakage of nanoparticles from tumor tissues. In addition, charged nanoparticles have a lesser penetration depth and shorter circulation time than that of neutral nanoparticles [39]. Moreover, tumor-targeting antibodies can be conjugated to the nanoparticles to achieve the enhanced permeability and retention effect (EPR) [36].

6. Multifunctional Nanoparticle Systems

A considerable development in the field of cancer immunotherapy has been introduced during the last few years. However, clinical trials of cancer vaccines could not receive significant success. In addition to several other factors, this unremarkable accomplishment could be due to the fact that traditional methods of drug delivery techniques were not safe. In recent years, new opportunities, especially nanoparticle-based modalities, have been explored for the treatment of cancer [40]. Particularly, cancer vaccines have been promisingly delivered using multifunctional nanoparticles, which exhibit several benefits, including targeted delivery of immunotherapeutics (such as immune checkpoint inhibitors) using stimuli-sensitive materials resulting in the reduced off-target effects and increase in drug efficacy. Other advantages of nanoparticle system is the simultaneous delivery of multiple therapeutic moieties, where treatment and imaging agents can be integrated in the core and on the surface of multifunctional nanoparticles for cancer targeting [41]. Some representative examples of multifunctional nanoparticulate systems for cancer immunotherapy are presented in Figure 4. Current studies have revealed that nanoparticles have multifaceted functions for (a) working as an effective substitute for generation and transduction of CAR- (chimeric antigen receptor-) T cell, (b) inculcating tumor-suppressing activity to TAM (tumor-associated macrophages), and (c) knocking down Kras oncogene addition by using nano-Crisper-Cas9 delivery system [42]. In addition, nanomedicine platform can be repurposed for the improvement of cancer therapy function by using multifunctional nanoparticles.

7. Nanoparticle-Mediated Delivery of Tumor Antigens

The induction of tumor immunity requires the effective transfer of tumor antigens to APCs. The researchers have introduced two important classes of antigens, i.e., TAAs (tumor-associated antigens) and TSAs (tumor-specific antigens, also called neoantigens). Although TAAs are mainly expressed on cancer cells, normal and differentiating cancer cells also contain TAA contents. Thus, an autoimmune reaction might be caused when these antigens are used as immunotherapeutic targets. Alternatively, the autoimmune problem is not observed in the case of TSAs, since they are expressed in cancer cells only. However, the human enzyme system easily degrades these innate tumor antigens. In addition, these antigens are less efficiently transferred to immune cells; thus, they are known as weak immunogenic species. Since secondary lymphoid organs primarily home the immune response, an effective anticancer immune response can only be initiated when the lymph nodes are efficiently accessed by tumor antigens. In view of that, a nanoparticle-mediated safe delivery of tumor antigens to lymph nodes has been extensively investigated [51]. These studies have revealed two main benefits, i.e., tumor antigen protection against biodegradation and their targeted delivery to the lymph nodes. Afterward, successfully and safely delivered nanoparticles undergo an effective internalization into APCs [52]. Most of the abovementioned problems have been solved by using nanoparticles for the delivery of tumor antigens. However, the synthesis and use of the nanoparticles for this purpose are required to comply with many considerations.

Nanoparticle delivery to lymph nodes is delicately affected by several factors such as water solubility, shape, size, and surface charge of nanoparticles [53–56].Hydrophobic polymers (for instance, chitosan) or polymers having hydrophobic component exhibit intrinsic adjuvant activity and show potential to activate immune cells even in the absence of additional signals [56, 57]. For example, the increase in side chain lipophilicity of PGA (poly(gamma-glutamic acid)) nanoparticles results in ameliorated uptake of antigen, increased activation of dendrites, and improved cellular response [58].

In addition to size, particle shape also affects nanoparticle drainage from lymph nodes. Nanoparticles have been prepared in a variety of shapes such as spherical, discs, rods, and stars [58]. However, spherical nanoparticles have better properties than other shapes in respect of migration effect, infiltration capacity, and circulation time [59–61].

Furthermore, transportation of antigen-loaded nanoparticles depends on their size. Nanoparticle size neither should be lesser than 5 nm (termed as small-size nanoparticles) to

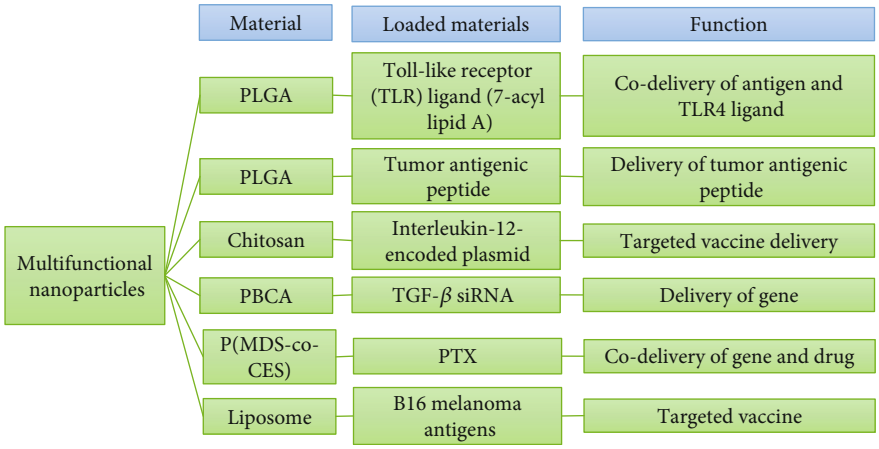

Figure 4: Representative examples of multifunctional nanoparticulate systems studied *in vivo* for cancer immunotherapy [43–50]: PLGA-Poly(lactide-o-glycolic acid), PBCA-Polybutyl cyanoacrylate, and P(MDS-co-CES)-A triblock polymer.

prevent their leakage from the circulatory system nor greater than 100 nm (termed as large-size nanoparticles) to avoid their entrapment in ECM and lymph nodes. Nanoparticles having a size of approximately 5–100 nm (termed as medium size nanoparticles) exhibit a prolonged circulation time and can be used to target the lymphatic system. For instance, PPS (poly(propylene sulfide)) nanoparticles having a size range of 20-45 nm persisted in the lymphatic system for about five days [57]. Additionally, APCs, lymph nodes, and dendritic cells contained almost half of these nanoparticles [15, 57]. A study on the comparison of nanoparticles having a size of 25 nm (smaller) and those with 100 nm (larger) after intradermal administration reported more efficient delivery of smaller nanoparticles to lymph nodes through the lymphatic system [15, 55]. Nonetheless, the optimum size of antigen-loaded nanoparticles for efficient delivery to lymph nodes is 5–100 nm. These nanoparticles can be chemically modified via attaching suitable ligands such as mannose for their active transport to the lymph nodes.

Furthermore, the nanoparticle surface charge not only influences the cellular internalization but also affects the immune response activation [62]. In general, positively charged nanoparticles exhibit a higher immune response but a lower tissue permeability than the negatively charged or inert ones. The reduced permeability could be attributed to their immobilization in the oppositely charged ECM [63]. As compared with the negatively charged or inert nanoparticles, positively charged nanoparticles are easily taken up by the dendritic cells localized at the site of injection. On the other hand, hemolysis and platelet aggregation and thus the premature antigen release are the critical problems associated with lymphatic transport of cationic nanoparticles [64, 65].

8. Nanoparticle-Mediated Delivery of Therapeutic Supplements

Therapeutic supplements (TS), also known as adjuvants, are used in combination with tumor antigens to enhance their mutagenicity. TS have a resemblance to pathogenic molecules which are identified by pattern recognition receptors (PRRs) [66–69]. An example of TS used in cancer immunotherapy is lipopolysaccharide. The internalization of TS with tumor antigens into APCs results in an ameliorated immune response against cancer through the induction of a strong antigen-specific T cell response [70–73]. In addition, the combination of nanoparticle-mediated delivery of tumor antigen with immune checkpoint blockade improves the immune response against cancer. Therefore, different types of solid tumor and blood cancer can be potentially treated by using nanoparticulate systems.

A recent study described the simultaneous delivery of tumor-specific antigens (TSAs) and TS using nanoliposomes (Figure 5) having a multifaceted immunomodulatory effect [74]. Nanoliposome size was reported as 100 nm and denoted by the term "tumosomes". It contained two immunostimulatory TS, i.e., MPLA (3-O-desacyl-4′-monophosphoryl lipid A) and DDA (dimethyldioctadecylammonium) as a danger signal and a cell-invasion domain, respectively. The findings revealed an enhanced anticancer immunity, reduction in tumor growth, and improved survival of mouse tumor models treated with the tumosomes. In this approach, self-antigens may face a condition of autoimmunity that could be overcomed by using TSAs. In addition, the therapeutic efficacy of this modality can be further improved by using it with other therapeutic approaches including chemotherapy.

9. Nanoparticle-Mediated Delivery of Immunomodulators

Tumors can create immunosuppressive tumor microenvironment which can enhance cancer growth and metastasis. Thus, cancer can be potentially treated by immunomodulation of tumor microenvironment [75].

One of the potential examples of immunosuppressive T cells is Tregs (Figure 6) which can suppress the activity of anticancer T-effector cells. Tregs are involved in the prevention of autoimmune disease via the establishment of immune tolerance against autoantigens. However, in cancer, Tregs can exert a suppressive effect on immune cells in the tumor

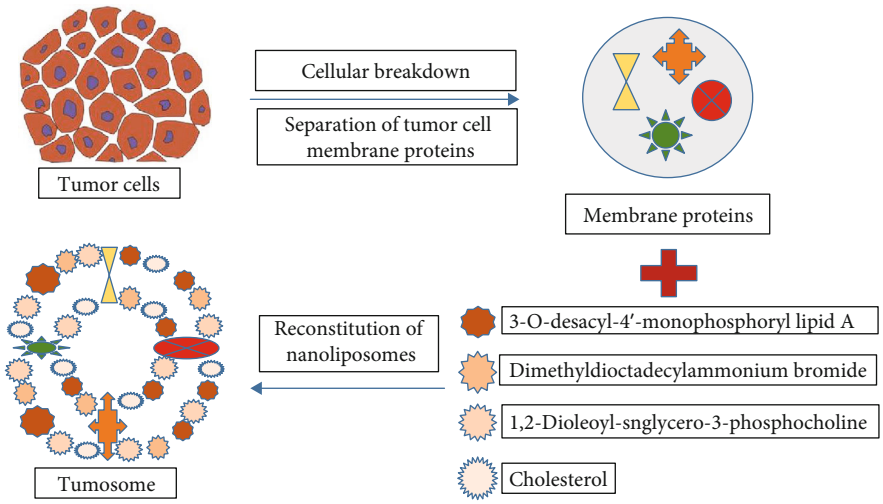

Figure 5: Diagrammatic presentation of multifaceted tumosome for cancer immunotherapy.

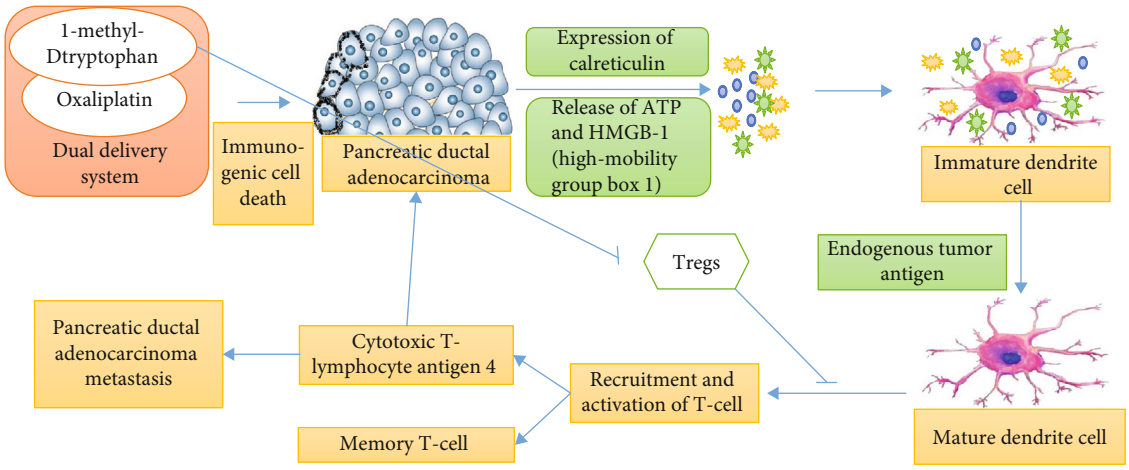

Figure 6: Diagrammatic presentation to express the combined effect of 1-methyl-D-tryptophan and oxaliplatin on immune response in pancreatic ductal adenocarcinoma. A vehicle was prepared for the codelivery of two chemotherapeutics, i.e., 1-methyl-D-tryptophan and oxaliplatin. 1-Methyl-D-tryptophan plays a role in causing immunogenic cell death via expression of calreticulin and release of ATP and HMGB-1 (high-mobility group box 1), while the interference of oxaliplatin with the indoleamine 2,3-dioxygenase 1 pathway is reported. After receiving adjuvant stimuli and uptaking the dying tumor cells, dendrite cells undergo a maturation process along with crosspresentation of tumor antigens. Afterward, primary and metastasized cancer cells are killed by CD+ T cells through granulysin and perforin. This codelivery system influences the indoleamine 2,3-dioxygenase 1 pathway, interferes Treg development, and controls other immunomodulatory activities, resulting in the strengthening of the apoptotic effect by the immune system. The immunogenic cell death pathway involves the activation memory T cells and helper cells which helps in the prevention of disease recurrence.

microenvironment resulting in the reduced anticancer immunity. Antitumor immunity can be induced by inhibition of elimination of Tregs [76]. For instance, anti-CTLA-4 is a checkpoint blockade that is utilized for the control of Tregs' activity in cancer immunotherapy. Moreover, Tregs can be removed from the tumor microenvironment by the engineering of Treg-targeted nanoparticles [77].

Tumor microenvironment contains a high level of TAMs. These are the immune cells which generate an excess of immunoregulatory cytokines such as TGF- (transforming growth factor-) β and IL-10. In addition, TAMs produce inflammatory cytokines such as IL-6 leading to the suppression of anticancer immune responses. Thus, effective cancer immunotherapy requires targeting and killing TAMs in the tumor microenvironment utilizing surface-modified nanoparticles.

Hepatic, lung, and breast cancer exhibit overexpression of various cytokines including TGF-β which suppresses activation, maturation, and differentiation of immune cells. Therefore, an immune response in cancer might be induced through the suppression of the TGF-β in the tumor microenvironment. In a recent study, nanoparticles were prepared by the process of microencapsulation for the delivery of TGF-β inhibitors to the tumor microenvironment. It resulted in the induction of both innate and adaptive immune activities leading to the inhibition of tumor

growth as well as an improvement in the survival of mice having metastatic melanoma.

Tumor microenvironment of hepatic, gastrointestinal, and breast cancer contains high levels of tumor-suppressor cells such as MDSCs which generate various cytokines such as IL-10 for the activation of Tregs and inhibition of other immune cells. In this context, effective cancer immunotherapy requires MDSC elimination in the tumor microenvironment. Nanoparticle-mediated delivery of immunomodulators to the tumor microenvironment can be accomplished via active or passive transport. Thus, the ameliorated anticancer immune effect and the reduced undesired effects can be acquired through nanoparticle-mediated delivery of immunomodulators to the tumor microenvironment.

The recent studies combined various therapeutic approaches (such as checkpoint blockade immunotherapy and nanoscale metal-organic structure-aided radiotherapy) with nanotechnology to overcome the immunosuppressant microenvironment of tumor-facilitating effective treatment of tumor [78–82]. The researchers are very optimistic to overcome the drawbacks of currently used cancer immunotherapy by utilizing these combined modalities.

10. Localized Anticancer Immunotherapy

The hundreds of studies have reported the synthesis of nanoparticles for the treatment of cancer; however, the majority of the developed nanoparticulate systems could not be translated into clinical use. A review published in 2016 on the nanoparticle-based studies conducted during the last 10 years revealed the delivery of <1% of the intravenously administered dose to solid tumors [83]. It could be due to the tumor microenvironment which comprises heterogeneous structure and the distorted vasculature system, resists the entrance of drug molecules into the tumor site, and thus suppresses antitumor efficacy. In this context, novel nanoparticulate systems have been developed for local administration which have greatly attracted the attention of cancer clinicians [84].

Interventional radiology is a branch of interventional oncology which deals with the use of image guidance for the localized diagnosis and treatment of cancer using a minimum surgical procedure [85]. Anticancer therapeutics can be delivered to various malignant areas using medical imaging technology, for instance, conjunction of MRI (magnetic resonance imaging) and catheters. The image guidance approach can be used in local therapy to achieve various benefits such as reduced dose, cost-effectiveness, lesser undesired effects, and swift response [86].

Nanoparticles have several versatile features which pave their use in the fabrication of various imaging agents. For instance, ferric oxide nanoparticles [87, 88] and gold nanoparticles [89] are widely used as contrast agents in MRI and CT scan, respectively. Consequently, such functional nanoparticles can be utilized in the development of injectable medicines for their local use in medical imaging.

The currently available anticancer immunotherapeutic agents are directly administered to the circulatory system of the patients which leads to low efficacy and high toxicity. For instance, a high dose of an immune checkpoint suppressor is required when it is administered as an intravenous infusion. However, a stronger anticancer T cell activity with a low risk of side effects can be induced through local administration of an immune checkpoint suppressor, even at low doses [90, 91]. Nonetheless, the efficacy of cancer immunotherapeutics can be improved while its associated side effects can be reduced through local immunomodulation [92]. Even, the systemic anticancer immunity can be promoted by activation of the locally injected immune cells. In addition, the situation in which systemic infusion is associated with the production of large amounts of serum antibodies can be avoided by using local immunotherapy. It leads to the reduced activity of nonspecific immune cells, diminished side effects, and suppressed inflammatory processes [93].

Thus, the locally administered nanoparticles which have imaging characteristics and can exert effective immunotherapeutic effect against cancer have gained promising importance. Nanoparticles loaded with low-dose immunotherapeutics can be developed by combining traditional interventional oncology approach with image-guided local immunotherapy against cancer to safely target immunological organs or solid tumors. One of the important features of this modality is the use of imaging devices for the confirmation of immunotherapeutic delivery to the target area.

In image-guided local immunotherapy, the disposition of immunomodulatory agents can be monitored by imaging the nanoparticles loaded with cancer immunotherapeutics such as cancer antigens, cytokines, and adoptive cell therapeutic moieties. Consequently, conventional anticancer therapies can be replaced with more efficacious cancer therapy comprising cancer immunotherapy, nanotechnology, and interventional oncology.

11. Conclusion

The current research has revealed the application of biomaterial-based nanoparticles in the amelioration of anticancer immunity. Nanoparticles can improve antigen presentation via efficient delivery of cancer antigens and therapeutic supplements to APCs in immunological organs, for example, lymph nodes. Therefore, a vaccine-like prolonged and broader immune effect can be yielded by utilizing nanoparticle-loaded cancer immunotherapeutics as compared with free immunotherapeutic agents. For instance, neoantigens based on mRNA (mRNA-nAg) are less immunogenic but its translation in the cytoplasm can enhance T cell activity [94]. However, ubiquitous nucleases can degrade such agents and hinder their delivery into APCs. It is a valuable approach to deliver mRNA-nAg to immune cells by using nanoparticles [95]. Furthermore, nanoparticle-mediated delivery of immunomodulators to the tumor microenvironment can initiate the process of immune surveillance [41]. Such drugs can be efficiently delivered to the tumor site by using characteristic nanoparticles which respond to the tumor microenvironment. Furthermore, nanoparticulate systems can be combined with other modalities such as radiotherapy [96], chemotherapy [97], and phototherapy [98, 99] to improve the therapeutic efficacy of

cancer immunotherapy. A few years back, for nanoparticle-loaded cancer immunotherapeutics, systemic administration was the preferred route of administration which caused toxic effects because of high doses. A few years back, nanoparticle was used to deliver cancer immunotherapeutics into systemic circulation; however, it required high doses of immunotherapeutics which caused toxic effects. Therefore, a new modality, named as image-guided local immunotherapy, is developed by combining the traditional interventional oncology approach with local cancer immunotherapy. This new modality produces therapeutic effectiveness even at low doses of immunotherapeutics due to their site-specific delivery and thus is associated with reduced toxicity [100]. In addition, immune cells or antibodies can be mimicked by using the synthesized nanoparticle based on the advance knowledge of mechanisms involved in cancer immunity. Recent advancement in the field of cancer immunotherapeutics is the development of nanoparticle-based artificial APCs [101], which can be used instead of natural APCs for the activation of the adaptive anticancer immune response.

The abovestated literature reveals that the interdisciplinary research, especially the union of various biomedical approaches, has evolved into current cancer immunotherapy. However, the development of biomaterial-based anticancer immunotherapy requires a detailed knowledge of how biomaterials interact with the immune system. For cancer immunotherapy, nanoparticle development using biomaterials has played an important role in achieving therapeutic efficacy at comparatively low doses and avoiding toxicity. In short, cancer patient's life quality and span can be improved by developing cancer vaccines based on nanoparticles.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

Manal Ali Buabeid and Ghulam Murtaza equally contributed.

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Review Article

Immunotherapy Deriving from CAR-T Cell Treatment in Autoimmune Diseases

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Chimeric antigen receptor T (CAR-T) cells are T cells engineered to express specific synthetic antigen receptors that can recognize antigens expressed by tumor cells, which after the binding of these antigens to the receptors are eliminated, and have been adopted to treat several kinds of malignancies. Autoimmune diseases (AIDs), a class of chronic disease conditions, can be broadly separated into autoantibody-mediated and T cell-mediated diseases. Treatments for AIDs are focused on restoring immune tolerance. However, current treatments have little effect on immune tolerance inverse; even the molecular target biologics like anti-TNFα inhibitors can only mildly restore immune balance. By using the idea of CAR-T cell treatment in tumors, CAR-T cell-derived immunotherapies, chimeric autoantibody receptor T (CAAR-T) cells, and CAR regulatory T (CAR-T) cells bring new hope of treatment choice for AIDs.

1. Introduction of Autoimmune Diseases

Autoimmune diseases (AIDs) are a spectrum of chronic disease conditions originating from an abnormal-activated autoimmune system and involve certain organ (organ-specific AIDs, i.e., type I diabetes, T1D) or multiple organ systems (systematic AIDs, e.g., systemic lupus erythematosus, SLE), displaying as autoimmune intolerance and leading to tissue injury [1–3]. Broadly, AIDs can be separated into two categories according to pathogenic mechanism: self-reactive antibody- or "autoantibody-" mediated AIDs in which antibodies are produced by plasma cells from the B lymphocyte lineage and self-reactive T lymphocyte-mediated AIDs. The incidence of AIDs is 80 cases per 100000 people, and the prevalence is over 3% globally, while in the USA, the prevalence reaches to 5%-8% [4, 5]. Women accounting for 65% of all patients, AIDs mainly occur in young and middle-aged women and have been the primary cause of death in the affected women. Currently, nearly a hundred kinds of AIDs have been reported, and the most common ones are T1D and autoimmune thyroid disease, followed by rheumatoid arthritis (RA), inflammatory bowel disease, SLE, and multiple sclerosis (MS) [6]. The definite etiologies of AIDs are unclear but may have association with genetic predisposition containing both monogenic and multiple genetic factors and environmental factors like nutrition, hormone level, diet, pathogens, drugs, insufficiency of vitamin D, and toxins [2, 7–9].

The pathogenesis of AIDs is not clear, but according to current study, the breakage of immune tolerance demonstrated when B or T lymphocytes fail to distinguish self from nonself with involvement of autoantibodies and/or self-reactive T lymphocytes is related to AIDs [2, 10]. The explanatory mechanisms to autoreactive B or T cells can be proposed as "molecular mimicry," the most common mechanism, which is when the sequence of pathogen-derived peptides is similar with self-peptides, which causes cross-reactivity of antigen receptors and results in autoimmune response; "epitope spreading," caused by virus infection, which is the change from the primary epitope to other epitopes or the generation of multiple neoepitopes on antigen-presenting cells; "bystander activation" which means the activation of preexisting autoreactive immune cells; and "viral persistence and polyclonal activation," explained by

continuous existence of viral antigen prompting immune response or epitope spreading. Moreover, other factors involved in regulating innate and adaptive immunity, like autoantigens released by apoptosis, microbiota, and insufficient vitamin D, may also contribute to loss of tolerance. All these mechanisms finally progress to reactive B or T cells and cause loss of immune tolerance and organ-specific or systemic autoimmune diseases [2, 3].

Autoantibody-mediated tissue destruction is a common feature of AIDs, which can be used to diagnose and classify AIDs [11]. Autoantibodies play a pathogenic role in cytotoxic damage by attacking a cell's functional structures through cell surface binding and lysis, and during the process, the most common damage pathways are complement activation and antibody-dependent cell-mediated cytotoxicity [2, 12]. SLE, Sjogren's syndrome (SS), and autoimmune hepatitis (AIH) are examples of autoantibody-mediated AIDs. Antigen-antibody immune complex-mediated tissue damage is also a critical pathogenic mechanism, and AIDs of SLE, RA, and SS are the illustrations. In addition, the selective pathways can be activated or blocked by autoantibodies after binding to cell surface receptors, and the activated selective disease Graves' disease and blocked selective disease myasthenia gravis are the instances. Self-reactive T lymphocyte-mediated AIDs are caused by cytotoxic effects. After recognizing a target cell by matching the T cell receptor (TCR) to the major histocompatibility complex I (MHCI) and autoantigen-originated peptides, autoreactive cytotoxic T cells directly kill target cells by secreting cytotoxic granules, like perforin and granzyme B, or activating the Fas-Fas ligand to induce cell apoptosis, and release cytokines like anti-tumor necrosis factor alpha (TNFα) and interferon gamma (IFN-γ) to cause tissue injury [2, 12, 13].

The key to treat AIDs is to restore immune tolerance. Traditionally, the typical immune suppressors are the disease-modifying antirheumatic drugs (DMARDs), like methotrexate, mycophenolate mofetil, cyclosporine, and so on, which give a general suppression of the immune system, thus causing increased risks of serious infection, developing lymphoma and other malignancies, but cannot significantly inverse immune tolerance. Recently, new immunosuppressants called biologics targeting localized targets or pathways rather than the whole immune system have been developed, like belimumab and rituximab depleting B cells, abatacept suppressing T cell activation, anti-TNFα inhibitors targeting TNFα, tocilizumab blocking interleukin 6 (IL-6), and ustekinumab inhibiting IL-12. The biologic agents are a class of monoclonal antibodies or fusion proteins targeting the receptors expressed by B cells or T cells or the key cytokines that involve regulation of B or T cells' differentiation. There are several ways to treat AIDs by targeting B lymphocytes, like eliminating B cells which is the direct method to wipe out the production of pathogenic antibodies, impeding B cells' activation by binding the inhibitory receptors expressed by B cells, or neutralizing key cytokines that participate in B cell activation, differentiation, or maturation [14]. Nevertheless, B cell elimination is the most widely used strategy to treat a series of AIDs, like RA, SLE, MS, and vasculitis.

Biologics can lower the toxicity and side effects in contrast with DMARDs, being better for long-term treatment. Nevertheless, they cannot restore the immune tolerance permanently [2, 15], thus requiring continuous administration, which brings new challenges, like weakened immunization of humanized antibodies [10, 15, 16]. Therefore, a precisely targeted treatment strategy that can restore immune tolerance is urgently needed. Fortunately, with the advances in adoptive cellular therapy for cancer, the extended use reaches AIDs.

2. Introduction of CAR-T Cells

The concept of adoptive cellular therapy (ACT) was first introduced when T cells were administrated to treat tumors, which benefited from the ability of IL-2 to grow human T cells ex vivo, therefore leading to the production of tumor-specific cells in a large scale. ACT has the advantages of expanding antitumor T cells ex vivo, high selection affinity towards target antigen, and modulating the host tumor microenvironment to a relative optimum condition prior to receiving T cell treatment. Later on, further use of ACT made the T cells have the engineered specific antitumor specificity by introducing conventional $\alpha\beta$ TCR or synthetic constructs, chimeric antigen receptors (CARs), to recognize the antigen expressed by a tumor cell [17]. The structure of a TCR is more complex than a CAR. A TCR is composed of an $\alpha\beta$ heterodimer which binds to peptide MHC, CD3 subunits, and a coreceptor CD4 or CD8 while a CAR consists of a single-chain molecule containing a single-chain variable fragment (scFv), a hinge, intracellular signaling domains from CD3ζ, and a costimulatory molecule [18–20]. So according to the structure, TCRs have lower affinities for their ligands than CARs, and antigen recognition of TCRs is dependent on MHC [21], while antigen recognition of CARs is not restricted by MHC which allows CAR-T cell treatment to have wider use by targeting more antigens, like proteins, carbohydrates, or glycolipids [19, 22, 23]. Therefore, more attention has concentrated on synthetic receptor CARs.

Chimeric antigen receptor T (CAR-T) cells are T cells engineered to express specific CARs that can recognize antigens expressed by tumor cells, and after the binding of antigens to receptors, the tumor cells are eliminated. The CAR-T cells can proliferate and survive in vivo for several years, which is the prerequisite for the treatment effect that keeps remission and controls or delays the relapse or deterioration of diseases [24]. To improve the antitumor efficacy and reduce T cell activation-accompanied toxicity of CAR-T cells, CARs went through an update, and the disparity for the different generations was presented in the intracellular signaling domain, the costimulatory domain. The first-generation CARs only have the CD3ζ intracellular domain, the second generations have both CD3ζ and one of the two costimulatory domains CD28 or 4-1BB (CD137), and third generations have two of the costimulatory domains such as CD27, CD28, ICOS, 4-1BB (CD137), or OX40 (CD134) in addition to CD3ζ [25, 26]. Compared to the third generation, the fourth-generation CAR-T cells, also called TRUCK T cells, are the CAR-T cells having a

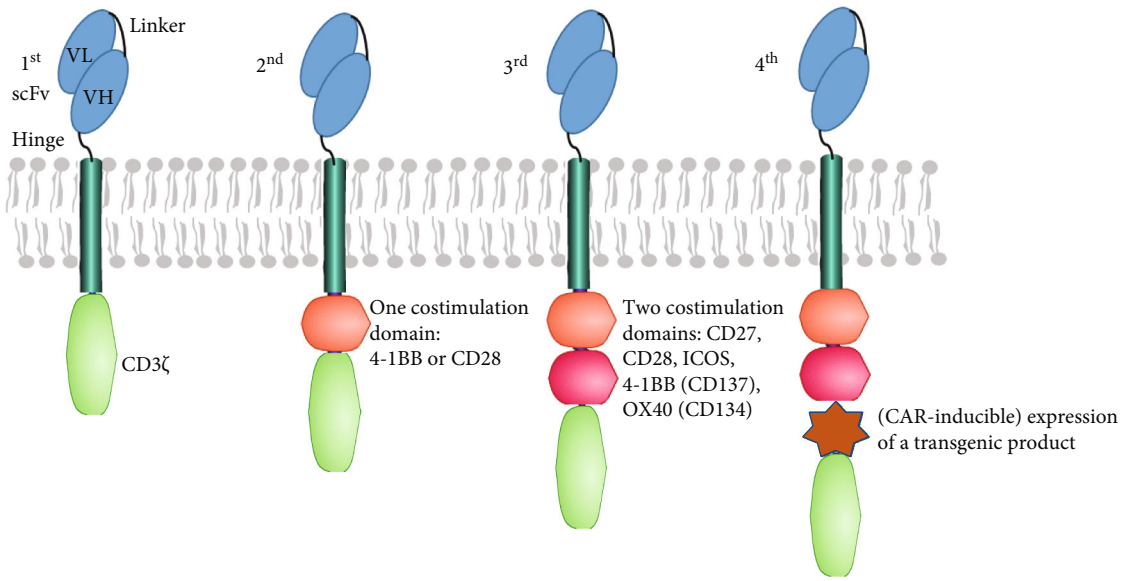

Figure 1: Generations of chimeric antigen receptors (CARs). CARs typically have an extracellular antigen recognition domain represented by an antibody-derived single-chain variable fragment (scFv) which contains a variable heavy (VH) chain and a variable light (VL) chain connected by a linker, a hinge, a transmembrane domain, with or without one or two costimulatory domains, and a CD3ζ. The fourth-generation CARs additionally have a "nuclear factor of activated T cell-responsive expression" element for an inducible transgenic product.

transgenic "payload" that is a "nuclear factor of activated T cell-responsive expression" element for an inducible transgenic product [27, 28] (Figure 1).

The application of CAR-T cells to treat B-lineage surface antigen CD19 is a huge forward step in cancer immunotherapy. The classic clinical use of CAR-T cells was to treat relapsed or refractory B cell acute lymphocyte leukemia (ALL), refractory B cell lymphoma, and non-Hodgkin lymphoma [29–32]. And now, the CAR-T cell is designed to have wider use that is to treat B cell malignancies beyond ALL, like chronic lymphocytic leukemia and multiple myeloma [24, 33, 34]. Also, CAR-T cells were used to target other hematological B cell, T cell, or myeloid malignancies; antigen molecular targets like BCMA, CD20, CD30, CD33, CD70, and CD123; and solid cancers like renal cell carcinoma (targeting CAIX), neuroblastoma (targeting L1-CAM or GD2), colon adenocarcinoma (targeting ErbB2), mesothelioma and pancreatic adenocarcinoma (targeting mesothelin) [17], sarcoma, antigen-targeted molecules like B7H3 [22, 25, 35, 36], and relapsed or refractory B cell precursor ALL [37].

Although CAR-T cell treatment has achieved huge success in hematologic malignancies, reaching high remission rate and response rate, treatment-related toxicity should be a concern, which is caused by high levels of inflammatory cytokines during the T cell activation and proliferation. Among the CAR-T cell treatment-related adverse events, cytokine release syndrome (CRS) is the most common one [38–41]; other adverse events like neutropenia, anemia, thrombocytopenia, and neurologic events are also common [29, 30].

3. CAR-T Cell-Derived Immunotherapy in AIDs

Application of CAR-T cells in AIDs to reach target treatment is promising [16, 42–44].

Similar to tumor treatment by targeting tumor-associated antigens expressed on the surface of tumor cells, CAR-T cells can be modified to treat AIDs by targeting specific autoantigens or antibodies expressed on the pathogenic cell surface. CAR-T cell-derived immunotherapy for AIDs can be classified as treatments of chimeric autoantibody receptor T (CAAR-T) cell and CAR-Treg based on a pathogenic mechanism. Several preclinical studies have been performed to investigate the application of CAR-T cells in AIDs (Table 1), and a few clinical trials are ongoing (Table 2).

CAAR-T cells are modified from CAR-T cells where chimeric autoantibody receptors are harbored by T cells instead of chimeric antigen receptors to target cells secreting antibodies, the autoreactive B cells [15], so the construction of a CAAR-T cell is composed of a specific antigen, a transmembrane domain, and intracellular signaling domains. After the specific antigen of the CAAR-T cells recognize and bind to the cognate autoantibodies expressed by the specific antibody producing B cells, the B cells will be eliminated. Using CAAR-T cells to treat antibody-mediated AIDs, two preconditions are needed [15]. One is that the sequence and molecular structure of the specific antigens are clear to guarantee the engineered key epitopes of the CAAR are correct to make sure the engineered epitopes can be recognized by cognate autoantibodies from patients. The other is the role of autoantibodies in the pathogenesis of a disease should be well investigated to make sure their pathogenicity.

Dsg3 CAAR-T cells were the human T cells engineered to express a CAAR that consisted of the pemphigus vulgaris (PV) autoantigen and desmoglein 3 (Dsg3), fused to CD137-CD3ζ signaling domains, and were effective for PV relief without any off-target toxicity, specifically eliminating Dsg3-specific B cells, thus obviously decreasing Dsg3 serum autoantibody titers [42]. Using Dsg3 CAAR-T cells to treat

Table 1: CAR-T cell-derived immunotherapy in preclinical models of AIDs.

CARs/generations	Construction of specific T cells or Tregs	Disease model	Specific autoantigen	Outcome	Reference
Dsg3 CAAR-T cells/2nd	Human T cells were engineered to express a CAAR that consisted of the PV autoantigen, Dsg3, fused to CD137-CD3ζ signaling domains	PV	Dsg3	Effective for PV relief, obviously decreased Dsg3 serum autoantibody titers	Ellebrecht et al. [42]
CD19-targeted CAR-T cells/2nd	CD8+ T cells were modified to express CD19-targeted CARs with CD28-CD3ζ signaling domains	Lupus	CD19	Eliminated autoantibody production, reversed disease phenotype, and prolonged survival time	Kansal and Richardson [45]
287-CAR-T cells/2nd or 3rd	CD8+ T cells were modified to express monoclonal antibody 287 CARs (287-CARs) harboring CD28-CD3ζ or CD28-CD137-CD3ζ signaling domains	T1D	I-A^{g7}-B:9-23 (R3) complex	Delayed but did not prevent the onset of T1D	Zhang et al. [49]
Insulin-specific CAR-Tregs/2nd	CD4+ T cells were transduced with retroviral particles encoding the CAR plasmid including Foxp3 to convert CD4+ T cells into Tregs	T1D	Insulin	Failed to prevent spontaneous diabetes	Tenspolde et al. [54]
MOG CAR-Tregs/2nd	CD4+ T cells transduced to Tregs expressing CARs with MOG, the FoxP3 gene, and CD28-CD3ζ signaling domains	MS	MOG	Inhibited EAE demonstrated as reduced cytokine expression and diminished disease symptoms	Fransson et al. [55]
TNP-TPCR Tregs	TNP-TPCR Tregs were isolated from transgenic mice expressing the TNP-specific chimeric receptor under the CD2 promoter	Colitis	TNP	Alleviated acute TNBS colitis	Elinav et al. [56]
CEA-specific CAR-Tregs/2nd	CD4+CD25+ Tregs were transduced with the CEA-specific SCA431 CAR that was fused to CD28-CD3ζ signaling domains	Colitis and colorectal cancer	CEA	Ameliorated colitis and prevented development of colitis-associated colorectal cancer	Blat et al. [57]

PV: pemphigus vulgaris; T1D: type I diabetes; Dsg3: desmoglein 3; MS: multiple sclerosis; MOG: myelin oligodendrocyte glycoprotein; EAE: encephalomyelitis; TPCR: tripartite chimeric receptor; TNP: 2,4,6-trinitrophenol; TNBS: 2,4,6-trinitrobenzenesulphonic acid; CEA: carcinoembryonic antigen.

Table 2: Clinical trials of CAR-T cell treatment for AIDs.

Intervention	Disease condition	Phase	Status	ClinicalTrials.gov identifier	Institute
Descartes-08 CAR-T cells	Generalized myasthenia gravis	I and II	Recruiting	NCT04146051	University of Miami
Anti-CD19 CAR-T cells	Systemic lupus erythematosus	I	Recruiting	NCT03030976	Shanghai Jiaotong University School of Medicine, Renji Hospital

PV is representative for applying CAR-T cells targeting antibody-mediated AIDs. Nevertheless, owing to short-term observations, the safety and efficacy are yet to be confirmed.

In murine lupus, CD8+ T cells were modified to express CD19-targeted CARs with CD28-CD3ζ signaling domains. A single use of CD19-targeted CAR-T cells was highly effective to treat lupus, manifested as complete and sustained CD19+ B cell depletion, terminated autoantibody production, reversed disease phenotype, and prolonged survival time, and the treatment effect was sustained for up to one year. Transferring splenic T cells from the mice after CD19+ B cell depletion by CAR-T cell treatment to lupus prone mice alleviated disease severity in adoptive autoimmune mice [45]. The persistence and function of CD19-targeted CAR-T cells were quite long in vivo after a single administration, reaching up to one year, and meanwhile, persistent B cell depletion was observed. This CD8+ T cell-originated CD19-targeted CAR-T cells targeted cell death in a direct mechanism way that depleted B cells effectively without the help from other cell types, which is superior to antibody-mediated cytotoxicity that requires a binding antibody for complement-dependent cell lysis. For instance, treatment of rituximab, an anti-CD20 antibody, requires repeated use to reach a therapeutic dose, with an insufficient required dose resulting in incomplete B cell depletion and failure treatment. Also, in this study, no obvious side effects were observed with the treatment of CD19-targeted CAR-T cells. But studies are required to further figure out the trait and function of plasma cell population and residual IgM^{lo} B cell in the murine lupus model. Therefore, CD19-targeted CAR-T cell treatment seems to be a new hope for SLE patients by targeting depletion antibody-producing B cells [46–48].

In the T1D NOD mouse model, I-A^{g7}-B:9-23 (R3) refers to a pathogenic complex wherein the MHC class II molecule I-A^{g7} in register 3 (R3) binds to the B:9-23 peptide which is a primary initiating epitope located between residues 9 and 23 of the insulin B chain, and a monoclonal antibody named mAb287 that can selectively bind to this complex was generated. 287-CAR-T cells were CD8+ T cells modified to target the I-A^{g7}-B:9-23 (R3) complexes, which could only delay the onset of T1D for about 6 weeks with a single infusion but could not prevent the disease development owing to short-time persistence of the transferred cells, having no detectable cells at 25 weeks of age [49]. This is the first study to demonstrate that CAR-T cells can be used to selectively target pathogenic T cell epitopes associated with autoimmunity presented by antigen presentation cells (APCs). The 287-CAR-T cells seemed unable to proliferate or survive in the spleen; however, they could home in on and expand in pancreatic lymph nodes where they possessed their cognate antigens as expressed by APCs. Also, those adoptively transferred 287-CAR-T cells could migrate to the target tissue, which are the inflamed islets. Application of 287-CAR-T cells did not show any adverse metabolic side effects and the gross change of immune cell populations and percentages.

Regulatory T cells (Tregs) also play a critical role in regulating the immune system by inhibiting the function of immune cells to keep immunologic self-tolerance and immune homeostasis, and an AID will occur when the specific transcription factor Forkhead box protein P3 (Foxp3) of Tregs is mutated or the CD4+CD25+ T cells are eliminated [50]. Therefore, applying Treg therapy in AIDs after being engineered to CAR-Tregs having antigen specificity may be a new choice [16, 21, 51, 52]. CAR-Tregs can induce antigen-specific cytolysis of the targeted cell in a granzyme B-dependent way, suppressing antigen-specific effector T cells' (Teffs) response, and releasing immunosuppressive cytokines, like transforming growth factor β1 (TGF-β1) and IL-10 [53]. CAR-Tregs are transduced from T cells and expanded ex vivo with normal expressing levels of Foxp3 to keep the expanding ability of reaching the therapeutic number, but transformation from CAR-Tregs to effector CAR-T cells in an inflammatory milieu is the major safety issue [44]. Nevertheless, CAR-Tregs can suppress Teffs by the following mechanisms: releasing immunosuppressive cytokines, such as IL-10, IL-35, and TGF-β; competing binding molecules CD80/CD86 on APCs with cytotoxic T lymphocyte antigen 4 expressed by CAR-Tregs to CD28 expressed by Teffs; and inducing apoptosis of Teffs through Fas-ligand or granzyme B/A and perforin produced by CAT-Tregs [52].

Insulin-specific CAR converted Tregs (CAR-cTregs), the first CAR-Tregs for T1D, are engineered CAR-Tregs where CD4+ T cells were transduced with retroviral particles encoding the second-generation CAR plasmid including Foxp3 to convert CD4+ T cells into Tregs. In the presence of insulin, proliferation of CAR-cTregs in vitro was normal and the suppressive capacity was similar to natural Tregs [54]. Although CAR-cTregs have a long existence in diabetic mice that they could be detected as long as at the 17th week after the adoptive transfer, they could not prevent spontaneous diabetes in NOD/Ltj female mice. The possible explanation for prevention failure of diabetes is that the soluble hexamer rather than the soluble monomer can activate CAR-T cells. As the specificity of CAR-cTregs is high with the antigen insulin, off-target effects are supposed to be small [54].

The engineered MOG CAR-Tregs are CD4+ T cells transduced to Tregs expressing CARs with myelin

oligodendrocyte glycoprotein (MOG), the FoxP3 gene, and CD28-CD3ζ signaling domains. MOG CAR-Tregs were effective to treat autoimmune encephalomyelitis (EAE), a model that mimics multiple sclerosis in humans after a single intranasal delivery and which homes in on various regions in the brain. The treatment reduced disease symptoms and decreased mRNA expressions of cytokines IFN-γ and IL-12. The EAE scores were instantly decreased upon intranasal administration, and the reduction of clinical disease symptoms was continuous, even becoming symptom-free on the 25th day. Intranasal delivery of CAR-Tregs was addressed when treating EAE as a cell numbers which would be decreased when homing in on the target tissue. So transplantation of cells into the brain through intranasal delivery can reduce cell dose and systemic exposure [55].

2,4,6-Trinitrophenol (TNP) tripartite chimeric receptor (TPCR) natural Tregs (TNP-TPCR Tregs) are natural Tregs isolated from transgenic mice expressing the TNP-specific chimeric receptor under the CD2 promoter with a maintained high Foxp3 level. TNP-TPCR Tregs could repeatedly expand when stimulated by cognate antigen ex vivo in a costimulation-independent and contact-relying way and were effective to alleviate acute 2,4,6-trinitrobenzenesulphonic acid- (TNBS-) induced colitis in a dose-dependent manner. After recovery from the first TNBS stimulation for three weeks, the second time TNBS colitis was inducted in TNP-TPCR Treg-treated mice showed 75% survival of mice, higher than the 33% survival in the wild-type Treg-treated group, indicating the development of persistent tolerance. After activation by dendritic cells preloaded with TNP, TNP-TPCR Tregs could inhibit proliferation of Teffs in a dose-dependent way [56].

Carcinoembryonic antigen- (CEA-) CAR-Tregs were the CD4+CD25+ Tregs transduced with the CEA-specific SCA431 CAR that was fused to CD28-CD3ζ signaling domains, and about 90% of the CAR-Tregs were FoxP3-positive cells. CEA-CAR-Tregs were effective for T cell transferred colitis relief and in inhibiting the development of the azoxymethane-dextran sodium sulfate- (AOM-DSS-) induced colitis-associated colorectal cancer [57]. CEA-CAR-Tregs could be homed in on and accumulated to the CEA-expressed sites, with the highest detection in the inflamed colon and to a much lesser extent in the small intestines and no detection in other visceral organs. The persistence of the CEA-CAR-Tregs was short, only accumulating and expanding in the colon for roughly 7 days and then quickly fading away in that it could not be detected at the 9th day after injection [57].

The clinical trial of NCT04146051 is a single-group nonrandomized study planning to enroll 18 participants to assess the safety and preliminary efficacy of CAR-T cells engineered from autologous T cells containing descartes-08 drug targeting B cell maturation antigen in patients with generalized myasthenia gravis. The trial has two phases, Ib and IIa. Phase Ib is a dose-escalation phase to measure the outcomes of the maximum tolerated dose with follow-up time for 28 days, and phase IIa is an expansion phase to observe the change of the daily living score during the 168-day follow-up period.

The clinical trial of NCT03030976 is a single-arm open-labeled nonrandomized study to assess the safety and efficacy of CD19-CAR-T cells engineered from autologous T cells with a second CAR containing 4-1BB as a costimulator in patients with CD19 positive B cell SLE. The trial is a phase I study intending to enroll 5 patients, and two days ahead of an initial infusion of (1-10) E6 CAR-positive T cells/kg, cyclophosphamide (0.5 g/m^2/d) is applied to reduce B cells. Assessment of safety is to report the number of adverse events, and efficacy is the overall response rates and the persistence of infused CAR-T cells in the circulation detected by quantitative PCR during the 6-week follow-up period.

4. Future Prospects

With advances in updating of constructing CARs and accumulating preclinical studies, application of CAR-T cell-derived immunotherapy in AIDs is feasible. Although preclinical studies have been performed, there is still a long way before we can apply CAR-T cells to clinical treatment in AIDs. Before clinical use, safety, effectiveness, persistence, and manufacture of CAR-T cells must be guaranteed. Using CAR-T cells to treat AIDs, CARs can be tailored according to specific antigens or antibodies in different AIDs, so CAR-T cells have unique specificity. Therefore, treatment of CAR-T cells theoretically does not cause side effects. Nevertheless, finding the specific antigens to construct antigen-specific CARs is not easy in some disease conditions. As CAR-T cells recognize cell surface molecules without the help of human leukocyte antigen expression, antigen recognition of CARs is not restricted by MHC, and CAR-T cell may recognize almost all types of antigens like carbohydrates, lipids, and proteins [27]. Expansion CAR-T cells in a large scale for clinical use may be challengeable, which can be solved by a cell culture platform [58, 59].

Exhaustion of CAR-T cells limits their functions in immunoregulation. As the costimulatory domains play a key regulatory role in determining functionality and persistence of CAR-T cells both in vitro and in vivo, costimulatory domains are the targets to improve the persistence. Although CARs with CD28 are related to enhanced expansion, persistence, and antitumor effect [60, 61], CARs with CD28 are not as good as CARs with 4-1BB (CD137) [62–64]. The persistence and antitumor effect of CARs with CD27 are similar to the CARs bearing CD28 or CD137 [65]. CD28-OX40 CARs can enhance specific cytolysis and improve antitumor response [66]. CARs with ICOS can further increase persistence and antitumor response in contrast with CD28- or CD137-alone CARs [67]. However, the suitable combinations of those costimulatory domains to reach the best persistence need more studies. In addition to modulating costimulatory domains in the first three-generation CARs to improve the persistence and expansion of CAR-T cells in vivo, the fourth-generation CARs are promising [28], as they have an inducible expression cassette for a transgenic protein, so those factors of IL-2 receptor β-chain, mRNA-encoding telomerase reverse transcriptase, or PI3K inhibitor should be considered when constructing CARs [68–70].

For the use of CAR-Tregs, attention should be paid to several issues. On the on hand, the immunosuppressive phenotype of Tregs will change after losing Foxp3 expression under an inflammatory microenvironment, from the immunosuppressive state to effector cells that aggravate disease symptoms [10]. To maintain the immune inhibitory phenotype of Tregs, several methods can be tried, like treating the Tregs with the vitamin A derivative all-trans retinoic acid that can sustain the stability and functionality of Tregs [71], administrating a Treg-favoring microbiota to the gut [72], and inducing ectopic expression of the Foxp3 gene to a stable regulatory phenotype of Tregs [73]. On the other hand, CD28 is the most critical costimulatory pathway to keep Tregs homeostasis plays a critical role in Tregs proliferation, differentiation, and survival and can upregulate IL-2 production and Foxp3 expression [74]. So CD28 should be adopted in CARs when constructing CAR-Tregs.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Anti-PLA2R1 Antibodies Containing Sera Induce In Vitro Cytotoxicity Mediated by Complement Activation

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The phospholipase A2 receptor (PLA2R1) is the major autoantigen in idiopathic membranous nephropathy (MN). However, the pathogenic role of anti-PLA2R1 autoantibodies is unclear. Our aim was to evaluate the in vitro cytotoxicity of anti-PLA2R1 antibodies mediated by complement. Forty-eight patients with PLA2R1-related MN from the prospective cohort SOURIS were included. Anti-PLA2R1 titer, epitope profile, and anti-PLA2R1 IgG subclasses were characterized by ELISA. Cell cytotoxicity was evaluated by immunofluorescence in HEK293 cells overexpressing PLA2R1 incubated with patient or healthy donor sera in the presence or absence of rabbit complement or complement inhibitors. Mean cytotoxicity of anti-PLA2R1 sera for HEK293 cells overexpressing PLA2R1 was $2 \pm 2\%$, which increased to $24 \pm 6\%$ after addition of rabbit complement ($p < 0.001$) ($n = 48$). GVB-EDTA, which inhibits all complement activation pathways, completely blocked cell cytotoxicity, whereas Mg-EGTA, which only inhibits the classical and lectin pathways, highly decreased suggesting a limited role of the alternative pathway. A higher diversity of IgG subclasses beyond IgG4 and high titer of total IgG anti-PLA2R1 were associated with increased cytotoxicity ($p = 0.01$ and $p = 0.03$ respectively). In a cohort of 37 patients treated with rituximab, high level of complement-mediated cytotoxicity was associated with less and delayed remission at month 6 after rituximab therapy (5/12 vs. 20/25 ($p = 0.03$) in 8.5 months $\pm$ 4.4 vs. 4.8 ± 4.0 ($p = 0.02$)). Kaplan-Meier analysis demonstrated that high level of cytotoxicity ($\geq$40%) ($p = 0.005$), epitope spreading (defined by immunization beyond the immunodominant CysR domain) ($p = 0.002$), and high titer of anti-PLA2R1 total IgG ($p = 0.01$) were factors of poor renal prognosis. Anti-PLA2R1 antibodies containing sera can induce in vitro cytotoxicity mediated by complement activation, and the level of cytotoxicity increases with the diversity and the titer of anti-PLA2R1 IgG subclasses. These patients with high level of complement-mediated cytotoxicity could benefit from adjuvant therapy using complement inhibitor associated with rituximab to induce earlier remission and less podocyte injury.

1. Introduction

Membranous nephropathy (MN) is an autoimmune disease and a major cause of nephrotic syndrome in adults [1]. It is defined by the presence of subepithelial immune complex deposits with alteration of the glomerular membrane and podocyte injury [2]. Most MN cases are associated with autoantibodies directed against podocyte antigens such as the M-type phospholipase A2 receptor (PLA2R1) [3] and thrombospondin type 1 domain-containing 7A (THSD7A) [4, 5] in 70% and 3% of adult patients, respectively. Disease evolution is highly variable from spontaneous remission to persistent proteinuria or end-stage renal disease [6]. Treatment remains controversial [7, 8]. Kidney Disease Improving Global Outcomes (KDIGO) guidelines recommend a supportive symptomatic treatment (Renine angiotensine system blockers and diuretics) in all patients and immunosuppressive therapy only in the case of persistent nephrotic syndrome or renal function deterioration [9]. Therefore, immunosuppressive treatments are often started only after significant and potentially irreversible complications. New KDIGO guidelines will probably modify this recommendation using new markers to start immunosuppressive therapy [10].

While the pathogenic role of anti-THSD7A antibodies has been proven by the formation of immune deposits and the onset of proteinuria in mice injected with human anti-THSD7A antibodies [11], no such study has been performed for PLA2R1 due to the absence of PLA2R1 expression in mouse or rat podocytes. Nevertheless, PLA2R1 antibody titers rise during clinical activity and decrease before remission [12], and MN recurrence after kidney graft is associated with high titers [13]. Anti-PLA2R1 titers could also predict outcome after immunosuppressive treatment in MN [14]. PLA2R1 epitopes have been identified in three domains of the protein (CysR, CTLD1, and CTLD7), and a mechanism of epitope spreading from the immunodominant CysR domain to CTLD1 and/or CTLD7 domains has been associated with poor prognosis [15–17] corresponding to later stages of the disease [18].

The complement system forms an important part of the innate immune system. It is involved in host defense, but also in autoimmune diseases, and is made up of over 30 proteins that can be sequentially activated in a complex enzymatic cascade. Three major pathways of complement have been described: classical, alternative, and lectin pathways, which are activated by different stimuli [19]. All three pathways converge on complement C3. Cleavage of C3 and C5 successively leads to the production of the membrane attack complex (C5b-9), which is the final common effector [20]. C5b-9 has been shown to have a major role in the Heymann nephritis rat model of MN [21, 22]. The role of the different complement pathways upstream of C5 activation in human and experimental MN remains largely unknown. Primary MN associated to PLA2R1 and THSD7A is characterized by predominant IgG4 (in deposits and in serum) and low amounts of IgG1 and IgG3 [23–25]. Immune complexes typically bind C1q and activate the classical pathway. However, IgG4 does not bind C1q and is considered to be unable to activate the classical pathway [26, 27]. The presence of IgG1 in early deposits could possibly activate the classical pathway, but C1q deposits are very weak in MN [28]. This suggests that the alternative or lectin pathways might be involved in complement activation. Hayashi et al. detected glomerular deposits of C4d, C3d, and C5b-9 in all patients while mannose-binding lectin (MBL) and C1q were detected in only 43% and 46% of patients, respectively [29]. One or more complement pathways may be activated after the formation of immune deposits and can vary among MN patients. Hayashi et al. also described that MN associated with glomerular MBL deposits is more severe. On the other hand, Bally et al. reported cases of PLA2R1-associated MN in patients having a complete MBL deficiency with complement activation mainly due to the alternative pathway [30].

The aim of this study was (i) to evaluate the pathogenic role of anti-PLA2R1 antibodies and the contribution of complement in a simple but routinely used *in vitro* cellular assay using HEK293 cells overexpressing PLA2R1, (ii) identify factors associated with complement mediated-cytotoxicity, and (iii) analyze prognosis value of this complement mediated-cytotoxicity. This study may provide the molecular basis to better understand MN pathogenesis and develop alternative therapeutic strategies.

2. Materials and Methods

2.1. Patients. Forty-eight patients with PLA2R1-associated MN from the prospective cohort SOURIS were included (NCT02199145) approved by our local Ethic committee. Patients from the SOURIS cohort are primary MN patients (defined by the absence of antinuclear antibodies, negative hepatitis B and C serologies, and negative cancer workup), adults, with MDRD > 30 ml/mn/1.73 m^2 and anti-PLA2R1 antibodies at diagnosis. The aim of the SOURIS cohort is to demonstrate that epitope profile at diagnosis could guide therapeutic strategy. Patients were enrolled from four French centers from 2015 to 2018 and had a median follow-up of 14 months. Baseline characteristics and follow-up data were recorded until 18 months. Serum anti-PLA2R1 levels, serum creatinine, serum albumin, and proteinuria were measured every 3 months for 18 months. Sera from 20 healthy donors, age- and gender-matched, were collected. Information and written consent were obtained from all patients.

Serum and morning spot urine samples were prospectively collected at the first infusion and every 3 months after the first rituximab infusions (i.e., month 3, month 6). Remissions were defined according to the 2012 KDIGO guidelines. Complete remission was defined by a urinary protein/creatinine ratio < 0.3 g/g accompanied by a normal serum albumin concentration and a preserved kidney function. Partial remission was defined by urinary protein/creatinine ratio < 3.5 g/g with over 50% reduction of proteinuria accompanied by an improvement or normalization of the serum albumin concentration and preserved kidney function.

2.2. Anti-PLA2R1 ELISA. The entire extracellular domain of human PLA2R1 or its CysR, CTLD1, and CTLD7 domains bearing a C-terminal HA tag were produced as secreted proteins as described previously [15]. Briefly, pcDNA3.1

expression vectors coding for each protein were transfected into HEK293 cells by calcium phosphate transfection. Three days after transfection, cell medium containing the secreted proteins was collected and centrifuged to remove cell debris. Anti-HA antibodies (Sigma-Aldrich) were coated for one night at 4°C. Plates were then washed three times with PBS/0.02% Tween 20, then blocked for two hours with Seramun Block (Seramun Diagnostica) and then washed again three times. Cell medium from HEK293 cells transfected with the soluble forms of the three PLA2R1 domains or the entire PLA2R1 tagged with HA was then added and incubated for one hour at room temperature on a plate shaker, then washed three times. Patients' sera were diluted at 1 : 100 in PBS/0.1% milk and added to the ELISA plates for two hours. After three washes, anti-human IgG4 (1 : 7500; Southern Biotech) or anti-human IgG1, IgG2, and IgG3, all conjugated with horseradish peroxidase (1 : 5000; Southern Biotech) and diluted in SeramunStab ST plus (Seramun Diagnostica), were incubated for one hour at room temperature on a plate shaker. After three washes, tetramethylbenzidine was added and the reaction was stopped with HCl 1.2 N after 15 minutes. Plates were read at 450 nm. A negative control as well as a highly positive index patient serum was used in each plate to generate a standard curve. The cut-off was optimized by receiver operating characteristic (ROC) curve analysis using 20 healthy controls.

Epitope spreading was defined by anti-CysR reactivity with additional anti-CTLD1 and/or anti-CTLD7 activities.

2.3. Measurement of Anti-PLA2R1 Antibodies by ELISA. Serum levels of total IgG anti-PLA2R1 antibodies were measured by the ELISA test developed by EUROIMMUN AG (Lübeck, Germany) [31]. Participants were considered as anti-PLA2R1-positive when levels were higher than 14 RU/ml.

2.4. Immunofluorescence Cytotoxicity Assay and Complement Activation Pathway. HEK293 cells overexpressing PLA2R1 with a Tetracycline-Regulated Expression System (PLA2R1/-HEK293 T-REx) were obtained as follows. Full-length membrane-bound human PLA2R1 bearing a C-terminal cytoplasmic HA tag was inserted into the pcDNA4/TO expression vector (InvitroGen) and transfected into HEK293 T-REx cells (cultured in DMEM, 10% fetal calf serum with blasticidin 5 μg/ml) using the calcium-phosphate procedure (InvitroGen). One day after transfection, stably expressing cells were selected with zeocine (0.8 mg/ml) for 3 weeks and PLA2R1 expression was induced with tetracycline (1 μg/ml) and validated by western blot as described [15] for 48 hours. Cells were collected by gentle dissociation with trypsin, centrifuged for 3 minutes at 600 rpm, and diluted in OptiMEM to obtain a concentration of 3×10^6 cells/ml.

Cell cytotoxicity was measured as described by Terasaki and McClelland [32]. Three microliters of serum from anti-PLA2R1-positive patients or healthy donors were incubated with 3×10^3 of either induced or noninduced PLA2R1/-HEK293 T-REx cells (added in 1 μl) for 1 hour at room temperature in 60-well Terasaki plates (Dutcher©, Strasbourg, France) in duplicates. Cytotoxicity was measured with serial serum dilutions (1 : 1, 1 : 3, and 1 : 10). Serum dilutions were associated to decreased (dilution 1 : 3) or negative (dilution 1 : 10) cytotoxicity (Figure Sup 1A). Standard rabbit complement (5 μl/well, Cedarlane©, Ontario, Canada) or OptiMEM was added and incubated for 1 hour at room temperature. Dead cells were revealed after adding 2.5 μl/well of Fluoroquench AO/EB staining/quench (Ingen©Chilly-Mazarin, France) for 10 minutes in the dark. The percentage of dead cells was estimated using a fluorescent microscope (Videomicroscope Zeiss LSM780). Two investigators read plates blindly with a good correlation (rs = 0.81, $p < 0.0001$) and an excellent concordance (ICC = 0.89 (0.85; 0.92)) (Figure sup 1B and 1C).

Various versions of gelatin veronal buffer (GVB) were used to determine the complement pathway involved in anti-PLA2R1 cytotoxicity. GVB supplemented with EDTA (GVB-EDTA) (ComplementTech©) was used to inhibit all complement pathways activation, whereas GVB supplemented with magnesium and EGTA (Mg-EGTA) (ComplementTech©) was used to inhibit the classical and lectin pathways, but not the alternative one. Three microliters of three PLA2R1-positive sera were diluted in 5 μl of GVB supplemented with GVB-EDTA or Mg-EGTA and 5 μl of standard rabbit complement. Cytotoxicity was assessed as described above. We confirmed in these conditions the inhibition of the classical pathway activity by measuring CH50 (Total Haemolytic Complement Kits Binding Site©) (Figure Sup 2).

All cytotoxicity assays were performed using the same batch of HEK293 T-Rex cells. A minimum of 50 cells per well was necessary for reading.

2.5. Western Blot Analysis. The expression of PLA2R1 in HEK293 cells overexpressing PLA2R1 with a Tetracycline-Regulated Expression System (PLA2R1/HEK293 T-REx) after induction with tetracycline was analyzed by SDS-PAGE under nonreducing conditions. A control without induction with tetracycline was performed. Total proteins (10-50 μg/well) were run on 4-15% precast TGX SDS-PAGE gels (Bio-Rad) and transferred to methanol-soaked PVDF membranes (Bio-Rad) under semidry conditions using Trans-blot Turbo (Bio-Rad) at 25 V constant for 12 min. Membranes were blocked overnight at 4°C in 5% milk with PBS-Tween (PBS-T) 0.05% and then incubated with primary and secondary antibodies for 2 h at room temperature. Primary antibodies were diluted with 0.5% dry milk in PBS-T. Membranes were prepared in replicates and probed with a serum of patient with anti-PLA2R1 antibodies diluted at 1 : 100. Secondary antibody for iMN sera was HRP-conjugated mouse anti-human IgG (Southern Biotech #9200-05) diluted 1 : 30,000 in PBS-T. Membranes were washed three times for 5 min in PBS-T after incubation with primary and secondary antibodies. Detection of protein bands was performed with a chemiluminescent substrate (Millipore) and a Fuji LAS3000 imager.

2.6. Statistical Analyses. Data are presented as mean ± SD (for variables with normal distribution) or median and

Table 1: Clinical characteristics of patients in the study.

Clinical characteristics	Cohort SOURIS (48 patients)
Age (years)	59 ± 15
Gender ratio (M/F)	3.3
LOCF (months)	14 (3; 18)
Proteinuria at baseline (g/g)	5.1 (3.5; 7.2)
Serum albumin at baseline (g/l)	23.9 (21.2; 29.7)
Serum creatinine at baseline (μmol/l)	103 (84.7; 135)
Proteinuria at LOCF (g/g)	1.9 (0.3; 3.4)
Patients treated with rituximab	37
Patients treated with other immunosuppressors (Ponticelli)	4

Data are presented as mean ± SD or as median and interquartile range. LOCF: last observation carried forward.

interquartile range (for variables with nonnormal distribution). We used the Shapiro-Wilk test to determine if a variable has a Gaussian distribution. Wilcoxon-Mann–Whitney test and Kruskal–Wallis test were used for comparison between groups. ROC curve analysis was used to define the threshold of each test. Survival curves for remission were calculated using Kaplan–Meier estimates for survival distribution. Correlation between the two readers was assessed by Spearman rank correlation coefficient. Concordance between the two readers was assessed by intraclass correlation coefficient (ICC should be over 0.80 for an excellent concordance). Adjusted analysis was performed using logistic regression. All statistics were performed using the Prism and SAS software. p values < 0.05 were considered as statistically significant.

3. Results

3.1. Patients and Anti-PLA2R1 Antibodies. A total of 48 PLA2R1-positive patients were enrolled in this study (Table 1). Thirty-six patients were included for a first course of MN and 12 patients for a relapse. All patients received conservative therapy with angiotensin-converting-enzyme (ACE) inhibitors and/or angiotensin II receptor blockers. During follow-up, 37 patients were treated with rituximab 1 g at 2-week interval. All patients were positive for the CysR domain of PLA2R1 at baseline, while 30 out of 48 patients (62.5%) had additional antibodies targeting CTLD1 and/or CTLD7 domains (epitope spreading) (Figure 1(a)). At last observation carried forward, epitope spreading was only identified in 6 out of 37 (16.2%) patients. At baseline, all patients had IgG4 anti-PLA2R1, while 24 patients (50%) also had IgG1, IgG2, or IgG3 autoantibodies. Anti-PLA2R1 IgG3 antibodies were the second most represented subclass with 22 (44%) positive patients (Figure 1(b)). Moreover, IgG1, IgG2, IgG3, and IgG4 anti-PLA2R1 antibodies were found in most samples at the first course of the disease, while at relapse, IgG4 anti-PLA2R1 was the predominant subclass ($p = 0.02$) (Figure 1(c)).

3.2. Cytotoxicity of Anti-PLA2R1 Autoantibodies Is Dependent on Complement Activation. We choose to test complement-mediated cytotoxicity with a simple, validated, and routinely used test in renal transplantation (the cross-match test), using HEK293 cells overexpressing PLA2R1 with a Tetracycline-Regulated Expression System (instead of lymphocytes in cross-match test).

Addition of anti-PLA2R1 serum and rabbit complement to HEK293 cells overexpressing PLA2R1 after induction with tetracycline (PLA2R1/HEK293 T-REx$^+$) led to strong cytotoxicity, while all other conditions were barely cytotoxic (Figures 2(a) and 2(b)). In PLA2R1/HEK293 T-REx$^+$, the mean cytotoxicity without complement was 2 ± 2% while it increased to 24 ± 6% with complement ($p < 0.001$). In HEK293 cells not induced with tetracycline (PLA2R1/-HEK293 T-REx$^-$), the mean cytotoxicity was 7 ± 2% and 2 ± 1% with and without complement, respectively. Addition of healthy donor serum was barely cytotoxic, no matter the conditions. The decreased cell viability of PLA2R1/HEK293 T-REx$^-$ in the presence of complement was probably caused by a minimal expression of PLA2R1 even in noninduced cells, as determined by western blot (Figure 2(c)). Using ROC curve analysis, we identified a threshold > 10% of cytotoxicity associated with a positive test with a sensitivity of 87.5% and specificity of 84.6% (AUC = 0.90 (0.83 to 0.98) $p < 0.0001$) (Figure Sup 3).

3.3. Analysis of the Complement Activation Pathways Involved in Cytotoxicity Mediated by Anti-PLA2R1 Autoantibodies. The complement activation pathways involved in anti-PLA2R1 cytotoxicity were studied with three anti-PLA2R1 serum samples showing a robust level of cytotoxicity (Figure 2(d)). The first two patients were positive for both IgG3 and IgG4 anti-PLA2R1 antibodies, while the third patient was only positive for IgG4. For the first patient, the addition of GVB-EDTA totally inhibited anti-PLA2R1-mediated cytotoxicity (from 65 to 0%) as addition of Mg-EGTA (from 65 to 10%). In the presence of Mg-EGTA, we confirmed a complete blockade of the classical pathway (CH50 < 10%, Figure Sup 2), suggesting that the alternative pathway mediates cytotoxicity for the first patient. For the second patient, the addition of GVB-EDTA strongly inhibited anti-PLA2R1-mediated cytotoxicity (from 50 to 10%), while the addition of Mg-EGTA only decreased anti-PLA2R1-mediated cytotoxicity to lower but still detectable levels (from 50% to 25%). For the third patient, the addition of GVB-EDTA strongly inhibited anti-PLA2R1-mediated cytotoxicity (from 40 to 10%) whereas the addition of Mg-EGTA only inhibited anti-PLA2R1-mediated cytotoxicity by 2-fold (from 40 to 20%) (Figure 2(a)).

In conclusion, GVB-EDTA, which is known to inhibit the three pathways, strongly inhibited anti-PLA2R1-mediated cytotoxicity in all three patients, which confirm the role of the complement, while Mg-EGTA, which is known to inhibit only the classical and the lectin pathways, partially inhibited the anti-PLA2R1-mediated cytotoxicity, suggesting a potential limited activation of the alternative pathway in some serum samples ($p = 0.007$) (Figure 2(e)).

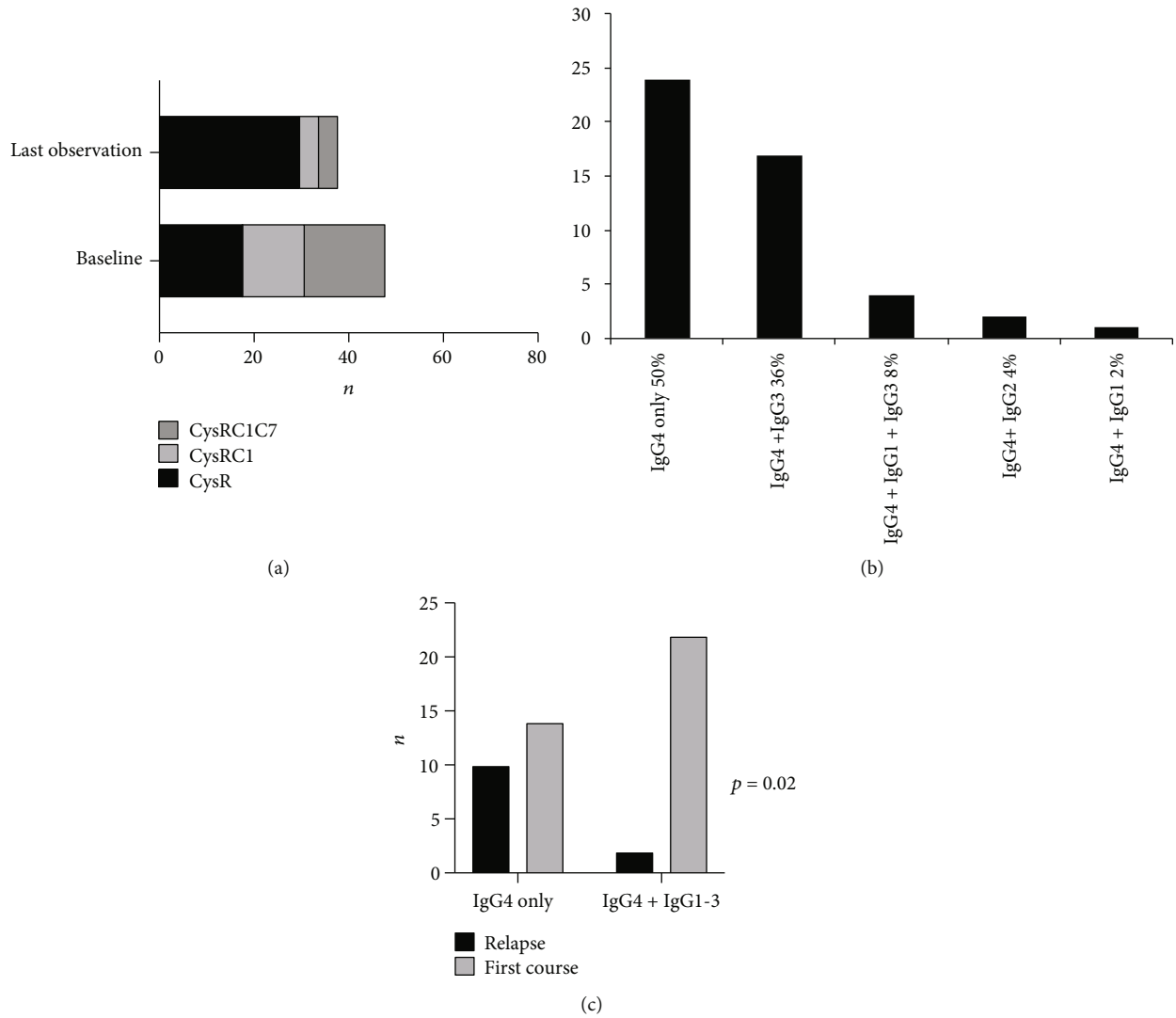
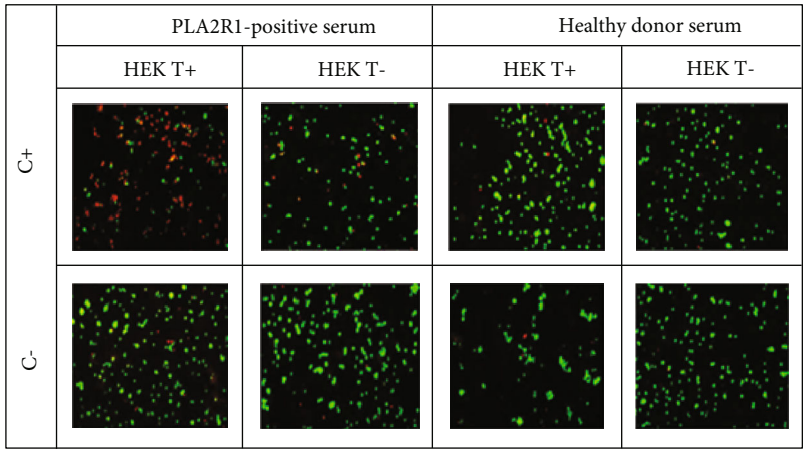


Figure 1: Description of samples with anti-PLA2R1 antibodies tested. (a) Epitope spreading profiles of patients at baseline ($n = 48$) and at last observation ($n = 36$). Follow-up was missing for 12 patients. C1: CTLD1; C7: CTLD7. CysR: immunized against CysR domain alone; CysRC1: immunized against CysR and CTLD1 domains; CysRC1C7: immunized against CysR, CTLD1, and CTLD7 domains. (b) IgG subclass profiles of patients at baseline ($n = 48$). (c) IgG subclass profiles of patients according to disease course: first course or relapse ($n = 48$). Note that patients with the first course of MN have a larger diversity of IgG subclasses ($p = 0.02$).

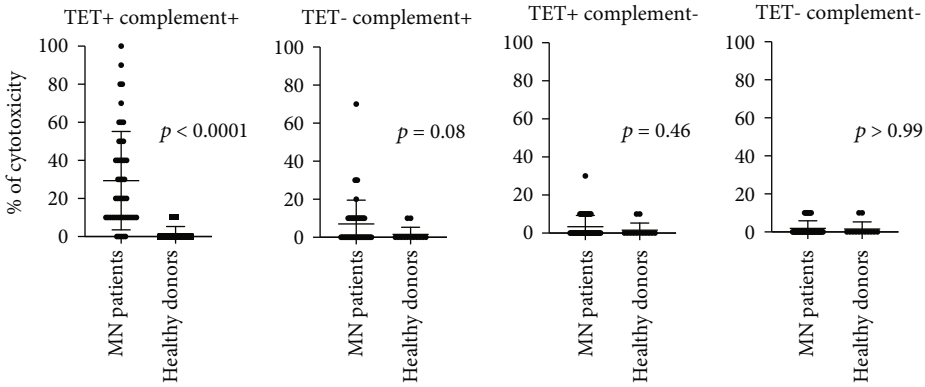
Because Mg-EGTA induces inhibition of more than half of the complement-mediated cytotoxicity, the activation of the lectin or the classical pathway seems to be predominant.

3.4. Factors Associated with Cytotoxicity Induced by Anti-PLA2R1 Autoantibodies and Mediated by Complement. We analyzed the factors that may be associated with cytotoxicity (>10%) among the different serum samples from the 48 patients. Epitope spreading as well as high IgG4 anti-PLA2R1 titers was not associated with decreased cell viability ($p = 0.72$ and $p = 0.50$, respectively) (Figures 3(a) and 3(b)) while high level of total anti-PLA2R1 total IgG titer was associated with cytotoxicity ($p = 0.03$, Figure 3(c)); moreover, these two variables were correlated (rs = 0.45, $p < 0.001$) (Figure 3(d)). As suggested, the presence of anti-PLA2R1 IgG1, IgG2, or IgG3 antibodies was associated with increased cell cytotoxicity ($p = 0.03$) (Figure 3(e)). Median complement-mediated cytotoxicity was significantly higher for serum samples containing different IgG subclasses versus those containing only anti-PLA2R1 IgG4 antibodies (30% (10; 40) versus 10% (0; 30), $p = 0.02$). There was an inverse relationship for complement-mediated cytotoxicity between serum samples containing multiple IgG subclasses and those containing only IgG4, suggesting that IgG subclasses other than IgG4 drive the observed cytotoxic effects ($p = 0.015$) (Figure 3(f)). We failed to identify a correlation between titers of each subclass of IgG and cytotoxicity (Figure Sup 4) suggesting that it is the whole of subclasses that is responsible for the cytotoxic effect.

3.5. Prognostic Factors for Remission. During follow-up, 37 patients were treated with rituximab 1 g at 2-week interval, 25 entered into remission at month 6 (67.6%). Time to remission after treatment was 6.1 ± 4.4 months.



(a)



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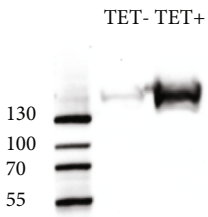
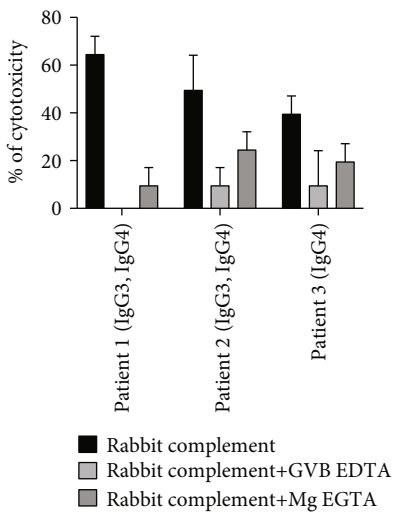
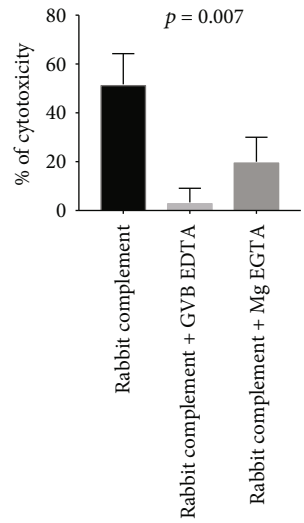


(c)



(d)

Figure 2: Continued.



(e)

Figure 2: Anti-PLA2R1-mediated cytotoxicity depends on complement in immunofluorescence cytotoxicity assay. (a) Anti-PLA2R1-mediated cytotoxicity depending on complement for a PLA2R1-positive serum and a healthy donor serum. HEK T+: HEK293T-REx cells transfected with PLA2R1 and induced with tetracycline to overexpress PLA2R1. HEK T-: HEK293T-REx cells transfected with PLA2R1 but not induced with tetracycline. C+: addition of rabbit complement. C-: no addition of rabbit complement. Dead cells appear in red; living cells appear in green. (b) Anti-PLA2R1 complement-mediated cytotoxicity in a cohort of 48 patients and 20 healthy donors in different conditions. Note a low level of cytotoxicity in noninduced HEK that tended to be significant ($p = 0.08$) caused by a minimal expression of PLA2R1 even in noninduced cells, as determined by western blot (c). All samples were tested using the same batch of HEK293 cells. A minimum of 50 cells per well was necessary for reading. (c) Expression of PLA2R1 in noninduced and induced HEK293 cells with tetracycline. Note a minimal expression of PLA2R1 in noninduced cells. (d) Analysis of complement activation pathways involved in anti-PLA2R1-mediated cytotoxicity for 3 PLA2R1-positive patients using two inhibitors of complement pathways. Patients 1 and 2 were positive for both IgG3 and IgG4 anti-PLA2R1 antibodies, while patient 3 was only positive for IgG4 anti-PLA2R1. An excess of GVB-EDTA (inhibitor of the three pathways of the complement) or Mg-EGTA (inhibitor of the classical and lectin pathways) was added in serum + complement and the complement-mediated cytotoxicity was measured. All samples were tested using the same batch of HEK293 cells. A minimum of 50 cells per well was necessary for reading. (e) Analysis of complement activation pathways involved in anti-PLA2R1-mediated cytotoxicity for 3 PLA2R1-positive patients. Note that GVB-EDTA strongly inhibits anti-PLA2R1-mediated cytotoxicity in all 3 patients, while Mg-EGTA, which is known to inhibit only the classical and lectin pathways, inhibits only partially the anti-PLA2R1-mediated cytotoxicity, suggesting a potential activation of the alternative pathway in some serum samples. A minimum of 50 cells per well was necessary for reading. HEK293 T-REx cells transfected with PLA2R1 and induced with tetracycline to express PLA2R1 were used. Negative controls (noninduced HEK T-REx cells) are not shown. All cytotoxicity assays were performed using the same batch of HEK293 T-Rex cells. A minimum of 50 cells per well was necessary for reading.

We compared clinical characteristics of patients with the highest level of cytotoxicity (the third tertile defined by a threshold of 40% or more of complement-mediated cytotoxicity) (Table 2). Patients with complement-mediated cytotoxicity ≥ 40% were similar at diagnosis for age and sex ratio but have the highest level of proteinuria (6.65 (5.75; 9.86) vs. 4.90 (3.23; 7.68), $p = 0.03$) and total IgG anti-PLA2R1 titer at diagnosis (192 (120; 686) vs. 94 (33; 199), $p = 0.01$) while IgG4 anti-PLA2R1 titer and level of epitope spreading were similar (1840 (322; 3990) vs. 1338 (220; 3461), $p = 0.62$, and 9/12 (75%) vs. 13/25 (52%), $p = 0.08$, respectively). After treatment with rituximab, fewer patients with high level of complement-mediated cytotoxicity entered into remission (5/12 (42%) vs. 20/25 (80%), $p = 0.03$) in longer time (8.5 months ± 4.4 vs. 4.8 ± 4.0, $p = 0.02$) with higher proteinuria at month 6 (3.10 (2.51; 5.60) vs. 1.21 (0.30; 2.32), $p = 0.001$).

We then analyzed factors associated with remission at month 6 (Table 3) (using univariate analysis): high anti-PLA2R1 total IgG titer, epitope spreading, and high level of cytotoxicity (≥40%) were associated with active disease at month 6 after 2 pulses of rituximab (185.0 (147.0; 893.0) vs. 73.0 (39.5; 209.0) ($p = 0.01$), 10/12 (83%) vs. 11/25 (44%) ($p = 0.04$), and 7/12 (58%) vs. 5/25 (20%) ($p = 0.03$), respectively). As all these factors were correlated (Figure 1(c)and Figure Sup 5), multivariate analysis failed to identify an independent prognosis factor. Because each remission occurred at different time points, we performed a time-to-event analysis of renal survival. Renal event was defined by achieving remission within the year after the first course of rituximab. The rate of remission was significantly lower for patients with high level of cytotoxicity (≥40%) ($p = 0.005$, Figure 4(a)), with epitope spreading ($p = 0.002$, Figure 4(b)), and high titer of total IgG anti-PLA2R1 ($p = 0.01$, Figure 4(c)).

Patients with high level of cytotoxicity (≥40%) had six fold decreased chance to achieve remission (Odds ratio = 6.33 (1.39; 28.69)) with a mean time to achieve remission of 8.5 months ± 4.4 months vs. 4.8 months ± 4.0 months ($p = 0.02$).

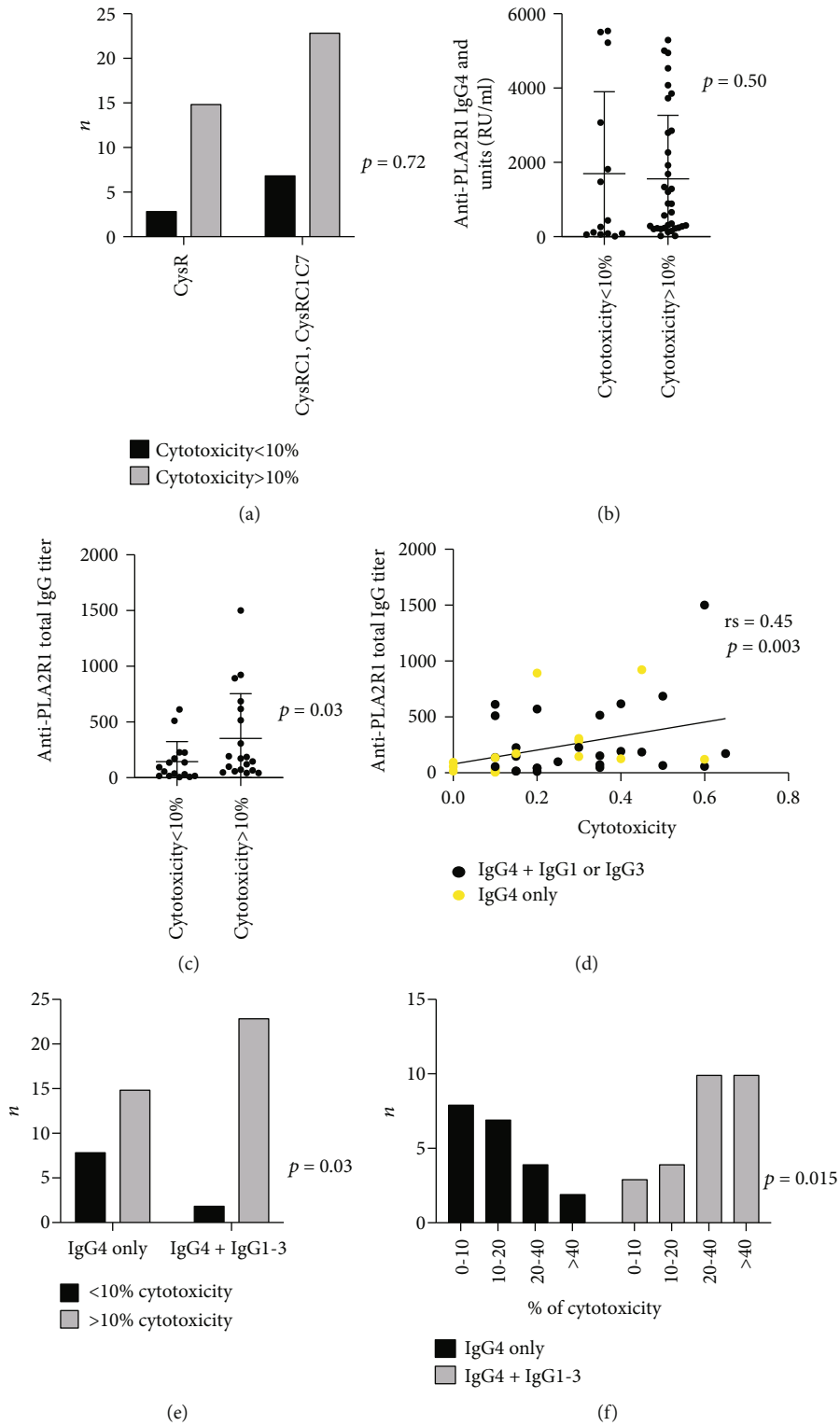


Figure 3: Predictive factors of anti-PLA2R1-mediated cytotoxicity in immunofluorescence cytotoxicity assay. (a) Relationship between anti-PLA2R1-induced cytotoxicity mediated by complement and epitope spreading profile at baseline ($n = 48$). CysR: immunized against CysR domain alone; CysRC1: immunized against CysR and CTLD1 domains; CysRC1C7: immunized against CysR, CTLD1, and CTLD7domains. (b) Relationship between anti-PLA2R1-induced cytotoxicity mediated by complement and Anti-PLA2R1 IgG4 (RU/ml) titers at baseline ($n = 48$). (c) Relationship between anti-PLA2R1-induced cytotoxicity mediated by complement and Anti-PLA2R1 total IgG titer at baseline ($n = 48$). (d) Correlation between Anti-PLA2R1 total IgG titer and complement-mediated cytotoxicity ($n = 48$). (e) Anti-PLA2R1-induced cytotoxicity mediated by complement according to anti-PLA2R1 IgG subclasses at baseline ($n = 48$). (f) Anti-PLA2R1-induced cytotoxicity mediated by complement regrouped by levels of cytotoxicity according to anti-PLA2R1 IgG subclasses at baseline ($n = 48$). Note that patients with high level of cytotoxicity (≥40%) are predominantly IgG4+IgG1-IgG3.

Table 2: Factors associated with cytotoxicity ≥ 40% ($n = 37$ patients treated with rituximab).

	Cytotoxicity ≥ 40% ($n = 12$)	Cytotoxicity < 40% ($n = 25$)	p value
Age	60 ± 11	57 ± 16	0.46
Sex ratio (M/F)	10/2	19/6	>0.99
Proteinuria at diagnosis (g/g)	6.65 (5.75; 9.86)	4.90 (3.26; 7.68)	0.03*
Anti-PLA2R1 total IgG (RU/ml) at diagnosis	192 (120; 686)	94 (33; 199)	0.01*
Anti-PLA2R1 IgG4 (RU/ml) at diagnosis	1840 (322; 3990)	1338 (220–3461)	0.62
Spreaders at diagnosis	10/12 (83%)	12/25 (48%)	0.07
Proteinuria at month 6 (g/g)	3.70 (2.51; 5.60)	1.21 (0.30; 2.32)	0.001*
Remission at month 6	5/12 (42%)	20/25 (80%)	0.03*
Time to remission (months)	8.5 ± 4.4	4.8 ± 4.0	0.02*

Data are presented as n (%) or mean ± SD or as median with interquartile range. Remission is defined as proteinuria < 3.5 g/g and serum albumin > 30 g/l. Spreaders are defined by samples with anti-CysR reactivity with additional anti-CTLD1 and/or anti-CTLD7 activities additional to anti-CysR reactivity.

Table 3: Factors associated with remission at month 6 in patients treated with rituximab ($n = 37$).

	Remission at month 6	No remission at month 6	p value
Age	55 ± 15	64 ± 14	0.12
Sex ratio (M/F)	2.3	11	0.21
Proteinuria at diagnosis (g/g)	4.9 (3.3; 10.4)	7.2 (4.6; 11.4)	0.08
Anti-PLA2R1 total IgG (RU/ml) at diagnosis	73.0 (39.5; 209.0)	185.0 (147.0; 893.0)	0.01*
Anti-PLA2R1 IgG4 (RU/ml) at diagnosis	885.0 (220.5; 2669.0)	2824.0 (420.5; 5538.0)	0.12
Spreaders at diagnosis	11/25 (44%)	10/12 (83%)	0.04*
Complement-mediated cytotoxicity ≥ 40%	5/25 (20%)	7/12 (58%)	0.03*

Data are presented as n (%) or mean ± SD or as median with interquartile range. Remission is defined as proteinuria < 3.5 g/g and serum albumin > 30 g/l. Spreaders are defined by samples with anti-CysR reactivity with additional anti-CTLD1 and/or anti-CTLD7 activities additional to anti-CysR reactivity.

4. Discussion

Our results demonstrate that anti-PLA2R1 antibodies induce in vitro cytotoxicity mediated by complement activation as suggested by Kistler et al. who analyzed TRPC6 protective role from complement-mediated podocyte injury [33]. Samples with high titer of anti-PLA2R1 total IgG have higher level of complement-mediated cytotoxicity, and these patients had exhibited a bad prognosis. In a previous work, we used human podocyte line in our standard conditions of western blot (using 10-50 μg/ml of total protein) to determine the approximate expression of PLA2R1 in human podocytes and observed a signal using positive control MN sample with PLA2R1 antibodies [13] as we observed in similar conditions using HEK293 cells overexpressing PLA2R1. This suggested that the level of PLA2R1 expression in HEK 293 cells overexpressing PLA2R1 is approximately comparable with the level of PLA2R1 expression in human podocyte.

We demonstrate that the level of cytotoxicity correlates with total IgG anti-PLA2R1 titer and not with IgG4 anti-PLA2R1. Patients with high level of cytotoxicity have more active disease at diagnosis, lower chance of remission after rituximab therapy, and longer time to enter into remission. Complement inhibitor (GVB-EDTA) strongly inhibited this cytotoxicity. If our results are confirmed in other studies, MN patients with high level of anti-PLA2R1 antibodies and complement-mediated cytotoxicity could benefit from adjuvant therapy using complement inhibitor associated with rituximab, which could probably induce less podocyte injury and earlier remissions.

There is a large body of evidence that anti-PLA2R1 IgG4 is predominant in MN [31, 34]. All patients were positive for IgG4 anti-PLA2R1. However, in addition to anti-PLA2R1 IgG4 antibodies, we observed in some patients the presence of IgG1, IgG2, or IgG3 anti-PLA2R1, as previously described [35, 36]. Moreover, IgG4 was the predominant subclass in relapsing MN. This result is in accordance with the IgG subclass switch from IgG1, IgG2, and IgG3 to IgG4 observed in renal biopsies during disease progression by Huang et al. [25]. A similar IgG subclass switch was observed in other IgG4-mediated autoimmune diseases such as pemphigus vulgaris and idiopathic thrombotic thrombocytopenic purpura [25, 37, 38].

The exact contribution of each of the three complement pathways in MN remains unknown. The lectin pathway may play an important role, since the prevalence and staining intensity of mannose-binding lectin (MBL) deposits were much higher in PLA2R1-positive patients than in patients without MBL deposits [29]. The staining intensity of MBL in glomeruli also correlated with the IgG4 staining intensity and was an unfavorable predictor for remission of proteinuria and renal dysfunction. It is unclear how the lectin pathway (that is known to be an antibody-independent pathway) is involved in MN (that is mediated by anti-PLA2R1

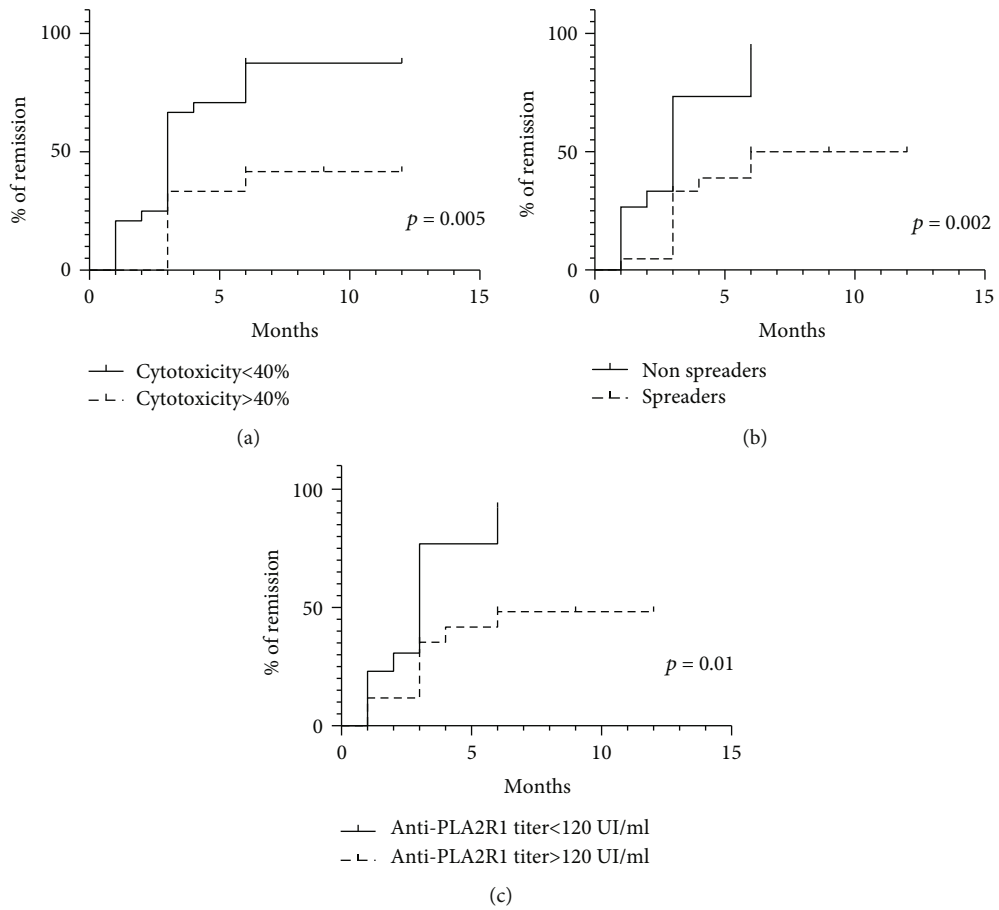


Figure 4: Factors associated with remission. Renal event is defined by remission (partial or complete) one year after diagnosis of MN. (a) Percent of patients achieving remission according to the level of complement-mediated cytotoxicity ($n = 37$). (b) Percent of patients achieving remission according to epitope spreading profile ($n = 37$). (c) Percent of patients achieving remission according to the level of total IgG anti-PLA2R1 ($n = 37$).

antibodies). Some authors hypothesize that abnormalities of galactosylation of IgG anti-PLA2R1 could activate the lectin pathway [29]. We demonstrated that inhibition of the classical and lectin pathways by Mg-EGTA inhibited the majority of complement-mediated cytotoxicity (completely for one patient and partially for two other patients). This partial inhibition would suggest a limited role of the alternative pathway in anti-PLA2R1-mediated cytotoxicity, which is still active in the presence of Mg-EGTA. Classical or lectin pathways are probably the predominant pathways implicated in MN. Bally et al. reported that PLA2R1-associated MN could develop in patients with IgG3 kappa anti-PLA2R1 antibodies having a complete MBL deficiency but capable of residual complement activation mainly due to the alternative pathway [30]. Moreover, Seikrit et al. described a case of rapidly progressive renal failure in a patient with membranous nephropathy, related to the appearance of antibodies against the complement regulatory protein, factor H. Inhibition of factor H led to hyperactivation of the alternative complement pathway [39]. This mechanism could explain the alternative pathway activation in some MN patients.

We described that during the first course of MN, several serum samples contained anti-PLA2R1 IgGs not restricted to the IgG4 subclass and these samples could induce more cytotoxicity than those containing only anti-PLA2R1 IgG4 autoantibodies. Thus, we suggest that in the presence of several anti-PLA2R1 IgG subclasses, several complement pathways may be activated, and their contribution will depend on the respective antibody titers and ratios between the different IgG subclasses. Segawa et al. also demonstrated that the activation of the different complement pathways varied according to the IgG subclasses presented in MN biopsy specimens [40]. It would be interesting to test which complement pathway can be activated by each individual IgG subclass of anti-PLA2R1 antibodies.

In different forms of human MN and animal models mediated by other antigens, each pathway of complement seems to be involved. Vivarelli et al. demonstrated a predominant role of the classical pathway for neutral endopeptidase protein-associated MN [41]. In a mouse model of MN, Luo et al. showed a role of the alternative pathway in the pathogenicity induced by glomerular subepithelial immune complexes [42]. However, no C3 deposition has been found in the renal tissue of mice following immunization with rabbit anti-THSD7A antibodies or purified human anti-THSD7A while these antibodies induced proteinuria and IgG deposits.

These results suggest that complement activation is not vital in the initiation of podocyte injury and proteinuria in this model [43, 44].

Based on our data, we can hypothesize a multistep mechanism of anti-PLA2R1 cytotoxicity: at disease onset, sera containing multiple IgG subclasses (including IgG4) induce cytotoxicity mediated by various complement pathways, then anti-PLA2R1 IgG4 which becomes the predominant subclass leads to the inhibition of PLA2R1 interaction with collagen from the glomerular basement membrane [45–47] at this time, complement-mediated cytotoxicity is not the main pathogenic mechanism. A similar scenario has been described in other autoimmune diseases mediated by IgG4, like in idiopathic thrombotic thrombocytopenic purpura, where an IgG4 subclass switching is associated with increased inhibition of ADAMTS13 enzymatic activity by anti-ADAMTS13 IgG4 antibodies [38]. These findings should now be confirmed in vitro with a podocyte model but will be more difficult to confirm in *in vivo* animal models due to the lack of expression of PLA2R1 in the mouse kidney [48, 49].

Our results suggest a potential benefit of the use of eculizumab (a monoclonal anti-C5) in membranous nephropathy. As we demonstrated, all samples were not equivalent in complement mediated-cytotoxicity. This treatment could be beneficial in only a part of MN patients with a large diversity of IgG subclasses, high level anti-PLA2R1 total IgG, and high level of complement-mediated cytotoxicity.

This study has several limitations. First, we could not demonstrate the role of complement in all sera, probably because of the lack of sensitivity of our system but we choose a simple, validated, and routinely used test as performed in cross-match test before kidney transplantation. We cannot exclude other mechanisms of cytotoxicity not mediated by complement. Secondly, we indirectly demonstrated a limited role of the alternative pathway with inhibition assays. Third, we did not study the lectin pathway that would probably play a role in MN, which is an IgG4-related disease [50].

In conclusion, we have made several observations likely relevant to the mechanism of pathogenesis in MN. First, our in vitro data showed that anti-PLA2R1 antibodies induce complement-mediated cytotoxicity. Second, this complement-mediated cytotoxicity is associated with high level of anti-PLA2R1 total IgG and more severe disease. Third, classical and/or lectin pathways are predominant. However, the role of each individual anti-PLA2R1 IgG subclass relative to each of the three complement pathways needs to be addressed in further studies, with a possible sequential scenario where the IgG subclass switch would also be associated with a switch of the complement activation pathways, from the classical and alternative pathways to the MBL pathway, as both the autoimmune response and the disease activity progress. Finally, if our results are confirmed, our findings would suggest that MN patients could benefit not only from targeted therapy on B cells but also from a therapy using complement inhibitors and eventually combining the two therapeutic strategies towards a more effective treatment for some MN patients with high level of cytotoxicity to reduce podocyte injury.

Abbreviations

MN: Membranous nephropathy
PLA2R1: M-type phospholipase A2 receptor
THSD7A: Thrombospondin type 1 domain-containing 7A
KDIGO: Kidney Disease Improving Global Outcomes
MBL: Mannose-binding lectin.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request (after ethics approval).

Ethical Approval

The local ethics committee approved the study protocol (NCT02199145).

Consent

Information and written consent were obtained from all patients.

Disclosure

Gérard Lambeau and Barbara Seitz-Polski are co-last authors. An abstract of this study have been presented during the 56th ERA-EDTA congress.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

B.S.P. and M.L. participated in the research design, in the analysis of the data, and in the drafting and revising of the article. M.L. and C.P. performed the assays under the supervision of G.L. and B.S.P. B.S.P., H.O., T.C., M.Z., S.B.S., T.C., C.C., N.J.C., B.K., and V.E. collected the clinical data. V.B. and G.D. produce HEK cells. All authors revised and gave final approval of the last version of this article. All the authors have validated the manuscript for publication.

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Supplementary Materials

Figure Sup 1A: serum dilutions were associated to decreased (dilution 1 : 3) or negative (dilution 1 : 10) cytotoxicity. Standard rabbit complement (5 μl/well, Cedarlane©, Ontario, Canada) or OptiMEM was added and incubated for 1 hour at room temperature. Dead cells were revealed after adding 2.5 μl/well of Fluoroquench AO/EB staining/quench (Ingen©Chilly-Mazarin, France) for 10 minutes in the dark. The percentage of dead cells was estimated using a fluorescent microscope (Videomicroscope Zeiss LSM780). Figure Sup 1B and 1C: two investigators read plates blindly with a good correlation (rs = 0.81, $p < 0.0001$) (Figure 1(b)) and an excellent concordance (ICC = 0.89 (0.85; 0.92)) (Figure 1(c)). Figure Sup 2: various versions of gelatin veronal buffer (GVB) were used to determine the complement pathway involved in anti-PLA2R1 cytotoxicity. GVB supplemented with EDTA (GVB-EDTA) (ComplementTech©) was used to inhibit all complement activation pathways, whereas GVB supplemented with magnesium and EGTA (Mg-EGTA) (ComplementTech©) was used to inhibit the classical and lectin pathways, but not the alternative one. Three microliters of three PLA2R1-positive sera were diluted in 5 μl of GVB supplemented with GVB-EDTA or Mg-EGTA and 5 μl of standard rabbit complement. Cytotoxicity was assessed as described above. We confirmed in these conditions the inhibition of the classical pathway activity by measuring CH50 (Total Haemolytic Complement Kits Binding Site©). All cytotoxicity assays were performed using the same batch of HEK293 T-Rex cells. A minimum of 50 cells per well was necessary for reading. Figure Sup 3: ROC curve of cytotoxicity between MN patients and healthy donors. Using ROC curve analysis, we identified a threshold > 10% of cytotoxicity associated with a positive test with a sensitivity at 87.5% and specificity at 84.6% (AUC = 0.90 (0.83 to 0.98), $p < 0.0001$). Figure Sup 4: correlation between anti-PLA2R11 titer and epitope spreading. *(Supplementary Materials)*

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Research Article

Amiselimod (MT-1303), a Novel Sphingosine 1-Phosphate Receptor-1 Modulator, Potently Inhibits the Progression of Lupus Nephritis in Two Murine SLE Models

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Amiselimod (MT-1303) is a novel and selective sphingosine 1-phosphate receptor-1 ($S1P_1$) modulator with a more favorable cardiac safety profile than other $S1P_1$ receptor modulators. In this study, we evaluated the effects of MT-1303 on the progression of lupus nephritis in two well-known murine systemic lupus erythematosus (SLE) models, MRL/*lpr* and NZBWF1 mice, compared with those of FK506. Daily oral doses of 0.1 and 0.3 mg/kg MT-1303 not only inhibited the development of lupus nephritis when administered before onset in MRL/*lpr* and NZBWF1 mice but also improved symptoms of lupus nephritis when administered after onset in MRL/*lpr* mice. Its efficacy in these models was more potent or comparable to that of FK506 (1 and 3 mg/kg). In histological analysis, treatment with MT-1303 inhibited infiltration of T cells into the kidneys, mesangial expansion, and glomerular sclerosis. MT-1303 treatment resulted in a marked reduction in T cells and B cells in the peripheral blood and significantly inhibited increases in the number of plasma cells in the spleen and T cells in the kidneys. In addition, administration of MT-1303 suppressed elevations in serum anti-dsDNA antibody levels in MRL/*lpr* mice, but not in NZBWF1 mice. Our findings show that MT-1303 exhibits marked therapeutic effects on lupus nephritis in two SLE models, likely by reducing the infiltration of autoreactive T cells into the kidneys. These results suggest that MT-1303 has the potential to be used as a therapeutic agent for patients suffering from SLE, including lupus nephritis.

1. Introduction

Amiselimod (MT-1303) is an oral selective sphingosine 1-phosphate receptor-1 ($S1P_1$) modulator [1] that is currently being developed for the treatment of various autoimmune diseases. A phase I study demonstrated that MT-1303 has a more favorable cardiac safety profile than fingolimod, the first $S1P_1$ receptor modulator approved for the treatment of relapsing-remitting multiple sclerosis (RRMS) [2]. A phase II study that enrolled more than 400 patients with RRMS reported that MT-1303 at doses up to 0.4 mg had superior efficacy over the placebo control as well as a benign safety profile [3]. MT-1303 is converted to its active metabolite, MT-1303 phosphate (MT-1303-P), and acts as a functional antagonist of the $S1P_1$ receptor. MT-1303-P induces $S1P_1$ receptor internalization in lymphocytes, inhibits lymphocyte egress from secondary lymphoid organs by reducing the S1P responsiveness of lymphocytes, and consequently exerts immunomodulatory activity by markedly reducing the number of circulating lymphocytes [1].

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by the production of a wide variety of autoantibodies and immune complex-mediated tissue inflammation [4–7]. Patients with SLE are susceptible to organ damage accrual caused by both active disease and medication toxicities [8, 9]. Development of an effective treatment with an acceptable safety profile, particularly one with high disease activity, and corticosteroid reduction is warranted [10, 11]. Belimumab, which inhibits the B cell activating factor (BAFF), a key survival cytokine for B cells, is

currently the only approved biological agent for SLE [12]. However, the efficacy of drugs targeting B cells is limited, and other approaches, including those targeting T cells, are required to improve treatment options for SLE patients [13, 14].

$S1P_1$ receptor modulators suppress infiltration of autoreactive T cells into sites of inflammation by inhibiting lymphocyte egress from secondary lymphoid organs [15] and have proven their therapeutic potential in RRMS and ulcerative colitis [3, 16, 17]. In addition, fingolimod reportedly reduces the production of high-affinity, class-switched antibodies by reducing the formation of the germinal center in the T cell-dependent antibody formation system in mice [18]. Therefore, $S1P_1$ receptor modulators are expected to have therapeutic potential against SLE by inhibiting infiltration of autoreactive T cells into sites of inflammation and by affecting autoantibody production. In fact, studies have reported that $S1P_1$ receptor modulators are efficacious in reducing proteinuria and improving kidney histology in MRL/*lpr*, NZBWF1, and BXSB mouse models of SLE [19–23].

In the present study, we evaluated the prophylactic and therapeutic effects of MT-1303 on lupus nephritis progression in MRL/*lpr* and NZBWF1 mice compared with FK506. MRL/*lpr* and NZBWF1 mice, well-known animal models of SLE, develop lupus nephritis spontaneously [24, 25]. In addition, we investigated the effects of MT-1303 on infiltration of T cells into the kidneys and autoantibody production in these mice.

2. Materials and Methods

2.1. Mice. Male MRL/*lpr* mice and female NZBWF1 mice were purchased from the Shizuoka Laboratory Animal Center (Shizuoka, Japan). Three to five mice were housed per plastic cage under specific pathogen-free conditions. They were kept at a constant temperature of 23 ± 3°C and relative humidity of 30–70% under a 12 h light/dark cycle. Food and water were available *ad libitum*. All animal experiments were performed using experimental protocols approved by the ethics review committee for animal experimentation of the Research Division of Mitsubishi Tanabe Pharma. All experimental procedures were as humane as possible.

2.2. Agents and Antibodies. Amiselimod (MT-1303; 2-amino-2-{2-[4-(heptyloxy)-3-(trifluoromethyl)phenyl] ethyl}propan-1,3-diol hydrochloride) was provided by Mitsubishi Tanabe Pharma (Osaka, Japan) and was dissolved in 0.5% hydroxypropylmethyl cellulose (HPMC) solution for oral administration. FK506 (Prograf Injection) was purchased from Astellas Pharma (Tokyo, Japan) and diluted with distilled water. The monoclonal antibodies (mAbs) used were obtained from BD Biosciences (Franklin Lakes, NJ, USA) or eBioscience (San Diego, CA, USA) and comprised the following: anti-CD3 mAb (clones 145-2C11 and 555273), anti-CD4 mAb (GK1.5), anti-CD8 mAb (53-6.7), anti-CD45R/B220 mAb (RA3-6B2), anti-CD38 mAb (90), and anti-CD138 mAb (281-2).

2.3. Study Design

2.3.1. MRL/lpr Study. To evaluate the prophylactic effect, MRL/*lpr* mice at 8 weeks of age without proteinuria (score of 0 or 1) were divided into 4 groups ($n = 12$ each) using the simulation method so that each group had equal mean and variance of body weight and proteinuria score. MT-1303 (0.1, 0.3, or 1 mg/kg) or vehicle was orally administered to the mice daily for 18 weeks, and the effects on the development of lupus nephritis, splenomegaly, and lymphadenopathy were evaluated. Additionally, MT-1303 (0.1 or 0.3 mg/kg) or vehicle was administered to MRL/*lpr* mice at 8 weeks of age for 12 weeks ($n = 12$ each), and levels of anti-dsDNA Ab in serum ($n = 12$) and plasma cell counts in the spleen ($n = 4$) were determined. To evaluate the therapeutic effect, MRL/*lpr* mice at 14 or 16 weeks of age with a proteinuria score of 2 were pooled, randomized by body weight, and divided into 5 groups ($n = 16$ each). MT-1303 (0.1 or 0.3 mg/kg), FK506 (1 or 3 mg/kg), or vehicle was administered for 6 weeks, and the effect on the progression of lupus nephritis was assessed.

2.3.2. NZBWF1 Study. NZBWF1 mice at 30 weeks of age without proteinuria (score of 0 or 1) were divided into 5 groups ($n = 12$ each). MT-1303 (0.1 or 0.3 mg/kg), FK506 (1 or 0.3 mg/kg), or vehicle was administered for 10 weeks, and the effects on the development of lupus nephritis were evaluated. Histological evaluation of the kidneys was conducted in half of the mice in each group ($n = 6$ each) except for the FK506 3 mg/kg group after the final administration. Additionally, MT-1303 (0.3 mg/kg) or vehicle was administered to NZBWF1 mice at 28 weeks of age for 13 weeks, and infiltration of lymphocytes into the kidneys, plasma cell counts in the spleen, and levels of anti-dsDNA Ab in serum were determined ($n = 8$ each).

2.4. Evaluation of Lupus Nephritis. The severity of renal disease was monitored by measuring proteinuria. Proteinuria was assessed once a week using Ames urinalysis strips (Albustix®; Siemens Healthcare Diagnostics, Tarrytown, NY, USA) and scored on a scale of 0–4 based on urinary protein concentrations, as follows: 0, <30 mg/dL; 1, 30–100 mg/dL; 2, 100–300 mg/dL; 3, 300–1000 mg/dL; and 4, >1000 mg/dL. In the NZBWF1 study, for more precise and objective evaluation, urinary protein concentrations were measured with a microplate spectrophotometer (SpectraMax 190; Molecular Devices, Sunnyvale, CA, USA) using the Coomassie Plus (Bradford) Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and scored on a scale of 0–4. A proteinuria score of 2 and above was defined as positive for proteinuria. Animals that were euthanized or died due to lupus nephritis progression were assigned a subsequent urinary protein concentration of 10 mg/mL, the maximum value.

2.5. Histology and Immunohistochemical Staining. Kidney tissue was fixed in 10% buffered formalin, embedded in paraffin, cut into 3 μm thick sections and stained with hematoxylin and eosin (HE) and periodic acid-Schiff (PAS). Kidney sections were evaluated and scored for multiple morphological features of lupus nephritis (mesangial expansion,

glomerular sclerosis, interstitial infiltrates, and perivascular infiltrates) by an independent pathologist. Kidney sections were scored using a 4-point scale: 0, no change; 1, mild; 2, moderate; and 3, severe, and the average score was calculated per animal per group.

For immunohistochemical staining, kidney tissue was fixed in zinc fixative, embedded in paraffin, and cut into 3 μm thick sections. The sections were incubated with rat anti-mouse CD3 mAb (555273) followed by secondary mAb conjugated to amino acid polymer and peroxidase (Histofine®, Nichirei Biosciences, Tokyo, Japan). The colorimetric reaction was performed using diaminobenzidine in the presence of hydrogen peroxide, and sections were counterstained with hematoxylin.

2.6. Lymphocyte Analyses. Using the IMMUNOPREP™ Reagent System (Beckman Coulter, Brea, CA, USA), 0.1 mL of peripheral blood was hemolyzed and fixed. The number of lymphocytes, T cells, B cells, CD3/B220 double-positive T cells, CD4 T cells, and CD8 T cells was measured using a Cytomics™ FC500 flow cytometer (Beckman Coulter) with Flow-Count™ (Beckman Coulter) as the internal standard. Single-cell suspensions were prepared from the spleen. Red blood cells were lysed, cell suspensions were reconstituted in phosphate-buffered saline, and the number of plasma cells ($CD138^{+}B220^{low}$) was measured using a flow cytometer [26]. Kidneys were minced and digested for 30 min at 37°C with 400 U/mL collagenase IV (Worthington Biochemical, Lakewood, NJ, USA). The kidney cell suspensions were subjected to a Lympholyte M gradient (Cedarlane, Burlington, Ontario, Canada) and spun at 2300 rpm for 20 min. The number of lymphocytes, T cells, B cells, CD4 T cells, CD8 T cells, $CD3^{+}CD4^{-}CD8^{-}$, and double-negative (DN) T cells was measured.

2.7. Anti-dsDNA Antibody. Serum levels of the anti-dsDNA antibody were measured using a mouse anti-dsDNA ELISA kit (Shibayagi, Gunma, Japan).

2.8. Statistical Analyses. The results are expressed as the mean ± S.E.M. Statistical differences were analyzed using Student's *t*-test, Williams test, or Shirley-Williams test. Statistical differences in proteinuria incidence were calculated using Fisher's exact test with Hommel's multiple comparison test by comparing with the control. Differences between groups were considered significant at $p < 0.05$.

3. Results

3.1. MT-1303 Strongly Inhibits the Development of Lupus Nephritis in MRL/lpr Mice. To determine the effects of MT-1303 on the development of lupus nephritis, MRL/*lpr* mice at 8 weeks of age were administered vehicle or MT-1303 (oral doses of 0.1, 0.3, and 1 mg/kg) daily for 18 weeks. The time course of changes in proteinuria scores are shown in Figure 1(a). In the vehicle-treated control group, mean proteinuria scores increased with time from 13 weeks of age, and all mice developed lupus nephritis at 21 weeks of age. In contrast, proteinuria scores in all MT-1303-treated groups were significantly lower than those in the control group, and no mice treated with MT-1303 at doses greater than 0.3 mg/kg developed lupus nephritis during the study period. Immunohistochemical staining with anti-CD3 mAb revealed that T cells had infiltrated the kidneys of vehicle-treated control mice at 26 weeks, particularly the periglomeruli area (Figure 1(b)). However, administration of MT-1303 markedly reduced this infiltration of T cells into the kidneys (Figure 1(c)). Moreover, in all MT-1303-treated groups, the weight of the axillary lymph nodes and spleen was significantly lower than that in the control group on the day after the final administration (Figures 1(d) and 1(e)), indicating that MT-1303 also inhibited the development of lymphadenopathy and splenomegaly. To confirm the effects of MT-1303, the number of peripheral blood lymphocytes was measured the day after the final administration (Figures 1(f) and 1(h)). In all MT-1303-treated groups, the number of T cells, CD4 T cells, B cells, and MRL/*lpr*-mouse-specific abnormal T cells was markedly lower than that in the control group.

3.2. MT-1303 Suppresses the Production of Anti-dsDNA IgG and Plasma Cells. MT-1303 at 0.1 or 0.3 mg/kg was administered to 8-week-old MRL/*lpr* mice for 10 weeks, and serum levels of anti-dsDNA IgG and the number of plasma cells were determined. Treatment with MT-1303 dose-dependently suppressed the increase in levels of anti-dsDNA IgG, with levels in the 0.3 mg/kg MT-1303 group decreasing significantly to around 40% of that in the control group (Figure 2(a)). The number of plasma cells in the spleen was dose-dependently and significantly lower than that in the control group (Figure 2(b)).

3.3. MT-1303 Has Therapeutic Effects on Lupus Nephritis in MRL/lpr Mice. Next, we directly compared the therapeutic effects of MT-1303 and FK506 on lupus nephritis in MRL/*lpr* mice. MT-1303 (0.1 and 0.3 mg/kg) or FK506 (1 and 3 mg/kg) was orally administered to MRL/*lpr* mice with established lupus nephritis for 6 weeks. The time course of changes in proteinuria scores and incidence of proteinuria are shown in Figures 3(a) and 3(b) and 3(c) and 3(d), respectively. In the control group, the mean proteinuria score gradually increased, confirming the progression of lupus nephritis. In contrast, from 2 weeks after the initiation of administration, mean proteinuria scores in all MT-1303-treated groups were significantly lower than those in the control group. In addition, the incidence of lupus nephritis, which was 100% at the initiation of administration, decreased to 56.3% and 37.5% at 6 weeks after administration of 0.1 and 0.3 mg/kg MT-1303, respectively. Additionally, treatment with FK506 significantly inhibited the progression of lupus nephritis, and the therapeutic efficacy of MT-1303 was comparable with that of FK506. Histological analysis of kidney sections showed glomerular lesion, proliferation of mesangial cells, and infiltration of T cells in the control group, while the severity of these lesions was reduced following treatment with MT-1303 (Figures 3(e)–3(h)).

3.4. MT-1303 Prevents Progression of Lupus Nephritis in NZBWF1 Mice. Thirty-week-old female NZBWF1 mice, divided into 5 groups according to their proteinuria scores

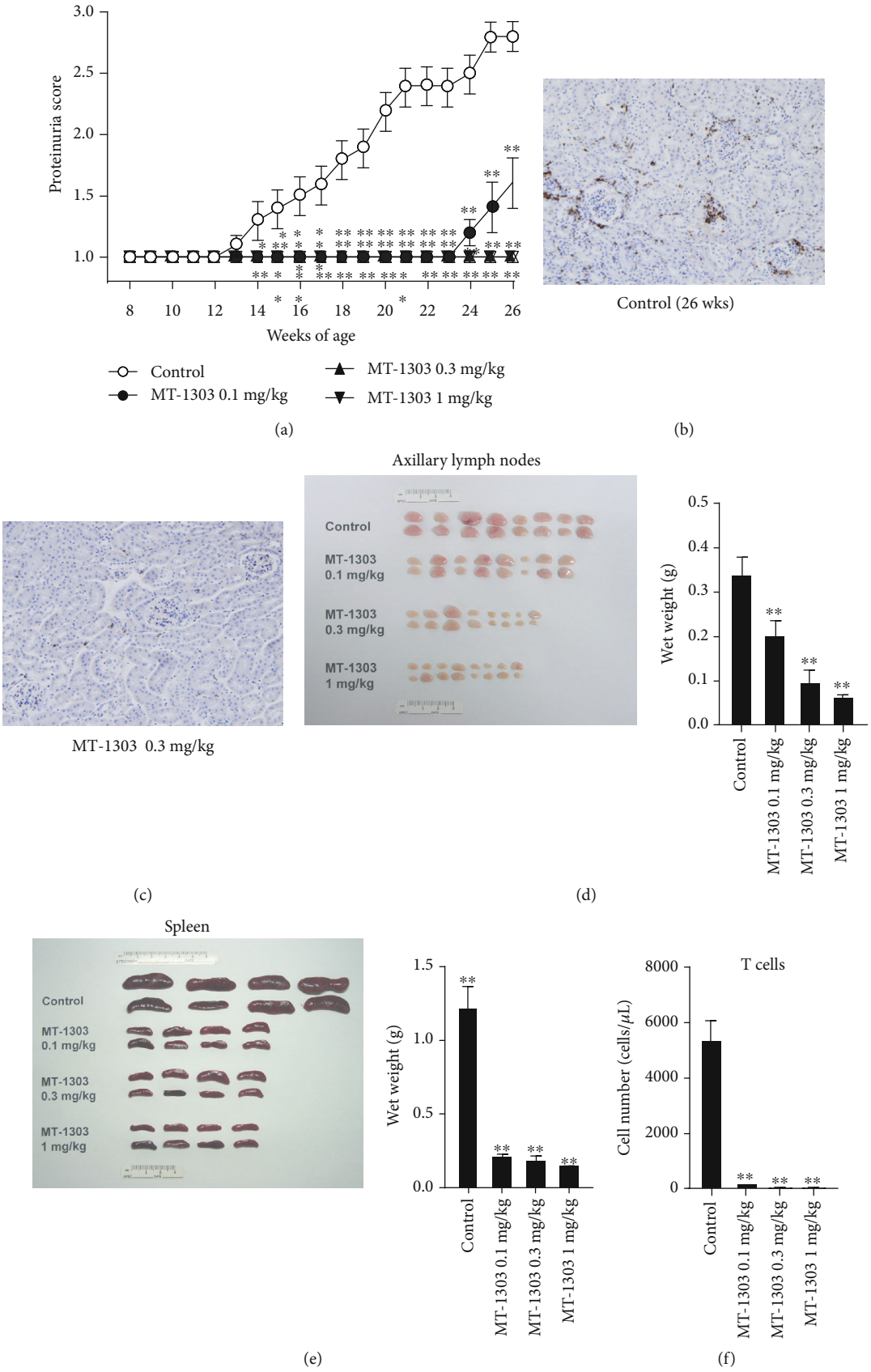

Figure 1: Continued.

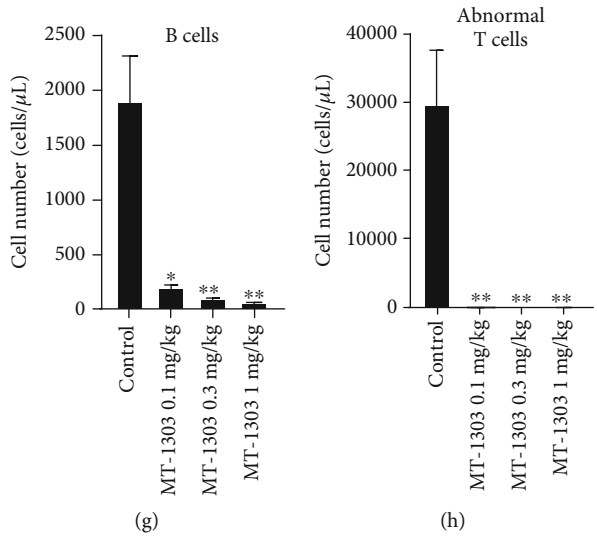

Figure 1: Prophylactic effect of MT-1303 on the development of proteinuria in MRL/*lpr* mice. MT-1303 was administered to MRL/*lpr* mice for 18 weeks from 8 weeks of age. (a) Proteinuria was assessed once a week using Ames urinalysis strips and was scored on a scale of 0–4 based on urinary protein concentrations as described in Materials and Methods. Each symbol represents the mean ± S.E.M. score of 12 animals. Statistical significance was calculated using the Shirley-Williams test by comparison with the vehicle-treated control group ($^{*}p < 0.05$, $^{**}p < 0.01$). (b, c) Kidney sections from vehicle- (b) or MT-1303 0.3 mg/kg- (c) treated mice were labeled with anti-mouse CD3 mAb. (d, e) The axillary lymph nodes (d) and spleen (e) were weighed on the day after final administration. Results are expressed as the mean ± S.E.M. of 8 mice. (f–h) The number of T cells (d), B cells (e), and abnormal T cells ($CD3^{+}B220^{+}$) (f) was measured by flow cytometry. Results are expressed as the mean ± S.E.M. of 4 mice. (d–h) Statistical differences were calculated using the Williams test by comparison with the control ($^{*}p < 0.05$, $^{**}p < 0.01$).

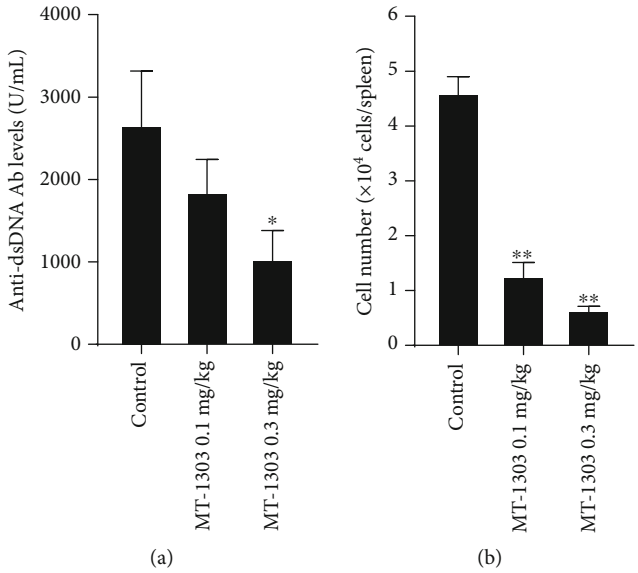

Figure 2: Effect of MT-1303 on anti-DNA antibody levels and plasma cells in MRL/*lpr* mice. MT-1303 was orally administered to MRL/*lpr* mice daily from 8 to 18 weeks of age. The serum and spleen were obtained the day after the final administration, and serum anti-dsDNA antibody levels (a) were determined by ELISA. Results are expressed as the mean ± S.E.M. of 12 mice. The number of plasma cells in the spleen (b) was determined by flow cytometry. Results are expressed as the mean ± S.E.M. of 4 mice. Statistical differences were calculated using the Williams test by comparison with the control ($^{*}p < 0.05$, $^{**}p < 0.01$).

and body weight, were orally treated with vehicle, MT-1303 (0.1 and 0.3 mg/kg), or FK506 (1 and 3 mg/kg) daily for 10 weeks until 40 weeks of age, and the effects of these drugs on lupus nephritis were evaluated. Survival rates during the study are shown in Figures 4(a) and 4(b). The number of animals that were euthanized or died due to worsening conditions from lupus nephritis progression during the observation period was 3, 1, 0, 2, and 3 in the vehicle control, MT-1303 0.1 mg/kg-treated, MT-1303 0.3 mg/kg-treated, FK506 1 mg/kg-treated, and FK506 3 mg/kg-treated groups, respectively. In the vehicle-treated control group, mean proteinuria scores increased with time and the score was

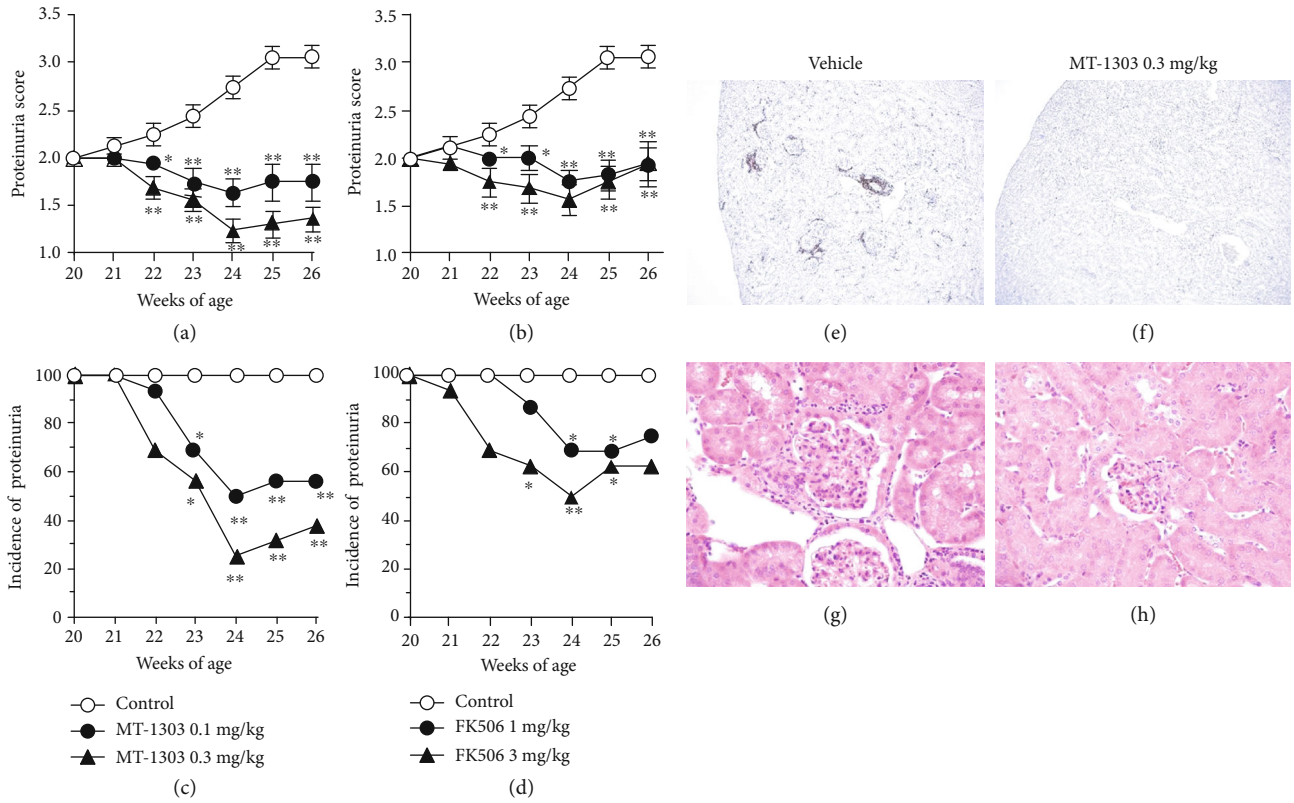

Figure 3: Therapeutic effects of MT-1303 and FK506 on established proteinuria in aged MRL/*lpr* mice. MT-1303 and FK506 were orally administered to MRL/*lpr* mice with a proteinuria score of 2 from 20 to 26 weeks of age. Proteinuria was assessed once a week using Ames urinalysis strips. (a, b) Proteinuria scores following administration of MT-1303 (a) and FK506 (b) are expressed as the mean ± S.E.M. of 16 mice. Statistical differences were calculated using the Shirley-Williams test by comparison with the control ($^{*}p < 0.05$, $^{**}p < 0.01$). (c, d) The incidence of proteinuria in MT-1303 groups (c) and FK506 groups (d) is expressed as the proportion of proteinuria-positive mice (proteinuria score ≥ 2) from a total of 16 mice. Statistical differences were calculated using Fisher's exact test with Hommel's multiple comparison test by comparison with the control ($^{*}p < 0.05$, $^{**}p < 0.01$). (e–h) Kidney sections from vehicle- (e, g) or MT-1303 0.3 mg/kg- (f, h) treated mice were stained with anti-mouse CD3 mAb (e, f) or HE (g, h).

significantly higher at the end of treatment than at the start of treatment, indicating that lupus nephritis had progressed (Figures 4(b) and 4(c)). Mean proteinuria scores in the MT-1303-treated groups remained lower than those in the control group and were significantly lower at the end of administration in all MT-1303-treated groups than in the control group. In contrast, no significant difference in proteinuria scores was observed between the FK506-treated and control groups. In addition, urinary neutrophil gelatinase-associated lipocalin (NGAL), which is reported to be elevated in both acute and chronic kidney disease [27], was measured, and urinary NGAL level increased over time in the control group. On the other hand, urinary NGAL levels remained lower in the MT-1303 groups than in the control group (Figure S1).

Histological analysis of kidney sections was performed in half of the mice in each group except for the FK506 3 mg/kg group (Table 1). In MT-1303-treated groups, the severity of several histological changes including mesangial expansion, glomerular sclerosis, and interstitial infiltrates was lower than that in the control group, with MT-1303 at 0.3 mg/kg significantly reducing the severity of mesangial expansion and glomerular sclerosis. However, no significant changes were noted in perivascular infiltrates in MT-1303-treated groups. In the FK506 0.1 mg/kg group, while the scores for mesangial expansion and glomerular sclerosis were lower than those in the control group, there were no significant differences between the groups. These results indicate that MT-1303 significantly suppresses lupus nephritis progression in NZBWF1 mice and that MT-1303 is more potent than FK506.

3.5. MT-1303 Reduces Infiltration of T Cells into the Kidneys of NZBWF1 Mice. To elucidate the effects of MT-1303 on infiltration of T cells into the kidneys in NZBWF1 mice, kidneys were obtained from NZBWF1 mice administered with MT-1303 (0.3 mg/kg) or vehicle for 13 weeks until 41 weeks of age, and the lymphocytes that infiltrated into the kidneys were analyzed by flow cytometry. In the vehicle-treated control group, infiltration of lymphocytes including T cells and B cells into the kidneys was detected at 41 weeks (Figures 5(a)–5(f)). Meanwhile, treatment with MT-1303 significantly reduced the number of infiltrating T cells, CD4 T cells, CD8 T cells, and $CD4^{-}CD8^{-}$ double-negative (DN) T cells. Further, CD4 T cell and CD8 T cell counts decreased to less than 20% of those in the control group. Additionally, B cell

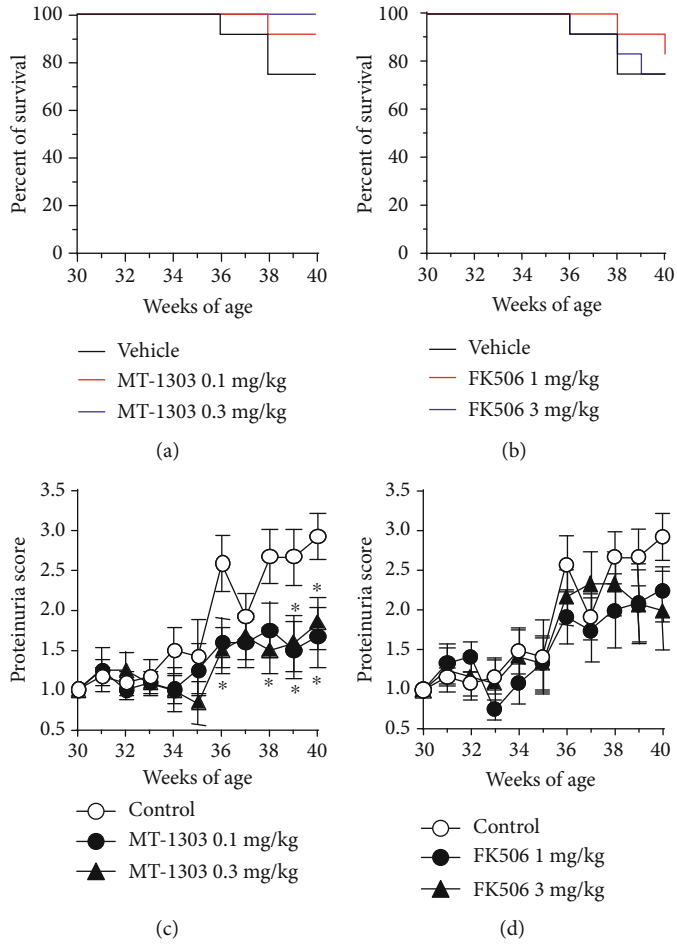

Figure 4: Effects of MT-1303 and FK506 on the development of proteinuria in NZBWF1 mice. MT-1303 and FK506 were orally administered to NZBWF1 mice daily from 30 to 40 weeks of age. (a) Percent survival in each group was shown over the study period. (b, c) Urinary protein concentrations were measured using the Bradford method once a week and scored on a scale of 0–4. Results are expressed as the mean ± S.E.M. of 12 mice. Statistical differences were calculated using the Shirley-Williams test by comparison with the control ($^*p < 0.05$).

Table 1: Effect of MT-1303 and FK506 on histological scores in the kidneys.

Treatment	Histological score (mean ± S.E.M.)			
	Mesangial expansion	Glomerular sclerosis	Interstitial infiltrates	Perivascular infiltrates
Control	1.8 ± 0.5	1.3 ± 0.4	0.2 ± 0.2	1.0 ± 0.3
MT-1303 0.1 mg/kg	1.0 ± 0.5	0.7 ± 0.3	0.2 ± 0.2	1.2 ± 0.3
MT-1303 0.3 mg/kg	0.3 ± 0.3*	0.2 ± 0.2*	0.0 ± 0.0	1.5 ± 0.2
FK506 1 mg/kg	1.0 ± 0.5	0.8 ± 0.5	0.2 ± 0.2	1.3 ± 0.2

n = 6, Shirley-Williams test ($^*p < 0.025$, control group vs. MT-1303 groups), Wilcoxon test (control group vs. FK506 group).

counts in the MT-1303-treated group were lower than those in the control group, although there was no significant difference between the groups.

In addition, we compared plasma cell counts in the spleen and serum levels of anti-dsDNA IgG in NZBWF1 mice after treatment for 13 weeks. The number of B cells and plasma cells in the spleen was significantly lower than that in the control group (Figures 6(a) and 6(b)); however, there was no significant difference in antibody levels between control and MT-1303-treated mice (data not shown).

4. Discussion

In the present study, oral administration of MT-1303 at 0.1 and 0.3 mg/kg was efficacious in MRL/*lpr* and NZBWF1 mice, two well-known murine SLE models that spontaneously develop lupus nephritis. MT-1303 not only inhibited the development of lupus nephritis when administered before onset but also improved the symptoms of lupus nephritis when administered after onset. Its efficacy in these models was superior or comparable to that of FK506, an

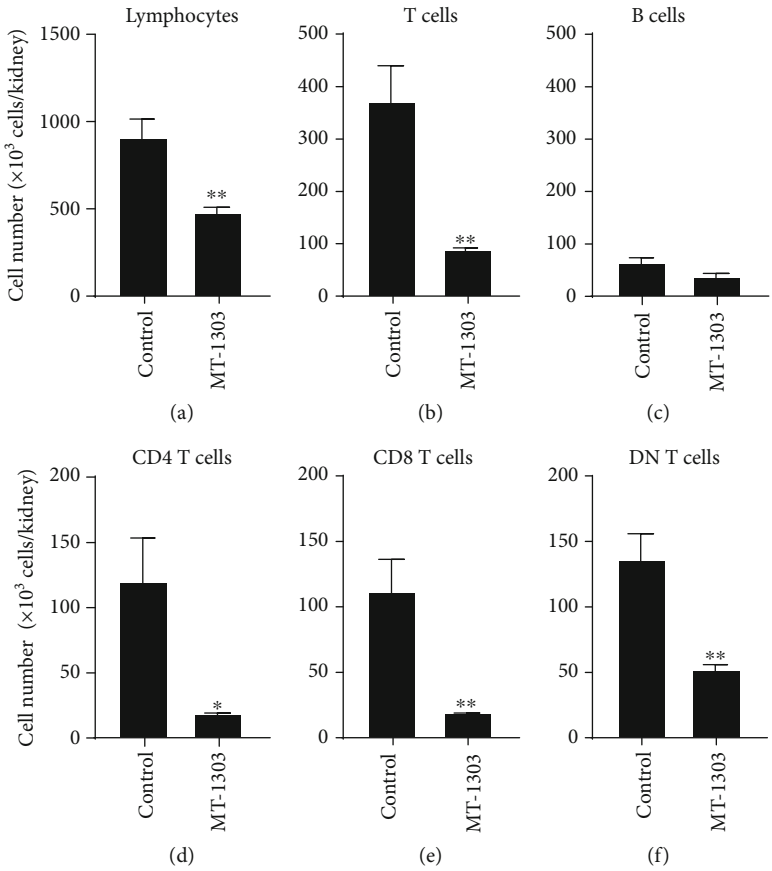

Figure 5: Effect of MT-1303 on the number of lymphocytes, T cells, B cells, CD4 T cells, and CD8 T cells in the kidneys of NZBWF1 mice. MT-1303 at 0.3 mg/kg was orally administered to NZBWF1 mice for 13 weeks from 28 weeks of age. The day after the final administration, the number of lymphocytes (a), T cells (b), B cells (c), CD4 T cells (d), CD8 T cells (e), and double-negative (DN) T cells (f) was determined by flow cytometry. Each column represents the mean ± S.E.M. cell count of 8 mice in each group. Statistical differences were calculated using Student's t-test ($^{*}p < 0.05$, $^{**}p < 0.01$).

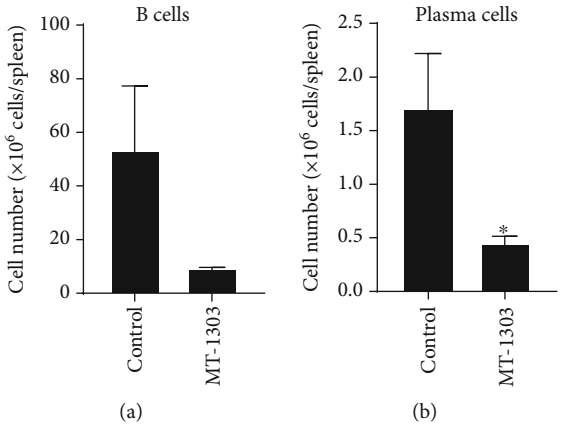

Figure 6: Effect of MT-1303 on the number of B cells and plasma cells in the spleen of NZBWF1 mice. MT-1303 at 0.3 mg/kg was orally administered to NZBWF1 mice for 13 weeks from 28 weeks of age. The spleen was obtained the day after the final administration. The number of B cells (a) and plasma cells (b) in the spleen was determined by flow cytometry. Results are expressed as the mean ± S.E.M. of 8 mice. Statistical differences were calculated using Student's t-test ($^{*}p < 0.05$).

already used treatment for lupus nephritis. Histological analysis additionally revealed that MT-1303 administration inhibited infiltration of T cells into the kidneys, mesangial expansion, and glomerular sclerosis.

Based on flow cytometric analysis, the number of T cells that infiltrated the kidneys increased in parallel with the progression of lupus nephritis in aged NZBWF1 mice. Immunohistochemical staining revealed T cell infiltration into the

periglomeruli area in addition to the tubulointerstitium in MRL/*lpr* mice with lupus nephritis. Treatment with MT-1303 decreased the number of T cells infiltrating the kidneys and the number of lymphocytes in the blood. Th1 and Th17 cells reportedly mediate glomerulonephritis in MRL/*lpr* mice, and a deficiency in the chemokine receptor CXCR3 significantly improves morphology and function of nephritic kidneys via interference with the trafficking of both Th1 and Th17 cells into the inflamed kidneys [28]. In addition, DN T cells and CD4 T cells have been reported to infiltrate the kidneys of patients with lupus nephritis and produce the inflammatory cytokines interleukin-17 and interferon-γ [29]. We have reported that fingolimod, the first $S1P_1$ receptor modulator, reduces infiltration of myelin antigen-specific Th17 and Th1 cells into the spinal cord [15]. Further, we have also confirmed that MT-1303 inhibits infiltration of colitogenic Th1 and Th17 cells into the colon in a mouse IBD model [30]. These results suggest that MT-1303 decreases infiltration of autoreactive Th1, Th17, and DN T cells into the kidneys by reducing the number of circulating lymphocytes in the blood, thereby eliciting a marked therapeutic effect on the progression of lupus nephritis in two SLE models.

In the mouse antibody response, fingolimod reportedly reduces the production of high-affinity, class-switched antibodies without affecting T cell-independent antibody production by reducing the formation of the germinal center in the T cell-dependent antibody formation system [18]. Additionally, treatment with fingolimod reduces the specific antibody production reaction against vaccination of anticipated novel antigens or recall antigens in MS patients [31]. In the present study, the effects of MT-1303 on blood anti-dsDNA antibody levels were contradictory. Administration of MT-1303 suppressed the increase in the number of plasma cells in the spleen of both strains, but the increase in anti-dsDNA antibody levels in the blood was suppressed only in MRL mice. However, these results are consistent with those observed with other $S1P_1$ receptor modulators in MRL/*lpr* and NZBWF1 mice [19–22]. A multicenter, open-label phase Ib trial in patients with SLE has been conducted to evaluate the safety, pharmacodynamics, and exploratory efficacy of MT-1303, with 17 subjects administered with a low or high dose of MT-1303 for 24 weeks (ClinicalTrials.gov NCT02307643). Currently, this study has been completed, and the results are now undergoing finalization. Since the changes of biological markers in this clinical trial have been monitored, the results will reveal the effect of MT-1303 on anti-dsDNA antibody levels in SLE patients.

$S1P_1$ receptor modulators have been developed for several autoimmune diseases, including MS, and MT-1303 has been evaluated in clinical trials for MS, psoriasis, Crohn's disease, and SLE. In a phase II study in more than 400 patients with RRMS, MT-1303 showed superior efficacy over the placebo control [3]. In inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), there is some clinical evidence supporting the use of the $S1P_1$ receptor modulators [17], and IBD is the next promising area of investigation after MS. Although the results of the phase II study of MT-1303 in CD patients were not as expected [32], we are developing MT-1303 for UC. In psoriasis, anti-IL-17 antibodies and the anti-IL-23p19 antibody are highly effective [33, 34], but the results of the phase II trial of MT-1303 were inferior to the effects of these antibodies (EudraCT number 2012-005750-27). On the other hand, although B cell-targeted belimumab is the only approved biological agent for SLE, its efficacy is limited [12], and a more effective agent with a novel mechanism of action is required. We expect that MT-1303, which affects trafficking of T and B cells, may have potential as a therapeutic agent for SLE; as noted above, a clinical trial for this condition has been completed and the results are now undergoing finalization.

Another $S1P_1$ receptor modulator, cenerimod, has been evaluated in a phase II clinical trial in 64 patients with SLE (ClinicalTrials.gov NCT02472795). Although the results of this study have not yet been reported, a phase IIb study in 500 patients with active SLE (ClinicalTrials.gov NCT02472795) has been initiated. In the phase I clinical study of cenerimod, it was reported that heart rate decreased in healthy subjects after the initial administration from the lowest dose used in the SLE clinical trials [35]. MT-1303 has been reported to have no clinically significant bradyarrhythmia after the first dose at the anticipating clinical dose [2, 3], suggesting that MT-1303 may be superior to cenerimod in terms of cardiac effects, which is a concern with $S1P_1$ receptor modulators.

5. Conclusions

Overall, this study demonstrates that MT-1303 shows superior or comparable therapeutic efficacy to FK506 in two SLE animal models. Moreover, it is likely that MT-1303 sequesters lymphocytes in secondary lymphoid tissues and the thymus and, thereby, not only inhibits infiltration of autoreactive T cells into inflammation sites but also affects the activation of autoreactive B cells and their differentiation to plasma cells by reducing the interaction between autoreactive T cells and B cells. Our nonclinical findings suggest that MT-1303 has the potential to be used as a therapeutic agent for patients suffering from SLE, including lupus nephritis.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

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Supplementary Materials

Supplementary Figure 1: effects of MT-1303 and FK506 on urinary levels of NGAL in NZBWF1 mice. *(Supplementary Materials)*

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Review Article

A Comprehensive Review and Update on the Pathogenesis of Inflammatory Bowel Disease

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Inflammatory bowel disease (IBD) is a chronic and life-threating inflammatory disease of gastroenteric tissue characterized by episodes of intestinal inflammation. The pathogenesis of IBD is complex. Recent studies have greatly improved our knowledge of the pathophysiology of IBD, leading to great advances in the treatment as well as diagnosis of IBD. In this review, we have systemically reviewed the pathogenesis of IBD and highlighted recent advances in host genetic factors, gut microbiota, and environmental factors and, especially, in abnormal innate and adaptive immune responses and their interactions, which may hold the keys to identify novel predictive or prognostic biomarkers and develop new therapies.

1. Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the gastrointestinal tract, which clinically contains Crohn's disease, ulcerative colitis, and other conditions [1]. The inflammation of the intestinal mucosa in IBD is characterized by episodes of abdominal pain, diarrhea, bloody stools, weight loss, and the influx of neutrophils and macrophages that produce cytokines, proteolytic enzymes, and free radicals that result in inflammation and ulceration [1, 2].

IBD is a lifelong disease occurring early in life in both males and females. The incidence and prevalence of IBD markedly increased over the second half of the 20th century, and since the beginning of the 21st century, IBD has been considered one of the most prevalent gastrointestinal diseases with accelerating incidence in newly industrialized countries [3–5]. The highest prevalence of IBD was reported in Europe (ulcerative colitis 505 per 100,000 persons in the southeast of Norway; Crohn's disease 322 per 100,000 persons in Hesse, Germany) and North America (ulcerative colitis 286.3 per 100,000 persons in Olmsted County, USA; Crohn's disease 318.5 per 100,000 persons in Nova Scotia, Canada) [5]. Since 1990, the incidence rate of IBD in Western countries was shown to be stable or started to drop, but the incidence rate in newly industrialized countries of Asia, Africa, and South America was increasing [5].

Crohn's disease usually involves the terminal ileum, cecum, perianal area, and colon, but it can affect any region of the intestine in a discontinuous pattern [6–8]. In contrast, ulcerative colitis involves the rectum and can affect part of the colon or the entire colon in a continuous pattern [6–8]. Crohn's disease exhibited histologically a thickened submucosa, transmural inflammation, fissuring ulceration, and granulomas, whereas the inflammation in ulcerative colitis is limited to the mucosa and submucosa with cryptitis and crypt abscesses [7–9].

Although the cause of IBD remains unknown, considerable progress has been made in recent years to unravel the pathogenesis of this disease. Studies have provided evidence that the pathogenesis of IBD is associated with genetic

susceptibility of the host, intestinal microbiota, other environmental factors, and immunological abnormalities [10, 11].

2. Pathogenesis of IBD

2.1. Genetic Factors. Genome-wide association studies (GWAS), next generation sequencing studies, and other analysis have identified over 240 nonoverlapping genetic risk loci, of which around 30 genetic loci are shared between Crohn's disease and ulcerative colitis [12–14]. The analysis of the genes and genetic loci identified in IBD indicates that several pathways play important roles in maintaining intestinal homeostasis, such as epithelial barrier function, innate mucosal defense, immune regulation, cell migration, autophagy, adaptive immunity, and metabolic pathways associated with cellular homeostasis [8, 15–17]. The permeability of the epithelial barrier enables microbial incursion, which is recognized by the innate immune system, which then launches appropriate tolerogenic, inflammatory, and restitutive responses partially by secreting extracellular mediators that recruit other cells, including adaptive immune cells [8].

Nucleotide-binding oligomerization domain 2 (NOD2) is the first gene found to be associated with Crohn's disease, which is frequently mutated in patients with Crohn's disease, occurring in around one-third of the patients [18, 19]. For instance, Crohn's disease patients associated with 1007fs mutation in the NOD2 gene show a much more severe disease phenotype than other Crohn's disease patients, while R702W and G908R mutations lead to increase inflammatory cytokine responses [6]. NOD2, a member of the cytosolic Nod-like receptor (NLR) family based on their triggers and the signaling pathways that they control, is one of the two important and distinct detection systems to sense microbial invaders [6]. NLR proteins are found in the cytoplasmic compartment, and the other detection systems are membrane-bound receptors, termed toll-like receptors (TLRs). NOD2 can recognize the minimal bioactive fragment of peptidoglycan found in the cell wall of both Gram-negative and Gram-positive bacteria, called muramyl dipeptide (MDP) [6, 20, 21]. Thus, NOD2 is thought to be important as an intracellular sensor of bacterial components [6, 20, 21]. Upon binding to its ligand—MDP, a conformational change of NOD2 occurs that allows it to bind the caspase recruitment domain of the adaptor protein RIP2 [6, 20]. RIP2 then induces the polyubiquitination of nuclear factor kappa B (NF-κB) essential modulator—I$\kappa\kappa\gamma$, which is the key scaffolding protein of NF-κB [20]. It then activates NF-κB, leading to secretion of some proinflammatory cytokines, such as IL-12. It can also activate the MAPK signaling pathway [6, 20].

NOD2 has also been implicated in the initiation of autophagy [8, 22]. Autophagy is a highly conserved recycling process involving the degradation of cytosolic contents and organelles, as well as resistance against infection and the removal of intracellular microbes [8, 22]. MDP stimulation can activate the autophagy process leading to confinement of intracellular bacteria within autophagosomes and subsequent control of infection [23]. Following bacterial recognition, NOD2 serve as molecular scaffolds for the nucleation of the autophagy machinery by interacting with ATG16L1 [23]. ATG16L1 is essential for all forms of autophagy. Interestingly, ATG16L1 polymorphisms are also linked to Crohn's disease like NOD2 [23]. The variant encoding the T300A substitution in *ATG16L1* increases the susceptibility of the protein ATG16L1 to caspase-3 cleavage and decreases its function [17, 24]. In patients with Crohn's disease who are homozygous for the T300A substitution in *ATG16L1*, they have abnormal TLR signaling and Paneth cell function [17]. Selective deletion of *ATG16L1* in T cells in mice results in spontaneous intestinal inflammation characterized by aberrant Th2 responses to dietary and microbiota antigens and decreasing Foxp3$^+$ Treg cell number [25]. These impaired T cell responses contribute to the disruption of the mucosal barrier through breaking the tolerance to intestinal antigens and promoting the secretion of IgG and IgA against commensal microbiota [17, 25].

GWAS has identified numerous single-nucleotide polymorphisms (SNP) in *IL-23R*, with high association for Crohn's disease and ulcerative colitis [26, 27]. Of interest, Arg381Gln, an uncommon allele at a highly conserved amino acid polymorphism, confers a protective effect in patients with Crohn's disease or ulcerative colitis through modulating IL-23R recycling and cytokine production by macrophages [27, 28].

The majority of risk-associated loci are shared across populations, but some loci show heterogenicity between populations; for example, *NOD2* and *IL23R* variants are present in the majority of European patients, but not in East Asian ancestry patients [29]. Also, although many individuals carry IBD-associated risk loci, only a small population develops IBD. Therefore, additional environmental factors and alterations to the interactions between the gut microbiota and mucosal immune system are required for the development of IBD.

2.2. Gut Microbial Factors. IBD appears to result from abnormal host immune responses to the intestinal microbiota [30–32]. Intestinal microbiota is the major environmental driver of IBD. The gastrointestinal tract of the human body is colonized at birth by a vast range of microorganisms that numerically exceed host cells by around 10 times [32, 33]. The gut contains 1000-5000 different species, with 99% coming from Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria [30]. These microorganisms contain around 100-fold as many genes present in the human genome [30, 32, 33]. The gut microbiota can be influenced by diet, probiotics, prebiotics, antibiotics, exogenous enzymes, fecal microbiota transplantation, and other environmental factors [31].

This gut microbiota is necessary for intestinal homeostasis and function, health, and disease [32–34]. Tolerance to gut microbiota must be maintained to benefit from their coexistence; on the contrary, colonization with specific pathogenic microbes might be detrimental to the host, leading to disease [33]. The coexistence with the microbiota can be beneficial to host metabolism and gastrointestinal development [32, 33]. In addition, the commensal microorganisms are required for the development and differentiation of the local and systemic immune system and nonimmune

components [32, 33]. They can protect the host from enteric pathogenic infections via colonization resistance and via synthesis of factors promoting mutualism [32]. For example, induction of a transforming growth factor- (TGF-) β-rich environment by indigenous *Clostridium species* enhances regulatory T cell (Treg) numbers and function in the colon and the resistance to DSS-induced murine colitis [32, 35]. Therefore, the host has evolved numerous mechanisms to maintain the homeostasis.

Both commensal and pathogenic microorganisms determine the consequence of an infection. Microbes can be detected by recognition by pattern recognition receptors (PRRs, including TLRs, NLRs, C-type lectin receptors, and RIG-like receptors) of pathogen-associated molecular patterns (PAMPs), which are found in many species of microorganisms [31–33]. The recognition of PRRs activates the innate immune system, leading to the activation of NF-κB and inflammasome, which stimulates the production of proinflammatory cytokines and chemokines, and this can also enhance tissue homeostasis and mucosal tolerance in the absence of barrier broken [33, 36]. The PAMPs are small molecular motifs conserved within many species of non-pathogenic and pathogenic microbes. Therefore, PRRs recognition is largely unable to distinguish between non-pathogenic and pathogenic microbes [33, 37]. It leads to the recognition of innate immunity to commensal microorganisms, having a crucial role in the maintenance of intestinal homeostasis, and is critical for the protection against gut injury and associated mortality [33, 37]. The imbalance of these interactions contributes to the development of intestinal inflammation.

In the complexity and multiplicity of the gut microbiota, our understanding on the roles of commensal and pathogenic microorganisms in establishing a healthy intestinal epithelial barrier and in disrupting the intestinal homeostasis has been greatly increased in the past decades [31, 36]. Animal studies have demonstrated that the intestinal microbiota play both proinflammatory and anti-inflammatory roles in the pathogenesis of IBD, and in most animal colitis models, the intestinal microbiota is indispensable for driving pathogenesis [36]. However, in human, it is difficult to demonstrate a definitive cause-effect relationship between intestinal microbiota and IBD [36]. Based on the studies in human and animal infection models, it is unlikely that a single infection causes or triggers the IBD in humans. But the intestinal microbiota clearly promotes the development of IBD [32, 33]. For instance, the presence of *Mycobacterium avium* subsp. *paratuberculosis* and adherent-invasive *Escherichia coli* is increased in Crohn's disease patients; the presence of *Clostridium difficile* is increased in both Crohn's disease and ulcerative colitis patients in relapse and remission states [38]. The increased mucosal bacterial counts and decreased anti-inflammatory commensal *Faecalibacterium prausnitizii* are also found in Crohn's disease patients [38].

In summary, microbial factors play important roles in the pathophysiology of IBD through impacting the immune systems in major ways and affecting host metabolism and gastrointestinal development [31–33, 36].

2.3. Environmental Factors. The important role of environmental factors in the pathogenesis of IBD is supported by recent studies on IBD epidemiology. The frequency of Crohn's disease has significantly increased in the more developed countries over the past 50 years, and the recognition of the disease corresponding with progressive industrialization in the less developed countries has also increased [39, 40].

Food intake is an important environmental factor that affects the development of IBD [41]. Studies have provided evidence that intake of fruit and vegetable has been associated with decreased risk of Crohn's disease [42]; intake of fast foods containing many fat and sugar-rich foods may exacerbate the development of Crohn's disease [41]. One study also shows that medium-chain fatty acids are more effective in accelerating intestinal inflammation than long-chain fatty acids [43]. In most of Western developed countries, sugar-rich foods have been recognized as one of the risk factors for Crohn's disease [41], and artificial food additives prevalent in Western diets may promote intestinal inflammation by interfering with barrier function in the gut [42].

Smoking is another example of a disease-specific modifier that seems to worsen Crohn's disease while being protective against ulcerative colitis [39, 40]. Smoking has been shown to affect cellular and humoral immune responses and to promote colonic mucus production [39, 41]. Nicotine, an essential content of cigarettes, has an inhibitory effect on Th2 cell function, but has no effect on Th1 cell function [39]. Evidence also suggests that smoking impairs autophagy, a process thought to be involved especially in Crohn's disease [44].

There are other environmental factors that influence the development of IBD, including but not limited to psychological stress, appendectomy, diet, and medications [45]. For example, appendectomy is an independent risk factor for developing Crohn's disease, while it is protective for ulcerative colitis [46]. Although many epidemiological studies already identified those environmental factors with disease evolution of IBD, it is still facing challenges to explore the mechanism studies of how environmental factors impact IBD disease progress [45]. One study shows that diet rich in animal protein promotes proinflammatory macrophage responses and exacerbates murine colitis [47].

2.4. Immunological Abnormalities. The immunological dysregulation in IBD is characterized by epithelial damage (abnormal mucus production, defective repair); expansion of inflammation driven by intestinal flora and a large number of cells infiltrating into the lamina propria including T cells, B cells, macrophages, dendritic cells (DCs), and neutrophils; and a failure of immune regulation to control the inflammatory response [3, 48, 49]. The activated lamina propria cells produce high levels of proinflammatory cytokines in the local tissue, including TNF, IL-1β, IFN-γ, and cytokines of the IL-23/Th17 pathway [3, 7, 48].

The intestinal immune system is divided into innate immunity and adaptive immunity. Innate immunity includes the barrier function of the intestinal mucosa, antibacterial proteins (complement, defensins, etc.), the acid PH value of stomach to limit microbial growth, innate immune cells

(neutrophils, macrophages, DCs and natural killer T cells, etc.), and innate cytokines and molecules (IL-1, TNF, and defensins) [49]. Adaptive immunity is pathogen-specific and is usually initiated under the circumstances in which the innate immune responses cannot circumvent the stimulation of a pathogen [49]. After exposure to a pathogen, it usually takes several days to finally activate adaptive immune responses, including T and B cells [49]. The initiation of immune response to intestinal flora is tightly regulated, and this regulation determines the occurrence of immune tolerance or a defensive inflammatory response. Disturbance of the balance of these responses can cause IBD [7].

2.4.1. Dysregulation of the Innate Immune System

(1) Intestinal Epithelial Barrier. The 400 mm^2 single layer of intestinal epithelial cells (IEC) is the primary cellular barrier. It functions as a selective barrier to confine the entry of antigens to the mucosal immune system for the aim of inducing oral tolerance to commensal microorganisms or food antigens and for the aim of host defense against pathogens [18, 50]. Therefore, the IEC play important roles in the gut in an immunological context through providing the antigen-sampling machinery, expressing PPRs (e.g., TLR and NLR) and involving the establishment of the tolerogenic environment in the intestine and controlling of the immune system in the gut-associated lymphoid tissue (GALT) [51]. The tight junctions between epithelial cells allow for the selective penetration of nutrients, fluids, and microorganisms. Normal gastrointestinal permeability relies on the intact epithelium, surface mucus, and peristalsis and the production of host protective factors [52].

Epithelial integrity is disturbed in IBD patients, and mice that have deficient epithelial barrier functions develop colitis [18]. The absorptive cells in colonic crypts expressing the proton channel OTOP2 and the satiety peptide uroguanylin, which can sense pH, is dysregulated in IBD [53]. In IBD, intestinal epithelial goblet cells are positionally remodeled and coincided with the downregulation of WFDC2, which is an antiprotease molecule with the ability to preserve the integrity of tight junctions between epithelial cells and prevents invasion by bacteria and mucosal inflammation [53]. One GWAS study identifies 3 susceptibility loci related to the epithelial barrier function in ulcerative colitis patients: *HNF4A* regulating the expression of cell junctions; *CDH1* encoding E-cadherin, a main component of adherent junctions; and *LAMB1* encoding laminin beta 1 subunit, expressed in the intestinal basement membrane [48]. Proinflammatory cytokines, secreted during intestinal inflammation such as TNF or IFN-γ, can increase the epithelial permeability by regulating tight junctions and promoting apoptosis [18]. IFN-γ increases paracellular permeability and induces endocytosis of tight junction transmembrane proteins [54]. Increased permeability to macromolecules has been found in IBD patients [55]. The high apoptotic rate of epithelial cells also leads to diminished epithelial barrier function observed in IBD. Studies have shown that apoptotic rate is increased in mildly to moderately inflamed colon of Crohn's disease and ulcerative colitis [18]. Also, apoptosis allows the loss of ions and water and the entry of small antigens [56]. IL-13, a key effector Th2 cytokine in ulcerative colitis, also shows the ability to impair epithelial barrier function by affecting epithelial apoptosis, tight junctions, and reconstitution velocity [57]. The reduced velocity of restitution can play a role in the response of an epithelial layer to naturally occurring or pathogen-induced small lesions [57].

The intestinal epithelium is also responsible for electrolyte transport. Disrupted electrolyte transport may lead to diarrhea [18]. Around 50% of Crohn's disease patients and almost 100% of ulcerative colitis patients have diarrhea as a symptom. The deficiencies of electrolyte transport in IBD contain hyporesponsiveness of electrogenic anion secretion, reduced synthesis of epithelial sodium channels, reduced NaCl absorption, and alteration of electrochemical gradient [18].

The intestinal epithelium may be improved, protected, and repaired by growth factors and cytokines [58]. These growth factors and cytokines play vital roles in the regulation of cell proliferation, differentiation, angiogenesis, inflammation, intestinal defense mechanisms, and intestinal wound repairs [18]. Currently, at least 30 different peptide growth factors have been shown to be involved in the maintenance of intestinal mucosal integrity, including epidermal growth factor, the TGF-β family, the insulin-like growth factor family, the fibroblast growth factor family, and the colony-stimulating factor family [58]. Of these factors, epidermal growth factor, insulin-like growth factor family, fibroblast growth factor family, and colony-stimulating factor family appear promising in the treatment of IBD and are being evaluated in clinical trials [18].

(2) Dendritic Cells. DCs are hemopoietic bone marrow progenitor-derived leukocytes, which are widely distributed throughout the body in small numbers [59, 60]. Although DCs were first described by *Paul Langerhans* in the late nineteenth century, their role as a central coordinator was not established until 1973 by *Ralph Steinman* et al. [61, 62]. DCs are professional antigen-presenting cells (APCs) specialized in antigen capture, process, and presentation to T cells. DCs are considered to be the most potent APCs that orchestrate innate and adaptive immune responses [63].

DCs are found throughout the gut, including the lamina propria, isolated lymphoid follicles, Peyer's patches, and mesenteric lymph nodes (MLNs) [60]. DCs have been documented both in the maintenance of immune tolerance to the commensal microorganisms and food antigens and in the initiation of host defense against pathogens [64, 65]. In the intestine, DC subtypes have been characterized into conventional DCs and plasmacytoid DCs, similar to those in other peripheral lymphoid organs [51, 59]. Conventional DCs are further divided into the following: $CD11b^+CD8\alpha^-$ DCs in the subepithelial dome, preferentially secreting IL-10 and inducing Th2 cells; $CD11b^-CD8\alpha^+$ in the interfollicular regions; and $CD11b^-CD8\alpha^-$ subsets in both areas, preferentially secreting IL-12 and inducing Th1 cells [51, 59]. Plasmacytoid DCs are specialized in the production of type I

interferons [65]. In the steady-state lamina propria, two major DC subsets have been characterized based on the reciprocal expression of CD103 and CX_3CR1 [51, 59].

DCs are present in an immature state with high phagocytic ability localized in peripheral tissues and in discrete regions of organized secondary lymphoid organs [59]. Immature DCs constitutively acquire foreign and self-antigens from the intestinal lumen through the following: (1) microfold (M) cells which transcytose antigens from the lumen to the mucosa [59]; (2) CX_3CR1^+ DCs extending dendrites between IEC and into the intestinal lumen to directly capture antigens and present them to $CD4^+$ T cells, which differentiate into effector T cells and secret proinflammatory cytokines [59, 60, 64]; (3) direct sample antigens as a result of breaches in the epithelial integrity as seen in intestinal inflammation [59, 64]; (4) mechanisms mediated by the fetal Fc receptor [59, 64]; and (5) lamina propria $CD103^+$ CX_3CR^- DCs receiving conditioning from epithelial cells and serving as the inducer of Treg cells [59, 60, 64].

After capturing antigens, immature DCs migrate from the Peyer's patch and lamina propria to the draining MLN, where they present the antigens to naïve T cells [51]. During the migration, DCs gradually become mature with the expression of costimulatory molecules. In addition, the lamina propria DCs constitutively transport antigens from apoptotic IEC or commensal microorganisms to the draining MLN to interact with T and B cells to initiate tolerogenic responses [51]. In particular, $CD103^+$ DCs isolated either from the lamina propria or from the MLN promote the development of $Foxp3^+$ Treg, which rely on retinoic acid and TGF-β [60]. Also, DCs conditioned in the presence of IEC-secreted thymic stromal lymphopoietin (TSLP) are less capable of secreting IL-12 and promoting Th2 responses [51].

In the presence of pathogens, the migration of DCs to the MLN increases. Activated DCs trigger a protective immune response including activating effector cells and determining which $CD4^+$ T helper cells (e.g., Th1, Th2, or Th17) will predominate [64].

In patients with IBD, DCs are attracted by the upregulated chemokines such as CCL20 or addressins such as mucosal vascular addressin cell adhesion molecule-1 and accumulate at inflammatory sites. Correlating with large amounts of DCs accumulation in the intestine, plasmacytoid DCs and myeloid DCs are downregulated in the peripheral blood of patients with active IBD [60, 64]. In the lesions of Crohn's disease, the numbers of $CD83^+$ DC and DC-specific ICAM-3 grabbing nonintegrin $(DC\text{-}SIGN)^+$ populations are significantly increased, while IL-12 and IL-18 are only detected in $DC\text{-}SIGN^+$ DC and not in $CD83^+$ DC [66]. DCs from MLN of patients with Crohn's disease preferentially induce the Th1 response [67]. Three types of DC are identified in the MLN of Crohn's disease and ulcerative colitis patients, including mature DCs, myeloid DCs, and plasmacytoid DCs [67]. Myeloid DCs from MLN of patients with Crohn's disease produce high levels of IL-23 and low levels of IL-10 [67]. While plasmacytoid DCs are shown to infiltrate intestinal mucosa of IBD patients, one recent murine study shows that plasmacytoid DCs are largely dispensable in the pathogenesis of intestinal inflammation during IBD [68].

Besides reacting inappropriately to captured antigens, intestinal DCs might also receive inappropriate signals from IEC during intestinal inflammation [60]. IEC isolated from about 70% of patients with Crohn's disease do not express TSLP mRNA and cannot control the DC-mediated proinflammatory response, leading to upregulated production of IL-12 by DCs, which then polarizes Th1 responses [69]. NOD2 expression on DCs may also play a critical role in their responses to microbes, because DCs derived from NOD-2 deficient Crohn's disease patients have an impaired ability to induce IL-17 production upon MDP challenge [70].

Evidence from animal models also demonstrates the role of DCs in the chronic intestinal inflammation. Large amounts of activated DCs accumulate in the lamina propria and MLN in murine models of colitis [60, 64]. In the $CD45RB^{hi}$ $CD4^+$ T cell transfer model of colitis, large amounts of $CD11c^+$ DCs expressing activation marker OX40 ligand (OX40L) are found in the MLN, and transferred T cells create aggregates with $CD11c^+$ DCs in the lamina propria. Blocking OX40-OX40L interaction ameliorates colitis [71, 72]. Analysis of the DC phenotype in murine colitis has shown that colonic lamina propria mature DCs express higher levels of costimulatory molecules (CD40, CD80, and CD86) and increase productions of IL-12p40 and IL-23p19 upon CD40 ligation [73]. IL-12p40 and IL-23p19 form IL-23, which is important for the stabilization of Th17 cell activation. When DCs are selectively ablated in mice before developing dextran sodium sulfate- (DSS-) induced colitis, the colon inflammation is exacerbated when compared with that of untreated mice [59].

Taken together, these data indicate that DCs play an important role in the pathogenesis of IBD through influencing the tolerance to the commensal microflora and dietary antigens and affecting immune responses [64].

(3) Myeloid-Derived Suppressor Cells (MDSC). MDSC are a heterogeneous population of cells that expand during cancer and other pathogenic conditions and have a remarkable ability to suppress various T cell responses and promote Treg expansion [74, 75]. MDSC suppress immunity by perturbing both innate and adaptive immune responses through secreted soluble mediators and induction of Treg cell expansion [74, 76]. MDSC contribute to the failure of immune therapy in patients with cancer and have been considered a therapeutic target for the treatment of cancer [74, 77, 78]. The expansion and functional importance of MDSC in non-cancer pathogenic conditions have been recently recognized. MDSC numbers are imbalanced and they act as a downregulation mechanism of immune responses in many diseases, such as autoimmune diseases [79], transplantation [80, 81], and asthma [82, 83, 84].

The roles of MDSC in IBD have been demonstrated in IBD patients and animal models. MDSC are increased in the peripheral blood of IBD patients [85, 86]; $CD14^+HLA\text{-}DR^{-/lo}$ monocytic MDSC have the ability to suppress T cell proliferation [85], while $CD33^+CD15^+$ granulocytic MDSC fail to suppress T cell response but instead enhance T cell proliferation [86]. We and others report that MDSC are

increased in TNBS- or DSS-induced murine colitis, and the percentage of MDSC in tissue is correlated with the severity of intestinal inflammation [87, 88]. Sorted MDSC from murine colitis can suppress T cell proliferation in vitro, and adoptive transfer of MDSC sorted from murine colitis, generated in vitro, or exosome released by granulocytic MDSC can decrease intestinal inflammation and reduce the secretion of proinflammatory cytokines [87, 89].

Patients with inflammatory bowel disease (IBD) are at increased risk for developing colorectal cancer. It is been found that MDSC accumulation is further increased in the lesions during the progression from colitis to colorectal cancer [90]. Antibody-mediated depletion of MDSC in mice during colitis reduces colon tumor formation [91]. These results indicate that MDSC may play a role in the progression from colitis to colon cancer.

(4) Macrophages and Natural Killer T (NKT) Cells. Macrophages are white blood cells that reside in the tissues, which have critical roles in the host immune defenses [92]. Macrophages are differentiated from monocytes after emigrating from blood vessels in response to different stimuli [92, 93].

Intestinal macrophages are the most abundant mononuclear phagocytes in the intestine, especially in the large intestine, where they account for around one-fifth of all leucocytes [64, 94, 95]. Most of the macrophages are found underneath the epithelium of lamina propria of the intestine where they surveil the environment, phagocytose potential harmful antigens, and promote epithelial cell renewal by producing several mediators [95, 96], and some macrophages can also extend transepithelial dendrites into the intestinal lumen [95]. $\alpha 4\beta 7$ integrin is important for homing of nonclassic monocyte to the gut, and impaired $\alpha 4\beta 7$-dependent gut homing is associated with reduced and delayed wound healing and reduces perilesional presence of wound healing macrophages [97]. Intestinal macrophages play critical roles in maintaining intestinal homeostasis and are also drivers of the pathology associated with IBD [65, 94]. Resident macrophages in the lamina propria immediately capture and clear the bacteria that breach the epithelial layer without initiating an inflammatory response and thus are vital for maintaining homeostasis [94]. For instance, they efficiently eradicate phagocytosed enteric bacteria such as *Salmonella typhimurium* and *Escherichia coli*. They might also eliminate apoptotic and senescent cells and other cellular debris [64, 94]. Moreover, resident macrophages in the lamina propria have a unique surface marker's expression pattern-high expression of CX3CR1 and low expression of costimulatory molecules, Fc receptors for IgA and IgG, complement receptors, and integrin $\alpha 2\beta 1$ [64, 98]. It suggests that these macrophages do not function as professional APC, unlike macrophages from other body compartments [64, 93, 99, 100]. These macrophages do not secret proinflammatory cytokines in reaction to cytokines or PAMPs or following phagocytosis of apoptotic cells [64, 93].

On the other hand, many inhibitory receptors are expressed on intestinal macrophages, including CD172a, CD200R1, IL-10R, and TGF-β receptors [94]. So the function of intestinal macrophages is influenced by corresponding soluble factors, such as IL-10 and TGF-β, secreted by a wide range of cell types, including epithelial cells, fibroblasts, subepithelial myofibroblasts, and lymphocytes [64]. Macrophages also have roles in tolerance through inducing anergic T cells or Treg and can impact the differentiation of naïve T cells into Th1, Th2, or Th17 cell types [101].

Studies have indicated the role of macrophages in the pathogenesis of IBD. In IBD patients, the number of macrophages increases in the inflamed mucosa with the major $CD14^{hi}HLA\text{-}DR^{dim}$ macrophages which can initiate a rapid response to luminal microbial antigens, unlike the resident macrophages [64, 102]. Also, the phenotype and functions of the macrophages in the inflamed sites differ from those in physical conditions. For instance, they express high levels of costimulatory molecules, such as CD40, CD86, CD80, and CD40 [103]. The $CD14^{hi}$ macrophages in inflamed Crohn's ileum exclusively contain $CD163^{lo}CD11c^{hi}$ subset, while $CD14^{hi}$ macrophages from noninflamed colon tissue contain both $CD163^{lo}CD11c^{hi}$ and $CD163^{hi}CD11c^{lo}$ subsets [98]. In addition, aberrant CD14-expressing macrophages isolated from the mucosa of IBD patients produce high levels of IL-12 and IL-23 in vitro under the microbial stimulation [104].

Animal models of IBD also support the role of dysregulated macrophages in the pathogenesis of IBD [64]. Murine colitis models show increased infiltration of CCL2 or MCP-1-mediated recruitment of monocytes and immature macrophages into the gut mucosa, which are arrested for further differentiation during inflammation, and they produce a large amount of proinflammatory mediators, such as TNF, IL-6, and nitric oxide [96]. $IL\text{-}10^{-/-}$ mice spontaneously develop colitis in which macrophages preferentially differentiated into proinflammatory subsets that produce high levels of IL-12 and IL-23. Deficiency of macrophages in $IL\text{-}10^{-/-}$ mice prevents the progression of colitis [105]. These results demonstrate that macrophages favor the development of intestinal inflammation.

NKT cell is another cell type involved in the pathogenesis of IBD [11]. NKT cell is a subset of lymphocytes that coexpress TCR along with typical surface receptors of natural killer cells and share the features of both innate and adaptive immune cells [106, 107]. NKT cell recognizes phospholipids or glycolipids that are presented by CD1d on the APC resulting in a rapid innate response through producing large amounts of Th1, Th2, and Th17 cytokines that then initiate most branches of the innate and adaptive immune systems [11]. NKT cell can be activated through multiple mechanisms, including direct activation by the recognition of CD1d on self- or microbial-derived lipids and indirect activation via cytokines, such as IL-12 and IL-18 [106]. Increased numbers of T cells expressing the NK marker CD161 are found in the inflamed lamina propria of ulcerative colitis patients, not in Crohn's disease. These cells can respond to CD1d with increased production of IL-13 [108]. In consistent with this, deficiency of CD1d and NKT cell prevents the development of oxazolone-induced murine colitis, resembling ulcerative colitis [109].

(5) Innate Immune Cytokine Pathways. In IBD, there is a markedly increased local production of various nonspecific inflammatory mediators, such as free radicals, leukotrienes, chemokines, and proinflammatory cytokines (e.g., TNF and TNF-related cytokines and IL-6 family of cytokines: IL-12, IL-23, IL-17, IL-18, and TGF-β) which follow the influx of inflammatory cells into the intestinal tissue [10]. Targeting those proinflammatory cytokines via monoclonal antibody or peptide-based virus-like particle vaccine strategy has been tested to be effective in the treatment of murine colitis and/or IBD patients [110–120].

(6) TNF and TNF-Related Cytokines (TL1A). TNF is a 17 kDa proinflammatory cytokine mainly secreted by monocytes, macrophages, and T cells that can impact proliferation, differentiation, and functions of multiple types of cells [121]. TNF has multiple biological functions, including stimulation of the acute phase response, cachexia, cytotoxicity, and potentially lethal shock [121]. TNF can also promote the production of IL-1 and IL-6, enhance the expression of adhesion molecules, and stimulate fibroblast proliferation [121]. TNF exists as a transmembrane protein, named membrane-bound TNF, where it is cleaved to a soluble form by TNF-converting enzyme [122]. Secreted TNF employs its biological functions via binding to two distinct cell surface receptors, the 55 kDa TNFR1 (p55) and the 75 kDa TNFR2 (p75) [122]. The binding of TNF to its receptors leads to activation of one of the three pathways: a death domain pathway results in apoptosis; another activates JNK, which is involved in cell differentiation and proliferation; and the third pathway activates NF-κB [123].

TNF has been implicated as an inflammatory mediator in many autoimmune diseases, such as rheumatoid arthritis, IBD, and multiple sclerosis [124]. Evidence has shown that the levels of TNF are increased in the intestinal mucosa, stool, and blood samples of IBD patients [123]. Moreover, the levels of TNF are correlated with clinical disease activity of Crohn's disease patients [123]. Several animal colitis models also demonstrate the role of TNF in the pathogenesis of intestinal inflammation. Anti-TNF monoclonal antibodies induce beneficial responses in some patients with IBD [123]. Anti-TNF blockade can not only promote the apoptosis of activated T cells but can also protect epithelial cells from apoptosis and tight junction compromise in the gastrointestinal epithelium [123].

More recently, TNF-like ligand 1A (TL1A) has been shown to be an important mediator of intestinal inflammation [125]. TL1A secretion is induced in APC by TLR ligands and FcR cross-linking, in CX_3CR1^+ mononuclear phagocytes by IBD-associated adherent microbiota and in T cells by TCR stimulation [126, 127]. The signaling pathway of TL1A is mediated through DR3, a TNF-family receptor that is mainly expressed on T cells [126]. TL1A synergistically increases the capacities of IL-12, IL-4, or IL-23 in the differentiation of Th1, Th2, and Th17 cells [125]. For instance, DR3 is selectively increased on Th17 cells, and TL1A enhances the proliferation of Th17 effector cells, whiles DCs derived from TL1A-deficient mice show a reduced capacity in promoting Th17 differentiation and proliferation [128].

The role of TL1A in the pathogenesis of IBD has been indicated [126]. The levels of TL1A are increased in IBD patients. Lamina propria $CD14^+$ macrophages in Crohn's disease patients produce a higher level of TL1A, and TL1A promotes alloantigen-induced IL-17 and IFN-γ production from T cells [129]. Furthermore, it has been demonstrated that polymorphisms in the TL1A gene (TNFSF15) are associated with increased risk for IBD [130]. Reducing the expression of TL1A/TNFSF15 on monocytes and macrophage is associated with susceptibility to IBD [131].

Consistent with studies of TL1A in IBD patients, animal studies also demonstrate a role for TL1A [132, 133]. TL1A promotes group 3 innate lymphoid cells to produce IL-22 which can protect acute colitis by promoting mucosal healing [127]; furthermore, TL1A induces OX40L expression on group 3 innate lymphoid cells which stimulates T cell activation and is required for T cell-driven murine colitis [127]. TL1A can impair the intestinal epithelial barrier and regulate tight junction protein expression via several pathways in DSS colitis [134]. TL1A may promote the differentiation of Th9 cells and enhance IL-9 secretion by upregulating the expression of TGF-β, IL-4, and PU.1, thus exacerbating DSS-induced murine colitis [135]. Administration of exogenous TL1A to mice with DSS-induced colitis upregulates both Th1 and Th17 responses in inflamed colonic tissue [129]. The expression level of TL1A affects the expansion and function of Treg in modulating murine colitis [136]. Administration of anti-TL1A antibodies partially ameliorates DSS-induced murine colitis, completely prevents the development of TNBS-induced murine colitis, and reduces the intestinal fibrosis in a chronic colitis model [132, 133, 137]. Taken together, TL1A is indicated in IBD pathogenesis, modulating the severity of intestinal inflammation and fibrosis.

(7) IL-6. There is accumulating evidence that IL-6 plays a pivotal role in the pathogenesis of IBD [122, 138]. Studies have shown that the levels of IL-6 are increased in the serum and the intestinal mucosa of patients with active Crohn's disease [139]. Moreover, the level of IL-6 is positively correlated with the clinical disease activity, frequency of relapses, and the severity of endoscopic and histopathological signs of inflammation in Crohn's disease [122, 140, 141]. Macrophages and T cells in lamina propria are likely to be the main producers of IL-6 [122, 142].

In intestinal inflammation, IL-6 exerts its effect through binding to the soluble form of its corresponding receptor (sIL-6R), not through the membrane-bound receptor for IL-6 (IL-6R) [122, 143]. The levels of sIL-6R and IL-6/sIL-6R complex are increased in the serum of IBD patients. Then the IL-6/sIL-6R complex activates gp130-positive T cells lacking IL-6R, leading to the translocation of STAT-3 and subsequent activation of transcription of the antiapoptotic genes Bcl-2 and Bcl-xl [144]. Therefore, this pathway confers resistance against apoptosis of intestinal T cells in IBD patients and in animal models of colitis as well [122]. A clinical trial shows that tocilizumab, a humanized anti-IL-6R monoclonal antibody, induces significantly higher clinical response rate in active Crohn's disease than that of the

placebo group [145]. It indicates that anti-IL-6R antibody may represent another therapeutic strategy for the management of IBD [122, 138].

2.4.2. Adaptive Immune System Dysregulation. Dysregulation of the innate immune system causes functional abnormalities of the adaptive immune system, which reveals many characteristics of chronic inflammatory processes in IBD [64].

$CD4^+$ Th cells play a critical role in orchestrating adaptive immune responses to various infections microbes [146, 147]. They are also involved in the pathogenesis of autoimmune and allergic diseases. Upon activation by T-cell receptor complex, naïve $CD4^+$ T cells may differentiate into different types of Th cells in the presence of different cytokines, including Th1, Th2, Th17, and Th9, and inducible regulatory T (iTreg) cells. They can be characterized by their special cytokine production profiles, transcription factors, and their functions [148]. Under the stimulation of IL-12, naïve $CD4^+$ T cells differentiate into Th1 cells, mainly producing IFN-γ and vital for protective immunity against intracellular viral and bacterial infections [148]. Under the stimulation of IL-4, naïve $CD4^+$ T cells differentiate into Th2 cells, producing IL-4, IL-5, IL-13, and IL-25, and are critical for eliminating extracellular parasites such as helminths [148]. In the presence of IL-4 and TGF-β, naïve $CD4^+$ T cells may differentiate into Th9 cells secreting IL-9, IL-10, and IL-21, which regulate allergic inflammation, autoimmune inflammation, and antitumor immunity [149]. TGF-β and IL-6 induce naïve $CD4^+$ T cells to differentiate into Th17 cells, producing IL-17, IL-17F, IL-21, and IL-22, important for controlling extracellular bacterial and fungi infections [148]. In the presence of TGF-β without IL-6, naïve $CD4^+$ T cells differentiate into iTreg cells. iTreg cells together with naturally occurring regulatory T (nTreg) cells are vital for the maintenance of immune tolerance and regulation of lymphocyte homeostasis, activation, and function [148]. Transcription factors also play important roles in the differentiation of Th cells and production of cytokines. The vital transcription factors of Th lineage are T-bet/Stat4 for Th1, GATA-3/Stat5 for Th2, PU.1/Smad/Stat6 for Th9, RORγt/Stat3 for Th17, and Foxp3/Stat5 for iTreg [148, 149].

It has been widely accepted that Crohn's disease is caused by an overly aggressive Th1 immune response and recently found excessive IL-23/Th17 pathway activation to bacterial antigens in genetically predisposed individuals [10, 52, 150–152]. The resulting infiltration of the bowel by granulocytes and macrophages leads to a release of enzymes, reactive oxygen intermediates, and proinflammatory cytokines, all of which cause discontinuous ulceration and full thickness bowel wall inflammation often including granulomas [153, 154]. On the contrary, ulcerative colitis is usually considered a "Th2-like" disease characterized by increased amounts of IL-5 and IL-13 [126]. Furthermore, Th17 and Treg are implicated in both Crohn's disease and ulcerative colitis, while Th9 cells are predominately involved in the pathogenesis of ulcerative colitis.

(1) Th1 Cells. A number of observations indicate Th1 cells are involved in the pathogenesis of Crohn's disease [126, 155]. T cells in the colonic lamina propria of Crohn's disease patients produce large amounts of IFN-γ and increase the expression of IL-12Rβ2, T-bet, and STAT4 [155]. IFN-γ-producing lamina propria lymphocytes are accumulated in the mucosa of patients. Macrophages in Crohn's disease patients produce high levels of IL-12 [155]. At the initial phase of Crohn's disease, mucosal T cells mount a typical Th1 response that resembles an acute infectious process and gradually disappear with progression to late Crohn's disease [156]. In addition, clinical responses are induced in a subcohort of patients with Crohn's disease treated with anti-IFN-γ antibody [126, 155]. In an animal colitis model, abrogation of IFN-γ in the $CD4^+$ $CD45RB^{hi}/Rag^{-/-}$ transfer model potently prevents the development of colitis; T-bet-deficient $CD4^+CD45RB^{hi}$ cell cannot induce the colitis in $Rag^{-/-}$ recipients [157]. These results indicate that Th1 plays a role in the pathogenesis of Crohn's disease.

(2) Th9 Cells. The role of Th9 and IL-9 in the pathogenesis of ulcerative colitis has been identified recently [158, 159]. The percentages of $PU.1^+IL\text{-}9^+Th9$ cells are significantly increased in colonic lamina propria of patients with ulcerative colitis, especially in patients with active ulcerative colitis [158]; IL-9 mRNA expression is also increased in inflamed colon samples from patients with ulcerative colitis [159]. Consistent with increased IL-9, IL-9R is found overexpressed on gut epithelial cells [158, 159]. Adoptive transfer of Th9 cells results in exaggerating intestinal inflammation of $RAG^{-/-}$ mice, while deficiency in PU.1 and IL-9 in T cells prevents oxazolone-induced murine colitis [158]. However, one recent study shows that the cytokine and colitis-inducing potential of Th9 is controlled by CD96 expression: adoptive transfer of $CD96^{low}$ Th9 into $Rag1^{-/-}$ mice induces severe intestinal inflammation, while transfer of $CD96^{high}$ Th9 does not cause colitis and blockade of CD96 can restore the expansion and inflammatory properties of $CD96^{high}$ Th9 cells [160], which indicates a functional heterogenicity of Th9 cells. Deficiency of IL-9 suppresses TNBS-induced murine colitis and reduces the number of $PU.1^+T$ cells in the lamina propria [161]. Further, it shows that IL-9 exacerbates murine intestinal inflammation through regulating intestinal tight junction, mucosa permeability, and mucosal wound healing [158, 161]. Administration of IL-9 blocking antibody improved oxazolone-induced murine colitis [158]. These results suggest the proinflammatory role of the Th9/IL-9 pathway in IBD, especially in ulcerative colitis.

(3) Th17 Cells. With the finding of the IL-23/Th17 pathway, more recently, studies highlight the role of this pathway in the pathogenesis of IBD [162]. Studies have shown that large amounts of IL-17-producing cells are mainly accumulated in the lamina propria of ulcerative colitis patients and in the submucosa and muscularis propria of Crohn's disease patients [163]. Flow cytometry analysis of mucosal cells also shows that the number of IL-17 producing T cells is increased in Crohn's disease patients than in normal controls, but some of these cells coexpress IFN-γ [164]. Gut biopsies grown ex vivo and LPMC cultured in vitro also produce high levels of IL-17 in IBD patients than in controls [165]. Other Th17

cytokines, such as IL-21, IL-22, and IL-23, are also increased in the inflamed tissue of IBD patients [166]. Also, GWAS suggest at least over 20 SNPs are linked to loci associated with Th17-regulating intracellular networks and signal transduction, indicating an important role of Th17 towards the pathogenesis of IBD, including IL-23R, IL-12B, JAK2, STAT3, and CCR6 [167].

The role of Th17 cells in the pathogenesis of IBD has also been evaluated in animal models. IL-17 is shown to be elevated in the IL-10 knockout and RAG1 knockout mouse models of IBD, respectively [168, 169]. Anti-IL-17 Ab ameliorates the severity of intestinal inflammation in RAG1 knockout mice reconstituted with IL-10 knockout $CD4^+$ T cells [169]. Further, deficiency of IL-17R (receptor) prevents the development of TNBS-induced murine colitis, including improving body weight loss, decreasing productions of IL-6 and local macrophage inflammatory protein-2, ameliorating colonic inflammation, and reducing tissue myeloperoxidase activity [170]. IL-17F-deficiency improves the development of DSS-induced murine colitis, whereas IL-17-deficiency exaggerates the development of DSS-induced murine colitis, indicating that IL-17F rather than IL-17A is important in sustaining DSS colitis [171]. This has been shown to be important clinically: monoclonal antibody against IL-17A, secukinumab, is ineffective in treating Crohn's disease but causes a higher rate of adverse events and increases disease severity [48, 172].

Enhanced production of IL-17 in the gut is also found in the C3H/HeSnJ SCID transfer colitis model, and adoptive transfer of IL-17-producing T cells to SCID recipients leads to severe colitis [173]. In the model of $CD8^+$ T cell-dependent colitis, it shows that a single adoptive transfer of naïve $CD8^+$ T cells into syngeneic RAG-deficient mice results in severe colitis, with rapid spontaneous proliferation of these $CD8^+$ T cells in MLN [174]. These $CD8^+$ T cells in the MLN coexpress IL-17 and IFN-γ. Also, adoptive transfer of naïve $CD8^+$ T cells isolated from either IL-17- or IFN-γ-deficient mice induced a remarkably less severe colitis, suggesting IL-17 and IFN-γ can cooperate to cause colitis in this model [174].

A role for IL-21 in the murine colitis is also indicated [175]. DSS colitis and TNBS-relapsing colitis are significantly decreased in IL-21-deficient mice, which is associated with reduced expression of Th17 cell-related genes (IL-17, IL-17F, and RORγt) in the colon tissue [175]. Furthermore, blockade of IL-21 using a specific IL-21R-fusion protein improves intestinal inflammation and downregulates Th17 responses during the course of DSS colitis [175]. Taken together, these data indicate that the Th17 pathway plays an important role in the pathogenesis of IBD.

(4) Treg Cells. The GALT is believed to be the primary site where naïve conventional $CD4^+$ T cells convert to iTreg after exposure to oral antigens in a lymphogenic environment [176, 177]. This conversion is dependent on TGF-β and retinoic acid producing $CD103^+$ DCs in the GALT. It has been supposed that nTreg mainly protects against autoimmunity in situ, but iTreg primarily inhibits immune responses against environmental and food antigens in the gut [178].

The dysfunction of Treg in IBD is usually believed to be due to the defective numbers of Treg or their suppressive function which cannot control the intestinal inflammation [177]. For instance, patients with a *FOXP3* gene mutation have defective Treg and always suffer from intestinal inflammation [179]. When compared with healthy controls, the numbers of Treg are decreased in peripheral blood but increased in inflamed colons of patients with IBD [177]. Also, the ratio of Treg to Th17 in peripheral blood is reduced in IBD patients when compared with controls [180]. However, the increased number of Treg in the colon lamina propria of IBD patients is still lower than that of patients with infectious enteritis or diverticulitis [181]. Treg isolated from inflamed colon or peripheral blood maintained normal cell contact-dependent, cytokine-independent suppressive capacity in vitro [181–183]. But effector T cells from IBD patients display relative resistance to Treg-mediated suppression, because effector T cells express high levels of Smad7 which is an inhibitor of the TGF-β signaling pathway [184]. These data indicate that Treg dysfunction might be due to an extrinsic milieu of activated cells that are resistant to suppression [177].

Studies on animal models of colitis also demonstrate the role of Treg in the control of intestinal inflammation [177]. Adoptive transfer of naïve T effector cells in the absence of Treg into SCID mice leads to colitis, whereas cotransfer of T effector cells and Treg does not induce colitis [177]. Furthermore, adoptive transfer of $CD4^+CD25^+$ Treg cures established $CD4^+CD45RB^{hi}$ transfer colitis [185]. In this model, Treg are capable of suppressing colonic inflammation by downregulating Th1 and Th17 responses depending on the presence of IL-10 and TGF-β [186, 187].

More recently, a new type of iTreg, called iTR35, has been identified which mainly produces the suppressive cytokine IL-35, not IL-10 or TGF-β [188, 189]. Adoptive transfer of IL-35-deficient Treg cannot cure $CD4^+CD45RB^{hi}$-induced murine colitis [189], whereas adoptive transfer of iTR35 generated in vitro can significantly improve the intestinal inflammation [188]. IL-35 also shows strong function in controlling intestinal inflammation. Administration of recombinant IL-35 significantly reduces the development of several forms of experimental colitis and reduces levels of cytokines of Th1 and Th17 cells [190].

Both iTreg and Th17 differentiations require TGF-β which induces Foxp3 and RORγt, so there is a fine balance existing between these two types of cells under the control of many factors [11]. For instance, low concentrations of TGF-β together with IL-6 and IL-21 induce the expression of IL-23R and promote the differentiation of Th17 cells [11]. On the contrary, high concentrations of TGF-β inhibit the expression of IL-23R and promote the development of iTreg [11]. Foxp3 directly interacts with RORγt to suppress its function, but IL-6, IL-21, and IL-23 downregulate the Foxp3-mediated suppression of RORγt [11, 191]. On the other hand, there is a close relationship between these two types of cells. Recent data have documented that memory Treg can convert into Th17 cells under inflammatory conditions, in which IL-1 is the key molecule in promoting conversion [192, 193]. A hybrid subpopulation of memory Treg

coexpressing Foxp3 and RORγt has been found which exert suppressive functions but concomitantly secret IL-17 ex vivo [194–196]. In the presence of IL-1, IL-2, IL-23, and TGF-β, human Th17 cells preferentially differentiate from natural naïve regulatory cells, rather than from conventional $CD4^{+}CD25^{-}$ naïve T cells [197]. Together, these findings illustrate that Treg deficiency may be associated with the pathogenesis of IBD.

3. Summary

IBD is a chronic and life-threating disease characterized by episodes of intestinal inflammation. Substantial progress in the past several decades has greatly increased our understanding of the pathophysiology of IBD especially in the field of immunology and increased the opportunities to explore other therapeutic pathways/targets. However, there are still unknown questions on pathogenesis, disease behavior, and intestinal inflammation drivers in different IBD patient subgroups which require further exploration. Associations between proinflammatory and anti-inflammatory immune cells and molecules and various genetic susceptibility, intestinal microbiota, and environmental factors (e.g., diet, smoking, and physiological stress) are continuously being evaluated to allow for a comprehensive understanding of pathogenesis of IBD. These investigations are critical not only for developing novel treatment strategies including the selection of the right targets to optimally manage IBD such as fecal microbiota transplantation, antisense oligonucleotide targeting proinflammatory molecules (such as NF-κB and Intercellular adhesion molecule 1), and monoclonal antibody/biologics to neutralize proinflammatory cytokines (such as TNF and IL-12/IL-23p40) but also for identifying biomarkers for diagnosis, monitoring, and prognostics or prediction of disease progress and treatment outcome. With this knowledge, we may have the ability to develop novel personalized treatments for IBD patients.

Abbreviations

Ab: Antibody
APC: Antigen-presenting cells
DC: Dendritic cells
DSS: Dextran sodium sulfate
IBD: Inflammatory bowel disease
IEC: Intestinal epithelial cells
IL: Interleukin
GALT: Gut-associated lymphoid tissue
GWAS: Genome-wide association studies
MDSC: Myeloid-derived suppressor cells
MLN: Mesenteric lymph nodes
NOD2: Nucleotide-binding oligomerization domain 2
NLR: Nod-like receptor
NF-κB: Nuclear factor kappa B
NKT: Natural killer T
PAMPs: Pathogen-associated molecular patterns
PRRs: Pattern recognition receptors
SNP: Single-nucleotide polymorphisms
TGF-β: Transforming growth factor beta
Th: T helper cells
Treg: Regulatory T cell
TLR: Toll-like receptor
TL1A: TNF-like ligand 1A
TNBS: Trinitrobenzenesulfonic acid
TSLP: Thymic stromal lymphopoietin.

Conflicts of Interest

The author declares no financial conflict of interest.

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Review Article

Programmed Cell Death Pathways in the Pathogenesis of Systemic Lupus Erythematosus

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Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disease characterized by excessive inflammatory and immune responses and tissue damage. Increasing evidence has demonstrated the important role of programmed cell death in SLE pathogenesis. When apoptosis encounters with defective clearance, accumulated apoptotic cells lead to secondary necrosis. Different forms of lytic cell death, including secondary necrosis after apoptosis, NETosis, necroptosis, and pyroptosis, contribute to the release of damage-associated molecular patterns (DAMPs) and autoantigens, resulting in triggering immunity and tissue damage in SLE. However, the role of autophagy in SLE pathogenesis is in dispute. This review briefly discusses different forms of programmed cell death pathways and lay particular emphasis on inflammatory cell death pathways such as NETosis, pyroptosis, and necroptosis and their roles in the inflammatory and immune responses in SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is a highly heterogeneous autoimmune disease that affects almost all organs and tissues [1]. It is characterized by production of abundant autoantibodies, deposition of massive immune complexes, upregulation of inflammatory and immune responses, and damage of different tissues [2]. Disruption of immune tolerance and sustained generation of autoantibodies against nuclear autoantigens are two major hallmarks of SLE. Since the first programmed cell death, apoptosis, described in 1972 by Kerr and his two colleagues [3], other programmed cell death pathways have been defined and intensively investigated, including NETosis, necroptosis, pyroptosis, and autophagy [4, 5]. Indeed, dysregulated cell death in combination with defective clearance of dying cells has been suggested to contribute to the release of damage-associated molecular patterns (DAMPs), amplification of inflammatory and immune responses, production and release of autoantigens, and tissue damage in SLE [6–8]. In this review, we discuss various forms of programmed cell death pathways with particular emphasis on inflammatory cell death such as NETosis, pyroptosis, and necroptosis and their consequences in the inflammatory and immune responses in SLE. Further studies on the roles of these distinct cell death pathways will deepen our comprehension of SLE pathogenesis and promote the development of therapeutic strategies for SLE.

2. Apoptosis and Secondary Necrosis after Apoptosis

In 2008, we proposed a cell death recognition model for the immune system that the consequences of immune responses, tolerance or adaptive immune responses, are dependent on the ways of cell death [9]. Indeed, necrosis actively initiates immune response while apoptosis induces immune tolerance [10, 11]. Apoptosis is a form of programmed cell death that functions to clear aged, diseased, or obsolete cells. The principal features of apoptosis are cellular shrinkage, membrane blebbing, and chromatin condensation. Two distinct apoptotic signaling pathways, intrinsic and extrinsic pathways, have been identified. The extrinsic pathway can be activated by

death factors, including FasL, TNF-α, and TRAIL, while the intrinsic pathway is triggered by DNA damage, endoplasmic reticulum stress, cytokine withdrawal, or lack of nutrient support. Both apoptotic pathways require activation of caspase family members, caspase-8 and caspase-9 for the extrinsic pathway and the intrinsic pathway, respectively. Ultimately, the pro-caspase-3 is cleaved into caspase-3 and activated, resulting in the cleavage of the cellular substrates and eventual apoptosis [12]. Apoptotic cells release "find me" signals (such as adenosine triphosphate (ATP) and uridine triphosphate (UTP)) and express "eat me" molecules (including phosphatidylserine (PS), phosphatidylcholine (PC), and phosphatidylethanolamine (PE)) on the cell membrane, recruiting phagocytes readily to migrate towards and promptly engulf apoptotic cells before their membrane integrity is lost. Thus, cellular contents of apoptotic cells, especially the nuclear contents, are not released into the extracellular space. Recently, caspase-3 has been found to inhibit the production of type I interferon by the cleavage of cGAS, keeping apoptosis immunologically silent [13]. In addition, the immunosuppressive cytokines such as TGF-β and IL-10 are released during the phagocytosis of apoptotic cells [14]. And importantly, T cell activation could be inhibited by apoptotic cells in an in vitro experiment [15]. In a mouse bone marrow transplant model, intravenous infusion of apoptotic cells resulted in the expansion of regulatory T cells [16]. Therefore, apoptosis is generally considered as not only a noninflammatory but also a dominant immune tolerance-inducing form of cell death. However, accelerated apoptosis encountered with defective clearance in SLE may result in massive accumulation of apoptotic cells that undergo secondary necrosis [17]. Loss of plasma membrane integrity and release of the cellular contents by secondary necrotic cells can trigger autoimmunity and contribute to the development of SLE [18].

Glomerular apoptotic nucleosomes were targeted by anti-dsDNA autoantibodies in human lupus nephritis [19]. Apoptotic features were also detected in epidermal keratinocytes of skin biopsies from chronic cutaneous lupus erythematosus [20]. In SLE patients, apoptotic cells diffusely accumulated in the germinal centers (GCs) of the lymph nodes [21]. Moreover, downregulation of miRNA-98 induced apoptosis in $CD4^+$ T cells from SLE patients through the Fas-caspase axis [22]. Apoptotic T cells increased in SLE patients and showed a positive correlation with the SLE disease activity index [23]. In addition to T cells, excessive apoptosis has also been observed in phagocytes which are important for apoptotic cell clearance. SLE sera could induce apoptosis in monocytes and lymphocytes [24, 25]. Lupus T cells could also induce monocyte apoptosis via the apoptotic ligands [26]. Consistent with these findings, increased monocyte/macrophage apoptosis occurred in SLE patients and contributed to autoantibody formation and tissue damage [27]. Similarly, increased apoptotic neutrophils were detected in SLE patients and positively related with disease activity [28, 29]. In summary, patients with SLE show high levels of apoptotic cells that are at least partly attributed to the massive apoptosis in tissue cells or in phagocytes.

Apoptotic cells must be engulfed efficiently by phagocytes to prevent the release of cell ingredients that may activate the immune system. However, impaired clearance of apoptotic cells in SLE is thought to disrupt the balance of the immune system. Efficient clearance of apoptotic cells mainly involves the recognition and engulfment by professional phagocytes. Indeed, apoptotic cell receptors and bridging molecules related to the recognition and engulfment have been found to be defective in SLE. Tyro-3, Axl, and Mer (TAM) receptor protein tyrosine kinases are important receptors on phagocytes for the clearance of apoptotic cells by their recognition of ligands that are bound to PS exposed on the membrane of apoptotic cells [30, 31]. Mer-deficient mice displayed accumulation of apoptotic and secondary necrotic cells in peripheral tissues and developed SLE-like autoimmunity [32]. Moreover, mutant mice that lack TAM receptors developed a severe lymphoproliferative disorder accompanied by broad-spectrum autoimmunity and high titers of autoantibodies [33]. Prompt recognition and efficient clearance of apoptotic cells also require the opsonization mediated by bridging molecules, such as C-reactive protein (CRP) as well as complement C1q. In response to IL-6, the short pentraxin CRP is produced in the liver and generally binds to polysaccharides and phosphocholine exposed on apoptotic cells. CRP not only promotes the classical pathway of complement activation but also increases opsonization and phagocytosis of apoptotic cells by macrophages [34, 35]. SLE patients showed elevated levels of anti-CRP antibodies in association with disease activity and renal involvement [36–38]. Treatment with CRP protected mice from lupus nephritis and enhanced animal survival [39]. As an opsonin, the complement protein C1q can bind to apoptotic cells and further promote removal of the apoptotic cells. SLE patients showed increased anti-C1q antibodies that were positively correlated with nephritis, dermatitis, hypocomplementemia, anti-dsDNA antibodies, and circulating immune complexes [40, 41]. Accumulation of renal apoptotic cells, higher titers of autoantibodies, and glomerulonephritis were observed in C1q-deficient mice [42]. In addition to defects in recognition of apoptotic cells, SLE also has impaired ability to ingest the apoptotic cells. Since tingible body macrophages containing engulfed apoptotic nuclei were reduced in the lymph nodes of SLE patients, apoptotic cells that could not be engulfed accumulated in the germinal centers of the lymph nodes [21]. Consequently, the noningested apoptotic materials directly bound to follicular dendritic cells and may, therefore, serve as survival signals for autoreactive B cells [21]. Indeed, macrophages from SLE patients exhibited an impaired ability to phagocytose apoptotic cells [29, 43].

Owing to the impaired clearance of apoptotic cells, accumulated apoptotic cells may undergo secondary necrosis by which cellular components are released. Necrosis is characterized by loss of plasma membrane integrity, exposure of autoantigens, and release of DAMPs and therefore induces autoimmunity. Autoantibodies promoted the uptake of secondarily necrotic cell-derived material by phagocytes, accompanied by secretion of huge amounts of inflammatory cytokines [44]. Furthermore, immune complexes that

contained nucleic acid released by necrotic and late apoptotic cells induced production of IFN-α in plasmacytoid dendritic cells (pDCs) [45]. Collectively, necrosis secondary to apoptosis may be involved in the pathogenesis and development of SLE through releasing DAMPs as well as autoantigens.

3. NETosis

A hallmark feature of SLE is the presence of antibodies against various nuclear antigens, especially anti-double-stranded (ds) DNA antibodies. NETosis, a specialized cell death form in neutrophil, is considered as a major source of modified and/or externalized autoantigens in SLE [46]. In particular, nuclear material released during the process of NETosis seems to be more immunogenic than apoptotic material. Infectious or sterile stimuli including microcrystals, inflammatory cytokines, activated platelets, autoantibodies, and immune complexes result in NETosis. The neutrophils extrude large web-like structures of decondensed chromatin decorated with intracellular components, including neutrophil elastase (NE), myeloperoxidase (MPO), high mobility group protein B1 (HMGB1), proteinase 3 (PR3), and LL-37 [47]. Several pathways are involved in the process of NETosis [48]. Classically, the initiation of suicidal NETosis requires calcium release from the endoplasmic reticulum, the protein kinase C activation, and the assembly of the nicotinamide adenine dinucleotide phosphate- (NADPH-) oxidase complex. Then, the production of reactive oxygen species (ROS) mediated by the NADPH-oxidase complex activates the enzyme protein-arginine deiminase 4 (PAD4) that mediates the histone citrullination and promotes chromatin decondensation. In addition, the translocation of NE and MPO to the nucleus also contributes to the further unfolding of chromatin and disruption of the nuclear membrane [49]. Finally, the decondensed chromatin coated with cytoplasmic components is released to the extracellular space, forming neutrophil extracellular traps (NETs). Of note, monosodium urate crystals directly interact with lysosomes to induce NETosis in a NADPH oxidase-independent manner, with chromatin decondensation mediated by NE [50]. And thus, the second important form of NETosis dependent on autophagy, rather than NADPH oxidase, has drawn peoples' attention. Several inhibitors of autophagy could block autophagy-dependent NETosis stimulated by PMA or LPS [51, 52]. Differently, vital NETosis can be induced by the activation of TLRs and the C3 complement receptor and the interaction between platelets and β2 integrin in a ROS-independent manner [53]. As in conventional suicidal NETosis, NE is also moved to the nucleus to facilitate decondensation of chromatin and disruption of nuclear envelope in vital NETosis. However, the protein-decorated chromatin is released through nuclear envelope blebbing and vesicular export, and the neutrophil remains alive and retains several conventional functions [54–57]. Recently, a novel form of NETosis dependent on mitochondrial ROS production has been reported, in which mitochondrial DNA instead of nuclear DNA is released. The mitochondrial NETosis can be induced by C5a, lipopolysaccharide, or ribonucleoprotein immune complexes [58, 59].

NETosis leads to the exposure of autoantigens to the immune system and the release of DAMPs to activate the immune responses. Native and oxidized DNA bound to NETs can activate pDCs to produce higher levels of IFN in a Toll-like receptor 9-dependent or a STING-dependent manner, respectively [58, 60, 61]. NET-derived LL-37-DNA complexes can also activate B cells to promote the production of antibodies [62]. Additionally, NETs and LL-37 can activate NLRP3 inflammasomes, which results in the secretion of mature IL-1β and IL-18, further exacerbating the inflammatory responses. In turn, IL-18 can induce NETosis in human neutrophils, creating a proinflammatory feed-forward loop that may result in disease flares [63]. An additional immunogenic mechanism that links NETosis to autoimmune diseases is the activation of complement system [64]. Furthermore, MMP-9 contained in NETs activates endothelial MMP-2, resulting in the endothelial dysfunction [65]. NETs may contribute to SLE-associated cardiovascular disease through oxidation of high-density lipoprotein (HDL) [66]. Tissue factor-bearing and IL-17A-bearing NETs promote thrombin production and the fibrotic potential of cultured skin fibroblasts in SLE [67]. And thrombin directly cleaves pro-IL-1α and activates the immune system [68]. Based on these findings, NETosis may trigger autoimmunity and cause tissue damage in SLE.

SLE patients display a distinct subset of proinflammatory neutrophils, named low-density granulocytes (LDGs), in the peripheral blood mononuclear cell (PBMC) fraction [69]. LDGs show enhanced ability to spontaneously undergo NETosis [70]. Compared with normal-density neutrophils, LDGs exhibit enhanced capacity to secrete higher levels of proinflammatory cytokines, including TNF-α, IL-8, and IL-6 [71]. Functional studies of LDGs also demonstrated their enhanced capability of synthesizing IFN [70, 71]. In addition to the spontaneous NETosis, various stimuli can accelerate NETosis in SLE, including circulating microparticles, immune complexes, type I IFNs, and autoantibodies [60, 61, 72, 73]. In particular, IL-18 released by pyroptosis can also induce NETosis [63]. Meanwhile, SLE patients show decreased ability to degrade NETs that is closely associated with clinical manifestations in SLE [74]. On the one hand, C1q in SLE was found to inhibit degradation of NETs through a direct inhibition of DNase I [64, 75]. On the other hand, NET-bound autoantibodies also inhibit NET degradation by preventing the access of DNase I to NETs [75]. Indeed, high levels of NET deposition were detected in the skin and kidney of SLE patients and lupus-prone mice [73, 76]. The link between NET formation and drug-induced lupus erythematosus further emphasizes the importance of NETosis in SLE pathogenesis. Some specific drugs (for example, hydralazine and procainamide) have been reported to induce lupus-like symptoms through induction of enhanced NET formation [77]. Thus, enhanced NETosis combined with defective clearance of NETs may lead to persistent and prolonged existence of NETs in SLE. It is worth noting that the presence of autoantibodies such as antinuclear antibodies and anti-dsDNA antibodies may be a response to the nuclear material released from NETosis in patients with SLE [78].

Some animal studies provided further evidence for the role of NETosis in the pathogenesis and development of SLE. Inhibition of peptidylarginine deiminase blocked NETosis and protected against lupus-related damage to the vasculature, kidneys, and skin in various lupus-prone mouse models [76, 79]. MRL/lpr mice treated with a Janus kinase inhibitor tofacitinib showed reduced NET formation, significant reduction of lupus activity, and improvement in SLE-associated vascular damage [80]. Recombinant milk fat globule-EGF factor 8 (MFG-E8) reduced early inflammatory responses and attenuated tissue damage in pristane-induced lupus mice by inhibiting neutrophil migration and NETosis [81]. In addition, our results showed that polydatin significantly inhibited NETosis through downregulation of ROS expression, resulting in amelioration of lupus-like manifestations in both pristane-induced lupus mice and MRL/lpr mice [82]. Collectively, these researches in combination with previous studies provide a proof of concept that NETosis may be strongly implicated in the pathogenesis and development of SLE.

4. Pyroptosis

Pyroptosis is a lytic and inflammatory form of programmed cell death induced by a variety of danger signals. It is characterized by gasdermin family-mediated pore formation on the plasma membrane, cell swelling, and eventual lysis, followed by release of cellular contents, especially inflammatory mediators IL-1β and IL-18 [83]. Although pyroptosis was first described in macrophage infected with Shigella flexneri in 1992 [84], it can also occur in monocytes, dendritic cells, $CD4^+$ T cells, hepatocytes, vascular endothelial cells (VECs), tubular epithelial cells, and many other cell types [85–89]. To date, three pathways have been reported to participate in pyroptosis, including the caspase-1-dependent canonical pathway, the noncanonical pathway involving caspase-4,5 (for human) or caspase-11 (for mouse), and the newly discovered caspase-3-dependent pathway. In the caspase-1-dependent pathway, the canonical inflammasome sensors, including NLRP1b, NLRP3, NLRC4, AIM2, or Pyrin, are activated by the recognition of pathogen-associated molecular patterns (PAMPs) or DAMPs [90]. The activation triggers the assembly of the inflammasome sensor, the inflammasome adapter ASC, and pro-caspase-1, resulting in the self-cleavage of pro-caspase-1 into activated caspase-1. On the one hand, activated caspase-1 directly cleaves the precursor cytokines pro-IL-1β and pro-IL-18 into mature inflammatory cytokines IL-1β and IL-18, respectively. On the other hand, activated caspase-1 directly cleaves gasdermin D (GSDMD) and releases active N-terminus subunit that binds to phosphoinositides in the plasma membrane and forms pore (about 10-14 nM in size). The pore formation results in the loss of osmotic potential, cytoplasmic swelling, release of inflammatory factors, and finally cell explosion. In the noncanonical pathway, caspase-4,5 or caspase-11 in the host cytoplasm can directly recognize lipopolysaccharide (LPS) from gram-negative bacterial and then cleave GSDMD, leading to host cell pyroptosis [91]. More recently, caspase-3, conventionally recognized as the apoptotic executioner caspase, has also been reported to cleave GSDME and initiate pyroptosis [92]. This implies that excessive apoptotic cells with activated caspase-3 are able to proceed to pyroptosis.

Numerous studies have suggested that pyroptosis can potentiate the inflammatory reaction and enhance adaptive immune responses by the release of various cellular contents. IL-1β and IL-18, the most important inflammatory cytokines released by pyroptotic cells, can trigger a secondary inflammatory response in neighboring cells. IL-1β can activate the NF-κB pathway through the IL-1 receptor, leading to the generation of inflammatory cytokines including cyclooxygenase-2 (COX-2) and IFN-γ [93]. Meanwhile, IL-18 signals can induce increased production of IL-1α, IL-6, and IL-8 primarily via the MAPK p38 pathway [93]. In addition, mature IL-18 can potentiate the cytolytic activity of natural killer cells and Th17 cells and, in combination with other cytokines, also promote polarization of T cells towards Th1 or Th2 [94]. Importantly, activated IL-18 can stimulate neighboring neutrophils to undergo NETosis, further amplifying the inflammatory and immune responses [63, 95]. In addition to the release of the inflammatory cytokines, pyroptotic cells also release HMGB1 which can serve as a kind of DAMPs to induce the production of proinflammatory cytokines, to promote the maturation and migration of dendritic cell and the activation of B cells, and also to trigger pyroptosis of macrophages [96, 97]. The pyroptotic cells release large quantities of ATP that can also induce the activation of NLRP3 inflammasome, resulting in the release of proinflammatory cytokines [98, 99]. In neutrophils, GSDMD is activated by neutrophil proteases and then NETosis promoted in a feed-forward loop [100]. In the later stage of pyroptosis, pore formation disrupts the osmotic potential and eventually leads to the cell lysis, followed by the release of condensed but intact nucleus. The intact nucleus may provide a source of autoantigens for the generation of antinuclear antibodies [88].

Increasing studies strongly suggest the important role of pyroptosis in the pathogenesis and progression of SLE. NLRP3 inflammasome, one of the inflammasome sensors mediating pyroptosis, was found hyperactivated in patients with SLE and lupus nephritis (LN) [101, 102]. In the presence of anti-dsDNA antibodies, dsDNA can induce the activation of NLRP3 inflammasome. Similarly, NLRP3 inflammasome activation can also be triggered by the interaction of U1-small nuclear ribonucleoprotein (U1-snRNP) and anti-U1-snRNP antibodies [103–105]. By binding extracellular ATP, P2X7 receptor can mediate the activation of NLRP3 inflammasome, causing the secretion of IL-18 and IL-1β. Indeed, suppression of P2X7 receptor by its selective antagonist brilliant blue G reduced the severity of nephritis and improved the survival of MRL/lpr mice by inhibiting the NLRP3 inflammation activation and decreasing the production of proinflammatory cytokines [106, 107]. In lupus-prone mice, inhibition of NLRP3 with MCC950 ameliorated proteinuria and renal histologic lesions [102]. Fu et.al also demonstrated that NZB/W F1 mice treated with pim-1 inhibitor AZD1208 showed a suppression of NLRP3 inflammasome activation and a significant reduction in the severity of lupus nephritis [108]. The expression of AIM2, another

inflammasome sensing double-stranded nucleic acids in the cytoplasm, was positively correlated with disease severity in patients with SLE and lupus-prone mouse model. Furthermore, reduction of AIM2 expression markedly alleviated lupus-like symptoms through inhibiting macrophage activation and inflammatory responses in DNA-induced lupus mice [109]. In addition to inflammasomes, the role of caspase-1 was also investigated in lupus. Mice lacking caspase-1 were protected against lupus-like features in pristane-induced lupus model [110]. Elevated levels of serum IL-18 were observed in SLE patients, and the levels were significantly correlated with severity of renal involvement and disease activity [111, 112]. Furthermore, high levels of HMGB1 were not only presented in the blood but also in the kidney biopsy samples of SLE patients [113, 114]. And the serum levels of HMGB1 were correlated with SLE disease activity [115]. Indeed, anti-HMGB1antibodies also occur in SLE patients [116, 117]. Renal tubular cell pyroptosis can be induced by the miR-155/FoxO3a pathway [118]. Importantly, an animal experiment has demonstrated that piperine significantly reduced the pyroptosis of tubular epithelial cells, leading to the suppression of LN development in pristane-induced lupus mice [89]. Interestingly, vascular endothelial cells can also be induced to undergo pyroptosis through the miR-125a-5p/TET2 pathway, perhaps explaining one kind of mechanisms in tissue damage in SLE [119]. Surprisingly, caspase-3, generally believed an executioner in apoptosis process, can also cleave GSDME to induce pyroptosis [92]. The findings suggest that in SLE enhanced apoptotic cells come across with defective clearance can undergo secondary necrosis/pyroptosis, resulting in autoimmunity that further drive SLE pathogenesis. To our knowledge, gasdermin family members are essential for pyroptosis, but there has been no evidence for the presence of GSDMD/GSMDE in SLE patients.

5. Necroptosis

Necroptosis is a caspase-independent form of programmed necrotic cell death characterized by receptor-interacting protein kinase 3- (RIPK3-) mediated phosphorylation of mixed lineage kinase domain-like protein (MLKL) [120]. It is triggered by TNFR, TLR3, TLR4, IFNRs, or Z-DNA binding protein 1 (ZBP1, also known as DAI) [121]. DAI acts as a RIP homotypic interaction motif- (RHIM-) containing protein and can directly promote RIPK3 activation [121]. Consistently, the activation by other triggers can promote the association of RIPK1 with RIPK3 by RHIM-RHIM domain interactions, leading to the activation of RIPK3 [122]. RIPK3 activation further promotes the phosphorylation of MLKL, and then, the phosphorylated MLKL translocates to the plasma membrane and disturbs the cell integrity, leading to the release of cellular contents and exposure of DAMPs [123–125].

Certain evidence has demonstrated that necroptotic signaling could also induce the NLRP3 inflammasome activation and eventual pyroptosis, further amplifying the inflammatory response. RIPK3 that is essential for necroptosis can promote the NLRP3 inflammasome activation and IL-1β inflammatory responses [126]. Similarly, MLKL induces the activation of the NLRP3 inflammasome in a cell-intrinsic manner, resulting in IL-1β release [127, 128]. Furthermore, ATP released by necroptotic cells binds to the receptor P2X7, which can activate the NLRP3 inflammasome and generate mature IL-1β [121, 129]. In addition, mitochondria released by cells undergoing TNF-α-induced necroptosis can be engulfed by human macrophages and dendritic cells, leading to the secretion of macrophage cytokines and maturation of dendritic cell [130]. Both IL-33 and IL-1α belong to the IL-1 family and have the potential to induce inflammatory responses and to amplify immune responses [131]. High levels of IL-1α and IL-33 were found in the serum of RIPK1-deficient mice, but dependent on the presence of RIPK3 and MLKL [132]. Furthermore, elevated expression of LL-33 was observed in necroptotic epidermal keratinocytes [133]. In vivo, treatment of LPS or poly(I:C) combined with the pan-caspase inhibitor zVAD-fmk leads to necroptotic cell death of macrophages accompanied by secretion of proinflammatory cytokines (including IL-6, TNF, MCP-1, and IFN-γ) [134]. In addition, type I interferon can promote the assembly of RIPK1 and RIPK3, causing necroptosis of macrophages and release of proinflammatory mediators (including IL-1α, IL-1β, and IFN-γ) [135]. MicroRNA-500a-3p suppressed necroptosis in renal epithelial cells and decreased the production of proinflammatory cytokines TNF-α and IL-8 by targeting MLKL [136]. Similarly, by targeting of RIP3, miRNA-223-3p inhibited cell necroptosis and reduced inflammatory responses [137]. Necroptotic cells also release HMGB1 [129]. Together, these results indicated that necroptosis can potentiate inflammatory responses through the release of proinflammatory mediators.

"Natural" necroptosis mostly occurs in infectious pathological conditions. Recently, some studies have suggested that necroptosis may be implicated in the pathogenesis and development of SLE. Importantly, necroptosis was observed in B cells from SLE patients [138]. The finding that constitutive IFN signaling contributes to the steady-state expression of MLKL and the initiation of necroptosis provides proof of concept that elevated IFN signaling in SLE augments necroptosis, causing tissue damage [139]. As mentioned above, necroptosis can promote inflammatory responses by the release of DAMPs. These findings may provide certain evidence for the role of necroptosis in the pathogenesis and development of SLE.

6. Autophagy

Autophagy is a highly conserved lysosome-mediated catabolic and homeostatic process for digesting unnecessary or dysfunctional cellular organelles and recycling nutrients [140]. Based on the modes of cargo delivering to the lysosome, four most common forms of autophagy, macroautophagy, microautophagy, chaperone-mediated autophagy (CMA), and noncanonical autophagy, have been identified [141]. The best known macroautophagy is characterized by the fusion of the autophagosome with lysosomes, the formation of membrane-delimited autolysosome, and degradation

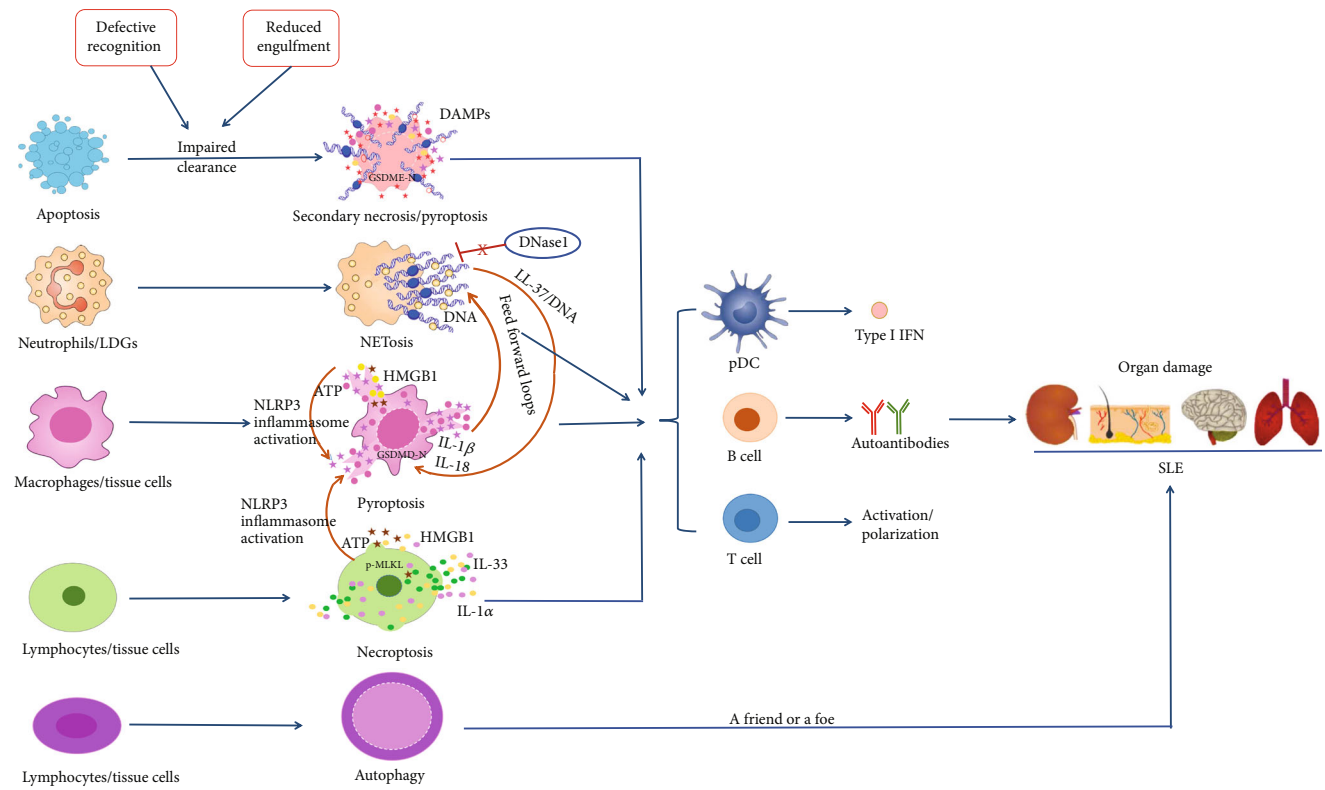

Figure 1: Programmed cell death pathways in the pathogenesis of systemic lupus erythematosus. Cell death recognition model for the immune system: consequence of immune responses, tolerance or adaptive immune responses, are dependent on the ways of cell death. Apoptosis results in immune tolerance, while lytic cell death (such as secondary necrosis, NETosis, pyroptosis, and necroptosis) contributes to the release of damage-associated molecular patterns (DAMPs), amplification of inflammatory and immune responses, production and release of autoantigens, and tissue damage in SLE.

of the cargoes such as proteins and invading microorganisms [142]. In terms of microautophagy, cytoplasmic entities targeted for degradation are captured by invaginations of the lysosomal membrane and then degraded. Differently, during the CMA, cytosolic proteins directly move to the lysosomal lumen by the protein translocation complex, independently of vesicles or membrane invaginations [143].

Increasing studies focus on the link between autophagy and autoimmune diseases, especially SLE. Genome-wide association studies (GWAS) have identified that several autophagy-related genes are correlated with susceptibility of SLE, including ATG5, CDKN1B, DRAM1, CLEC16A, and ATG16L2 [144]. In further studies, other autophagy-related genes including ATG7, IRGM, LRRK2, MAP1LC3B, MTMR3, and APOL1 were also found to be related with the susceptibility of SLE [145–147]. Collectively, these observations revealed a strong relationship between autophagy and SLE susceptibility. In addition, environmental factors contributing to the pathogenesis of SLE have been linked to autophagy. DNA damage mediated by ultraviolet radiation (UV) resulted in the destruction of important proteins, such as ULK1 and its regulator AMBRA1, in autophagy [148]. In addition, ultraviolet B radiation can suppress the activity of miR-125b and increase autophagy in PBMC of SLE patients [149]. Furthermore, autophagy plays an important role in MHC-II presentation of Epstein-Barr (EB) nuclear antigen 1 to T cells [150]. Notably, autophagy machinery has been found dysregulated in SLE. Autophagy activation found in early stages of B cells in SLE patients and in a lupus mouse model was required for plasmablast development, suggesting the essential role of autophagy in autoantibody production in SLE [151]. As previously reported, T cells from lupus-prone mice showed higher autophagy rate compared with those from control mice [152]. Macroautophagy was also increased in IFN-γ-producing T cells from SLE patients, and the percentage of autophagy positively correlated with the disease activity [153]. Moreover, increased autophagic vacuoles were detected in the cytoplasm of T cells from SLE patients, particularly in naive $CD4^+$ T cells [154]. Autophagic activation of peripheral Th17/Treg cells of SLE patients may result in increased proinflammatory response of Th17 cells and decreased function of Treg cells [155]. Autophagy has been found to be required for the secretion of IFN-α by pDCs [156]. MicroRNA-199a was identified to inhibit cell autophagy and reduce IFN-β production via targeting TANK-binding kinase 1 [157]. Loss of DC autophagy slowed disease progression and reduced IFN-α production in Tlr7.1 tg mice [158]. Importantly, autophagy promotes the formation of NETs, further amplifying inflammatory responses [51, 159, 160]. The most meaningful finding on the link among NETs,

Table 1: Possible therapeutic targets for SLE.

Targets	Inhibitors	Death pathway	References
Autophagy	Chloroquine	NETosis	[51, 66, 168]
$Ca2^{+}$	Cyclosporine A/tacrolimus	NETosis	[48]
ROS	N-Acetyl cysteine/polydatin	NETosis	[82]
Mitochondrial ROS	Mito TEMPO	NETosis	[48]
MPO	PF1355	NETosis	[48]
NE	Vitamin D	NETosis	[7]
PAD4	Cl-amidine	NETosis	[76, 79]
DNA	DNase	NETosis	[75]
P2X7 receptor	Brilliant blue G	Pyroptosis	[106]
NLRP3 inflammasome	MCC950/AZD1208	Pyroptosis	[102, 108]
AIM2	/	Pyroptosis	
Caspase-1	zVAD-fmk	Pyroptosis	[110]
GSDMD	LDC7559	Pyroptosis	[100]
GSDME	/	Pyroptosis	
IL-18	Monoclonal antibody of IL-18	Pyroptosis	[48]
RIPK1	Nec-1	Necroptosis	[122]
RIPK3	miRNA-223-3p	Necroptosis	[137]
MLKL	MicroRNA-500a-3p	Necroptosis	[136]
mTOR	Rapamycin/glucocorticoids	Autophagy	[165, 166]
Autophagic-lysosomal degradation	Chloroquine	Autophagy	[155]
Autophagosomes	Vitamin D	Autophagy	[161]

autophagy, and SLE is that the REDD1/autophagy pathway promotes thromboinflammation and fibrosis in human SLE by NETs coated with tissue factor and IL-17A [67]. Interestingly, both chloroquine and vitamin D exhibit therapeutic effects on SLE patients partly by suppressing autophagy [155, 161]. Collectively, these data strongly support a pathogenetic role of autophagy in SLE. However, there are several studies that provide other lines of evidence for a cytoprotective role of autophagy. As a noncanonical form of autophagy, microtubule-associated protein 1 light chain 3 alpha- (L3C-) associated phagocytosis (LAP) is of importance to efficiently degrade phagocytosed microbes. Mice that lack any components of the LAP pathway displayed lupus-like features including increased production of autoantibodies, deposition of immune complex, glomerulonephritis, and type I IFN signature [162]. Repeated injection of dying cells into LAP-deficient mice resulted in increased production of inflammatory cytokines and caused a lupus-like syndrome [162]. Interestingly, increased autophagosomes have been found in podocytes from MRL/lpr mice and in renal biopsies from SLE patients. Two recent studies have supported the cytoprotective effect of autophagy on podocyte damage [163, 164]. In vitro, complement inactivated serum, IgG, and IFN-α from patients with LN could induce autophagy in both murine and human podocytes. With regard to intervention, autophagy inhibitors exacerbated podocyte damage while its inducer relieved the injury. Consistently, the autophagy inducer, rapamycin, decreased disease activity and reduced prednisone requirement in refractory SLE patients in clinical practice [165, 166]. Similarly, the glucocorticoids were identified to induce autophagy by blocking IP3-mediated calcium signaling and mTOR [167].

7. Conclusions

A large number of researches have provided strong evidence for the concept that dysregulation in cell death pathways and defective clearance of death materials trigger autoimmunity and contribute to the pathogenesis and progression of SLE. In SLE, accelerated cell death occurs and dying cells cannot be cleared promptly and effectively, leading to the exposure of nuclear and cytoplasmic autoantigens and release of DAMPs that work together to induce autoimmune responses and inflammatory responses. Lytic and inflammatory cell death, including secondary necrosis after apoptosis, NETosis, necroptosis, and pyroptosis, plays important roles in SLE pathogenesis and progression. However, owing to the lack of markers of necroptosis, human disease processes that involve necroptosis in vivo are hard to investigate (Figure 1). Thus, further studies that link necroptosis and SLE are needed. Interestingly, autophagy may be either a friend or a foe in SLE and different drugs for SLE treatment have various effects on autophagy, making more careful studies urgently necessary to further decipher detailed mechanisms, key signaling molecules, and checkpoints. Given that inflammatory cell death pathways are closely involved in SLE pathogenesis, inhibiting the inflammatory cell death processes at different steps and promoting the clearance of death material may be promising therapeutic strategies for treating SLE (Table 1).

Disclosure

Fangyuan Yang and Yi He contributed equally to this work as co-first authors.

Conflicts of Interest

All authors declare that there are no conflicts of interest.

Authors' Contributions

F.Y.Y wrote Sections 1, 2, and 3 and Y. H wrote Sections 5, 6, and 7. Z.Q.Z wrote Section 4 and designed Figure 1. E.W.S developed the conception and polished the manuscript. All authors read and approved the final manuscript.

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Review Article

Advances in the Research on Anticardiolipin Antibody

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Anticardiolipin antibody (ACA) is a kind of autoantibody and is one of the antiphospholipid antibodies (aPLs). Phospholipids with a negative charge on platelets and endothelial cell membranes are ACA target antigens. ACA is common in systemic lupus erythematosus and other autoimmune diseases and is closely associated with thrombosis, thrombocytopenia, and spontaneous abortion. In 1983, Harris established a method for detecting ACA, and research on the antibody has gained worldwide attention and has developed rapidly. For this review, we browsed articles that cover most of the ACA-related studies in the last 25 years and extracted influential ideas and conclusions in this field.

1. Introduction

The first antiphospholipid antibody (aPL) was found in patients with syphilis in 1906, and its associated antigens were identified as phospholipids. The aPLs including anticardiolipin antibody (ACA), anti-beta2-glycoprotein I (β_2GPI), and lupus anticoagulant (LA) are a heterogeneous group of autoantibodies reacting against phospholipids, phospholipid-protein complexes, and phospholipid-binding proteins [1]. In 1941, cardiophospholipid was isolated from the heart of the cow and named, which has the highest concentration in the mammalian myocardium and skeletal muscle. Each cardiac phospholipid molecule contains four unsaturated fatty acids, which are easily oxidized or polymerized. The study found that ACA had cross-reactivity with most negatively charged phospholipids. ACA can be combined with negatively charged phosphodiesters in cardiac phospholipid molecules, and the fatty acids in phospholipid molecules are essential components of their antigenicity.

2. Brief Introduction of Anticardiolipin Antibody

ACA is a group of heterogeneous antibodies that are classified into IgG, IgA, and IgM. ACA can be combined with cardiac phosphatide, phosphatidyl serine, or phosphatidyl inositol. The target antigen of ACA is controversial. It can be phospholipids in the heart; phospholipid-binding proteins in the plasma, such as β_2GPI; or protein and phospholipid compounds. The target antigen may also be the phospholipids that are exposed by the conformation change after the formation of the complex or the new antigenic determinants on β_2GPI [2]. ACA can be detected in many diseases, including autoimmune disease, recurrent abortion, cerebrovascular disease, and infectious disease. The relationship between ACA and its clinical manifestation is divided into two categories. ACA in autoimmune disease and antiphospholipid syndrome (APS) is within the autoimmune range, and its target antigen is phospholipid-binding protein, which can cause coagulation disorder; syphilis and other infectious diseases contain nonimmune ACA, whose target antigen is cardiophosphate, and it does not depend on plasma proteins such as β_2GPI.

3. Clinical Laboratory Detection Assay

In 1983, Harris et al. first established the ACA test by using cardiolipin as an antigen, a mixture of gelatin/phosphate-buffered saline (PBS) to dilute patients' serum samples and radiolabeled antihuman IgG or IgM to detect bound ACA as a radioimmunoassay. Over the next few years, the test method evolved so that fetal cow serum or adult bovine serum replaced gelatin/PBS as a sample diluent to provide sufficient β_2GPI for a valid test, enzyme-labeled antihuman

IgG or IgM antibodies replaced radiolabeled antihuman IgG or IgM antibodies, and ACA enzyme-linked immunosorbent assay (ELISA) was established [3]. ACA from patients with APS requires β_2GPI as a cofactor for cardiolipin binding [4]. Thus, ACA assays may indirectly be dependent on the function and structure of β_2GPI.

Many scholars have been working on the improvement and standardization of ELISA detection methods. It was demonstrated that both discrepancies and the lack of interlaboratory agreement are mainly due to the way that laboratories perform the test, the way that the test is calibrated, and how the results are calculated. When laboratories use the standard ELISA kit and calculate in a uniform manner, the agreement between laboratories gains much improvement [5]. At the 8th International Antiphospholipid Antibody Symposium, experts pointed out that the routine use of an ELISA kit was the first choice for the diagnosis of APS. However, no standardized ELISA methodology is available and intra- and interlaboratory variability remains at a high level.

Recently, new methods have been proven to be useful for detecting ACA. Line immunoassays employing a novel hydrophobic solid phase for the detection of ACA seem to be an intriguing alternative [6, 7]. Chemiluminescence immunoassay (CLIA) has been available for ACA detection since 2010. A fully automated CLIA can use paramagnetic particles coated with cardiolipin or human β_2GPI to capture antibodies from the serum samples and then detect ACA. Meneghel et al. showed that CLIA had a significantly lower comparative sensitivity for IgM ACA but a significantly higher comparative specificity for IgM ACA with respect to a homemade ELISA [8]. Fluorescence enzyme immunoassay, which measured the fluorescence in the reaction mixture once the reaction was stopped, was compared with homemade ELISA. Mattia et al. found that the sensitivities of the two methods were similar with the exception of IgM ACA [9]. Collectively, fully automated systems of ACA assay should be developed for standardizing ACA testing as a result of the significant intra-assay and intra-assay variation.

4. The Clinical Significance of ACA Laboratory Detection

4.1. Autoimmune Disease

4.1.1. Systemic Lupus Erythematosus. Systemic lupus erythematosus (SLE) is a chronic, multisystemic, autoimmune inflammatory disorder that primarily affects premenopausal women [10]. In 2012, the Systemic Lupus International Collaborating Clinics group proposed new classification criteria that include clinical and serological contents to diagnose and classify SLE, and ACA is one of the six serological contents [11]. Hobbs et al. conducted a single-center, retrospective inception cohort study to evaluate clinicopathological features, including the ACA of pediatric patients with new-onset SLE nephritis. They found that 90% (19/21) of the patients were positive for ACA [12]. In another study including 390 SLE patients, 47% had an elevated level of ACA. SLE patients had prolonged activated partial thromboplastin time (APTT), thrombocytopenia, and positive Coombs' test results, but they did not have APS. Prolonged APTT was strongly associated with venous or arterial thrombosis [13]. A study found that platelet activation was not induced by ACA+LA (lupus anticoagulant)+plasma only but was significantly augmented by ACA+LA+ plasma in combination with adenosine diphosphate (ADP) at a low concentration that had only a modest effect on platelet activation. In contrast, none of the ACA+LA-, ACA-LA+, or ACA-LA- plasma samples were capable of enhancing platelet activation even in the presence of ADP stimulation, indicating that ACA and LA may cooperate to promote platelet activation and may be involved in the pathogenesis of arterial thrombosis and thrombocytopenia in patients with SLE [14]. Alharbi et al. evaluated the differences between systemic sclerosis systemic lupus erythematosus overlap syndrome (SSc-SLE) and SLE and found that the level of ACA was increased in patients with SSc-SLE [15].

4.1.2. APS. APS is an autoimmune disorder that is clinically characterized by recurrent venous and/or arterial thromboembolic events or pregnancy morbidity and is associated with aPLs [16–18]. Patients must have both clinical and laboratory criteria to be diagnosed with APS, and APS diagnosis requires the presence of at least one of the three aPLs [19]. Vascular thrombosis is defined as one or more clinical episodes of arterial, venous, or small-vessel thrombosis, involving any organ, and is confirmed by appropriate imaging and/or histopathological analyses [19]. The clinical classification criteria of pregnancy morbidity are as follows: (1) one or more unexplained deaths of a morphologically healthy fetus at or beyond the 10th week of gestation, with normal fetal morphology; (2) one or more premature births of a morphologically healthy neonate at or before the 10th week of gestation because of eclampsia or severe preeclampsia, or placental insufficiency; and (3) three or more unexplained consecutive spontaneous abortions before the 10th week of gestation, with maternal anatomic or hormonal abnormalities, in which maternal or paternal chromosomal abnormalities have been excluded. The updated Sydney classification scheme recommends (1) a lupus anticoagulant detection according to guidelines published by the International Society on Thrombosis and Hemostasis; (2) ACA (IgG or IgM) exceeding 40 IgG or IgM antiphospholipid units; or (3) anti-β_2GPI antibodies (IgG or IgM) at levels exceeding the 99th percentile measured by ELISA [20]. It was reported that ACA is positive in 84-90% of APS patients and in 25% of APS patients (without LA) [21]. The importance of ACA testing in the diagnosis of APS was challenged in 2002; however, Nash et al. demonstrated that the omission of ACA testing from the clinical investigation of APS could lead to a failure to diagnose the syndrome in a large proportion of patients. In their report, using 123 patients with persistent aPL, more than one-quarter of the cohort of patients' studies were positive for ACA and negative for LA and anti-β_2GPI [22]. Thus, the importance of ACA in the diagnosis of APS cannot be ignored.

4.1.3. Rheumatoid Arthritis. Rheumatoid arthritis (RA) is a connective tissue disease that influences organs of the whole

body. Articular inflammation is the most prominent manifestation, and skin involvement is often seen. Serologically, rheumatoid arthritis may show rheumatoid factor (RF) and/or other autoantibodies. Wolf et al. collected specimens from 173 consecutive patients with rheumatoid arthritis and analyzed the laboratory results and clinical manifestations. Abnormally elevated IgG and/or IgM ACA levels were detected by ELISA in the sera of 55 (32%) patients. They also found that of 36 patients with rheumatoid nodules, 17 had positive ACA levels (sensitivity 47%) indicating a statistically significant association [23]. In contrast, Bobbio-Pallavicini et al. investigated the effect of long-term infliximab treatment on various autoantibodies in patients with RA. The results showed an increase in the ACA titer without any related clinical manifestations [24]. Seriolo et al. suggested that the association between ACA positivity and decreased levels of free protein S in RA patients may represent one of the risk factors for thrombotic events [25].

4.1.4. Immune Thrombocytopenic Purpura. Immune thrombocytopenic purpura (ITP) is an acquired disorder characterized by isolated thrombocytopenia resulting from autoantibody-mediated peripheral platelet destruction and the absence of any obvious initiating and/or underlying cause of thrombocytopenia. Despujol et al. collected data from 215 patients with ITP and found that 54 (25%) patients had ACA, including 42 (20%) that were IgG-ACA positive [26].

Thrombocytopenia, as a manifestation of primary APS, has a reported prevalence of 20 to 46%. Although evidence suggests that ACA may bind activated platelet membranes and cause platelet destruction, the pathogenesis of thrombocytopenia related to ACA remains unclear. Carlos et al. detected the IgG and IgM of aPLs via ELISA in 21 patients with ITP and 33 with APS. The results showed that the frequencies of IgG antibodies in ITP were ACA (47.6%) > anti-β_2GPI (19%). In APS, conversely, anti-β_2GPI was the most frequent (73%) [27].

4.2. Thrombosis. ACA is one of the indicators of thrombosis. Naess et al. suggested that elevated ACA levels were not a risk factor for subsequent venous thrombosis in a general population via a large population-based nested case-cohort study containing 508 venous thrombosis cases and 1464 matched control subjects from a total 66 140 participants. Elevated ACA levels were still associated with a twofold increased risk of venous thrombosis in the presence of autoimmune disease (mainly systemic lupus erythematosus) [28]. Godoy et al. found that the serum ACA levels of 34 patients who suffered from deep vein thrombosis were above the normal reference range. However, in 6 patients with recurrent thrombosis, 4 tested as positive. Thus, patients with deep vein thrombosis who are positive for ACA had a higher risk of recurrent thrombosis [29].

ACA attracts monocytes into the vessel wall and induces their adherence to endothelial cells, which is mediated by adhesion molecules such as intercellular cell adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin [30]. A study confirmed that ACA derived from (NZW × BXSB) F_1 mice, a model of antiphospholipid syndrome with myocardial infarction, can cross-react with oxidized low-density lipoprotein, which is believed to induce the transformation of macrophages into foam cells and, in some cases, to cause endothelial cell damage [31]. Another study found that IgG ACA was able to enhance the expression of monocyte chemotactic protein-1 (MCP-1) at both the protein and mRNA levels, and the overexpression of MCP-1 has been implicated in several pathological conditions, including atherosclerosis, thrombosis, and inflammatory disease [32]. Alves et al. revealed that ACA induces nitric oxide production acutely through the increased expression of inducible nitric oxide synthase in both *ex vivo* and *in vivo* models which might lead to feedback inhibition of endothelium-derived nitric oxide and increase the risk of thrombosis [33]. The possible mechanisms of ACA causing thrombosis are as follows: (1) ACA reacts with the membrane phospholipids of platelets or vascular endothelial cells, thereby inhibiting the synthesis of prostacyclin in vascular endothelial cells (PGI_2). Thus, the factors contributing to thrombosis are increased [34]. (2) After ACA damages vascular endothelial cells, the release of plasminogen activator is reduced, thereby increasing the tendency of thrombosis [35]. (3) ACA-IgG can also cause direct immune damage to endothelial cells, triggering platelet adhesion, aggregation, and the activation of factor XII [36]. (4) ACA can inhibit thrombin regulation, reduce the activation of protein C, and increase blood coagulation activity *in vivo*, thereby promoting thrombosis.

4.3. Recurrent Abortion. Recurrent abortion refers to consecutive spontaneous abortions in women. A study aimed at evaluating the prevalence of high ACA in women with histories of at least two miscarriages found that high ACA levels were identified in 55.77% of the individuals. A systematic review and meta-analysis demonstrated a positive association between antiphospholipid antibodies and/or APS in patients with recurrent abortion [37]. A study analyzed 85 antenatal patients with recurrent fetal loss (cases) and an equal number of antenatal patients without recurrent fetal loss (control) matched for age. The conclusion was that the prevalence of aPL among antenatal patients with recurrent abortion was at least 3 times higher than that of the normal antenatal clients [38]. Previous studies showed that triple aPL positivity (ACA, anti-β_2GPI, and LA) is associated with pregnancy complications in aPL carriers [39, 40]. The above experimental data confirmed a positive correlation between ACA and recurrent abortion, especially in relation to late-stage recurrent abortion. Therefore, ACA can be used as one of the indicators to predict the abortion in high-risk women.

The possible mechanism of ACA resulting in recurrent abortion includes the following aspects: (1) ACA interferes with calcium-dependent phospholipid-binding protein V, which affects the flow of blood between the villi [41]. (2) The combination of ACA and vascular endothelial phosphatide can damage the vascular endothelium and cause local thrombus formation, resulting in insufficient blood supply for the decidual membrane and placenta, vascular lesions, placental embolism, and infarction. (3) ACA reacts with platelets or the membrane phospholipids of vascular

endothelial cells, causing local blood vessels to contract, platelet aggregation, and a decreased blood volume of the placenta, finally resulting in a pathological pregnancy [42]. (4) The serum total complement level decreased in APS patients, and the circulating immune complex increased. There is excessive activation of complement, which leads to fetal abortion and limited embryo development [43]. (5) In addition, ACA can cause placental vasculitis, which results in inadequate fetal oxygen supply and nutrition, causing fetal distress and death.

4.4. Cerebrovascular Disease. ACA is associated with cerebrovascular disease. Epidemiological studies of patients with acute nonhemorrhagic cerebral apoplexy showed that ACA was significantly elevated in patients with acute cerebral infarction and had increased before onset. ACA may be involved in the process of cerebral infarction. Studies have shown that the ACA level of multifocal cerebral infarction patients is significantly higher than that of patients with single cerebral infarction [44]. Cerebral infarction patients positive for ACA had a significantly increased risk of a second cerebral infarction. Therefore, ACA can provide a reference indexes for clinical treatment [45].

The relationship between ACA and cerebrovascular disease risk factors is generally considered as follows: (1) Age: in recent years, it is believed that ACA has a higher positive rate in young patients with cerebral infarction [46]. Therefore, in middle-aged and young people, if there is unexplained cerebral apoplexy, transient ischemic attack, deep vein thrombosis, etc., it can be further examined by analyzing ACA. A study of ACA-positive patients with cerebral infarction found that there were more female patients than males. Positive patients were more likely to have heart disease, blood disease, and neurological complications. In addition to other risk factors, the recurrence rate for female patients was higher than that for male patients [47]. (2) Diabetes: patients with diabetes have a disorder of glucose metabolism and lipid metabolism that can produce a large number of oxygen free radicals, which causes the vascular endothelial cells to be damaged, so that the platelet function is hyperactive, producing a large amount of ACA. ACA, in turn, affects the synthesis of PGI2 by vascular endothelial cells, interfering with thrombus adjusting element, fibrinolysis enzyme activator, and the activity of the protein C system and inhibiting thrombin III activity. In addition, ACA also promotes the activation of platelet function and microvascular disease, thereby involving thrombosis in the body. (3) High blood pressure: hypertension itself can lead to vascular endothelial cell injury. ACA can react with the membrane phospholipids in the endothelial cell membrane of the arterial wall, and the damage to the endothelial cells leads to the exposure of membrane phospholipids, inducing the production of ACA. On the other hand, ACA aggravates the damage to the endothelium, thus forming a vicious circle. (4) Smoking: the ACA of smokers is significantly higher than that of nonsmokers, and its mechanism may be as follows: smoking can cause LDL to be susceptible to oxidation, leading to an increase in oxidative products in the body. In addition, the synthesis and activity of superoxide dismutase and glutathione peroxidase can be reduced. Oxygen free radicals increase greatly, causing vascular endothelial cell injury to induce the production of ACA [48]. (5) Hyperlipidemia: the combination of ACA and the compound product of β_2GPI and phospholipid will reduce the removal of triglycerides, leading to hypertriglyceridemia [49]. At the same time, due to the increased body fat, the body will produce a large number of oxide free radicals and oxidation products and will also promote the formation of mural thrombi, thus causing the occurrence of cerebrovascular accidents and cerebral infarction.

4.5. Infectious Diseases and Other Symptoms. Since the association between aPL and syphilis was first described, many other viral, bacterial, and parasitic infections have been shown to induce antiphospholipid antibodies, notably ACA. A review of the literature shows that while ACA occurs frequently in viral infections, particularly in HIV (49.75%), HBV (24%), and HCV (20%), it is not correlated with thrombosis risk or hematological manifestations. Concerning bacterial infections, ACA is often present in leprosy (42.7%) [50].

The possible mechanism of infectious diseases inducing ACA is still not very clear, but molecular mimicry has been studied. Barton demonstrated that the two most broadly reactive HIV-1 envelope gp41 human monoclonal antibodies, 2F5 and 4E10, are polyspecific autoantibodies reactive with the phospholipid cardiolipin. ACA may be induced by autoantigen mimicry of the conserved membrane-proximal epitopes of the virus [51]. A previous study showed that immunological cross-reactivates between autoantigens and viruses, molecular mimicry, and immunomodulation by viral proteins may account for both cross-reactivity with autoantigens and abnormal T and B cell functions in autoimmune disorders [52].

A few articles studied ACA in other diseases, including epilepsy, preeclampsia, chorea, Budd-Chiari syndrome, and chronic pancreatitis (especially for autoimmune pancreatitis). There might be an autoimmune-mediated pathogenesis, especially via ACA, in the onset of these diseases [53–57].

5. Conclusion

We believe that with the continuous improvements in medicine and the rapid development of science and technology, the detection method of ACA will be more standardized. Considering that phospholipids with negative charge are the main constituents of the cell membrane and are widely distributed throughout the body, ACA may play a role in some stages of the pathogenesis of several diseases. These antibody/antibody complexes represented by ACA are mostly involved in complex systemic diseases involving multiple organs (SLE, thrombotic diseases, cardiovascular diseases, etc.). Because of this, insufficient research has been conducted on its molecular mechanism. We hope that through future research, we can find a messenger (such as effector cells) to organically link the "organ-antibody axis" and investigate ACA-related diseases from a new perspective. As a result, the study of the nature of ACA, its target antigen, and the method of ACA laboratory testing will help to advance the modern precision medicine.

Conflicts of Interest

The authors report no financial or commercial conflict of interest in this work.

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Research Article

miR-98 Modulates Cytokine Production from Human PBMCs in Systemic Lupus Erythematosus by Targeting IL-6 mRNA

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Objective. There is evidence that interleukin-6 (IL-6) upregulation plays a critical role in immunopathology of systemic lupus erythematosus (SLE). MicroRNA- (miRNA-) 98 was predicted to bind with the 3′-untranslated region (3′-UTR) of IL-6 gene. We hypothesized miR-98 through its regulation of IL-6 gene expression to influence cytokine production from peripheral blood mononuclear cells (PBMCs) in SLE. *Methods.* The expression of miR-98 and IL-6 mRNA in the PBMCs of 41 SLE patients and 20 healthy controls (HC) was detected by quantitative reverse transcription PCR (qRT-PCR). The correlations between miR-98 expression and clinical features were evaluated. Luciferase reporter assay was performed to identify miR-98 targets. miR-98 mimics, miR-98 inhibitor, and IL-6 overexpression vector were generated. Cell viability of PBMCs was assessed using MTT assay. Gene expression and protein level were determined by qRT-PCR and Western blotting. TNF-α, IL-8, IL-1β, and IL-10 levels in cultured supernatants were quantified using ELISA. *Results.* The expression of miR-98 was downregulated in PBMCs of SLE patients, and its expression is negatively associated with IL-6 levels. miR-98 expression was correlated with disease activity, lupus nephritis, and anti-dsDNA antibody. IL-6 mRNA was a target gene of miR-98. IL-6 overexpression promoted the proliferation of PBMCs and increased the levels of TNF-α, IL-8, IL-1β, and IL-10. Those effects were further enhanced by miR-98 inhibitor, while were suppressed by miR-98 mimics. miR-98 regulated the levels of STAT3 phosphorylation via its target gene IL-6. *Conclusion.* The current study revealed that miR-98 could ameliorate STAT3-mediated cell proliferation and inflammatory cytokine production via its target gene IL-6 in patients with SLE. These results suggest that miR-98 might serve as a potential target for SLE treatment and other IL-6-mediated diseases.

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic and incurable autoimmune disease with a breakdown of self-tolerance that leads to various immune abnormalities, including the production of autoantibodies to double-stranded DNA and other nuclear antigens and accumulation of immune complexes in the kidney and other important organs [1]. Until now, the etiology and pathogenesis of SLE are not well clarified. Genetic, epigenetic, cytokine, hormonal, and environmental factors might all be involved in the initial and development of SLE [2].

Cytokines are believed to play important roles in modulating the immune response against foreign or self-antigen [3, 4]. A number of studies have identified that many

cytokine levels including interleukin- (IL-) 6, IL-10, and tumor necrosis factor- (TNF-) α are significantly elevated in SLE [5, 6]. IL-6 is a proinflammatory cytokine produced by antigen-presenting cells. Data from several studies suggest that elevated levels of IL-6 are implicated in regulating disease activity and in the involvement of different organs in patients with SLE [7, 8]. However, the mechanisms governing the regulation of cytokines in SLE remain elusive.

MicroRNAs (miRNAs) are single stranded, small short noncoding RNA strands, usually 22 nucleotides in length, ubiquitously expressed in human cells and tissues [9]. During the last few years, it has become clear that miRNAs participate in numerous physiological and pathological processes. miRNAs regulate gene expression at the posttranscriptional level. Numerous studies have shown that miRNAs are critical for the development and function of the immune system [10–13]. However, the functional role of miRNAs in cytokines regulating in patients with SLE has not been previously investigated.

In the present study, we predicted specific miRNAs which could bind with the 3′ untranslated region (3′UTR) of IL-6 mRNA using the online software TargetScan (http://www.targetscan.org/vert_71/) and identified that miR-98 indeed targeted IL-6. Based on these findings, we aimed to investigate the expression and function of miR-98, especially its potential role in regulating cytokines in SLE.

2. Materials and Methods

2.1. Patients and Controls. Forty-one SLE patients classified according to the 1997 American College of Rheumatology (ACR) criteria for SLE [14] were recruited from Guangzhou First People's Hospital from March to May 2017. Twenty age- and sex-matched healthy controls (HC) from the same general population were recruited voluntarily. In the SLE group, there were 37 females and 4 males; the mean age was 34.1 ± 16.6 years. In the control group, there were 14 females and 6 males; the mean age was 32.6 ± 14.1 years. All the control samples were collected from the physical examination center. Approvals were obtained from the Ethics Committee of Guangzhou First People's Hospital and the Ethics Committee of Jinan University based on the ethical guidelines of the 2008 Declaration of Helsinki, and informed consent was obtained from all study participants.

Clinical and demographic information was collected from admission records, including gender, age, serological examinations, organ involvement, lupus disease activity, and therapeutic medications. Laboratory test results included erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), compliment 3, immunoglobulin G (IgG), serum creatinine (SCr), serum albumin (ALB), anti-cardiolipin antibody (aCL), anti-β2-glycoprotein 1 (β2-GP1) antibody, antinuclear antibody (ANA), anti-double-stranded DNA (ds-DNA) antibody, anti-Sm antibody, anti-RNP antibody, and anti-Ro/La antibody. Organs involved included skin, joints, serosae, renal, and central nervous system. Lupus nephritis (LN) was defined if clinical and laboratory manifestations met the ACR criteria [15]. Central nervous system involvement consisted of 12 manifestations of neuropsychiatry syndromes which were defined in ACR [16]. Disease activity was assessed on the basis of the SLE Disease Activity Index (SLEDAI) [17]. Medications including corticosteroids and additional immunosuppressive agents were also recorded.

2.2. Cell Isolation and Cell Culture. Human peripheral blood mononuclear cells (PBMCs) of SLE patients and HC were isolated from heparinized blood by density gradient centrifugation. After isolation, PBMCs were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin in U-bottom 24-well plates. The amount of cells/mL was adjusted at a density of 2×10^6 cells/well.

2.3. Plasmid Construction. A STAT3 promoter was cloned and inserted into pGL3-control vector using XhoI and MluI to construct pGL3-STAT3. An IL-6 ORF was cloned and inserted into pcDNA3.1 vector using HindIII and EcoRI to construct pcDNA3.1-IL6. Plasmids were transfected into PBMCs using Lipofectamine 2000.

2.4. MTT Assay. PBMC viability was assessed using an MTT assay according to the manufacturer's instructions. Briefly, PBMCs were transfected with miR-98 mimics, miR-98 inhibitor, IL-6 overexpression vector, and/or treated with S31-201 in a 96-well plate for 24 h. MTT reagent (0.5 mg/mL) was then added to each well. After a 4 h incubation at 37°C, the formazan crystals were dissolved in DMSO and the absorbance was recorded at 570 nm using a microplate reader (ThermoFisher).

2.5. RNA Isolation and Real-Time Quantitative RT-PCR. PBMC total RNA was isolated using the TRIzol reagents (Invitrogen, San Diego, CA, USA). cDNA was synthesized from 2 μg of total RNA using a reverse transcription kit from (ThermoFisher, MA, USA). TNF-α, IL-8, IL-1β, and IL-10 mRNA levels were determined by real-time PCR using SYBR Green mix (Takara, Dalian, Liaoning, China). Primers are listed in Table S1. RT of miR-98 was performed from 10 ng of total RNA using All-in-One™ miRNA qPCR Primer (HmiRQP0853, GeneCopoeia, Guangzhou, China) and the All-in-One™ miRNA qRT-PCR Detection Kit (QP015, GeneCopoeia, Guangzhou, China). cDNA obtained from this step was used for quantitative TaqMan PCR using the real-time primers provided, according to the manufacturer's instructions. Cq values were converted to fold expression changes ($2^{-\Delta\Delta Cq}$ values) following normalization to U6 small nuclear RNA. For mRNA analysis, RT was performed on total RNA using random primers (Promega), and β-actin was used to control for cDNA concentration in a separate PCR reaction for each sample. $2^{-\Delta\Delta Cq}$ values were used to calculate the expression of each mRNA.

2.6. Luciferase Reporter Assay. A luciferase reporter assay was conducted using HEK293T cells. After 48 h transfection with miR-98 mimics or inhibitor, 800 ng pcDNA3.1 vector or pcDNA3.1-IL6 plasmids, along with 1 ng pRL-TK and 200 ng pGL3-STAT3 plasmid, HEK293T cells were harvested for luciferase activity assessment using a dual-luciferase

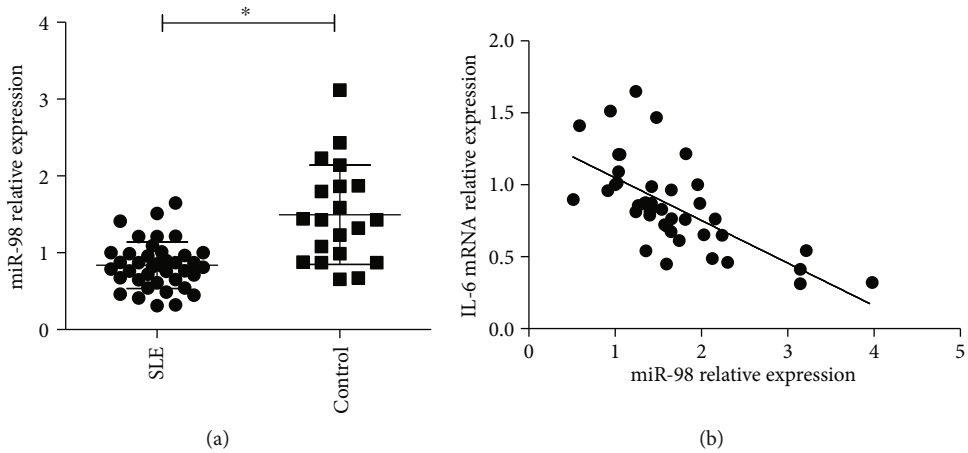

Figure 1: miR-98 expression is decreased in SLE. (a) The expression of miR-98 was detected in PBMCs of 41 SLE patients and 20 healthy controls using qRT-PCR; $^*p < 0.05$. (b) The correlation between the expression of IL-6 mRNA and expression of miR-98 in PBMCs of SLE patients was analyzed by two-tailed Pearson's correlation analysis, $r = -0.695$; $p < 0.001$.

reporter assay system (Promega). The final results were normalized to Renilla luciferase activity. The results are representative of at least three independent experiments.

2.7. Enzyme-Linked Immunosorbent Assay (ELISA). TNF-α (DTA00C), IL-8 (D8000C), IL-1β (QLB00B), and IL-10 (D1000B) levels in cultured supernatants were quantified using an ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Assays were performed in triplicate.

2.8. Western Blotting. PBMC proteins were extracted using RIPA lysis buffer with a proteinase inhibitor. The protein concentration in the lysates was measured by the BCA protein assay kit (#23227, Pierce, ThermoFisher), and 50 μg of the total protein mixed with 4x SDS loading buffer was loaded per lane. The proteins in the lysates were separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). In order to block nonspecific binding, the PVDF membranes were incubated with 5% skim milk powder at room temperature for 1 h. The PVDF membranes were then incubated for 12 h at 4°C with an antiserum containing antibodies against IL-6 (#12912, Cell Signaling Technology), Stat3 (#9139, Cell Signaling Technology), Phospho-Stat3 (Tyr705) (#9145, Cell Signaling Technology), and GAPDH (MA5-15738, Invitrogen). Primary was diluted at 1 : 1,000. A peroxidase-conjugated secondary antibody (1 : 5,000 dilution, BOSTER Biological Technology Co. Ltd, Wuhan, China) and enhanced chemiluminescence western blot detection reagents (NCI4106, Pierce, ThermoFisher) were used to visualize the target proteins, which were quantified with ChemiDoc XRS+ (Bio-Rad Laboratories, Inc.).

2.9. Statistical Analyses. The differences of continuous variables with nonnormal distribution and ordered categorical variables between two groups were compared by the Mann-Whitney U test. Student's t test was used to compare the differences of continuous variables with normal distribution, and chi-square for categorical variables. Mean ± SD or median and interquartile range was presented for continuous or ordinal data. Categorical variables were presented as the absolute count and percentage. Statistical analyses were performed using the SPSS 21.0 package. A p value less than 0.05 was considered to be statistically significant.

Table 1: Comparison of miR-98 expression in PBMCs between SLE patients and healthy controls (HC).

	miR-98 expression		χ^2	p
	Low expression	Normal and overexpression		
SLE	21	20	9.564	0.002
HC	2	18		

3. Results

3.1. The Expression of miR-98 Is Decreased in SLE PBMCs. The expression of endogenous miR-98 in PBMCs of 41 SLE patients and 20 HC was detected by qRT-PCR. The results showed that the expression of miR-98 was much lower in SLE PBMCs compared to that in HC PBMCs ($p < 0.05$) (Figure 1(a)). miR-98 levels were presented as mean and standard deviation (SD). In this study, miR-98 low expression was considered when the expression level of miR-98 was below or equal to mean-SD from HC PBMCs, miR-98 high expression was considered when the expression level of miR-98 was above or equal to mean + SD from HC PBMCs, and miR-98 normal expression was considered when the expression level of miR-98 was ranging from mean-SD to mean + SD. It was noted that the ratio of miR-98 low expression samples in the SLE group was significantly higher than that in the control group (Table 1), suggesting an underlying association between decreased miRNA expression and pathogenesis of SLE.

Table 2: Comparison of demographic and clinical characteristics between SLE patients with and without miR-98 low expression.

	Patients with miR-98 low expression ($n = 21$)	Patients without miR-98 low expression* ($n = 20$)	p
Female, n (%)	17 (81.0)	20 (100.0)	0.126
Age at SLE diagnosis, yrs, mean ± SD	34.27 ± 20.25	30.54 ± 7.37	0.606
Skin rash, n (%)	6 (28.6)	10 (50.0)	0.160
Oral ulcer, n (%)	7 (33.3)	9 (45.0)	0.401
Arthritis, n (%)**	16 (28.6)	10 (50.0)	0.001
Polyserositis, n (%)	4 (19.0)	3 (15.0)	0.732
Lupus nephritis, n (%)**	14(66.7)	6 (30.0)	0.019
Central nervous system involvement, n (%)	4 (19.0)	2 (10.0)	0.706
SCr (μmol/L), mean ± SD	121.80 ± 81.19	98.46 ± 63.64	0.093
ALB (g/L), mean ± SD	26.71 ± 6.32	28.42 ± 5.25	0.120
Complement 3 (g/L), mean ± SD	0.37 ± 0.14	0.44 ± 0.26	0.133
IgG (g/L), mean ± SD	12.72 ± 5.63	16.01 ± 6.68	0.154
ACL (IgM/IgG), n (%)	1 (4.8)	1 (5.0)	0.938
Anti-β2-GP1 (IgG), n (%)	2 (9.5)	1 (5.0)	0.720
ANA, n (%)	19 (90.5)	19 (95.0)	0.593
Anti-ds-DNA, n (%)**	13 (61.9)	3 (15.0)	0.004
Anti-Sm, n (%)	4 (19.0)	0 (0.0)	0.107
Anti-Ro, n (%)	12 (57.1)	7 (35.0)	0.105
Anti-La, n (%)	3 (14.3)	1 (5.0)	0.606
Anti-U1RNP, n (%)	4 (19.0)	2 (10.0)	0.663
Corticosteroid therapy, n (%)	21 (100.0)	19 (95.0)	0.773
IV CYC therapy, n (%)	14 (66.7)	9(45.0)	0.577
MMF therapy, n (%)	3 (14.3)	2 (10.0)	0.682
Hydroxychloroquine therapy, n (%)	19 (90.5)	19 (95.0)	0.593
Disease activity (SLEDAI, mean ± SD)**	14.93 ± 7.91	7.13 ± 6.68	0.032

*Patients with normal and overexpression of miR-98; **p value < 0.5.

3.2. miR-98 Expression Is Negatively Correlated with IL-6 Levels and miR-98 Low Expression Was Correlated with LN. The correlations between miR-98 expression and clinical features were evaluated. Compared with patients without miR-98 low expression, patients with miR-98 low expression showed significantly higher rates of LN and arthritis. There was no relationship between miR-98 expression and gender, age, central nervous system involvement, or skin rash (Table 2). With regard to the laboratory data, miR-98 low expression was found to be correlated with anti-dsDNA antibody. Other laboratory data were not significantly different between these patients (Table 2). Patients with miR-98 low expression had a significantly higher level of SLEDAI score than that without miR-98 low expression. Furthermore, a significant negative correlation was observed between miR-98 expression and IL-6 mRNA expression in SLE PBMCs ($r = -0.695$, $p < 0.001$) (Figure 1(b)).

3.3. miR-98 Directly Targets the 3′-UTR of IL-6 mRNA. Eukaryotic expression vectors of miR-98 mimics and miR-98 inhibitor were generated. The transfection efficacies of miR-98 mimics and inhibitor in PBMCs were detected and verified by qRT-PCR. Compared with the control vector (control) and anticontrol vector (anticontrol) transfected PBMCs, miR-98 production was increased in the miR-98 mimics group and decreased in the miR-98 inhibitor group (Figure 2(a)). By means of TargetScan6.0 online software, IL-6 was found to be a putative target of miR-98 (Figure 2(b)). We hypothesized miR-98 by binding IL-6 mRNA to perform its biological functions.

A dual-luciferase reporter assay was performed to demonstrate a direct interaction between IL-6 and miR-98 in healthy PBMCs. The group with cotransfected miR-98 mimics with luciferase vectors bearing WT IL-6 mRNA 3′-UTR target sequences showed the lowest luciferase activity. The luciferase activity was not significantly different between cotransfected control and miR-98 mimics with luciferase vectors bearing the mutated IL-6 mRNA 3′-UTR group (Figure 2(c)), suggesting that miR-98 is indeed binding to 3′UTR of IL-6 mRNA.

The effects of miR-98 on IL-6 production by SLE PBMCs using both qRT-PCR and Western blot analysis were evaluated. We found that IL-6 expression was significantly suppressed in the miR-98 mimics group at mRNA and protein

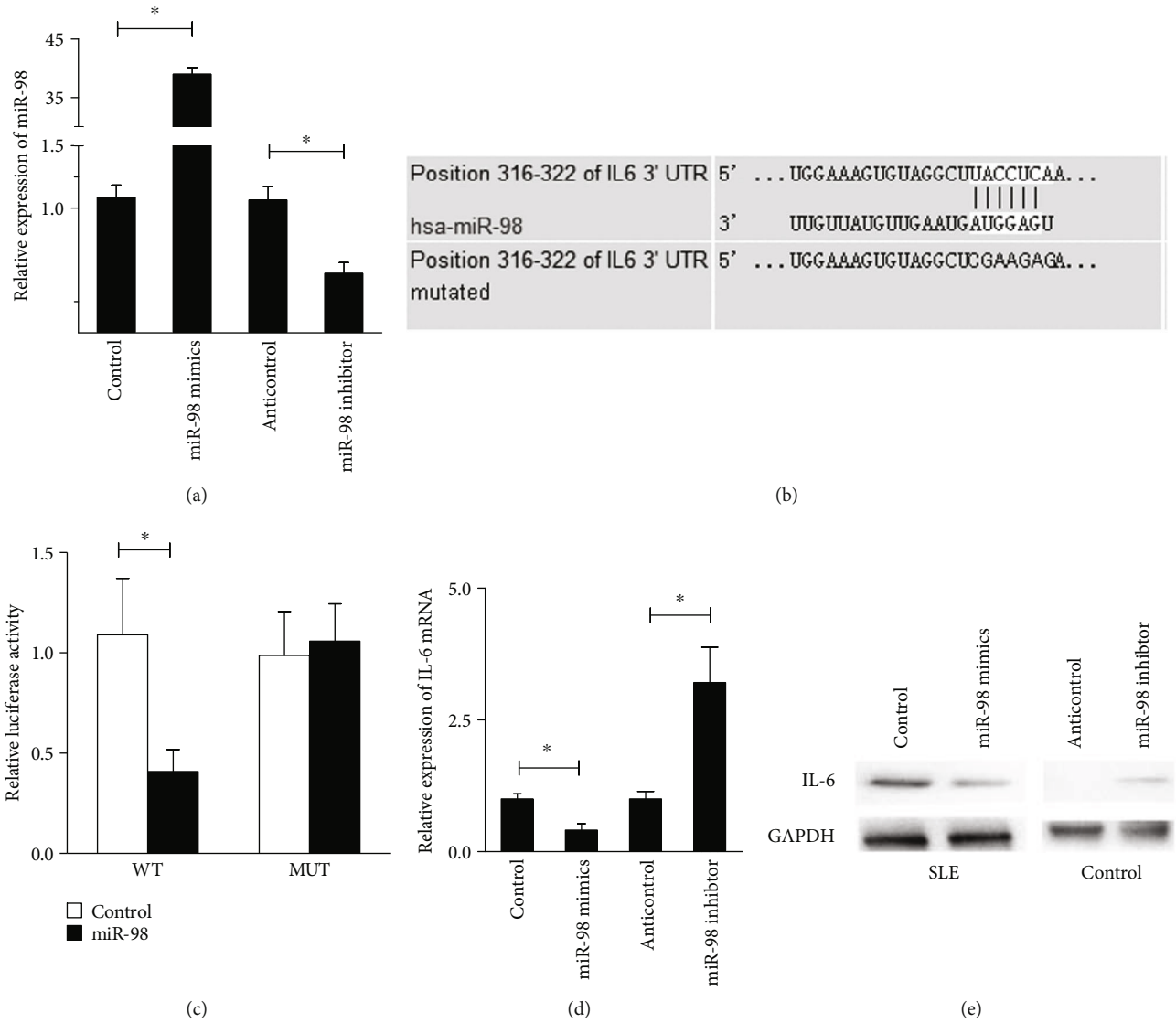

Figure 2: IL-6 is a target of miR-98 in SLE. (a) PBMCs of SLE patients were transfected with miR-98 mimics, inhibitor, or control sequences, then miR-98 levels were detected by qRT-PCR. U6 was used as an internal control. (b) The predicted miR-98 binding site within IL-6 mRNA 3′UTR and its mutated version by site mutagenesis are as shown. (c) Luciferase assay was performed in healthy PBMCs that were cotransfected with miRNA mimics or control sequences and reporter vectors carrying IL-6 mRNA 3′UTR with wild type (pMIR-IL-6-WT) versus mutated type (pMIR-IL-6-MUT), respectively. (d) PBMCs of SLE patients were transfected with miR-98 mimics or with unrelated sequences as controls, then PBMCs of SLE patients were transfected with anticontrol or miR-98 inhibitors, respectively. (e) Western blotting was used to test IL-6 expression; GAPDH was used as an internal control. Data are means of three separated experiments ± SD; $^{*}p < 0.05$.

levels, whereas it was boosted in the miR-98 inhibitor group (Figures 2(d) and 2(e)), indicating that the expression of IL-6 is negatively regulated by miR-98.

3.4. miR-98 Downregulates IL-6-Mediated PBMC Proliferation and Inflammatory Cytokine Production in SLE. To further investigate the function of miR-98 and whether the effects of miR-98 were mediated through IL-6, we transfected control vector or IL-6 overexpression vector into SLE PBMCs with miR-98 mimics or miR-98 inhibitor. The levels of IL-6 overexpression vector in SLE PBMCs were measured by ELISA (Figure 3(a)). MTT assay was used to measure the effect of miR-98 on the proliferation of SLE PBMCs. Proliferation of PBMCs was analyzed at 48 h. The results indicated that miR-98 mimics suppressed the proliferation of PBMCs and that overexpression of IL-6 reversed this inhibition (Figure 3(b)), while miR-98 inhibitor increased the proliferation of SLE PBMCs and was further enhanced by IL-6 overexpression (Figure 3(c)).

In addition, we found that the levels of TNF-α, IL-8, IL-1β, and IL-10 in SLE PBMCs were markedly downregulated by miR-98 mimic transfection (Figure 3(d)) and that

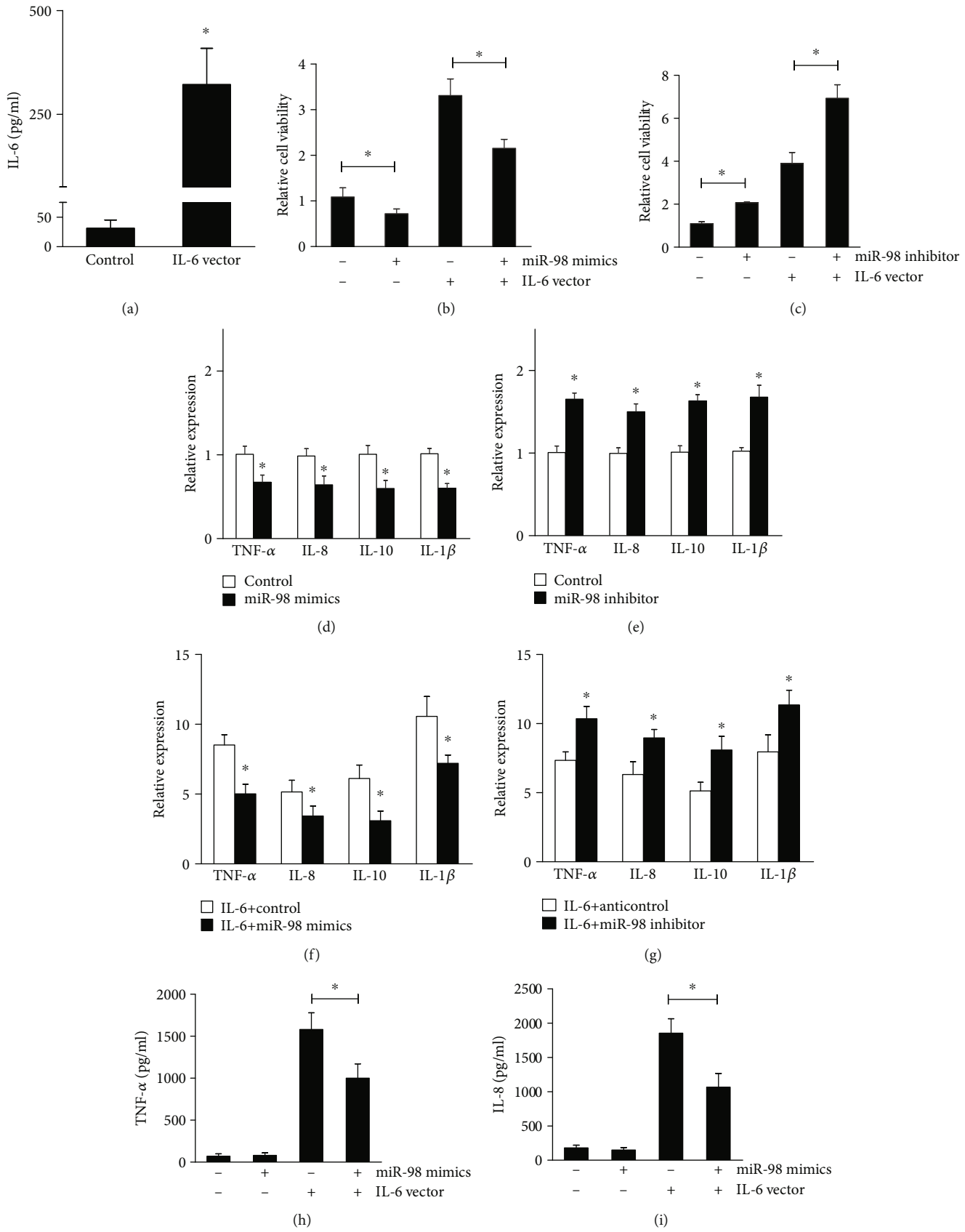

Figure 3: Continued.

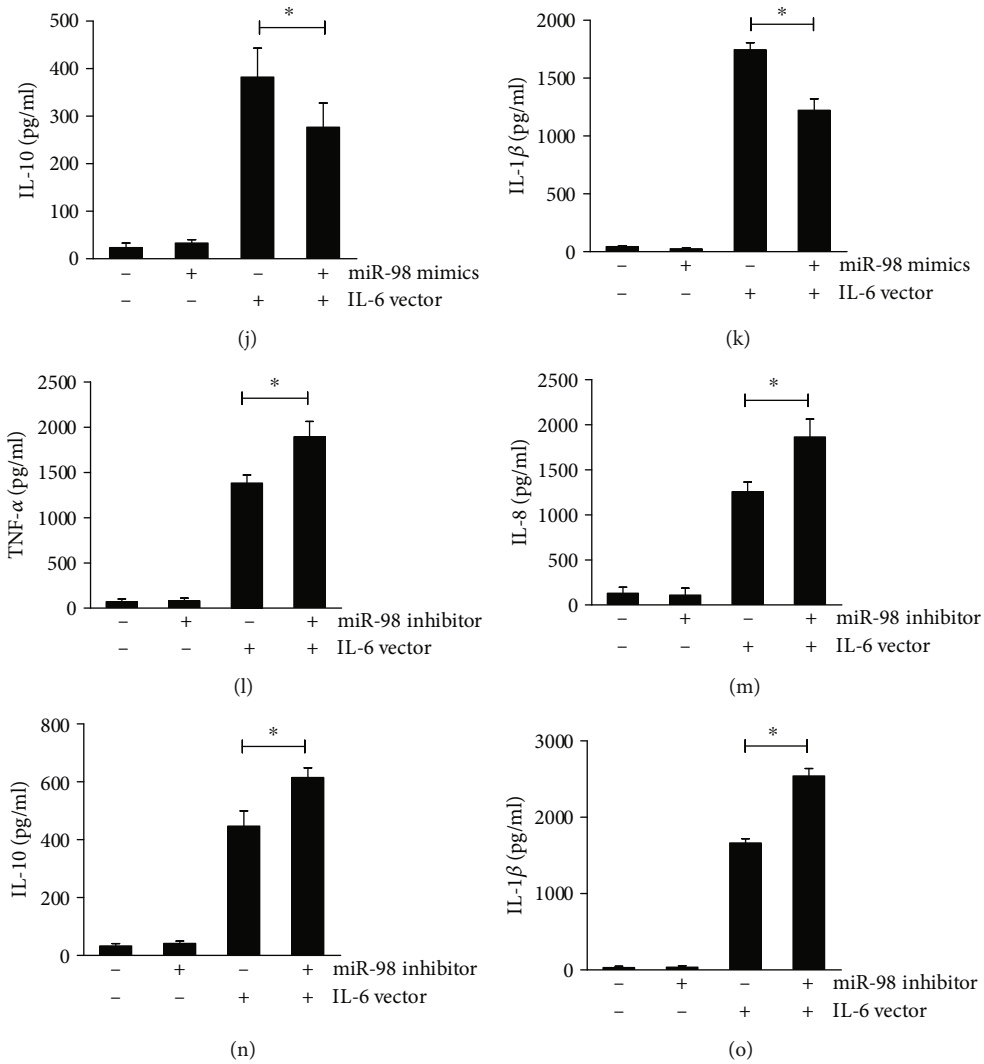

Figure 3: IL-6 reverses miR-98-mediated PBMC function in SLE. (a) PBMC cells of SLE patients were transfected with IL-6 overexpression vector or empty vector, then IL-6 protein levels in supernatants were detected by ELISA. MTT assay shows that overexpression of IL-6 could disrupt the inhibition effect of miR-98 mimics on PBMCs of SLE patients (b) and inhibition of IL-6 could enhance the effect of miR-98 inhibitor on PBMCs (c). PBMCs were transfected with miR-98 mimics and/or IL-6 overexpression vector; the expression of TNF-α, IL-8, IL-1β, and IL-10 in cells and their levels in a medium was detected by qRT-PCR (d, e) and ELISA (h–k), respectively. PBMCs were transfected with miR-98 inhibitor and/or IL-6 overexpression vector; the expression of TNF-α, IL-8, IL-1β, and IL-10 in cells and their levels in a medium was detected by qRT-PCR (f, g) and ELISA (l–o), respectively. Data are means of three separated experiments ± SD; $^*p < 0.01$.

were markedly upregulated when miR-98 was suppressed (Figure 3(e)). IL-6 overexpression increased the levels of TNF-α, IL-8, IL-1β, and IL-10, which were further enhanced in the miR-98 inhibitor group but were suppressed in the miR-98 mimics group (Figures 3(f)–3(o)). These results indicated that miR-98 could ameliorate IL-6-mediated cell proliferation and inflammatory cytokine production in patients with SLE.

3.5. miR-98 Regulates STAT3 Phosphorylation Level and STAT3-Mediated PBMC Proliferation and Inflammatory Cytokine Production via IL-6 in SLE. Signal transducer and activator of transcription 3 (STAT3) protein plays a central role in transmitting IL-6 signals. Our data showed that either miR-98 mimics or miR-98 inhibitor could not alter the luciferase activity of STAT3 (Figures 4(a) and 4(b)) and could not regulate the phosphorylation of STAT3 (Figure 4(c)). However, when the STAT3 activity was stimulated by IL-6, the miR-98 mimics could suppress IL-6-mediated STAT3 activation, and miR-98 inhibitor led to an opposite effect (Figures 4(a) and 4(b)). In addition, it was noted that the level of phosphorylated STAT3 was lower when IL-6 vector was cotransfected with miR-98 mimics than IL-6 vector transfection alone (Figure 4(c)), suggesting that binding to the IL-6 mRNA 3′-UTR is crucial for the regulation of STAT3 phosphorylation by miR-98.

Our data showed that either suppression of miR-98 or overexpression of IL-6 could promote PBMC proliferation,

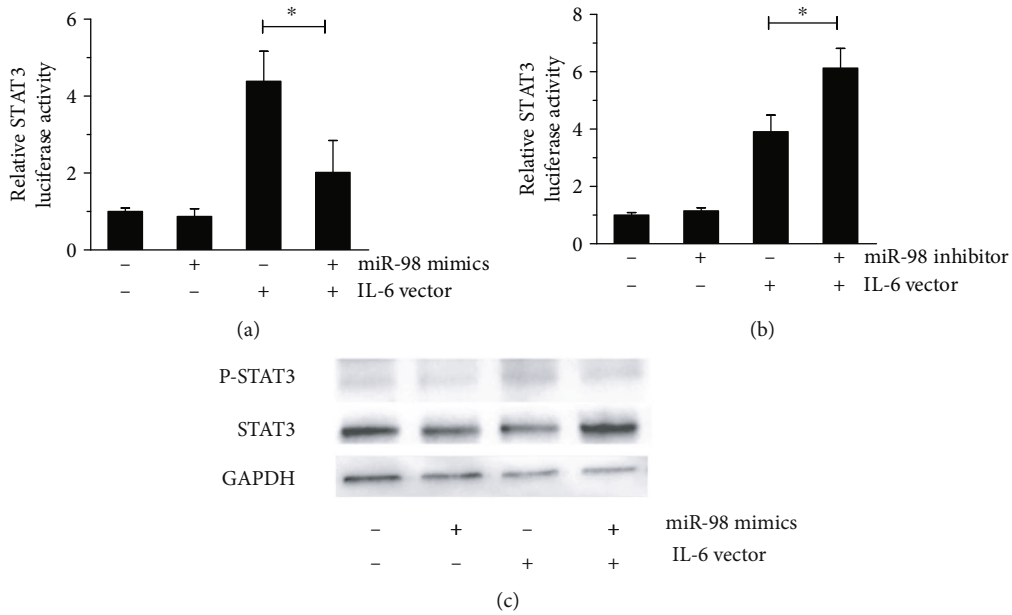

Figure 4: miR-98 can upregulate STAT3 levels in the presence of IL-6. HEK293T cells were transfected with pRL-TK, pGL3-STAT3, miR-98 mimics/inhibitor, and/or IL-6 overexpression vector; the activity of STAT3 was analyzed by luciferase reporter assay (a, b); the protein expression of STAT3 and phosphorylated STAT3 was detected by Western blotting (c). Data are means of three separated experiments ± SD; $^{*}p < 0.01$.

while STAT3 inhibitor S31-201 could abrogate the PBMC proliferative effect stimulated by miR-98 inhibitor cotransfected with IL-6 vector (Figure 5(a)). The levels of TNF-α, IL-8, IL-1β, and IL-10 from PBMCs were increased by miR-98 inhibitor combined with IL-6 vector transfection (Figure 5(b)), while STAT3 inhibitor S31-201 could reverse those effects (Figures 5(c)–5(f)). These results indicated that miR-98 could inhibit IL-6-mediated cell proliferation and inflammatory cytokine production via STAT3 in patients with SLE.

4. Discussion

Recent development in genetics and epigenetics has improved our understanding of SLE pathogenesis. Identifying differentially expressed genes and the mechanisms that regulate them will provide a comprehensive understanding of the initiation and development of SLE. Zhu et al. performed whole-genome transcription analysis using PBMCs from 30 SLE patients, including 15 with LN and 15 without LN, and 25 normal controls. They identified 552 upregulated genes and 550 downregulated genes in PBMCs of SLE, and of special note, they found IL-6 to be upregulated [18]. Our previous study had also shown that IL-6 was both upregulated and hypomethylated in SLE PBMCs [19].

In the current study, our aim was to investigate the regulatory mechanism of IL-6 in SLE. miRNAs can serve as novel biomarkers for SLE, both as useful diagnostic and prognostic tools [10]. TargetScan software predicted that miRNAs might bind to the 3′UTR of IL-6 mRNA. This prediction suggested that miR-98 was a potential regulator for the expression of IL-6. We found that miR-98 was significantly downregulated in SLE PBMCs relative to controls. miR-98 has been shown to be dysregulated in many different diseases. As noted by previous literature, miR-98 was downregulated in tumors, including non-small-cell lung cancer [20], leukemia [21], hepatocellular carcinoma [22], and breast cancer [23]. Chen et al. reported that the expression of miR-98 was upregulated in B cells isolated from mouse hearts with myocarditis [24]. In our study, the expression of miR-98 was remarkably lower in SLE PBMCs compared to that in HC PBMCs. We then examined the relationship between miR-98 levels and clinical parameters. The results indicated that low expression of miR-98 was correlated with disease activity, renal involvement, and anti-dsDNA antibody. Anti-dsDNA antibody was one of the hallmarks of the disease in terms of diagnosis and an important biomarker of SLE activity and kidney involvement or dysfunction [25, 26]. Our results indicated that miR-98 might be correlated with disease phenotype and serve as a new disease activity biomarker in SLE.

A significant negative correlation was observed between miR-98 expression and IL-6 mRNA expression in SLE PBMCs. Our study demonstrated that miR-98 was indeed binding to 3′UTR of IL-6 mRNA and the expression of IL-6 is negatively regulated by miR-98. IL-6 mRNA has been identified as a target of miR-98 in other diseases and tissues in previously reported experiments. Li et al. was the first to identify IL-6 mRNA as a target of miR-98 in melanoma [27]. Ji et al. identified that miR-98 was significantly downregulated in nucleus pulposus (NP) tissues in patients with intervertebral disc degeneration relative to controls. They

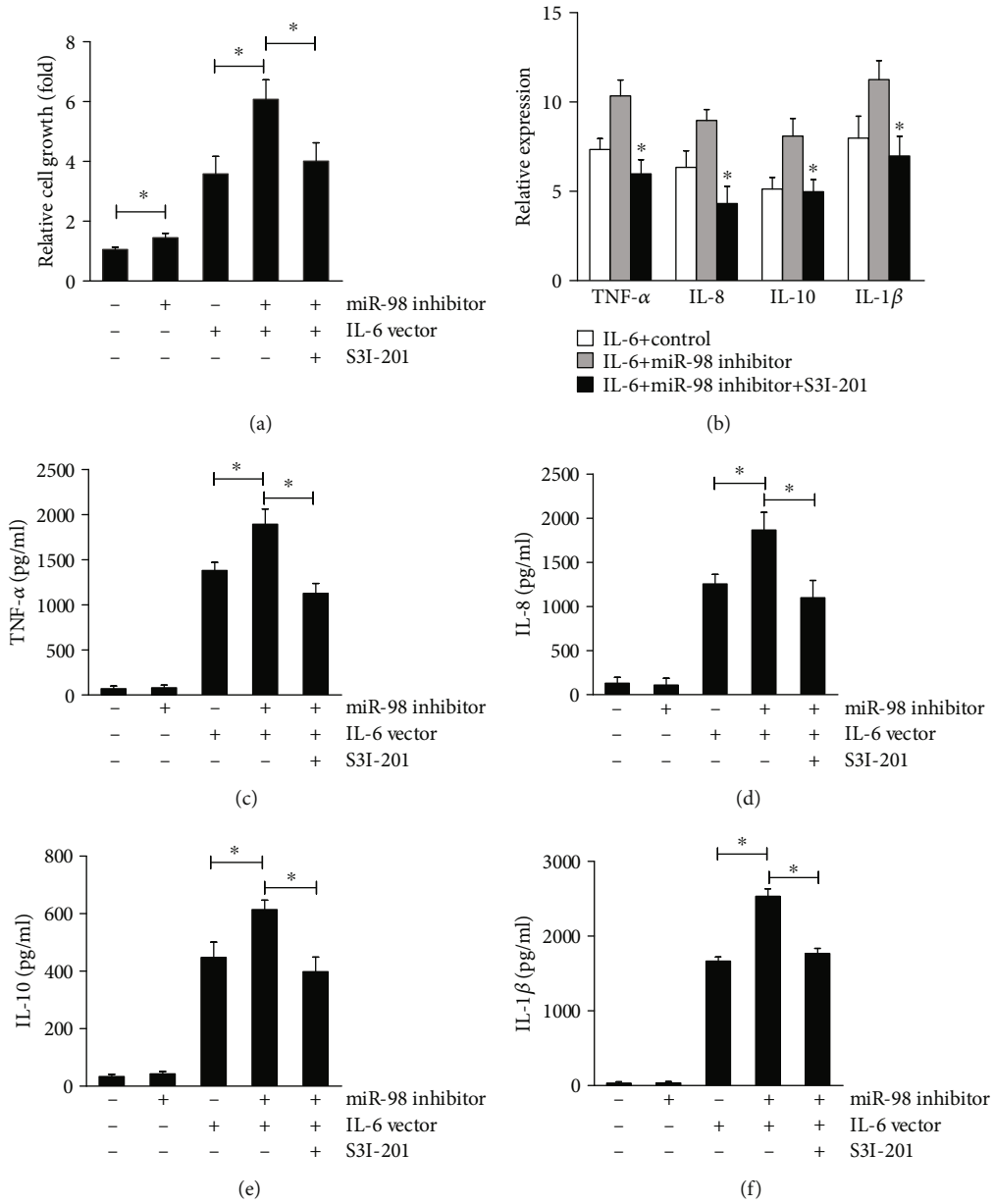

Figure 5: STAT3 regulates IL-6-mediated miR-98 functions in SLE PBMCs. PBMCs from SLE patients were transfected with miR-98 inhibitor or NC, IL-6 overexpression vector or empty vector, and/or S3I-201; cell growth was analyzed by MTT assay (a); the expression of TNF-α, IL-8, IL-1β, and IL-10 in cells was detected by qRT-PCR (b); the levels of TNF-α, IL-8, IL-1β, and IL-10 in a medium was tested by ELISA (c, d). Data are means of three separated experiments ± SD; $^{*}p < 0.01$.

found that miR-98 significantly promoted type II collagen expression in NP cells and that knockdown of IL-6 induced effects on NP cells similar to those induced by miR-98 [28]. Another study demonstrated that monocyte chemotactic protein-1 induced IL-6 expression in THP-1 macrophage cells via downregulating miR-98 [29].

The imbalance of proinflammatory cytokines, such as IL-6, TNF-α, IL-1β, and IL-10, was demonstrated that contributed to immune dysfunction and also mediated inflammation of the tissues and organ damage in SLE [30]. We further examined the effect of miR-98 on functional activity in PBMCs. We found that miR-98 inhibition could promote the proliferation of PBMCs, and inhibition of miR-98 combined with overexpression of IL-6 could increase the levels of TNF-α, IL-8, IL-1β, and IL-10 in PBMCs but were suppressed by miR-98 mimics. These results indicated that miR-98 could ameliorate IL-6-mediated cell proliferation and inflammatory cytokine production in patients with SLE. This finding suggested that enhancing the expression of miR-98 in SLE might have clinical benefits.

STAT3 protein plays a central role in transmitting cytokine signals. STAT3 signaling was identified to play a key role

in suppressing TNF-α synthesis by human monocytes in the course of systemic inflammation in vivo. IL-10 levels were enhanced in the serum and tissues of patients with SLE. STAT3 and recruitment to the IL-10 promoter had been demonstrated to induce IL-10 expression in SLE [5]. In this study, we identified that miR-98 could not regulate the expression and activation of STAT3 unless IL-6 was present. Furthermore, we found that the STAT3 inhibitor S31-201 could abrogate the combined effects of miR-98 inhibitor plus IL-6 overexpression vector. Therefore, we speculated that miR-98 might suppress PBMC cytokine production via IL-6/STAT3.

The current study systematically analyzed the expression and function of miR-98 in SLE PBMCs. However, it had its own limitations as an experiment in vitro from a heterogeneous cell population (PBMCs). Further research investigating the function of miR-98 from each PBMC subpopulations in SLE is required. Moreover, additional investigation on transgenic animal models would help us to identify the in vivo function of miR-98.

In conclusion, the expression of miR-98 is downregulated in SLE PBMCs. miR-98 might be correlated with disease phenotype and serve as a new disease activity biomarker in SLE. miR-98 downregulation contributes to IL-6-mediated PBMC proliferation and inflammatory cytokine production in SLE. miR-98 regulates the STAT3 phosphorylation level via IL-6 in SLE. miR-98 might serve as a potential target for SLE treatment.

Data Availability

The experimental and clinical data used to support the findings of this study are included within the supplementary information file.

Ethical Approval

This study is adhered to the highest ethical standards and expects research to comply with the appropriate guidelines for human studies. The study protocol has been approved by the research institute's committee on human research.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Dr. Shiwen Yuan, Dr. Chun Tang, and Dr. Dongying Chen contributed equally to this work and should be considered as co-first author.

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Supplementary Materials

The provided supplementary material is the primers for qRT-PCR in this study. *(Supplementary Materials)*

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Review Article

Autoimmune Hepatitis—Immunologically Triggered Liver Pathogenesis—Diagnostic and Therapeutic Strategies

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Autoimmune hepatitis (AIH) is a severe liver disease that arises in genetically predisposed male and female individuals worldwide. Diagnosis of AIH is made clinically applying diagnostic scores; however, the heterotopic disease phenotype often makes a rapid determination of disease challenging. AIH responds favorably to steroids and pharmacologic immunosuppression, and liver transplantation is only necessary in cases with acute liver failure or end-stage liver cirrhosis. Recurrence or development of de novo AIH after transplantation is possible, and treatment is similar to standard AIH therapy. Current experimental investigations of T cell-mediated autoimmune pathways and analysis of changes within the intestinal microbiome might advance our knowledge on the pathogenesis of AIH and trigger a spark of hope for novel therapeutic strategies.

1. Introduction

Autoimmune hepatitis (AIH) is a complex immune-mediated liver disease that is diagnosed histologically by interface hepatitis and high serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and immunoglobulin G (IgG) and the presence of autoantibodies [1]. The initial perception of AIH as a chronic inflammatory liver dysfunction which mainly affects young Caucasian women [2] has been amplified to both sexes of all age groups and all ethnic societies worldwide [3]. AIH can be asymptomatic or present in various forms from subclinical disease to acute liver failure and end-stage liver disease [4].

Specific diagnostic criteria and scoring systems have been established which include analysis of autoantibodies (ANA, SMA, anti-LKM1, and anti SLA), immunoglobulins (IgG), viral markers (IgM anti-HAV, HBsAg, HBV DNA, and HCV RNA) and histological findings [5]. According to the antibody profile, AIH can be divided into two subtypes. The presence of ANAs and or anti-smooth muscle antibodies (SMA) may indicate AIH type 1 (AIH-1), and anti-liver kidney microsomal antibody type one (LKM1) and anti-LKM3 and/or anti-liver cytosol type one antibody (LC1) are disease markers for AIH type 2 (AIH-2) [6].

The exact mechanisms for the immune tolerance breakdown in AIH have not been described yet, but there is growing

evidence that a genetic predisposition, molecular mimicry, and an imbalance between effector and regulatory immunity are key pathologic components for disease development. In this context, several lines of evidence support the central role of impaired T cell number and function [1].

The mainstays of AIH therapy are corticosteroids alone or in combination with azathioprine; however, new therapeutic interventions comprising the entire immunosuppressive armamentarium including biologics as well as cellular-based therapies have been proposed [7]. Liver transplantation (LT) can be a life-saving intervention for patients with acute liver failure (ALF) due to acute severe autoimmune hepatitis (AS-AIH) as well as patients with decompensated chronic AIH or hepatocellular carcinoma. Recurrent disease after LT has been reported in up to 10%-50% of patients, and an onset of de novo AIH has also been described for pediatric and adult liver transplant recipients [8].

This paper will mainly focus on the pathogenesis and diagnosis as well as treatment challenges of AIH. Based on our own empiric data and current standards, it furthermore tries to establish a treatment algorithm for patients with acute hepatitis suspected of having an autoimmune involvement.

2. Epidemiology

AIH occurs worldwide, with a variable clinical phenotype and a disparity in age-, gender-, ethnicity-, and geography-related incidence and prevalence [9]. Although uncertain, phenotypic variations and changes may in part rely on environmental, infectious, microbial, and genetic factors [10].

The annual incidence of AIH ranges from 0.67 to 2.0 cases per 100.000, and the annual prevalence ranges from 4.0 to 24.5 per 100.000 people depending on the geographical location [11, 12]. A significant increase in disease incidence has been recognized for Spain [13], Denmark [14], and the Netherlands [15] whereas a stable although permanently high incidence has been reported for New Zealand and the Asia-Pacific Area [16, 17]. This geographical escalation and differentiation can vaguely be explained by the "hygiene hypothesis," which proposes high sanitation standards, lack of microbial exposure, and hence altered microbiome compositions as the underlying cause of increased systemic immune and autoimmune responses within the population [18]. A dysbiosis of the microbiome which is shaped during infancy may also hypothetically be accounted for the different peaks of AIH onset which range from early childhood to mid- and late adulthood in the aforementioned countries. Further possible explanations for changes in peak age of onset may be the emergence of indigenous triggering antigens inducing immune reactivity in the elderly or disappearance of antigens that triggered autoimmune hepatitis in the young [9]. However, recent reports suggest that AIH might have been simply underdiagnosed outside of the age ranges initially described and might have always been uniformly present in all age groups [19, 20]. The female to male ratio is 4 : 1 and even higher (10 : 1) in AIH type 2 [21]. Mortality in AIH is highest during the first year of diagnosis and exceeds almost sixfold the mortality of the general population. The 10-year liver-related mortality which ranges from 6.2% to 10.2% is different in various ethnic subgroups and may be influenced by cultural and socioeconomic factors such as limited access to medical care [14, 22, 23]. Although the risk of carcinogenesis in AIH is lower than that in viral hepatitis, the occurrence rate of hepatocellular carcinoma (HCC) in cirrhotic patients with AIH ranges from 3.3% to 5.1% [17]. This increased incidence might in part be related to the long-standing course of disease. A large Japanese multicenter study reported a cumulative 1-year and 10-year survival rates of 85.4% and 39.4%, respectively, and the mean survival of these patients was reported to be 3.3 years [24]. Treatment options for HCC in AIH cirrhosis include (i) surgical resection and (ii) percutaneous and (iii) transarterial interventions which are roughly applied in thirds each, depending on the liver function, tumor location, and status of the patient.

The fact that AIH differs in occurrence, phenotype, and outcome suggests that several other triggers might exist that have not been described yet. More population-based studies are hence required to further improve management skills, individualize therapy, and enhance outcomes of patients with AIH.

2.1. Pathogenesis of AIH. Although all pathophysiologic mechanisms of AIH are not fully understood, there is growing evidence that a genetic predisposition, molecular mimicry, and an imbalance between effector and regulatory immunity in a particular autoimmune ecosystem are key pathologic factors for disease development [25] (Figure 1). In addition, several other extrahepatic autoimmune disorders have more frequently been described in patients with AIH [26]. It has been widely accepted that a genetic predisposition for autoimmune disease is related to genes within the HLA and non-HLA systems [27].

Although only investigated in small cohorts, susceptibility and resistance to AIH have been associated to DRB1 allelic variants within the HLA region of chromosome 6 [28, 29] and European, American, and Asian studies have described additional geographic variations within HLA alleles associated with AIH [30]. While a predisposition has been associated with the DRB1∗13 or DRB1∗03 and DRB∗07 or DRB1∗03 alleles in South America, Asian studies described an increase in susceptibility of AIH for both DRB1∗0405 and DRB1∗0401 [31, 32]. Particular aggressive courses of diseases have furthermore been attributed to DRB1∗0701 and DRB1∗03-DRB1-04 alleles [33]. In addition, a meta-analysis in the Latin American population revealed protective factors for alleles DRB1∗1302 and DQB1∗0301 [34].

Outside the HLA system, genetic studies identified several single nucleotide variants in the coding regions of tumor necrosis factor-induced protein 3 (TNFIP3) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), which have also been associated with the development of autoimmune disease, especially in the Chinese population [35]. A recent meta-analysis on CTLA-4 however only identified one study which showed a positive association of CTLA-4 and AIH [36].

Further putative triggers (e.g., viruses) for AIH have also been linked to the hypothesis of molecular mimicry and cross-reactivity between foreign epitopes and hepatic antigens [3]. This includes hepatitis A virus (HAV) [37], hepatitis C virus (HCV) [38], hepatitis E virus (HEV) [39], measles [40], Epstein-Barr virus (EBV) [41], and herpes

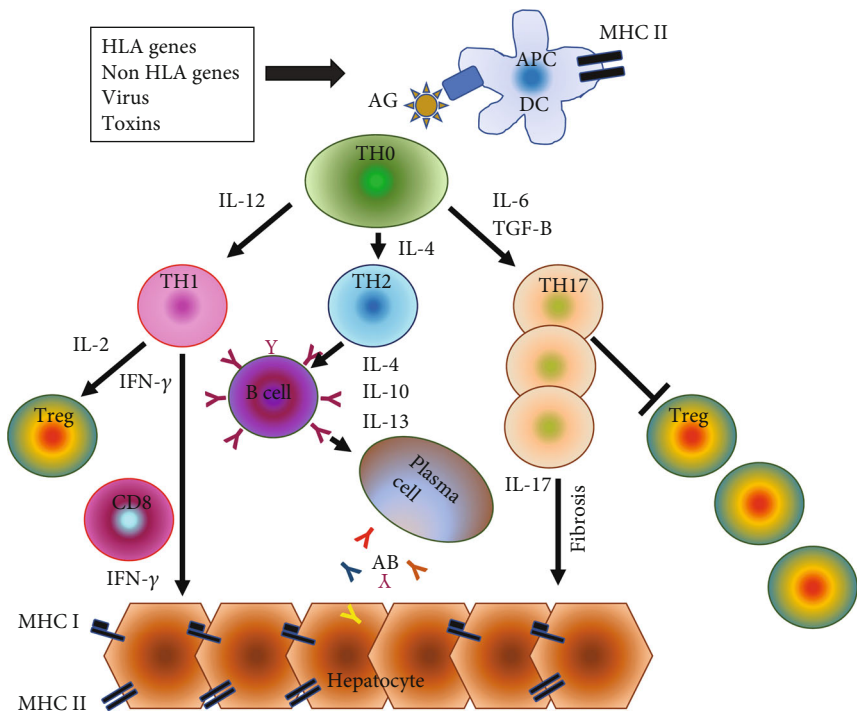

Figure 1: Pathogenesis of autoimmune hepatitis. HLA and non-HLA molecules as well as environmental triggers such as viruses, toxins, and the microbiome have been suggested as key components for a T cell-mediated immune response. The presentation of autoantigenic peptide (AG) to naïve $CD4^+$ T helper cells (TH0) by antigen-presenting cells (APC, dendritic cells (DC)) leads to a secretion of proinflammatory cytokines (IL-12, IL-6, and TGF-B) who give rise to the development of Th1, Th2, and TH17 cells. TH1 cells secrete IL-2 and IFN-γ, which stimulate $CD8^+$ cells to induce expression of HLA class I and HLA class II molecules on hepatocytes. Tregs and Th2 cells secrete IL-4, Il-10, and IL-13 thereby stimulating the maturation of B cells and plasma cells which themselves produce autoantibodies. TH17 cells, which increased number correlates with the degree of liver fibrosis, secrete proinflammatory cytokines and suppress T regulatory cells (Treg). The numerical decrease of Tregs leads to impaired tolerance to autoantigens which subsequently results in the initiation and perpetuation of autoimmune liver damage. The histological characteristics of interface hepatitis comprise an inflammatory cell infiltrate consisting of lymphocytes and plasma cells which is located around the portal tracts.

simplex virus [42]. Molecular mimicry is furthermore posed as a possible key element for microbiome-associated and drug-induced intestinal autoimmunity. Changes in the composition of the microbiome may lead to increased intestinal permeability, which subsequently facilitates transition of bacteria into the portal circulation [43]. This disruption of the gut barrier axis, which can be influenced by diet and antibiotic exposure, has been demonstrated to facilitate immune-mediated liver inflammation [44]. Pathognomonic changes in gut microbiota have furthermore recently been described in an experimental humanized mouse model of AIH [45]. Further noninvasive gene sequencing and microarray-based biomarker detection within the microbiome of patients with autoimmune disease might hence be of substantial scientific merit [46, 47].

In addition, research has identified several drugs (e.g., minocycline, nitrofurantoin, melatonin, diclofenac, statins, and ornidazole (Table 1)) which may be involved in precipitating AIH. It is important to clarify that drug-induced AIH is a completely different entity from drug-induced liver injury (DILI); however, overlap syndromes have been described in up to 9% of cases in which AIH and DILI are indistinguishable from each other [48]. The assessment of drug-induced AIH is complex; nonetheless, it has been shown that drug metabolites may stimulate the production of autoantibodies by acting antigenic [10]. In this context, priming of the immune system could occur years before apparent disease. Independent from the trigger mechanism, presentation of autoantigenic peptides to $CD4^+$ T helper cells (TH0 cells) in general leads to a rise in several TH subsets (TH1, TH2, and TH17) by generation of proinflammatory cytokines, which are moreover involved in complex autoimmune regulations [25]. In this context, experimental data demonstrated that cytokine-activated TH1 and TH17 cells foster an increase in hepatic C-X-C motif chemokine 9 (CXCL9) and C-X-C motif chemokine 10 (CXCL10)

Table 1: Drugs triggering AIH.

Author	Year published	Drug
Bjornsson et al. [199]	2010	Minocycline
Czaja [10]	2015	Nitrofurantoin
Hong and Riegler [200]	1997	Melatonin
Scully et al. [201]	1993	Diclofenac
Alla et al. [202]	2006	Statins
Kosar et al. [203]	2001	Ornidazole

expression, thereby triggering progression of AIH in mice [49]. TH17 cells are additionally involved in clearing pathogens during host defense reactions and have been reported to induce tissue inflammation in autoimmune disease through Treg suppression [50]. CXCL10 also known as interferon gamma-induced protein 10 (IP-10), which is secreted by several cell types in response to interferon-Y (IFN-y), has been advocated as a hepatic biomarker of inflammation and fibrosis and has furthermore been proposed as an indicator for disease severity in AIH [51]. On the other side, the divergence of high intrahepatic and low serum levels of Tregs in patients with untreated acute severe AIH [52] could be related to abnormal Treg homing into the inflamed liver, an issue also described in liver transplant patients suffering from acute cellular rejection [53]. In parallel, a decrease in intrahepatic Tregs has been described in AIH patients receiving steroid and azathioprine (AZA) treatment [52] (Figure 1).

2.2. Diagnosis of AIH. Diagnosis of AIH is demanding, and in particular, the detection of "acute newly formed" AIH is even more of a challenge [54]. AIH typically manifests as a chronic disease with an insidious onset of chronic liver disease symptoms, and patients may occasionally get diagnosed after incidental discovery of abnormal liver function test [55]. In contrast, up to 20% of patients present with an acute icteric hepatitis which in rare cases may end up with the development of an acute liver failure (ALF) [56]. Acute presentation of disease is generally more common in children, and a subtle onset of disease is vice versa more frequently observed in adults [57].

There is no single pathognomonic test for AIH, and diagnosis is solely based on several indicative clinical, serological, biochemical, and histological findings.

The first diagnostic criteria were established in 1992 by the Autoimmune Hepatitis Group [58] which were subsequently revised in 1999 [59]. The revised criteria however included complex and insufficiently validated parameters of questionable value, which were devised primarily to allow comparison of studies from different centers. In consequence, simplified criteria which enclosed 4 instead of 12 diagnostic parameters were proposed to facilitate a wider applicability in routine clinical practice. These criteria, upon which a score is calculated, include the measurement of autoantibody (titers of antinuclear antibodies ANA, anti-smooth muscle antigen (SMA), and anti-liver-kidney microsomal antibody type 1 (LKM-1)) and immunoglobulin G levels (serum concentrations of globulins or IgG above normal), the evaluation of liver histology (evidence of interface hepatitis, lymphoplasmacytic infiltrate, and rosetting of liver cells), and the exclusion of viral hepatitis (exclusion of viral markers for HAV, HBV, and HCV) (Table 2). A score of 6 is considered as probable AIH and a score of ≥7 as definite AIH [5]. Several data show that the simplified criteria retain a high sensitivity of >80% and a specificity of >95% for the diagnosis of AIH [60].

However, in cases with acute presentation [61] or overlap syndrome [62] where primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) could coexist in combination with AIH and patients present with clinical, biochemical, serological, and histological features of both a cholestatic liver disease and AIH, diagnostic criteria do not seem to perform so well [63]. However, due to its low frequency and lack of standardized diagnostic criteria, diagnosis of an AIH/PSC or PBC/AIH overlap is complicated. The pathogenesis of overlap syndromes is far from clear, and caution should be used with early diagnosis. Overdiagnosis may lead to unnecessary steroid treatment which in normal cases of PBC and PSC may not be applied. The gold standard for overlap syndrome diagnosis is clinical judgement, and the strongest independent predictor is the microscopic tissue examination of the liver [64].

The prevalence of AIH-PBC overlap is approximately 10% of adult patients, and special "Paris criteria" [65] exist that depending on the presence of at least two out of three key criteria (which for PBC include (i) increased alkaline phosphatase (ALP) or g-glutamyl-transaminase (g-GT) levels, (ii) presence of antimicrobial antibodies (AMA), and (iii) liver histology indicating florid bile duct lesions) provide a clinical diagnosis of an "AIH-PBC overlap syndrome" with high sensitivity (92%) and specificity (97%) [66].

In contrast, diagnostic criteria for the "AIH-PSC overlap," which has a prevalence of 7%-14%, are less well defined and rely primarily on typical magnetic resonance cholangiopancreatography (MRCP) findings [67].

In the emerging field of IgG4-associated autoimmune diseases, AIH might also be classified into an IgG4-associated and IgG4-nonassociated type, depending on its serological and immunohistochemical presence [68]. Interestingly, the number of T and B cells located in the liver of patients with IgG4-positive AIH is significantly increased, when compared to negative patients. This increased number of liver-specific T and B cell activity has been furthermore linked to better corticosteroid treatment responses in patients with IgG4-associated AIH [69].

In spite of diagnostic criteria, the final diagnosis of AIH needs to be made clinically. Physical examination does not add substantial value to the formation of a solid diagnosis, since symptoms like fatigue, abdominal pain, jaundice, and itching are nonspecific and solely indicate the presence of liver failure. However, AIH should be considered in any patient with acute or chronic liver disease and in particular when increased levels of serum aminotransferases, high immunoglobulins, high titers of circulating antibodies, and other autoimmune diseases are present [70].

2.3. Autoantibodies. The measurement of autoantibodies, which has been incorporated in all scoring systems, is a crucial step for both diagnosis and subclassification of AIH and should be performed in all patients suspect for AIH [71]. It is however unclear whether autoantibodies substantially contribute to the pathogenesis of the disease.

Type 1 AIH (AIH-1) which is the predominant type of AIH in both adults and children is characterized by positivity for antinuclear antibody (ANA) and/or anti-smooth muscle antibody (anti-SMA), which can be detected in 80% and 63% of patients, respectively. Concurrent positivity for both antibodies implies a relatively low sensitivity of 43%, with a specificity of 99% and accuracy of 74% [72]. ANAs have shown to be relatively variable markers during the course of

Table 2: Scoring systems for AIH.

Original criteria (minimum req. parameters)	Revised criteria	Simplified criteria
Sensitivity: 85%	Sensitivity: 100%	Sensitivity: 90%
Specificity: 90%	Specificity: 93%	Specificity: 95%
Accuracy: 80%	Accuracy: 82%	Accuracy: 92%
(1) Gender	(1) Female sex	
(2) Serum biochemistry ALP vs. AST	(2) ALP : AST (or ALT) ratio	
(3) Total serum globulin y-globulin or IgG	(3) Serum globulins, IgG	(1) IgG
(4) Autoantibodies	(4) ANA, SMA, LKM-1	(2) ANA, SMA, LKM-1
	(5) AMA	
(5) Hepatitis viral markers	(6) Hepatitis viral markers	(3) Absence of viral hepatitis
	(7) Drug history	
(6) Average alcohol intake	(8) Average alcohol intake	
	(9) Liver histology	(4) Liver histology
(7) Other etiological factors, history of hepatotoxic drug use, or exposure to blood products	(10) Other autoimmune disease	
(8) Genetic factors (other autoimmune disease in patients or first-degree relatives)	(11) Optional additional parameters, HLA-DR3 and HLA-DR4	
	(12) Response to therapy	
Score interpretation Pretreatment: Definite AIH > 15 Probable AIH 10-15 Posttreatment: Definite AIH > 17 Probable AIH 12-17	Score interpretation Pretreatment: Definite AIH > 15 Probable AIH 10-15 Posttreatment: Definite AIH > 17 Probable AIH 12-17	Score interpretation Maximum score: 10 >6: probable AIH >7: definite AIH
Additional parameters		
(9) Histology		
(10) Any defined liver autoantibody		
(11) Genetic factors HLA-DR3 and HLA-DR4		
(12) Response to therapy		

AIH, whereas higher titers of SMAs positively correlated with the histologic and biochemical disease activity. This is also true for anti-actin antibodies, which are a subset of SMAs that are present in 86% to 100% of patients with AIH-1 [73]. This means that although SMAs in AIH are mainly directed against filamentous (F) actin, a smaller fraction of 14% has a different molecular target [74].

Type 2 AIH (AIH-2), which only accounts for about 5%-10% of cases, occurs mostly in children and is characterized by the presence of anti-liver/kidney microsomal type 1 (anti-LKM-1) and/or anti-liver cytosol type 1 (anti LC-1) antibody. Incidences of antibodies to LKM 1 are subject to geographical variations. In this regard, they have been detected in up to 38% of pediatric patients in England [75] and only 2% in North American patients [76]. LKM-1 antibodies have a low sensitivity (1%) for AIH; however, their specificity and accuracy are 99% and 57%, respectively [72]. They can furthermore be detected in up to 10% of European patients with hepatitis C virus (HCV) infection [77, 78]. The molecular target of LKM 1 antibodies has been identified to as a short linear sequence in P450IID6 of the cytochrome monooxygenase [79, 80]. In 24% to 32% LC-1 antibodies may appear concurrently with anti-LKM-1 in young European AIH-2 patients [81, 82, 83] and they can also be detected in 12% to 33% of chronic HCV-positive patients [84, 85]. The target antigen has been identified to be a cytosolic enzyme named Formiminotransferase cyclodeaminase [86, 87, 88]. AIH-2 is furthermore characterized to be related with other drug-metabolizing enzymes as autoantigens which also include anti-LKM-2 antibodies directed against CYP2C9-tienilic acid, anti-LKM-3 against UGT1A, and anti-LC1 (liver cytosol antigen-1) and anti-APS (autoimmune polyglandular syndrome type-1) against CYP1A2, CYP2A6, and others [89].

In addition to all aforementioned nonorgan-specific autoantibodies (e.g., ANA, anti-SMA, and anti-LKM-1), several other autoantibodies have been described, which deliver important clues for the diagnosis, disease activity, and prognosis of autoimmune liver disorders [90]. These include the non-liver-specific heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1 [91, 92] and the liver-specific anti-soluble liver antigen (SLA) for AIH-1 and AIH-2 diagnosis, and positivity for the latter may be associated with a more severe clinical course and worse disease prognosis [93]. It is nowadays accepted that the presence of anti-SLA does not justify the definition of a

third subgroup of AIH and should rather allow to classify AIH in type 1 [94].

Since most liver autoantibody titers correlate with age, testing in pediatric patients demands special attention. Contrary to adults, repeated examination in pediatric AIH patients is reasonable, since titers of liver-related antibodies have been shown to correlate with disease activity, and treatment response may be paralleled by the reduction or even disappearance of serum antibodies [95]. Regular autoantibody testing in adults is currently not supported due to the lack of empiric data for antibodies being a surrogate marker for inflammatory disease activity in the older patient.

2.4. Liver Histology. In general, liver biopsy is considered a critical element in the differential diagnosis of liver disease and can be weighed as an independent factor to distinguish AIH from other liver illnesses [96]. Furthermore, liver histology is not only a prerequisite for applying the simplified AIH score but is also paramount in both initial diagnosis and long-term follow-up, since it allows for disease staging, therapy monitoring, and assessment for inflammation and fibrosis [3].

Current guidelines recommend liver biopsy at the time of the first presentation [97]; however, in patients with acute liver failure and compromised coagulation status, bleeding risk may be increased. In this context, transjugular or plugged biopsies may be a viable alternative approach in the procurement of a liver specimen for histologic evaluation [98]. A surgical approach to liver biopsy by explorative laparoscopy may be a rather invasive approach which requires general anesthesia.

Biopsy findings are laid out to distinguish typical (2 points), compatible (1 point), and atypical (0 points) histologic features of AIH, each contributing variable points to the simplified score. Typical lesions incorporate lymphoplasmacytic infiltrates, presence of rosettes, emperipolesis, and plasma cells, affecting the interface, hence commonly described as interface hepatitis. This nomenclature has been applied due to the apparent sharp contrast between the inflammatory zone and the normal hepatic parenchyma (Figure 2). Interface hepatitis is associated with the development of periportal fibrosis and may progress to bridging fibrosis and ultimately lead to cirrhosis. The accumulation of plasma cells in central areas reportedly occurs during the acute phase of AIH and is not visible in acute hepatitis caused by viruses and drugs.

On the other hand, rosette formation of hepatocytes and emperipolesis is not a "conditio sine qua non" for AIH diagnosis since these changes are commonly seen in other causes of acute lobular hepatitis [99]. Histological examination not only allows for differentiation between AIH and other autoimmune liver diseases (such as primary biliary cirrhosis, primary sclerosing cholangitis, and autoimmune cholangitis); it furthermore allows the diagnosis of up to 20% of AIH patients who do not have detectable autoantibodies. Patients presenting with fulminant hepatic failure histology may solely display massive necrosis and multilobular collapse [100]. Liver histology is not only essential to confirm the diagnosis of AIH; it also helps to assess disease severity and can be instrumental in guiding the intensity of immunosuppressive therapy or estimate the timepoint of setting the patient up for liver transplantation.

3. Pharmacological Treatment of AIH

3.1. Standard Frontline Treatment: Corticosteroids and Azathioprine. The overall goal of AIH treatment is to induce and maintain complete suppression of the inflammatory activity and to prevent disease progression to cirrhosis and liver decompensation [100]. In this context, treatment can basically be structured into an induction phase and maintenance phase [101]. Remission is achieved when (1) clinical symptoms are absent and (2) transaminases and (3) immunoglobulins have come to normal levels. In children (4), low autoantibody titers are an additional remission criterion.

Standard induction therapy in AIH includes a combination of high-dose prednisolone with or without azathioprine [102]. In case of monotherapy, starting steroid dose is 60 mg/day in adults and 1-2 mg/kg/day in children, not exceeding a maximum dose of 60 mg per day. With regard to combination treatment, differences between the EASL and AASLD guidelines exist, which lie mainly in the starting point of azathioprine which is generally administered at doses of 50 mg daily. While AASLD recommends a simultaneous starting of azathioprine and corticosteroids [103], EASL guidelines also suggest a staggered azathioprine treatment regimen, starting 2 weeks after the introduction of corticosteroids [104]. EASL guidelines furthermore advise that, in patients whom steroid-specific side effects are expected, remission can also be induced by replacing prednisolone with budesonide at a starting dose of 9 mg/day. A recent multicenter randomized controlled trial supported the fact that steroid-specific side effects were less frequent in patients treated with budesonide when compared to those treated with prednisolone. Budesonide furthermore has been shown to induce a higher complete remission rate (reduction to normal ALT) when compared to prednisolone [105].

However, it is important to highlight that budesonide is ineffective in the presence of cirrhosis, excluding at least one-third of AIH patients who have evidence of cirrhosis at timepoint of diagnosis [106]. Furthermore, it is important to stress that in contrast to previous guidelines, where remission was defined by achievement of transaminase levels below twice the upper limit of normal, current guidelines consider normal ALT, bilirubin, and IgG levels as complete remission. This makes the comparison of several retrospective studies difficult since they apparently focus on different study endpoints. Nevertheless, after a successful 4-week induction therapy in which tapering of steroids has already started, depending on the clinical course of the patient (normally at the beginning of week 3), a maintenance phase is initiated with continuous fixed doses of 10 mg of prednisolone and 50 mg of azathioprine daily, until normalization of serum transaminases, bilirubin, or IgG levels is achieved and resolution of histological abnormalities becomes evident [103]. Treatment is usually continued for at least two years [107], and subsequent decision to discontinue therapy generally balances the pros of long-term drug-free remission and cons of relapse-risk need for retreatment [108]. The

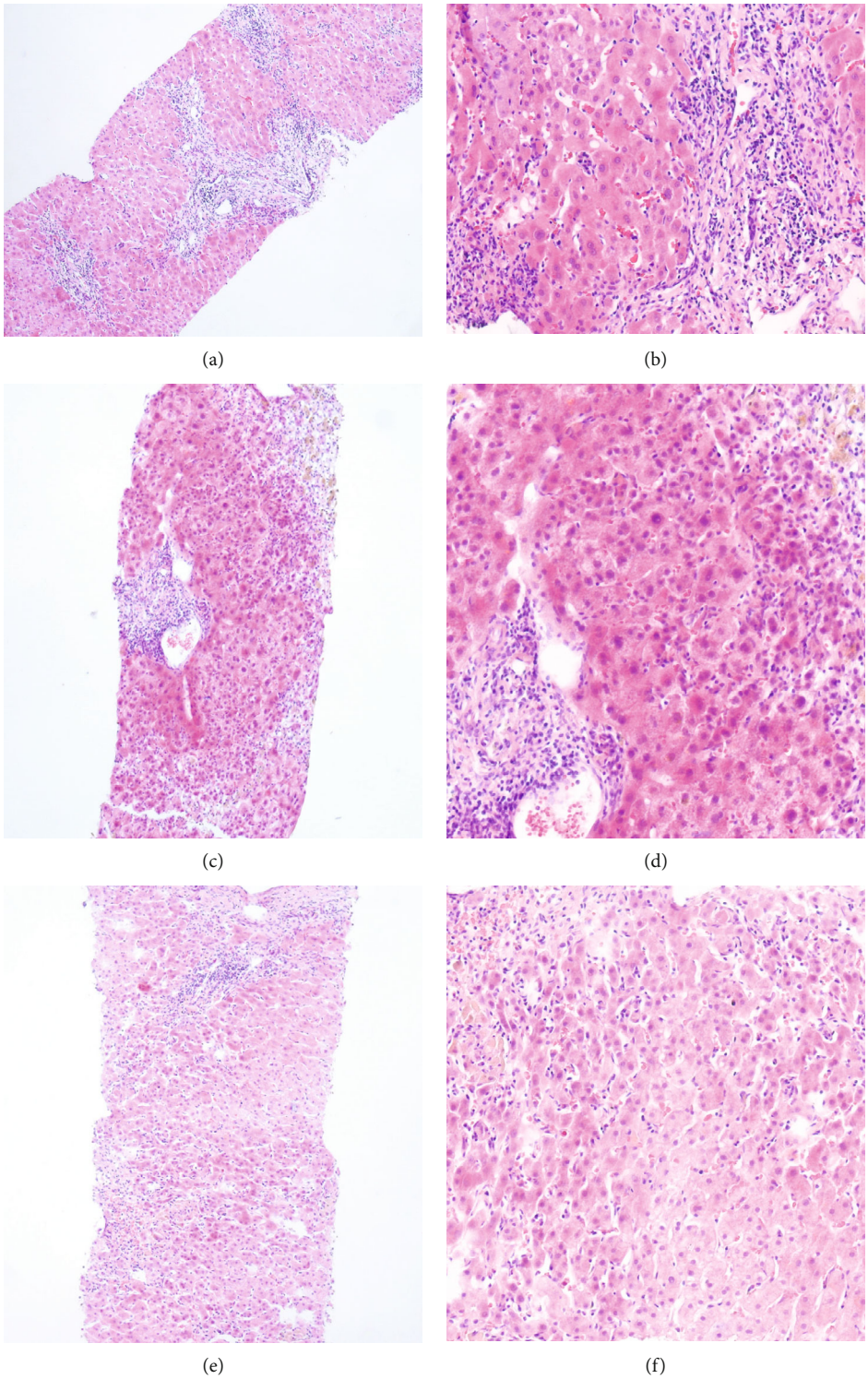

Figure 2: Example of histological features of autoimmune hepatitis (a, b), virus hepatitis C (c, d), and drug-induced liver injury (DILI) (e, f). Haematoxylin and Eosin (H&E) staining: (a, c, e) 20-fold magnification; (b, d, f) 40-fold magnification. (a, b) Interface hepatitis. Liver biopsy histology of a patient with autoimmune hepatitis typically reveals a dense portal and periportal mononuclear cell infiltrate including several plasma cells. The infiltration of lymphocytes and plasma cells in the central and periportal areas reflecting interface hepatitis occurs during the acute phase of AIH but is rather not present during acute hepatitis caused by hepatitis C virus (c, d) and drug-induced liver injury (e, f).

frequency of achieving a treatment-free state depends on the treatment duration, and recent studies have shown that treatment cessation after 3 to 5 years results in at least temporary remission rates of up to 40% [109]. However, relapse has been shown to be almost universal in patients with autoimmune hepatitis in remission [110].

4. Alternative and Second-Line Treatment

4.1. Mycofenolate Mofetil (MMF). Mycofenolate mofetil (MMF) is a next-generation noncompetitive inhibitor of inosine monophosphate dehydrogenase, which acts as the rate-limiting enzyme in purine synthesis. Since, in contrast to other cells, T and B cell proliferation predominantly relies on purine synthesis, MMF is effectively used as an antiproliferative immunosuppressant drug. In solid organ transplantation, MMF has surpassed azathioprine as first-line antirejection therapy and it is currently also used as a frontline or alternative treatment option for autoimmune hepatitis.

In treatment-naïve AIH patients, MMF in combination with prednisolone has been shown to be effective and save in inducing disease remission. A recent meta-analysis comparing standard treatment with MMF and prednisolone also proved MMF to obtain higher remission rates of aminotransferases and IgG levels and lower nonresponse rates [111]. A prospective head-to-head comparison of MMF to azathioprine is still in need; however, corresponding trials are under way (NCT02900443). In patients with corticosteroid-refractory disease or azathioprine intolerance, MMF has also been used successfully as second-line or salvage therapy [4]. On the flipside, MMF has several side effects including gastrointestinal symptoms, and due to its teratogenicity, it cannot be prescribed to a pregnant woman which is highly relevant since AIH predominantly affects women of young age [112]. From an economic standpoint, MMF seems to be 6-7 times more expensive than azathioprine which results in high treatment costs in patients with indefinite treatment length [113].

4.2. Calcineurin Inhibitors: Cyclosporine A and Tacrolimus. Cyclosporine A and Tacrolimus belong to the group of calcineurin inhibitors (CNI) which find widespread application as immunosuppressive drugs for solid organ transplant recipients in which scenario they act immunosuppressive by inhibiting Treg activation and IL-2 production [114].

The first clinical data show that Cyclosporine A can effectively be used as frontline therapy of AIH patients, and one small prospective clinical trial at least indicated equivalency to standard AIH therapy [115]. In parallel, Cyclosporine A has also been successfully used as alternative therapy of AIH patients not responding to azathioprine and steroids [116]. Predominantly due to the small number of patients, these studies warrant additional data before Cyclosporine A can confidently be recommended for AIH therapy. Cyclosporine A has been associated with serious side effects including nephrotoxicity, neurotoxicity, infection, and increased incidence of malignancy after long-term use. Animal data also suggest that Cyclosporine A may promote autoimmunity [117, 118] and may have more immunosuppressive capacity than anti-inflammatory activity, both actually inappropriate for AIH treatment.

Although Tacrolimus is a more potent calcineurin inhibitor than Cyclosporine with less nephrotoxic side effects, its use as frontline treatment in AIH is currently not supported by empiric data [119]. This is also due to the fact that the low number of retrospective case series used variable endpoints as remission criteria [120]. Few prospective studies in patients with steroid refractory disease however showed biochemical and histologic improvement with decreased inflammation and reduced fibrosis progression following Tacrolimus treatment [121].

4.3. mTOR Inhibitors: Sirolimus and Everolimus. Sirolimus and Everolimus have potent immunosuppressive and antiproliferative properties due to their ability to inhibit the mammalian target of rapamycin (mTOR), a specific intracellular protein kinase regulating cell proliferation, motility, and survival. Both substances have been effectively used in solid organ transplantation [122] and anticancer treatment [123] and on drug-eluting stents in patients with main coronary artery disease [124]. The role of mTOR inhibitors in AIH treatment has to be explored; however, first reports indicate the successful treatment of refractory AIH and recurrent or de novo posttransplant autoimmune hepatitis in a small number of patients [125, 126].

4.4. Biologicals: Rituximab and Infliximab. Only recently, monoclonal antibodies, e.g., Rituximab and Infliximab, have effectively been used in patients with refractory or difficult to treat AIH [127, 128, 129] [130].

Although AIH may be considered a T cell-mediated autoimmune disease, Rituximab, which is a monoclonal B cell-depleting antibody, has shown beneficial effects in refractory courses of AIH [131]. One possible explanation for this favorable effect might be the active role of B cells in antigen presentation and T cell suppression which has recently been demonstrated in an animal model of AIH [132]. Clinical side effects of Rituximab comprise infectious complications, which in some circumstances required treatment withdrawal in AIH patients. Although limited, this amount of data clearly supports further investigation of this monoclonal antibody for AIH treatment. This particularly includes the establishment of safety profiles, dosing guidelines, and monitoring strategies [4].

Infliximab is a humanized chimeric monoclonal antibody directed against the proinflammatory cytokine tumor necrosis factor alpha (TNF-a). Since its FDA approval more than 20 years ago, Infliximab has made substantial contribution in the treatment of different chronic autoimmune diseases including AIH [133]. Its clinical use, however, is limited to some small retrospective studies where it was mainly used as a salvage therapy in AIH patients [129, 134]. Infliximab can induce hepatotoxicity-resembling AIH symptoms as well as several other immune-mediated disorders [135]. This is why it should be applied with caution only in specialized centers with a large body of experience in AIH therapy and monitoring.

4.5. Thiopurines: Azathioprine (AZA), 6-Mercaptopurine (6-MP), Allopurinol, and 6-Thioguanine (6-TG). Thiopurines are a group of immunosuppressive drugs that act anti-inflammatory by inhibition of T cell activation and proliferation. Azathioprine (AZA) is a prodrug that is nonenzymatically converted to 6-mercaptopurine (6-MP) which ultimately leads

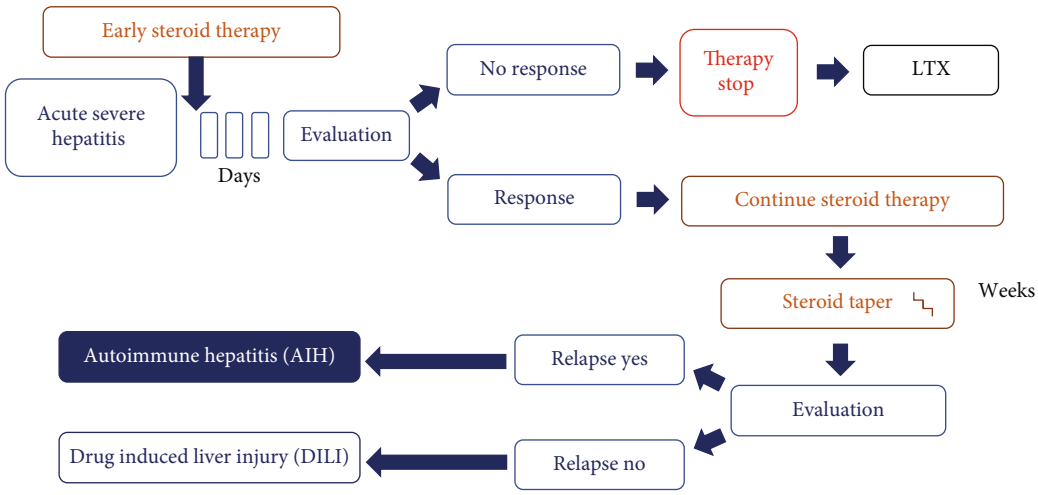

Figure 3: Proposed treatment strategy for indeterminate acute severe hepatitis based on the authors' experience. Patients with diagnosis of acute severe hepatitis receive early steroid treatment. Evaluation after 3 days is aimed at assessing a possible steroid response. If no response is seen, steroid therapy is stopped and alternative treatment strategies are considered. In case of therapy response, steroids are tapered accordingly. After steroid withdrawal, reevaluation may allow for discrimination between autoimmune hepatitis (AIH) and drug-induced liver injury (DILI), since early steroid tapering in AIH is commonly accompanied by a disease relapse.

to the generation of 6-thioguanine (6-TG). All three types of thiopurine metabolites can be routinely detected by drug metabolite monitoring and have been extensively used for inflammatory bowel disease (IBD) therapy [136]. In three AIH patients who were unresponsive or intolerant to AZA treatment, 6-MP has also been effective [137]. Another retrospective study on 22 AIH patients with azathioprine intolerance, 6-MP seemed to be beneficial and, in most cases, well tolerated as second-line treatment. Side effects included gastrointestinal symptoms and leukopenia requiring discontinuation of therapy in 23% of patients [138].

Under certain circumstances, AZA treatment can be detrimental due to an altered drug metabolism, which promotes the generation of hepatotoxic metabolites. In this circumstance, coadministration of Allopurinol can redirect thiopurine metabolism back to the physiologic pathway and reestablish effective AIH therapy [139]. Similar effects were recently confirmed in a retrospective case series of AIH patients with skewed thiopurine metabolism, in which Allopurinol was capable to reestablish normal AZA function [140].

6-Thioguanine (6-TG), which is approved for the treatment of acute and chronic myeloid leukemia and chronic lymphatic leukemia, was also evaluated as second-line therapy in AIH patients with failed MMF therapy and intolerance to azathioprine [141]. Although these findings were recently confirmed in a retrospective analysis in AIH patients with intolerance to AZA or 6-MP [142], 6-TG cannot be recommended for AIH treatment without reservations, due to its potential hepatotoxicity.

4.6. Ursodeoxycholic Acid (UDCA). Ursodeoxycholic acid (UDCA) has been widely used as treatment of choice in patients with PBC [143]. Through its choleretic action, UDCA hence reduces the retention of bile acid which in PBC and PSC is caused by a defect in hepatic bile acid excretion [144]. In addition, UDCA has been shown to have immunomodulatory properties [145] and can inhibit immune globulin production in a concentration-dependent manner [146]. In this context, UDCA has shown to be beneficial in patients with AIH allowing additional steroid tapering [147, 148]. A recent study on Japanese patients with a histological and serological mild course of AIH even revealed that UDCA monotherapy can achieve and maintain normalization of ALT levels in 71% [149]. However, this clinical value and the definition of further criteria for UDCA treatment indications must be confirmed in prospective studies.

5. Differential Diagnosis of AIH and DILI in Patients with Acute Severe Hepatitis and Liver Failure

Acute liver failure (ALF) is a rare syndrome, characterized by an acute disorder in liver coagulopathy, high serum aminotransferases, and hepatic encephalopathy, which rapidly leads to progressive multiorgan failure with unpredictable complications [150].

AIH typically manifests as a chronic illness; however, up to 20% of patients may present with an acute onset of disease which can be associated with the development of acute liver failure (ALF) [56]. The identification of AIH as the etiology of ALF is key to effective therapeutic intervention, because a delay in diagnosis and initiation of steroid therapy results in poor outcomes [151].

In parallel, drug-induced liver injury which is predominantly caused by acetaminophen overdosing, or other idiosyncratic drug reactions, accounts for 13% to 17% of cases of acute liver failure in both the United States and Europe [152, 153]. Differential diagnosis of AIH and DILI presenting with acute liver failure is extremely complicated

and frequently ends up with the unsatisfactory diagnosis of indeterminate acute liver failure.

While corticosteroids have been shown to be beneficial in treating acute severe courses [154] and even acute on chronic courses [155] of AIH, treatment of acute forms of DILI primarily focuses on identifying and withdrawing the offending agent. Administration of N-acetylcysteine (NAC), which is generally recognized as the antidote for acetaminophen overdose, has been also shown to significantly improve outcome of nonacetaminophen-induced acute liver failure [156]. Administration of corticosteroids in patients with DILI and ALF is still under debate. While early studies suggested no benefit or even an adverse effect [157, 158], recent publications prove corticosteroids to be safe in patients with severe DILI [159, 160]. In summary, studies suggest that corticosteroids may increase the risk of infection in patients with ALF for both disease etiologies (AIH and DILI) in the event of liver transplantation [161, 162].

Several data including our own data suggest that early steroid use in patients with indeterminate acute severe hepatitis is safe and is not accompanied by increased infectious complications. Subsequent dose tapering even allows for early discrimination between AIH and DILI due to specific transaminase flares in patients with AIH [163]. DILI often remains a diagnosis of exclusion. We therefore believe that early and short-term use of corticosteroids is not detrimental but instead beneficial for patients with indeterminate acute severe hepatitis likely developing ALF (Figure 3).

6. Liver Transplantation for Autoimmune Hepatitis

Autoimmune liver disease (AILD) represents one of the major indications for liver transplantation and accounts for approximately 24% of transplants performed in Europe and the US [164, 165]. Among the three major AILD entities, namely, AIH, PBC, and PSC, only AIH may present as acute liver failure and hence qualify for high-urgency (HU) liver transplantation [161]. Among AILD, AIH only accounts for a fraction of 3% for pediatric and 5% for adult transplants; nevertheless, in several Scandinavian countries, AILD is the leading indication for liver transplantation, presumably due to a relatively low prevalence of hepatitis C and alcoholic liver disease [166].

In general, transplantation is indicated for patients who present with acute fulminant liver failure which is unresponsive to steroid treatment. Best sets of diagnostic factors associated with outcome for liver transplantation for ALF comprise King's College [167] and Clichy criteria [168, 169]. Further indications include patients with end-stage chronic autoimmune liver disease with a Model of End-Stage Liver Disease (MELD) Score > 15 or higher who have or have not developed HCC. Although there is no single viable predictor for the necessity of LT, patients with a higher MELD score on administration, no improvements in bilirubin and INR levels within the first days of steroid treatment, and presence of necrosis on histology have an increased need for urgent transplantation [170].

Chronic courses of disease with an unacceptable quality of life due to treatment-resistant pruritus or severe hepatic encephalopathy may also benefit from transplantation. HCC occurs in AIH patients with cirrhosis with a variable incidence of 1.9% per year [171]. Accordingly, routine cancer screening and surveillance among this cohort for early detection and treatment is mandatory. The combination of prednisolone and a calcineurin inhibitor is currently the gold standard immunosuppressive treatment regimen for liver transplantation and can also be considered as highly effective in patients with AIH resulting in 1- and 5-year graft survival rates of 84% and 75%, respectively, 5- and 10-year patient survival rates of 90% and 75% [172].

7. Recurrent and *De Novo* Autoimmune Hepatitis

In up to 40% autoimmune disease can recur after liver transplantation, despite immunosuppressive therapy [173, 174]. The immunosuppressive regimen however does not seem to have a huge impact on recurrence rate [175]. Acute fulminant AIH is less likely to recur than chronic manifestations. If this is the case, graft dysfunction demonstrates identical features to those of classical AIH [176]. The severity of necroinflammatory activity in the native liver and high IgG levels at the time of transplantation are most reliable predictors of recurrence [176]. Other risk factors for return of AIH after liver transplantation remain unvalidated and controversial [177].

Recurrent AIH is responsive to the reintroduction or dose increase of corticosteroids and azathioprine [178]. Treatment-refractory patients can alternatively be managed with Cyclosporine [125], Sirolimus [179], or MMF [103]. It is important to distinguish recurrent AIH from other etiologies causing liver damage such as rejection, biliary problems, and viral hepatitis [180]. Although uncommon, retransplantation and even return of AIH in a second liver graft have been described [181, 182].

Manifestation of AIH in patients undergoing liver transplantation for other diseases than AIH is called *de novo* AIH [183]. Initially described in pediatric patients [184], de novo AIH has also been reported in adult patients with frequencies ranging from 2.1 to 6.6% [185] and a reported time to development ranging from 0.3 and 7 years post liver transplantation [186]. Although high IgG and autoantibody levels are commonly present in patients with de novo AIH, liver biopsies must be performed to confirm diagnosis [187, 188]. Histology is furthermore an essential element in the differential diagnosis between de novo AIH and graft rejection. The time interval between liver transplantation and onset of disease (de novo AIH) might be an important diagnostic clue [189]. There is also supporting evidence that de novo AIH might be a form of late graft rejection itself [190]. This hypothesis is supported by the fact that antibodies which arise in patients after episodes of acute rejection are directed against graft antigens and not self-antigens. Recurrent courses of rejection have subsequently been described as a risk factor for de novo AIH development [191].

Several studies investigated the genetic background of de novo AIH after liver transplantation and tried to find a relationship to the possession of specific major histocompatibility (MHC) antigens by the recipient and/or the donor [192]. In particular, the status of HLA-DR4 and HLA-DR3 was associated with a risk of recurrence in some research [184, 193]. An increased expression of DRB∗0301 or DRB∗0401 in either donor or recipient has furthermore been described; however, larger numbers of patients are needed to prove the genetic influence on the development of de novo AIH post liver transplantation [186].

Other risk factors associated with the development of de novo AIH include female gender and a donor age of >40 years [194], as well as glutathione S-transferase T1 (GSTT1) donor/recipient mismatch [195, 196].

Recommendations for the treatment of de novo AIH are similar to the standard treatment for recurrent AIH after liver transplantation and comprises the combination of corticosteroids and azathioprine. The majority of cases can be treated effectively, and only a small fraction may progress to graft failure and require retransplantation [197]. Long-term outcomes of de novo AIH have shown excellent results in a study of 31 patients, who reports no death after liver transplantation for de novo AIH after a median follow-up of 7.1 years [198].

8. Empiric Data on Diagnosis and Treatment of AIH Patients in Our Own Institution

We investigated the clinical outcome of patients with acute AIH in our single center. A retrospective analysis of data (from 10/2011 to 5/2016) identified 38 patients with eventually newly diagnosed AIH, who presented with clinical symptoms of acute severe hepatitis and high ALT levels (ALT > 5 × ULN, upper limits of normal).

Demographic data are presented in Table 3. Of note, patients had a median MELD score of 16 (range 6-23) at the time of presentation and cirrhosis was evident in 11%. Simplified diagnostic criteria for AIH were available for $n = 26$ patients, revealing definite or probable AIH in 27% and 31% of patients, respectively. 42% had a simplified AIH score of ≤5, indicative for no AIH at the time of presentation. Liver biopsy was performed in $n = 28$ patients (74%), out of which 29% demonstrated typical signs of AIH. However, interface hepatitis was only detectable in 11%.

All patients received tapered corticosteroids and azathioprine with median starting dosages of 60 mg and 100 mg, respectively. AZA was routinely added three weeks after corticosteroid start when tapering of steroid was initiated. 29% of patients were intolerant to AZA, requiring therapy cessation and switch to MMF. In another 5%, Everolimus was added to the MMF therapy plan. Steroid tapering at our institution was performed according to the EASL protocol and guidelines.

As depicted in Figure 4(a) , ALT values significantly decreased over time following corticosteroid treatment and in most cases returned to normal values after 6 months. A first full treatment response was detectable in one patient already two weeks after corticosteroid treatment, and response rates significantly increased over time (Figure 4(b)). ALT values reached normal levels in more than 70% of cases after 1 year of medical treatment. In this cohort, probably due to the sufficiently early and effective steroid treatment protocol, we register a 100% survival rate, with no requirement for high-urgency liver transplantation. Infectious complications (3%) were also comparatively low under immunosuppressive therapy in our cohort.

Table 3: Demographic and descriptive data of AIH patients treated at our institution between 10/2011 and 05/2016.

Demographic data	
Total number 10/2011-05/2016	$n = 38$
Age (median)	50 years
Male	$n = 16$ (42%)
Female	$n = 22$ (58%)
Cirrhosis	$n = 4$ (11%)
Ascites	$n = 3$ (8%)
Hepatic encephalopathy (grade 1)	$n = 1$ (3%)
MELD, median (range)	16 (6-23)
Other autoimmune disease	$n = 21$ (55%)
Simplified AIH score (available for $n = 26$)	
Definite AIH (points ≥ 7)	$n = 7$ (27%)
Probable AIH (points > 6)	$n = 8$ (31%)
No AIH (points ≤ 5)	$n = 11$ (42%)
Liver histology (available for $n = 28$)	
AIH typical	$n = 8$ (29%), interface hepatitis $n = 3$ (11%)
Inconclusive	$n = 7$ (25%)
Not performed	$n = 10$ (36%)
Immunosuppressive therapy	
Prednisolone (mg), median (range)	60 mg (50-100 mg)
Prednisolone therapy duration (days), median (range)	180 days (60-1080 days)
Azathioprine (mg), median (range)	100 mg (50-200 mg)
Azathioprine start after weeks, median (range)	3 weeks (1-12 weeks)
Azathioprine intolerance	$n = 11$ (29%)
Conversion to MMF	$n = 11$ (29%)
Conversion to Everolimus	$n = 2$ (5%)
Outcome	
Survival	$n = 38$ (100%)
Infection	$n = 1$ (3%)
Liver transplantation	$n = 0$ (0%)

9. Conclusion

Over the last decades, substantial progress in understanding the pathogenesis of AIH has been made. In this context, animal models have been vital instruments in detecting

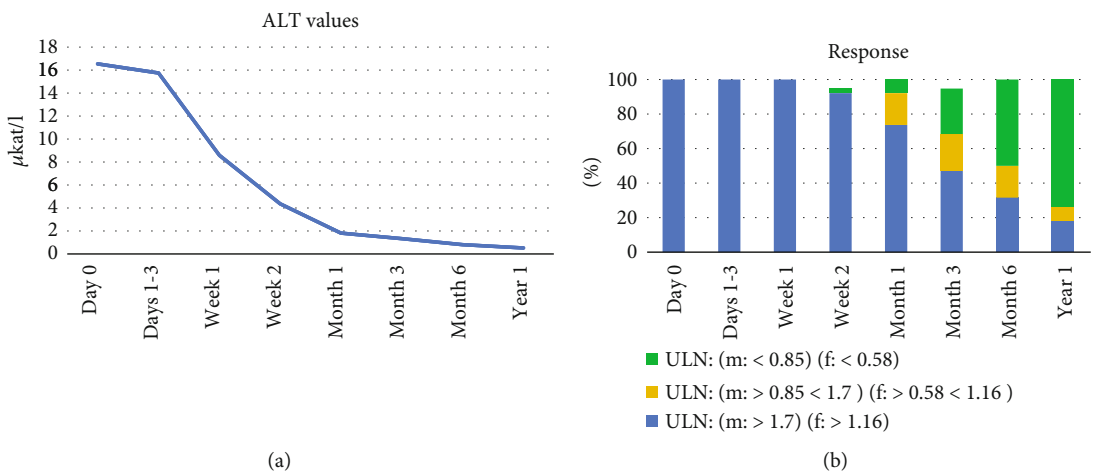

Figure 4: ALT values (a) and response rates in % (b) (indicated as ranges of upper limits of normal, ULN) of AIH patients who received early corticosteroid treatment at our institution. ALT < 0.85 μkat/l for male and <0.58 μkat/l for female patients were considered normal and hence classified as full response (green). ALT levels > 0.85 and <1.7 μkat/l for male and >0.58 μkat/l and <1.16 μkat/l for female were considered as partial response (yellow), and ALT > 1.7 μkat/l for male and >1.16 μkat/l for female were considered as no response (blue).

different immune cell and cytokine involvement in autoimmune-triggered liver damage. Large human genome studies have identified key predisposing HLA allelic variants associated with AIH development, and several genetic associations outside the HLA locus are under investigation. The currently used AIH scoring systems display acceptable sensitivity and specificity for clinical practice; however, firm diagnostic tools are still in demand. EASL and AASLD guidelines provide a fundamental overview about diagnostic and therapeutic approaches. Corticosteroid treatment with or without azathioprine still remains the gold standard, and liver transplantation is reserved for severe cases presenting with acute liver failure or patients with chronic end-stage liver disease. Novel emerging laboratory techniques may provide a better understanding of the pathogenesis of AIH and facilitate innovative and specific AIH therapy.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

Elisabeth Sucher and Robert Sucher contributed equally to this work.

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Research Article

Association of Melatonin Pathway Gene's Single-Nucleotide Polymorphisms with Systemic Lupus Erythematosus in a Chinese Population

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Objectives. This study was to investigate the association of melatonin (MTN) pathway gene's single-nucleotide polymorphisms (SNPs) with susceptibility to systemic lupus erythematosus (SLE). *Methods*. We recruited 495 SLE patients and 493 healthy controls, 11 tag SNPs in *MTN receptor 1a* (*MTNR1a*), *MTNR1b*, and arylalkylamine N-acetyltransferase (*AANAT*) genes were genotyped and analyzed. Serum MTN concentration was determined by enzyme-linked immunosorbent assay (ELISA) kits. *Results*. Two SNPs of *AANAT* gene (rs8150 and rs3760138) associated with the risk of SLE; CC carriers of rs8150 had a lower risk as compared to GG (OR = 0.537, 95% CI: 0.361, 0.799), whereas GG carrier in rs3760138 had an increased risk (OR = 1.823 , 95% CI: 1.154, 2.880) compared to TT. However, we did not find any genetic association between the other nine SNPs with SLE risk. Case-only analysis showed associations of rs2165667 and rs1562444 with arthritis, rs10830962 with malar rash, rs3760138 with immunological abnormality, and rs8150 with hematological abnormality. Furthermore, a significant difference between plasma MTN levels with different genotypes of rs1562444 was observed. Haplotype analyses revealed that haplotype of CCTAT, CTAGT, and GGG was significantly associated with the increased risk in SLE susceptibility, but TCTAT and CTG appeared to be a protective haplotype. *Conclusions*. The present study supported the genetic association of MTN pathway genes with SLE susceptibility and specific clinical manifestations, suggesting the potential role of MTN pathway genes in the pathogenesis and development of SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic and inflammatory autoimmune disease characterized by antinuclear autoantibody production and a multitude of immune-complex deposition, which is involved in multisystem; such as the skin, kidney, and brain; and caused organs/tissue destruction [1, 2]. Until now, the etiology of SLE is still not fully understood. A large body of literatures have suggested that the interactions between exogenous (infectious triggers, ultraviolet radiation, and dietary habit) and endogenous sources (hereditary susceptibility, endocrine disorders and

disturbed status of oxidative metabolism, and autoimmune responsiveness sympathetic nervous system) are responsible for the pathogenesis and development of SLE [3–9].

Melatonin (MTN), as one of the major neuroendocrine hormones, is mainly produced and secreted by the pineal gland; it allows to regulate the circadian day-night rhythm and seasonal biorhythms and is also a key player in the neuroendocrine-immune pathway [10, 11]. Several studies have suggested the important role of MTN on the regulation of the immune system; it can skew the immune response by repressing the production of several proinflammatory cytokines (such as tumor necrosis factor- (TNF-) α, interleukin- (IL-) 1β, and IL-6), as well as blocking the DNA-binding activity of NF-κB both *in vitro* and *in vivo*, exerting an anti-inflammatory effect [11–16].

Previous studies have investigated the underlying role of MTN in SLE. Lechner et al. observed an increased MTN level in MRL/MP-fas lupus-prone mice (represents an animal model for human autoimmune diseases, which spontaneously develops lupus-like glomerulonephritis, systemic vasculitis, arthritis, and sialadenitis) during the light phase; under the regulation of MTN, the levels of autoantibodies were reduced, and the histological changes were improved in female lupus MRL/MP-fas lupus-prone mice [17]. MTN is able to inhibit IgM, anti-dsDNA, and anti-histone antibodies, thus, decreases the levels of IL-6 and IL-13 and increases the IL-12 levels [18]. In patients with SLE, a lower daily MTN level was observed as compared to healthy controls, and this decreased daily MTN level inversely correlated with the systemic lupus erythematosus disease activity index (SLEDAI) [19]. In addition, study has also revealed the seasonal pattern of MTN levels in SLE, with an elevated daily plasma MTN levels in December than in June [20]. These findings provided the possible evidence that MTN might play a potential role in the pathogenesis of SLE.

The synthesis and function of MTN mainly depend on three MTN pathway genes, *MTN receptors 1a/1b* (*MTNR1a*/*MTNR1b*) are largely responsible for mediating the downstream effects of MTN, and arylalkylamine N-acetyltransferase (*AANAT*) is the major enzyme in MTN synthesis [21, 22]. The genetic association of MTN genes with some diseases has been demonstrated in several studies, including SLE, multiple sclerosis (MS), breast cancer, and major depression [23–26]. Nevertheless, associations between genetic variation in MTN pathway genes and SLE susceptibility have not been determined.

In the present study, we conducted a case–control study to comprehensively evaluate the role of common genetic variation in the *MTNR1a*, *MTNR1b*, and *AANAT* genes to SLE susceptibility in a Chinese population.

2. Materials and Methods

2.1. Study Subjects and Methods. This case–control genotyping study recruited a total of 988 subjects (495 SLE patients and 493 healthy controls). The sample size and power calculation of the study were computed by power and sample size program, where both the minor allele frequency (MAF) and statistical significant level were set as 0.05, odds ratio (OR) was 1.5, case and control ratio was 1 : 1 and when the statistical power was 0.8, the computed sample size for cases was 451. In addition, we also evaluated the statistical power for the 496 included cases, and the results showed that the computed statistical power was 0.833.

Patients with SLE were recruited from the Department of Rheumatology and Immunology at Anhui Provincial Hospital, The First Affiliated Hospital of Anhui Medical University. The diagnosis of SLE was established by the presence of four or more 1997 revised American College of Rheumatology (ACR) classification criteria [27]. Patients with viral infections and any history of cancer, pregnancy, and recurrent spontaneous abortions were excluded based on reviews of their appropriate history. The normal controls consisted of age, gender, and ethnicity-matched healthy individuals who belonged to the same geographical area as that of cases; normal controls were excluded if they had a family history of SLE or any other autoimmune disease and history of any chronic or lifestyle diseases like depression, hypothyroidism, hypertension, diabetes mellitus, and tuberculosis (TB). Demographics, clinical features, and related laboratory results were obtained from hospital medical records and then reviewed by experienced physicians.

The Ethical Committee of Anhui Medical University (Hefei, Anhui, China) approved this study. All the study subjects provided informed consent to participate in this study.

All studies on humans described in the present manuscript were carried out with the approval of the responsible ethics committee and in accordance with the national law and the Declaration of Helsinki 1975 (in its current, revised form).

2.2. MTN Pathway Gene's SNP Selection and Genotyping. Ensembl Gene Browser 37 (GRCh37) (http://grch37.ensembl.org/index.html) (Ensembl Archive Release 90) was implemented to acquire the genetic and location information of *MTNR1a*, *MTNR1b*, and *AANAT* genes [28], and linkage pedigree file (PED) and marker information file were downloaded. Then, the downloaded files were used to select the tag SNPs in Haploview 4.2 software (Broad Institute, Cambridge, MA, USA), with a MAF above 0.05 in Chinese Han population (CHB) of Beijing, linkage disequilibrium (LD) with an r^2 threshold of 0.8. A total of 46 tag SNPs (23 *MTNR1a* tag SNPs, 13 *MTNR1b* tag SNPs, and 10 *AANAT* tag SNPs) were captured for further evaluation. The function prediction for 46 tag SNPs was assessed by the online bioinformatics tools (https://snpinfo.niehs.nih.gov/snpinfo/snpfunc.html) [29]; the basic information of these tag SNPs is shown in Supplementary Table 1. In addition, the published literatures about the MTN pathway gene's polymorphisms were also carefully reviewed. Based on one or more of the following criteria: assumed functionality (located in the regulatory regions, for example, 3′-untranslated regions (UTR), 5′-UTR, or amino acid change), $r^2 \geq 0.80$ and MAF $\geq$ 5%, as well as previous studies reported SNPs), the five tag SNPs (rs10030173, rs2119882, rs2165667, rs4861722, and rs6847693) in *MTNR1a* gene, three tag SNPs (rs1562444, rs10830962, and rs3781637) in *MTNR1b* gene,

and three tag SNPs (rs8150, rs3760138, and rs12942767) in *AANAT* gene were finally chosen for further genotyping.

Genomic DNA was extracted from peripheral venous blood of patients and healthy controls using a QIAGEN kit (QIAGEN, Hilden, Germany) based on the manufacturer's instructions, and quantification and concentration of DNA was determined using NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA). Qualified sample requirements were shown as follows: concentration greater than 50 ng/μl, total amount greater than 600 ng, and no obvious degradation.

Genotyping was performed in using improved multiple ligase detection reaction (iMLDR), with technical support from the Center for Genetic & Genomic Analysis, Genesky Biotechnologies Inc., Shanghai. A multiplex PCR-ligase detection reaction method was used in the iMLDR. For each SNP, the alleles were distinguished by different fluorescent labels of allele-specific oligonucleotide probe pairs. Different SNPs were further distinguished by different extended lengths at the 3′ end. Two negative controls were set: one with double-distilled water as template and the other with DNA sample without primers while keeping all other conditions the same in one plate. Duplicate tests were designed, and the results were consistent. A random sample accounting for ~3% of the total DNA samples was directly sequenced using BigDye Terminator version 3.1 and an ABI3730XL automated sequencer (Applied Biosystems) to confirm the results of iMLDR.

2.3. Plasma MTN Determination. Blood samples were collected from 5 ml of whole blood of all study subjects and then stored at -80°C until assayed. Plasma MTN concentration was determined by enzyme-linked immunosorbent assay (ELISA) kits (the lower detection limit was 0.1 pg/ml) which were purchased from Anhui Xinle Biotechnology Co. Ltd.; the results of MTN were expressed as picograms per milliliter. The interassay and intraassay variation coefficients of the ELISA kit of our study were 6.9% and 7.6%, respectively.

2.4. Statistical Analysis. The allelic and genotypic association analyses between SLE patients and healthy controls were performed in using the Chi-square or Fisher's exact test. Logistic regression analyses were utilized to calculate odds ratios (ORs), and 95% confidence intervals (CIs) for the association between genotype and SLE susceptibility, additive, dominant, recessive, and allelic models were also considered. A nonparametric test was used to compare the difference in plasma MTN levels among patients with different genotypes. Statistical analysis was implemented with the use of the SPSS (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.).

Hardy–Weinberg equilibrium (HWE) among controls was assessed by comparing the observed-to-expected genotype frequencies using the Chi-square test. Online software SHEsis was used for haplotype analyses of each MTN pathway genes; all the haplotypes with a frequency < 0.03 were ignored in the analysis [30]. All results with a two-tailed $P < 0.05$ were considered to be statistically significant. The Bonferroni correction was used for multiple testing.

3. Results

3.1. Characteristics of the Study Population. The present study recruited 988 subjects, with 495 SLE patients and 493 healthy controls. In SLE patients, there were 57 males and 438 females with a median age of 37.00 (28.00, 46.00) years and the median disease duration was 4.10 (range from 1.05 to 9.06) years, while there were 55 males and 434 females with the median age of 38.00 (30.00, 47.00) in healthy controls. No significant differences in gender and age distribution were observed between SLE patients and normal controls (Table 1). The major clinical manifestations of SLE were immunological abnormality (73.1%), hematological abnormality (68.3%), arthritis (49.5%), malar rash (45.3%), photosensitivity (39.0%), and renal abnormality (37.2%). In control groups, the presence of observed genotype frequencies of all included tag SNPs was distributed in compliance with the HWE (all $P > 0.05$).

3.2. Association of MTNR1a/b and AANAT Gene's Polymorphisms with Susceptibility to SLE. There were no significant differences in allele and genotype distribution of 8 tag SNPs in *MTNR1a/b* genes between SLE and healthy controls (all $P > 0.05$) (Table 2). However, when analyzing the allele and genotype frequency of 3 tag SNPs in *AANAT* genes, the results showed that two SNPs of rs8150 and rs3760138 were associated with the risk of SLE susceptibility, where CC carriers of rs8150 had a lower risk as compared to GG (OR = 0.537, 95% CI: 0.361, 0.799) ($P = 0.002$), whereas GG carrier of rs3760138 had an increased risk as compared to TT (OR = 1.823, 95% CI: 1.154, 2.880) ($P = 0.010$), but we did not observe other positive findings regarding the SNPs of rs12942767 (Table 3).

3.3. Association of MTNR1a/b and AANAT Gene's Polymorphisms with Clinical Features in Patients with SLE. Case-only analysis was conducted to further explore the genetic association of *MTNR1a/b* and *AANAT* gene's polymorphisms with specific clinical features of SLE. In *MTNR1a/b* genes, a significantly increased AA genotype frequency of rs2165667 (*MTNR1a*) and A allele frequency of rs1562444 (*MTNR1b*) were found in patients with arthritis than those without (both $P = 0.024$). In addition, the frequency of CC/CG genotype in rs10830962 (*MTNR1b*) was significantly lower in patients with malar rash than in those without ($P = 0.018$). In terms of the genetic association of *AANAT* gene with clinical features of SLE, there was a higher GG/GT/TT genotype distribution of rs3760138 in patients with positive immunological abnormality than those with negative ($P = 0.024$); the C/G allele and CC/CG/GG genotype frequency of rs8150 appeared to have a significantly increased risk in patients with positive hematological abnormality compared with those with negative ($P = 0.039$, $P = 0.010$, respectively). Nevertheless, no other positive findings were revealed regarding the *MTNR1a/b* and *AANAT* gene's polymorphisms with SLE clinical features (Table 4).

3.4. Association of Plasma MTN Concentrations with Genotypes in Patients with SLE. The results indicated that, in patients with SLE, there was a significant difference of

Table 1: Demographic characteristics and clinical features of patients with SLE and control subjects.

Parameters	Patients with SLE ($n = 495$)	Healthy controls ($n = 493$)
Demographic characteristics		
Age (year)	37.00 (28.00, 46.00)**	38.00 (30.00, 47.00) **
Female, n (%)	438 (88.5)	434 (88.0)
Male, n (%)	58 (11.5)	59 (12.0)
Disease duration (year)	4.10 (1.05, 9.06) **	NA
BMI (kg/m^2)	21.48 (19.72, 23.43) **	NA
SLEDAI	11.40 ± 9.07*	NA
Disease manifestations		NA
Malar rash, n (%)	224 (45.3)	NA
Discoid rash, n (%)	94 (19.0)	NA
Photosensitivity, n (%)	193 (39.0)	NA
Oral ulcers, n (%)	119 (24.0)	NA
Arthritis, n (%)	245 (49.5)	NA
Serositis, n (%)	45 (9.1)	NA
Renal abnormality, n (%)	184 (37.2)	NA
Neurological abnormality, n (%)	21 (4.2)	NA
Hematological abnormality, n (%)	338 (68.3)	NA
Immunological abnormality, n (%)	362 (73.1)	NA

BMI: Body mass index; n: number; SLE: systemic lupus erythematosus; SLEDAI: systemic lupus erythematosus disease activity index. *Data with normal distribution were described as mean and standard deviation (SD). **Data with skewed distribution were described as median and interquartile range (IQR).

MTN level among genotype of AA, AG, and GG in rs1562444 (*MTNR1b*) ($P = 0.001$), which GG genotype showed an elevated MTN concentration than in AA and AG genotype (20.57 pg/ml *vs* 14.08 pg/ml *vs* 10.36 pg/ml). However, no significant differences of plasma MTN concentrations were observed in other SNPs between patients (Table 5).

3.5. Haplotype Analyses. The haplotype of tag SNPs in *MTNR1a/b* and *AANAT* genes was constructed by using SHEsis software. Haplotype analyses implied that haplotype CCTAT (*MTNR1a*), CTAGT (*MTNR1a*), and GGG (*AANAT*) were significantly associated with the increased risk in SLE susceptibility, but TCTAT (*MTNR1a*) and CTG (*AANAT*) appeared to be a protective haplotype (all $P < 0.05$). However, no positive findings of other haplotypes were observed (Tables 6–8).

4. Discussion

The neuroendocrine-immune system is regarded as a fundamental network supporting the health state that could play an important role in the development of autoimmune disorders [31, 32]. MTN, as one of the pineal gland-driven hormones, secreted in a circadian rhythm and regulated by photoperiod with the highest peak at midnight and lowest level after sunrise, acting mainly as a regulator for sleeping rhythm [33–36]. In recent years, the bidirectional associations between the pineal gland and the immune system have been suggested to depend on the immune-modulating effect of MTN and the pineal regulation by different lymphokines [37]. MTN might have a direct effect on immune-competent cells, thus, fulfilling critical roles on the development and progression of autoimmune diseases [38–40].

Our previous study has evaluated the level of MTN in patients with SLE as compared to healthy controls. Although there was no significant difference in MTN concentration between those two groups, we observed a slightly lower level of MTN in SLE patients than in healthy controls; in addition, an inverse correlation of MTN concentration with IgM was also revealed [41]. Similarly, Robeva et al. revealed that there was a decreased daily MTN level in women with SLE and found an inverse relationship between daily MTN concentrations and disease activity [19]. In the subarctic region, the study has demonstrated the presence of seasonal variations in daily MTN level, where the increased level of MTN was discovered in December than that in June [20]. These evidences suggest that the change of MTN concentration may be involved in the pathogenesis of SLE.

MTNR1a/b and *AANAT* play an important role during the pathway of MTN from biosynthesis to its functioning; the former *MTNR1a/b* genes are largely responsible for mediating the downstream effects of MTN, while the latter *AANAT* gene is the major enzyme in MTN synthesis. Several previous studies have investigated the underlying role of MTN pathway genes in a number of human diseases. In SLE, primary study, performed by Tanev et al., has demonstrated no significant differences in allelic and genotype distribution of *MTNR1b* gene (rs1562444, rs10830962, and rs10830963) polymorphisms between 109 patients with SLE and 101 controls, yet, in SLE patients, C/C genotype of rs10830963 in *MTNR1b* gene was related to increased prevalence of leucopenia compared to C/G and G/G genotype; the rs10830963 G/G carriers had a lower number of lupus

Table 2: Genotype frequencies of *MTNR1a*/*b* SNPs in SLE patients and healthy controls.

SNPs	Analyzed model		SLE	Control	OR (95% CI)	*P* value*
rs10030173 (*MTNR1a*)	Genotypes	CC	107	93	1.308 (0.916, 1.867)	0.139
		CT	242	234	1.176 (0.883, 1.565)	0.267
		TT	146	166	1.000	
	Additive model	CC	107	93	1.308 (0.916, 1.867)	0.139
		TT	146	166	1.000	
rs2119882 (*MTNR1a*)	Genotypes	CC	71	78	0.792 (0.542, 1.156)	0.226
		CT	217	235	0.803 (0.612, 1.054)	0.114
		TT	207	180	1.000	
	Additive model	CC	71	78	0.792 (0.542, 1.156)	0.226
		TT	207	180	1.000	
rs2165667 (*MTNR1a*)	Genotypes	AA	230	200	1.267 (0.849, 1.890)	0.246
		AT	206	228	0.995 (0.667, 1.484)	0.982
		TT	59	65	1.000	
	Additive model	AA	230	200	1.267 (0.849, 1.890)	0.246
		TT	59	65	1.000	
rs4861722 (*MTNR1a*)	Genotypes	AA	25	28	0.978 (0.548, 1.743)	0.939
		GA	165	189	1.243 (0.707, 2.183)	0.450
		GG	304	274	1.000	
	Additive model	AA	25	28	0.978 (0.548, 1.743)	0.939
		GG	304	274	1.000	
rs6847693 (*MTNR1a*)	Genotypes	CC	182	167	0.868 (0.658, 1.145)	0.318
		CT	229	242	0.918 (0.635, 1.326)	0.647
		TT	84	84	1.000	
	Additive model	CC	182	167	0.868 (0.658, 1.145)	0.318
		TT	84	84	1.000	
rs1562444 (*MTNR1b*)	Genotypes	AA	254	262	1.039 (0.659, 1.636)	0.870
		GA	199	186	1.146 (0.720, 1.826)	0.565
		GG	42	45	1.000	
	Additive model	AA	254	262	1.039 (0.659, 1.636)	0.870
		GG	42	45	1.000	
rs10830962 (*MTNR1b*)	Genotypes	CC	176	145	1.349 (0.951, 1.913)	0.093
		CG	220	236	1.036 (0.746, 1.438)	0.834
		GG	99	110	1.000	
	Additive model	CC	176	145	1.349 (0.951, 1.913)	0.093
		GG	99	110	1.000	
rs3781637 (*MTNR1b*)	Genotypes	CC	11	11	1.009 (0.420, 2.424)	0.984
		CT	111	110	1.003 (0.429, 2.341)	0.995
		TT	373	372	1.000	
	Additive model	CC	11	11	1.009 (0.420, 2.424)	0.984
		TT	373	372	1.000	

SLE: systemic lupus erythematosus; SNPs: single-nucleotide polymorphisms; OR: odds ratio; *MTNR1a*: *melatonin receptor 1a*; *MTNR1b*: *melatonin receptor 1b*.*The *P* values are not corrected for multiple testings, Bonferroni corrected $P = 0.0167$.

criteria than in those with C/C genotype [25]. The *MTNR1b* rs10830962 and rs10830963 polymorphisms have been predominantly investigated in the context of metabolic disorders, of which rs10830962 and rs10830963 G alleles were reported to associate with reduced insulin secretion, increased fasting plasma glucose concentrations, and increased risk for diabetes in different populations [42–44]. In autoimmune diseases of multiple sclerosis (MS), there were no significant allelic associations of SNPs rs4753426 and rs10830963 in *MTNR1b* gene with susceptibility to MS,

Table 3: Genotype frequencies of *AANAT* SNPs in SLE patients and healthy controls.

SNPs	Analyzed model		SLE	Control	OR (95% CI)	*P* value*
rs8150	Genotypes	CC	176	202	0.537 (0.361, 0.799)	**0.002**
		GC	232	236	1.128 (0.860, 1.480)	0.384
		GG	86	53	1.000	
	Additive model	CC	176	202	0.537 (0.361, 0.799)	**0.002**
		GG	86	53	1.000	
rs3760138	Genotypes	GG	271	223	1.823 (1.154, 2.880)	**0.010**
		GT	188	216	1.306 (0.820, 2.078)	0.261
		TT	36	54	1.000	
	Additive model	GG	271	223	1.823 (1.154, 2.880)	**0.010**
		TT	36	54	1.000	
rs12942767	Genotypes	GG	450	447	—	1.000
		GA	44	46	—	1.000
		AA	1	0	1.000	
	Additive model	GG	450	447	—	1.000
		AA	1	0	1.000	

SLE: systemic lupus erythematosus; SNPs: single-nucleotide polymorphisms; OR: odds ratio; *AANAT*: *arylalkylamine N-acetyltransferase*. *The *P* values are not corrected for multiple testings, Bonferroni corrected $P = 0.0167$.

Table 4: The positive findings on association of clinical characteristics with genotype and allele frequencies in *MTNR1a*/*b* and *AANAT* genes.

Gene (SNPs)	Allele (M/m)	Clinical features	Group	Genotypes (*n*)			*P* value	Alleles (*n*)		*P* value
				MM	Mm	mm		*M*	*m*	
rs2165667 (*MTNR1a*)	A/T	Arthritis	Positive	125	87	33	**0.024**	337	153	0.318
			Negative	105	119	26		329	171	
rs10830962 (*MTNR1b*)	C/G	Malar rash	Positive	77	90	57	**0.018**	244	204	0.055
			Negative	99	130	42		328	214	
rs1562444 (*MTNR1b*)	A/G	Arthritis	Positive	136	94	15	0.072	366	124	**0.024**
			Negative	118	105	27		341	159	
rs3760138 (*AANAT*)	G/T	Immunological abnormality	Positive	198	131	33	**0.024**	527	197	0.264
			Negative	73	57	3		203	63	
rs8150 (*AANAT*)	C/G	Hematological abnormality	Positive	131	155	51	**0.039**	417	257	**0.010**
			Negative	45	77	35		167	147	

SNPs: single-nucleotide polymorphisms; *MTNR1a*: *melatonin receptor 1a*; *MTNR1b*: *melatonin receptor 1b*; *AANAT*: *arylalkylamine N-acetyltransferase.*

but the rs10830963-rs4753426 G-T haplotype associated with the risk of MS in the progressive MS group [23]. Deming et al. analyzed the MTN pathway gene's polymorphisms in human breast cancer patients, and they supported that AA genotype of *MTNR1b* rs10765576 was associated with a decreased risk of breast cancer, the GG genotype in premenopausal women correlated with an increased risk for breast cancer, and however, in postmenopausal women, the GG genotype were related with a decreased risk of breast cancer; they did not observe any significant breast cancer associations for variants in the *AANAT* gene [24]. In patients with major depression, the two SNPs of *AANAT* (rs3760138 and rs4238989) were reported to be associated with an increased contribution to major depression [26].

In the present study, the tag SNPs of rs8150 and rs3760138 in *AANAT* gene were associated with genetic susceptibility to SLE, but no genetic association regarding the other nine tag SNPs with SLE susceptibility was found. Case-only analysis indicated that AA genotype frequency in rs2165667 (*MTNR1a*) AA genotype and rs1562444 (*MTNR1b*) A/G allele frequency were at increased risk for arthritis and rs10830962 (*MTNR1b*) CC/CG genotype was at decreased risk for malar rash. In *AANAT* gene, rs3760138 GG/GT/TT genotype associated with positive immunological abnormality than those with negative, and rs8150 CC/CG/GG genotype and its C/G allele appeared to have an increased risk for hematological abnormality. Moreover, we also found that there was a significant difference of MTN concentration samong the genotype of AA, AG, and

Table 5: Association of plasma MTN levels with genotype in *MTNR1a/b* and *AANAT*.

SNPs	Genotypes	Number	Plasma MTN levels (pg/ml) M (P_{25}, P_{75})	P value
rs10030173 (*MTNR1a*)	CC	19	14.22 (9.23, 20.57)	0.946
	CT	45	13.45 (9.91, 19.06)	
	TT	19	12.39 (10.27, 18.69)	
rs2119882 (*MTNR1a*)	CC	9	12.19 (9.54, 25.08)	0.312
	CT	44	13.79 (10.34, 20.00)	
	TT	30	10.82 (9.16, 16.33)	
rs2165667 (*MTNR1a*)	AA	32	10.69 (8.41, 16.12)	0.079
	AT	44	13.24 (10.39, 19.91)	
	TT	7	19.38 (10.49, 28.21)	
rs4861722 (*MTNR1a*)	AA	6	15.47 (8.24, 34.58)	0.640
	GA	30	11.74 (10.19, 16.35)	
	GG	47	13.71 (9.91, 21.46)	
rs6847693 (*MTNR1a*)	CC	25	10.62 (9.08, 16.41)	0.333
	CT	47	13.45 (10.36, 20.20)	
	TT	11	18.37 (8.59, 21.95)	
	CT	1	11.07	
rs1562444 (*MTNR1b*)	AA	41	14.08 (10.63, 20.08)	**0.001**
	AG	35	10.36 (8.94, 13.86)	
	GG	7	20.57 (18.37, 28.21)	
rs10830962 (*MTNR1b*)	CC	25	13.71 (10.32, 22.10)	0308
	CG	49	11.58 (9.80, 18.30)	
	GG	9	15.57 (10.05, 33.40)	
rs3781637 (*MTNR1b*)	CC	3	18.37 (13.71, 28.21)	0.392
	CT	16	13.12 (8.57, 20.27)	
	TT	64	12.55 (10.25, 17.83)	
rs8150 (*AANAT*)	CC	36	13.68 (10.28, 23.20)	0.518
	GC	34	11.88 (9.62, 18.45)	
	GG	13	13.04 (10.44, 14.82)	
rs3760138 (*AANAT*)	GG	45	12.19 (9.78, 17.09)	0.802
	GT	31	13.45 (10.05, 21.95)	
	TT	7	14.27 (12.01, 21.46)	
rs12942767 (*AANAT*)	GA	5	13.03 (9.88, 16.83)	0.878
	GG	78	13.07 (9.92, 19.59)	

SNPs: single-nucleotide polymorphisms; *M*: median; MTN: melatonin; *MTNR1a*: *melatonin receptor 1a*; *MTNR1b*: *melatonin receptor 1b*; *AANAT*: *arylalkylamine N-acetyltransferase.*

GG in rs1562444 (*MTNR1b*), where the GG genotype showed an elevated MTN concentration than in AA and AG genotype. We might hypothesize that the rs1562444 variant polymorphisms lead to the aberrant expression of MTN in patients with SLE. Later, the haplotype of *MTNR1a/b* and *AANAT* was identified; *MTNR1a* gene haplotype of CCTAT and CTAGT, and *AANAT* gene haplotype of GGG showed an increased risk in SLE susceptibility, but haplotype of TCTAT (*MTNR1a*) and CTG (*AANAT*) appeared to have a protective role.

The present study investigated the genetic association of SNPs in MTN pathway genes (*MTNR1a/b* and *AANAT*) with SLE susceptibility. However, there are some limitations in our study. First, the current study might be due to inherent selection biases such as a relative small sample size, the limited number of variables accounted, and the lack of information regarding body mass index (BMI) in healthy controls. Second, the potential confounding factors, such as type of treatments and concomitant infections, may have an effect on the level of MTN. Furthermore, although our

Table 6: Haplotype analysis of five SNPs in *MTNR1a* gene in SLE patients and healthy controls.

Haplotype	SLE (*n* (%))	Controls (*n* (%))	χ^2	*P*	OR (95% CI)
rs10030173, rs2119882, rs2165667, rs4861722, rs6847693					
CCTAT	44.05 (0.045)	27.21 (0.028)	4.135	**0.042031**	1.650 (1.014, 2.686)
CTAGC	341.42 (0.346)	331.12 (0.337)	0.241	0.623479	1.049 (0.866, 1.270)
CTAGT	38.30 (0.039)	19.92 (0.020)	5.983	**0.014466**	1.962 (1.133, 3.400)
TCTAT	135.01 (0.137)	180.70 (0.184)	8.071	**0.004512**	0.702 (0.549, 0.897)
TCTGT	117.73 (0.119)	118.09 (0.120)	0.001	0.974785	0.996 (0.757, 1.309)
TTAGC	217.53 (0.220)	216.35 (0.220)	0.003	0.954914	1.006 (0.811, 1.249)

SLE: systemic lupus erythematosus; SNPs: single-nucleotide polymorphisms; *MTNR1a*: *melatonin receptor 1a*; OR: odds ratio. Total $\chi^2 = 16.556$, $P = 0.005$. All the haplotypes with a frequency < 0.03 were ignored in the analysis.

Table 7: Haplotype analysis of three SNPs in *MTNR1b* gene in SLE patients and healthy controls.

Haplotype	SLE (*n* (%))	Control (*n* (%))	χ^2	*P*	OR (95% CI)
rs1562444, rs10830962, rs3781637					
CAT	296.17 (0.299)	260.80 (0.265)	2.573	0.108701	1.175 (0.965, 1.430)
CGC	130.72 (0.132)	127.39 (0.129)	0.014	0.906006	1.016 (0.782, 1.320)
CGT	144.94 (0.146)	137.77 (0.140)	0.123	0.725622	1.046 (0.813, 1.346)
GAT	408.55 (0.413)	442.60 (0.450)	3.208	0.073303	0.849 (0.710, 1.016)

SLE: systemic lupus erythematosus; SNPs: single-nucleotide polymorphisms; *MTNR1b*: *melatonin receptor 1b*; OR: odds ratio. Total $\chi^2 = 3.762$, $P = 0.288$. All the haplotypes with a frequency < 0.03 were ignored in the analysis.

Table 8: Haplotype analysis of three SNPs in AANAT gene in SLE patients and healthy controls.

Haplotype	SLE (*n* (%))	Control (*n* (%))	χ^2	*P*	OR (95% CI)
rs8150, rs3760138, rs12942767					
CGG	339.49 (0.344)	324.11 (0.329)	0.559	0.454842	1.074 (0.891, 1.295)
CTG	244.50 (0.247)	315.27 (0.320)	12.475	**0.000415**	0.701 (0.576, 0.854)
GGA	43.12 (0.044)	44.37 (0.045)	0.018	0.894335	0.971 (0.633, 1.492)
GGG	347.39 (0.352)	291.50 (0.296)	7.336	**0.006775**	1.299 (1.075, 1.571)

SLE: systemic lupus erythematosus; SNPs: single-nucleotide polymorphisms; *AANAT*: *arylalkylamine N-acetyltransferase*; OR: odds ratio. Total $\chi^2 = 14.211$, $P = 0.003$. All the haplotypes with a frequency < 0.03 were ignored in the analysis.

study represented significant genetic variations regarding *AANAT* gene in Chinese SLE patients, the detailed mechanism about potential effect of the *AANAT* gene variation on SLE is scarce.

In conclusion, our study demonstrated that, in the Chinese population, the genetic polymorphism of MTN pathway genes associated with the susceptibility to SLE, as well as with specific clinical manifestations, suggesting that the MTN pathway genes might be involved in the pathogenesis and development of SLE. However, further large sample size studies in other population are needed to further reveal the significance of MTN pathway gene's polymorphisms in SLE. In addition, related mechanism researches are necessary to better understand the function of the MTN pathway gene SNP in different immune cell types and to evaluate its correlation with clinical features.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical Approval

This study was approved by the Ethical Committee of Anhui Medical University (Hefei, Anhui, China). All the study subjects provided informed consent to participate in this study. All studies on humans described in the present manuscript were carried out with the approval of the responsible ethics committee and in accordance with national law and the Declaration of Helsinki 1975 (in its current, revised form).

Conflicts of Interest

The authors confirm that there are no conflicts of interest.

Acknowledgments

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Supplementary Materials

Supplementary Table 1: characteristics of the 46 Tag SNPs. *(Supplementary Materials)*

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Review Article

Autophagy in Immune-Related Renal Disease

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Autophagy is an important biology process, central to the maintenance of biology process in both physiological and pathological situations. It is regarded as a "double-edged sword"—exerting both protective and/or detrimental effects. These two-way effects are observed in immune cells as well as renal resident cells, including podocytes, mesangial cells, tubular epithelial cells, and endothelial cells of the glomerular capillaries. Mounting evidence suggests that autophagy is implicated in the pathological process of various immune-related renal diseases (IRRDs) as well as the kidney that underwent transplantation. Here, we provide an overview of the pathological role of autophagy in IRRDs, including lupus nephritis, IgA nephropathy, membrane nephropathy, ANCA-associated nephritis, and diabetic nephropathy. The understanding of the pathogenesis and regulatory mechanisms of autophagy in these renal diseases may lead to the identification of new diagnostic targets and refined therapeutic modulation.

1. Introduction: Current Perspectives on the Pathogenesis of Immune-Related Renal Diseases

Most immune-related renal diseases, or glomerulonephritis, frequently affect young people, often cannot be cured, and significantly lead to chronic kidney disease and end-stage renal failure, with associated morbidity and cost. In the past several years, there have been extensive researches focusing on its pathogenesis, which helps to gain increasing knowledge about cause and treatment. Traditionally, aberrant immunity is in the research spotlight for disease occurrence and progression and may also be relevant to other autoimmune diseases. Nevertheless, this organ is susceptible to various immunity-associated assaults, of which the underlying mechanisms are nowadays paid more attention to. Among these intriguing features, the process of autophagy in renal resident cells seems to serve as a protective role from certain injuries and toxic exposure, although research data are sometimes inconsistent (Table 1). The regulation and function of autophagy is likely cell type and context specific (Figure 1). Research into the role of autophagy in kidney physiology and pathogenesis still remains a largely understudied field.

2. Overview of Autophagy

Autophagy is a universal cell biology process in eukaryotic cells. It eliminates injured organelles and biological macromolecules. It is proven to be an important and highly conservative regulation mechanism to maintain intracellular homeostasis. Compared to the ubiquitin-proteasome system (UPS) that selectively degrades short-lived proteins, autophagy prefers aged and dysfunctional cytoplasmic proteins [1].

In general, baseline autophagy in mammalian is a physiological process but can be triggered by starvation or by various conditions, including ischemic, toxic, immunological, and oxidative insults. The process of autophagy consists of two major steps: induction of autophagosome and fusion of autophagosome with lysosome (for detailed description, please refer to expert reviews, i.e., [2, 3]). A large number of autophagy-related (ATG) proteins are involved in the process of autophagy (Figure 2). ATG proteins can be divided

Table 1: Autophagy's role in immune cells and renal resident cells.

	Cell type	Role of autophagy	Ref.
Immune cells	Macrophage	Helps control inflammation and contributes to caspase-independent cell death	[6, 7]
	Dendritic cell	Required for virus detection, antigen presentation, and inferon production	[8]
	T cell	Promotes survival and cytokine secretion	[11]
	B cell	Contributes to B cell differentiation and cell death	[12, 13]
Renal resident cells	Podocyte	Autophagy dysfunction is associated with clinical proteinuria and decreased renal function	[17, 24]
	Capillary endothelial cell	Protects endothelial from ROS	[26, 27]
	Mesangial cell	Protects mesangial cell from ROS and contributes to cell survival	[30, 31]
	TEC	Promotes TEC survival and helps eliminate toxins; contributes to renal fibrosis and nephropathic cystinosis	[32, 40]

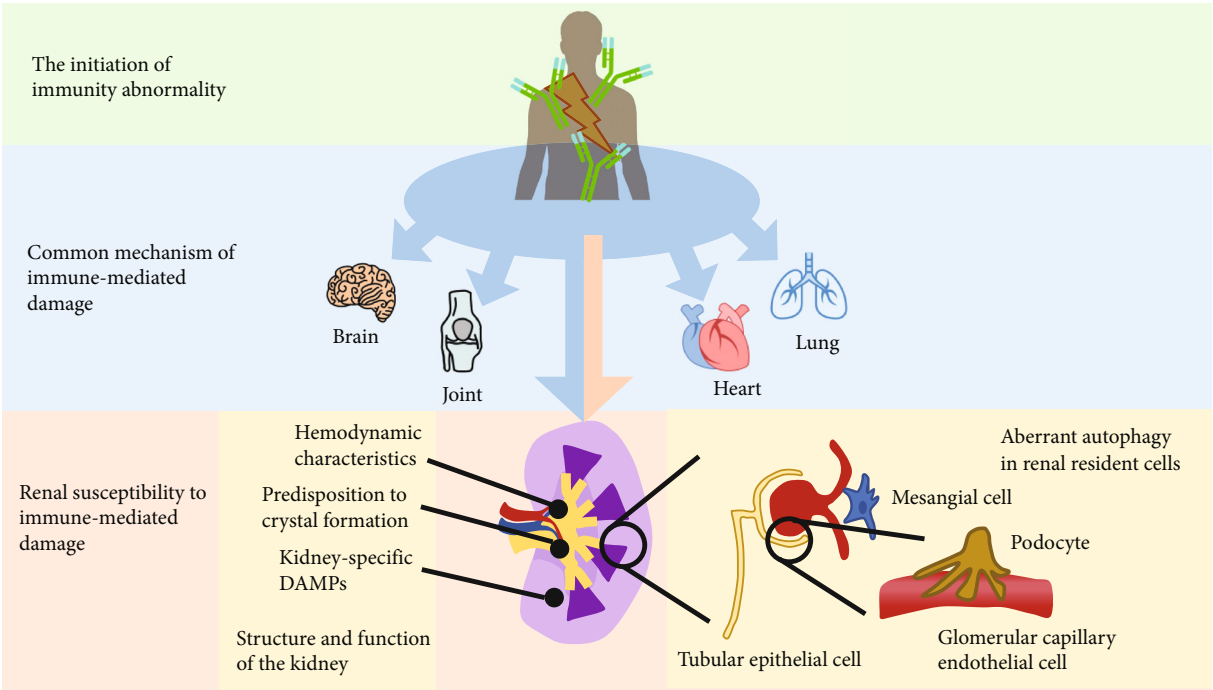

Figure 1: Immune-related renal disease. Aberrant immunity, such as autoimmune diseases, is a systemic disease. These immune disruptions, such as autoantibody production, immune complex formation, and disposition, can cause damage to any organ of our body, such as the heart, the lung, and the joints. However, the kidneys are susceptible to these immune-mediated damages, which results from its unique hemodynamic characteristics, kidney-specific DAMPs, and crystal formation in the tubule system. Besides, the renal resident cells, including podocytes, glomerular capillary epithelial cells, tubule epithelial cells, and mesangial, are also found to be susceptible to immune-mediated injuries.

into five groups, namely, ATG1 kinase complex (ATG1/Unc-51-like kinase (ULK) 1/2), ATG9, class III phosphoinositide 3-kinase complex (PI3KC3), ATG12 conjugation system, and ATG8 conjugation system [1].

3. Autophagy in Immune Cells

Autophagy in immune cells significantly alters immune activity. In innate immunity, autophagy achieves to augment immune cell activity and helps to fight against infection. Bacteria, viruses, and parasites can be degraded by autophagy, in which autophagy plays a protective role against these pathogens. Autophagy also restricts inflammation by interacting with certain signaling pathways and by engulfing inflammation triggers. In autophagic lysosomes, toll-like receptors (TLRs) might recognize damage-associated molecular pattern molecules (DAMPs) and pathogen-associated molecular pattern molecules (PAMPs) as autologous antigens and lead to an effective activation of immune reactions [4], which undermines immune tolerance and leads to the development of autoimmunity [5].

Several players in the innate immune system are regulated by autophagy to certain extent, such as macrophage and dendritic cell. Macrophage helps eliminate pathogens

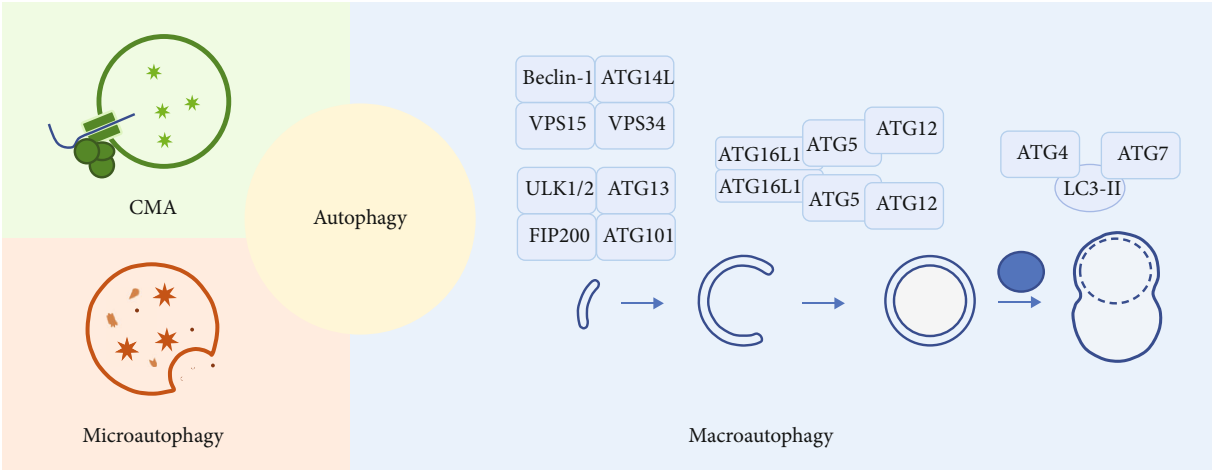

Figure 2: Classic autophagy pathway. There are three major types of classic autophagy process in mammalians: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). Macroautophagy includes four major steps: induction, elongation, and maturation of autophagosome and fusion with lysosome. Several protein complexes are involved in this process.

through intracellular digestion. Autophagy-deficient macrophage upregulates IL-18 and IL-1β production in the face of inflammatory stimulation through the TLR pathway [6]. Autophagy also contributes to caspase-independent macrophage cell death, which decreases inflammation [7]. In dendritic cells, autophagy is required for virus detection, antigen presentation, and inferon production [8, 9].

Several adaptive immune responses, such as lymphocyte development and antigen presentation, can be enhanced by autophagy activity [10]. Autophagy-mediated MHC class II presentation is a case in point. Extracellular antigens are captured into the autophagosomes of antigen-presenting cells. The autophagosome then degrades the antigens into immunogenic peptides and loads them onto MHC-II molecules to CD4+ T cells.

Accumulating evidence suggests that autophagy plays a pivotal role in T cell selection and survival. For example, in the selection of naïve T cell repertoires in the thymus, high autophagy activity in thymic epithelial cells achieves to deliver endogenous proteins to MHC-II molecules and contributes to TCR selection, consequently eliminating autoreactive CD4+ T cells [11]. Autophagy in activated T cells promotes survival and secretion of cytokines such as IL-2 and IFN-γ, thus influencing Th cell polarization.

As for B cells, autophagy plays a complex role. Unlike mature T cells, the survival of mature peripheral B cells seems not to necessarily require autophagy. On the one hand, autophagy is essential during B cell differentiation, i.e., *Atg5* deletion dramatically results in B-1 cell death [12]. On the other hand, autophagy can also induce autophagy-associated cell death [13]. Therefore, B cell receptor ligation-induced autophagy might be essential in eliminating self-reactive B cells, thereby reducing autoimmunity. In addition, recent data suggest that autophagy regulates ER homeostasis to control immunoglobulin (Ig) secretion in plasma cell, and yet deleting *Atg5* can lead to excessive Ig production [14].

4. Autophagy in Renal Resident Cells

4.1. Podocytes. Terminally differentiated podocytes are susceptible to various insults. From the perspective of pathology, the loss of podocytes is considered a key feature in progressive glomerular disease. Podocyte injury is the key factor in proteinuria, and loss of podocytes by cell death or detachment is a critical step for the progression of glomerular diseases and aging [15]. Autophagy appears to "monitor" the quality of podocytes under physiological and pathophysiological conditions. Podocytes from patients with minimal change disease (MCD) showed higher levels of Beclin 1-mediated autophagic activity than those from patients with focal segmental glomerulosclerosis (FSGS) [7, 16]. Furthermore, a high level of autophagy in podocytes of MCD patients often predicts a stable disease status, while MCD patients with decreasing levels of autophagy progressed to FSGS [17].

Loss of autophagy in podocytes results in a markedly increased susceptibility to various models of renal disease. Recent study showed that mice with *Atg5* or *Atg7* loss-of-function mutation developed histological and clinical characteristics of human FSGS. Silencing *Atg5* or *Atg7*, respectively, also showed significant podocyte alternations. One day post-nephrectomy, mice with *Atg7*-deficient podocytes exhibited much higher proteinuria, as well as foot process effacement and podocyte loss in renal biopsy [18]. Podocyte-specific *Atg5* knockout mice developed mild proteinuria by 8–12 months of age and defunction to degrade damaged mitochondria via mitophagy [19]. Inhibiting autophagy by silencing other ATG genes also undermines podocyte functions. Mice with podocyte-specific deletion of *Vps34* developed early proteinuria, progressive glomerulosclerosis, and renal failure by 9 weeks [20]. Podocytes from these knockout mice displayed a phenotype of impaired autophagic flux with accumulation of enlarged vacuoles, suggesting that Vps34 participates in maintaining autophagic flux in podocytes.

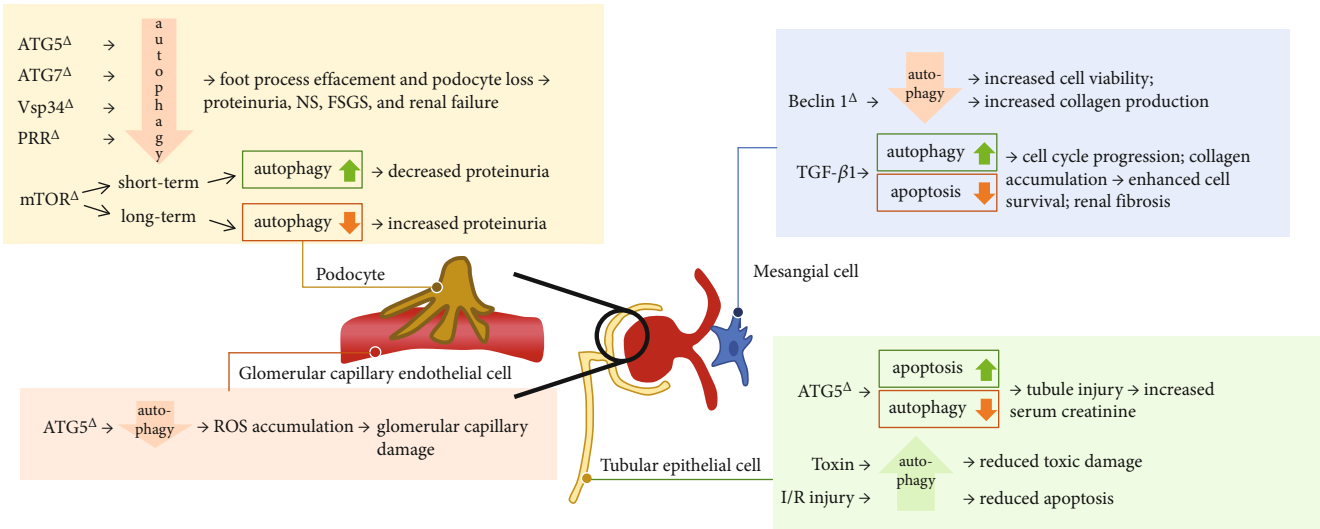

Figure 3: Autophagy in renal resident cells. The figure summarizes current studies of autophagy in renal resident cells, including podocytes, glomerular capillary epithelial cells, mesangial cells, and tubule epithelial cells. Upregulation or downregulation of autophagy activity through gene expression modulation or under certain stimulation can influence the survival of these cells and the overall function of the kidney. Taken together, autophagy plays a protective role in the physiology and pathophysiology of the kidney.

Similarly, podocyte-specific prorenin receptor- (*PRR*-) knockout mice developed nephritic syndrome within 2-3 weeks after birth and died by the 4th week. Electron microscopy revealed that the mice displayed progressive podocyte damage with foot process effacement and vacuolation and podocyte cell death [21, 22]. Dramatic accumulation of ubiquitinated protein and ubiquitin-binding scaffold protein p62/sequestosome 1 (SQSTM1) further suggested a block in autophagic clearance of the ubiquitinated protein aggregates [23]. Taken together, these studies highlight the particular importance of autophagy as a key homeostatic mechanism for podocytes under physiological and stress conditions.

Modulating mTOR to alter autophagy activity also influences podocyte structure and function. Podocyte-selective knockout of the *Mtor* gene mice developed proteinuria at 3 weeks of age with progressive podocyte damage and ultimately end-stage kidney failure by 5 weeks of age [24]. Similarly, in immortalized human podocytes, treatment with mTOR inhibitor rapamycin induced incomplete autophagy, showing a favorable cytoprotective effect. However, mTOR is also required to regenerate functional lysosomes and completion of autophagic process [25]. Thus, prolonged activation of mTOR may lead to lack of autophagy substrate, causing autophagy insufficiency. Given that disruption of the autophagic pathway may play a role in the pathogenesis of proteinuria, therapy with mTOR inhibitors can lead to both favorable and unfavorable consequences regarding to different time duration.

In short, autophagy is essential in podocyte survival and physiological functions. Blocking autophagy by inhibiting *ATG5*, *ATG7*, *mTOR*, *Vps34*, and *PRR* leads to podocyte cell injury, death, or dysregulated function and ultimately leads to decreased renal function (Figure 3).

4.2. Endothelial Cells of the Glomerular Capillaries. In a report of cultured cells from Fabry disease, cells from patients showed increased autophagy with a higher basal level of LC3. In the study, renal biopsies were obtained before and after 3 years of treatment. It showed that vacuole accumulation in endothelial cells and mesangial cells was drastically decreased after a 3-year therapy [26]. These data suggest that impairment in endothelial cell autophagic processes may contribute to Fabry disease in endothelial cell injury.

Recent study also demonstrated that autophagy protected glomerular endothelial cells in reacting to reactive oxidant species (ROS) [27]. Glomerulus endothelial and hematopoietic cell-specific *Atg5*-deficient mice presented with abnormal morphology in glomerulus and ROS accumulation, which can be attenuated by administration of ROS scavenger. These data suggest that autophagy in epithelial cells protects the glomerular capillary from oxidative stress and maintains its integrity.

4.3. Mesangial Cells. Glomerular mesangial cells are located in the centrilobular region called the mesangium, providing support for the glomerular structure as well as regulating glomerular filtration [28]. Mesangial cells also produce extracellular matrix that makes up the mesangium, in maintaining the homeostasis of kidney interstitial. However, they can also act as a deterioration factor in the development of a number of glomerular diseases, i.e., IgA nephropathy. When the kidney suffers from progressive kidney disease, mesangial cells proliferate and produce excessive extracellular matrix, leading to the development of glomerulosclerosis and kidney fibrosis.

It was observed that cadmium induced both autophagy and apoptosis in mesangial cells. But autophagy blockade resulted in increased cell viability without affecting apoptosis, suggesting that autophagy plays a role in cell death in mesangial cells exposed to cadmium [29]. It was reported that cadmium induced autophagic cell death through a calcium-extracellular signal-regulated kinase-dependent pathway

and, in part, through increased reactive oxygen species (ROS) production and activation of glycogen synthase kinase-3b (GSK-3b) [30].

Autophagy also contributed to survival of mesangial cells. Under the condition of serum deprivation, mesangial cells undergo apoptosis. Transforming growth factor-β1 (TGF-β1) promoted autophagy and enhanced cell survival by inhibiting mesangial cells from undergoing apoptosis. LC3$^{-/-}$ mesangial cells abrogated TGF-β1 rescue from serum deprivation-induced apoptosis, indicating a cytoprotective role of autophagy in mesangial cells [31]. Autophagy also plays a role in downregulating the production of matrix in mesangial cells by accelerating the process of degrading intracellular type I collagen (Col-I) produced by mesangial cells [31]. Beclin 1$^{+/-}$ mice presented with significantly increased collagen deposition in the kidneys. Mesangial cells isolated from Beclin 1$^{+/-}$ mice or transfected with Beclin 1 siRNA-expressed higher basal level of Col-I. Also, mesangial cells that were treated with an autophagy inhibitor showed an increased Col-I protein level. Accordingly, treatment with trifluoperazine, an inducer of autophagy, resulted in a decreased Col-I protein level induced by TGF-β1.

Consequently, it is suggested that autophagy may constitute an adaptive mechanism to glomerular injury by inhibiting apoptosis and promoting mesangial cell survival. The findings also implicate a novel role of autophagy as a cytoprotective mechanism to negatively regulate and prevent excess collagen accumulation in the glomeruli and hold promise for a new therapeutic target to mitigate pathogenesis of glomerulosclerosis and fibrosis.

4.4. Renal Tubular Epithelial Cell (TEC). Unlike podocytes, tubules display a low level of basal autophagy under normal conditions. Mice with *Atg5* deletion in proximal tubules gradually developed deformed mitochondria and accumulation of cytosolic inclusions, leading to proximal tubular cell hypertrophy and eventual degeneration. Mice with *Atg5* deletion in distal tubules also displayed a significant accumulation of p62/SQSTM1 and oxidative stress markers, without significant alteration in kidney function up to 12 months of age [32]. *Atg5* deletion in the entire tubule system resulted in accumulation of p62/SQSTM1 throughout the tubular segments, and at 5 months, there was a significant increase in serum creatinine [32]. Therefore, while ATG5 deficiency solely in proximal or distal tubular cells did not cause significant renal dysfunction, ATG5 deficiency in all tubule segments caused impairment of kidney function, suggesting tubular autophagy is important in the preservation of kidney function.

In facing exposure to environmental toxins, activated autophagy is also observed in the tubular cells. Upregulation of autophagy before apoptosis was detected in the proximal tubules of mice injected with cisplatin. Inhibiting autophagy enhanced the activation of caspases and apoptosis in cisplatin-treated proximal tubular cells. It suggested that autophagy protected tubular cells from apoptosis [33]. Proximal tubule-specific autophagy-deficient mice developed more severe AKI and increased apoptosis after cisplatin treatment [34, 35]. Moreover, autophagy-deficient proximal tubules exhibited with increased DNA damage, p53 and c-Jun N-terminal kinase (JNK) activation, and accumulation of toxic protein aggregates and ROS after cisplatin treatment. Similar phenomenon is also observed when TECs are treated with aristolochic acid or cyclosporin A. Autophagy activity is activated when toxin is administrated, and suppressing autophagy induced a higher level of apoptosis [36–38].

In models of obstructive nephropathy induced by unilateral ureteral obstruction (UUO), it was observed that mTOR was inhibited and cell autophagy activity was enhanced in order to remove abnormal intracellular components. Similarly, autophagy is confirmed as a protective role in the face of renal I/R injury. It demonstrated that blocking autophagy enhanced hypoxia-induced apoptosis in cultured renal proximal tubular cells [35]. Studies using mice with conditional *Atg5* or *Atg7* gene deletion in the proximal tubule confirmed that autophagy protected the proximal tubule from I/R injury. These results provide further support for the cytoprotective role of autophagy in the tubules.

In other circumstances, autophagy in the tubules serves as a contributing factor to the disease development. Another UUO model in mice revealed that autophagy is associated with renal fibrosis. Inhibiting autophagy resulted in suppressed renal fibrosis through downregulating profibrotic factors [39]. Nephropathic cystinosis is one of the lysosomal storage diseases that characterized with an abnormal function of the kidney tubules and progressive development of renal insufficiency. It was observed that autophagy was upregulated in these patients' fibroblasts and proximal tubular cells [40]. The apoptosis rate of proximal renal TECs of cystinosis can be reduced by inhibiting autophagy. Sodium arsenite induced autophagic cell death in renal tubular cells both *in vitro* and *in vivo*, and suppression of autophagy attenuated cell death. Autophagy is also implicated in the cytotoxicity of nanomaterials in proximal tubular cells. Cell death induced by fullerenol exposure at millimolar concentrations was associated with cytoskeleton disruption, autophagic vacuole accumulation, and mitochondrial dysfunction. Furthermore, autophagy inhibitor 3-MA ameliorated loss of mitochondrial membrane potential and ATP depletion, suggesting that autophagy may contribute to fullerenol-induced cell death.

In summary, autophagy is activated in various forms of renal tubular injury. At the current stage, the precise role of autophagy in tubular injury response and the pathogenesis of kidney fibrosis is not well understood. Some studies have investigated how autophagy is involved in the tubule pathogenesis yet findings seemed contradictory. There have been studies that provided evidence to support a cytoprotective role, and others that support deleterious effects of autophagy, which may suggest a context-dependent characteristic. Different types and severity of injuries may produce different outcomes of autophagy. For example, a certain degree of autophagic activity can maintain tissue homeostasis, while higher or excessive autophagic activity leads to apoptosis. Future investigations, for instance, by using targeted autophagic gene knockout mice, are necessary to elucidate the precise functional role of autophagy in tubular injuries.

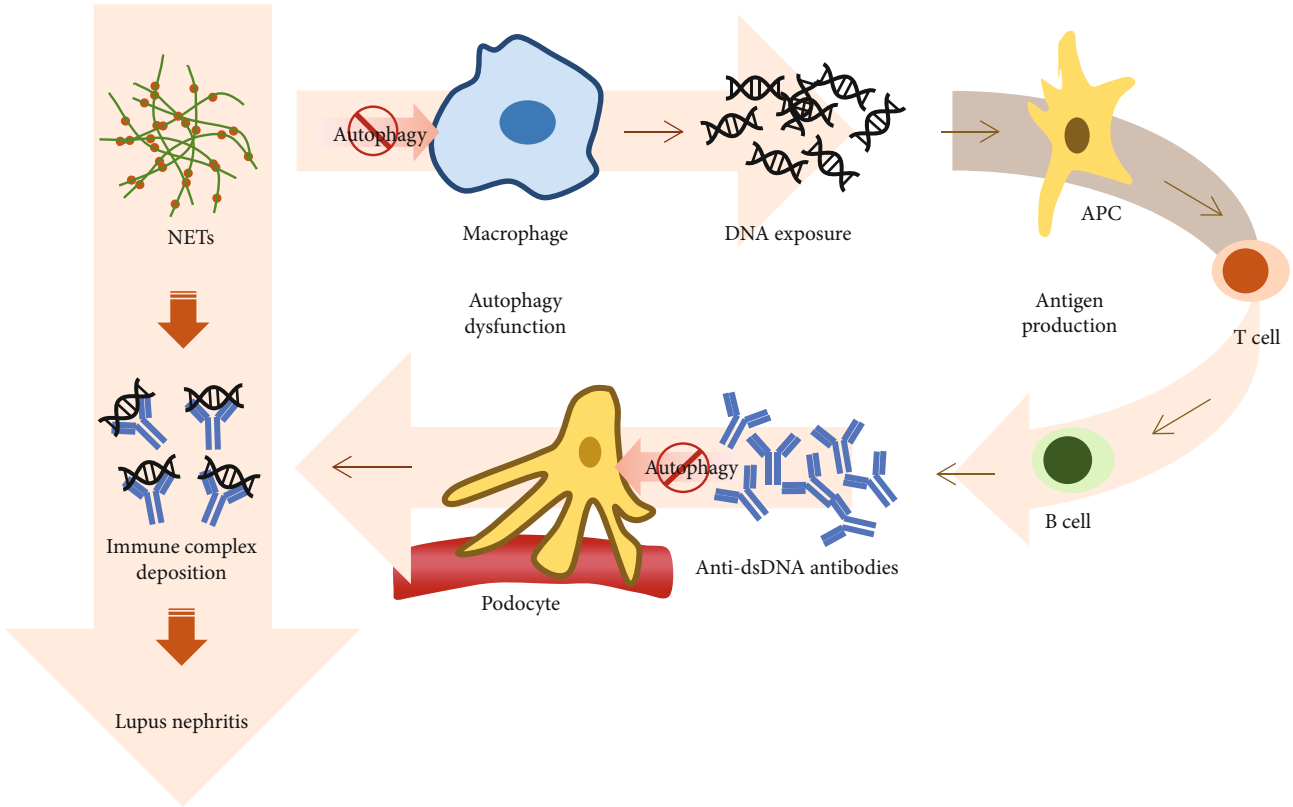

Figure 4: Autophagy dysfunction in lupus nephritis. Macrophagy eliminates neutrophil extracellular trap (NET) through autophagy. Autophagy deficiency in macrophagy leads to DNA exposure of NET, which activates adaptive immunity and produces anti-dsDNA antibodies. Autophagy dysfunction in podocytes fails to clear the autoantibodies, which subsequently binds to DNA fractions of NETs, forming immune complexes. These immune complexes will damage kidney and cause nephritis in lupus.

5. Autophagy in Human Immune-Related Renal Diseases

5.1. Lupus Nephritis (LN). Systemic lupus erythematosus (SLE; lupus) is a chronic autoimmune disease characterized by production and deposition of autoantibody, damaging multiple tissues and organs. One of the most common and severe complications is lupus nephritis (LN), presenting with proteinuria, hematuria, hypertension, and chronic kidney disease. Early renal involvement of SLE patients often indicates a poor prognosis [41]. More recent studies have revealed that dysregulated autophagy in certain cells are involved in the pathogenesis of LN (Figure 4).

Neutrophils release neutrophil extracellular trap (NET) to restrict the invasion of infectious pathogens. NETs include condensed chromatin and neutrophil proteins, and failing to remove NETs in time might result in autoantibody production. SLE/LN patients exhibited with significant impairment in NET degradation. Consistently, these patients had higher titers of anti-dsDNA antibodies [42]. MTOR inhibitor-treated neutrophils presented with a higher level of autophagy activity and NETs [43]. Thus, we may associate an increased level of autophagy with its impairment in degrading NETs, which may result in the exposure of intracellular antigens to trigger the production of autoantibodies.

A study observed an increased LC3-II expression in spleen and kidney macrophages from lupus mice. Transfer of Beclin-1 knockdown macrophages into macrophage-free lupus mice relieved renal pathological severity, decreased anti-dsDNA titer, and declined urine protein [44]. It indicated that autophagy in macrophage may assist LN development, yet the underlying mechanism deserves further investigation.

As for the renal resident cells, it was reported that podocytes were able to take up SLE patients' anti-dsDNA antibodies, subsequently initiating autophagy to degrade these intracellular aggregations. Chemical inhibition of autophagy in podocytes results in the accumulation of dsDNA antibodies and consequently cell injuries [45]. Likewise, in both lupus mice and human biopsy samples, it was reported that autophagy is only activated in podocytes [46]. Inhibition of autophagy, using 3-MA or ATG5-silencing, resulted in decreased podocyte functioning and increased podocyte layer permeability. Yet, rapamycin treatment to activate autophagy could alleviate podocyte injury.

However, another drug, namely, P140, a spliceosomal peptide, suppresses autophagic flux and increases the LC3 level in B cells. Administration of P140 in lupus-prone mice reduces proteinuria and decreases titer of anti-dsDNA antibodies [47]. Although P140 and rapamycin exhibit opposite effect on autophagy [48–50], they both achieve to attenuate the severity and symptoms of LN. Hence, it indicates that autophagy involves in the development of LN in a complicated way, which is in need of more researches to obtain the full picture and a better target to treat SLE and LN.

5.2. IgA Nephropathy (IgAN). In an earlier paper, it was observed that there were two types of autophagy in the kidney of patients with IgAN [51]. The first type of autophagy, defined as type I autophagy, has a condensed ribosome area with few lipid droplets and a limiting membrane originated from injured mitochondria. Type II autophagy characterized in a condensed ribosome and more lipid droplets, with a limiting membrane from rough ER. In the more recent study, it suggested that type I autophagy occurrence might predict a worse prognosis in IgAN [52]. However, the translational significance of autophagy in renal biopsy still need to be confirmed.

5.3. Membranous Nephropathy. In an experimental membranous nephropathy rat model, "passive Heymann nephritis," ER stress, and autophagy upregulation were observed in podocytes. A recent study also reported that mTORC1 was negatively correlated with autophagy in the passive Heymann nephritis model, where glomerular mTORC1 signaling activation and autophagy downregulation corresponded. Analysis with renal biopsy sample showed increased LC3-positive autophagosomes from patients with membranous glomerulonephritis [19]. ATG3 mRNA was also observed to be significantly higher in microdissected glomeruli from patients with FSGS and membranous glomerulonephritis compared to that in normal controls (pretransplant allograft biopsies).

5.4. ANCA-Associated Nephritis (AAN). ANCA-associated vasculitis (AAV) is an autoimmune disease that targets perivessel tissues and vessel walls of internal organs, characterized with the presence of antineutrophil cytoplasmic antibody (ANCA). The involvement of the kidney in AAV is termed ANCA-associated nephritis (AAN). Patients with AAN often present with hematuria, proteinuria, and cylindruria and might progress rapidly into end-stage renal disease [53]. There were also researches associating AAN with autophagy dysfunction. It was reported that neutrophils treated with ANCA presented with a higher level of autophagy and released more NETs [54]. Anti-LAMP-2 antibody-treated human neutrophils also exhibited with higher activity of autophagy and decreased apoptosis rate. These effects were attenuated by autophagy inhibitors but not by apoptosis inhibitors [55]. Thus, it may be possible that ANCA-stimulated NETosis is closely regulated by autophagy activity.

5.5. Diabetic Nephropathy (DN). Dysregulated autophagy has been suggested to play important pathogenic roles in a variety of disease processes. Inhibition of autophagy was observed in DN rat kidney as well as in type 2 diabetes patient renal biopsy. Possible mechanisms include overactivation of the mTOR pathway, AMPK activation, and decreased expression of silent information regulator 1 (SIRT1). High glucose level mediates the regulation of mTOR, AMPK, and SIRT1, the three nutrient-sensing signal pathways, which all result in the inhibition of autophagy. Inhibition of autophagy might fail to clear AGEs, ROS, or induce ER stress and eventually leads to kidney fibrosis. Therefore, autophagy serves as a protective role in the pathogenesis of DN.

5.6. Kidney Transplantation. During and after the transplantation surgery, the donated kidney often faces with various challenges, such as ischemic-reperfusion injury (I/RI) and rejection [56]. Several observations indicate that the kidney exhibited increased activity of autophagy after I/RI. However, it is still under debatable on whether autophagy plays a protective or detrimental role. Neither chemical modulation, such as 3-MA and chloroquine, nor genetic inhibition of autophagy, such as siRNA against *Atg5* and tubule-specific *Atg5* knock-out mice, shows consistent results [32, 35, 56, 57]. One possible reason is that autophagy often displays a dynamic and transient nature, which complicates the comparison between studies where researchers analyzed different durations of I/RI. Moreover, crosstalk of autophagy with other cell death pathways often results in unwanted and even unknown effects. For example, regulating the Bcl-2 protein family not only influences the activity of autophagy but also affects apoptosis since it is also an antiapoptosis protein [58, 59]. Current perspective holds that autophagy switches its role in the development of I/RI, depending on the duration and severity of I/RI [60]. It proposed that in the beginning of I/RI, autophagy is upregulated to exert a protective role against I/RI. When I/RI continues, autophagy increases and reaches a level that starts to contribute to the progress of I/RI. It provides a possible explanation that protective and destructive roles of autophagy are both observed in I/RI, yet further researches are needed to support this hypothesis.

Immune tolerance is another important factor that determines the allograft survival. In general, immunologic tolerance of the allograft includes autoreactive effector T cell deletion and the upregulation of Treg functioning [61]. Pim-2, a Pim family kinase, is found in alloreactive effector T cell and favors its survival and proliferation [62]. It was reported that the Pim-2 level was positively correlated with the severity of allograft rejection and that inhibition of Pim-2 prevented the rejection [63]. Interestingly, T cell regulation, which leads to immune tolerance, is found to be associated with autophagy. In autophagy-deficient mice that follows CD40-CD154 costimulatory blockade, T cell proliferation was enhanced and INF-γ production increased, leading to MHC-mismatched allograft rejection [64]. Treatment with rapamycin [65, 66] leads to a higher autophagy level and higher biological quality of Tregs. And higher Treg number is modestly associated with better renal function and lower serum creatinine [66]. It suggests that autophagy regulates T cells to induce immune tolerance and promote allograft survival.

To sum up, in either I/RI or immune rejection that the allograft might face, autophagy achieves to exert its effect, yet its exact role still remains investigation. In current clinical practice, rapamycin, as an autophagy activator, is frequently used to reduce allograft rejection. Hence, autophagy still appears to play a protective role on allograft survival in general. It is possible that autophagy is a double-edge sword in this circumstance and that its protective role outweighs its

harmful one, which results in allograft prolonged survival. Nonetheless, researches are needed for further elucidation.

6. Perspectives: New Diagnostic and Therapeutic Targets

Undoubtedly, autophagy seems to be a promising target for certain renal disease treatments. There are, however, obstacles that need to be solved before therapy can be used in the clinical setting.

Current autophagy inhibitors, including chloroquine, bafilomycin, rapamycin, and 3-methyladenine, affect more than just autophagy, therefore, it may end up paradoxically to be a deterioration to the sick kidney. More specific and selective autophagy modulator is needed to overcome this dilemma. Moreover, long-term modulation of autophagy might not be as effective and safe as intermittent regulation. The AMPK inducer metformin might meet this requirement, yet inadequate clinical data limits its further application. Another challenging is that the result of modulating autophagy is pretty uncertain, depending on several complex factors such as timing, duration, and intensity of autophagy induction. Under different circumstances, the exact same modulation of autophagy might end in either renal protection or renal cell apoptosis. Therefore, further studies are needed to determine an appropriate condition and therapeutic window where autophagy modulation would yield protective effects. The ability to monitor autophagy in the clinical setting as both a diagnostic tool and a therapeutic guide is still unsolved. Since autophagy is dynamic in its nature, regulated within short-lived and unstable protein-protein binding, it is virtually impossible to capture the dynamic autophagy flux using static biopsy specimens, which is incompatible with routine diagnostic tools.

7. Concluding Remarks

In summary, autophagy is activated in various forms of immune-related renal diseases. At the current stage, the precise role of autophagy in renal injury response and the pathogenesis is not well understood. There have been studies that provide evidence to support a cytoprotective role of autophagy, and others that support deleterious effects of autophagy. It is plausible that it is context dependent. The difference in the types of injuries and severity of injuries may produce different outcome of autophagy, in that, a certain degree of autophagic activity can maintain tissue homeostasis, whereas excessive autophagic activity results in cell death. Future investigations, for instance, by using targeted autophagic gene knockout mice, are necessary to elucidate and clarify the precise functional role of autophagy in immune-related renal diseases.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Clinical Study

Low-Dose Sirolimus Immunoregulation Therapy in Patients with Active Rheumatoid Arthritis: A 24-Week Follow-Up of the Randomized, Open-Label, Parallel-Controlled Trial

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Background. We have reported previously the insufficient absolute number or functional defects of regulatory T cells (Tregs) in patients with rheumatoid arthritis (RA), challenging conventional unspecific immunosuppressive therapy. Sirolimus, a mTOR inhibitor, is reported to allow growth of functional Tregs; here, we investigated the efficacy of low-dose sirolimus combined with conventional immunosuppressants (sirolimus immunoregulation therapy) for RA treatment with lower side effects and better tolerance. *Methods*. In this nonblinded and parallel-group trial, we randomly assigned 62 patients to receive conventional glucocorticoids and immunosuppressants with or without sirolimus at a dosage of 0.5 mg on alternate days for 24 weeks in a 2 : 1 ratio. The demographic features, clinical manifestations, and laboratory indicators including peripheral blood lymphocyte subgroups and $CD4^+T$ subsets were compared before and after the treatment. *Results*. Finally, 37 patients in the sirolimus group and 18 in the conventional treated group completed the 6-month study. By 24 weeks, the patients with sirolimus experienced significant reduction in disease activity indicators including DAS28, ESR, and the number of tender joints and swollen joints ($p < 0.001$). Notably, they had a higher level of Tregs as compared with those with conventional therapy alone ($p < 0.05$), indicating that sirolimus could partly restore the reduced Tregs. Concomitantly, their usage of immunosuppressants for controlling disease activity was decreased as compared with the conventional group with no difference in blood routine, and liver and renal functions both before and after the treatment of sirolimus and between the two groups ($p > 0.05$). *Conclusions*. Low-dose sirolimus immunoregulatory therapy selectively upregulated Tregs and partly replaced the usage of immunosuppressants to control disease activity without overtreatment and evaluable side effect. Further study is required using a large sample of RA patients treated with sirolimus for a longer period. This trial is registered at the Chinese Clinical Trial Registry (http://www.chictr.org.cn/showproj.aspx?proj=17245).

1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease, potentially leading to joint cartilage and bone damage and even disability due to profound inflammation [1]. Nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids, and immunosuppressants are conventionally used to treat RA patients [1]. However, a portion of patients still have

inadequate response to them or severe side effects. New therapies are urgently required for RA.

Many important immunological dimensions, especially the balance of effector T cells and regulatory T cells (Tregs), are altered in RA [2, 3]. Th17 cells, one of the effector T subsets among $CD4^+T$ cells, have been reported to mediate the inflammatory process by producing interleukin 17 (IL-17) as well as other effector cytokines and chemokines [4–6]. In contrast, Tregs actively suppress activation of the immune system and prevent autoimmune disease [7]. Recently, we have reported the reduction of the absolute number of peripheral Tregs but not the increase of effector T and Th17 cells in RA patients [8, 9], which argues and challenges the pathological foundation of conventional immunosuppressive therapy that also nonspecifically inhibits Tregs. Thus, one of goals of a new therapy for RA should be to maintain and restore a relative balance between effector T and Treg cells.

Sirolimus, also known as rapamycin, is a macrolide compound that inhibits its mechanistic target (mTOR), which regulates cell growth and metabolism in response to environmental cues. mTOR is also essential in driving abnormal lineage specification within the immune system in various rheumatic diseases [10]. Several studies have reported that sirolimus and its analogues reduced joint inflammation in animal models of arthritis [11] and in a few patients with RA [12–14] or JIA [15]. Further study revealed that this clinical benefit might accrue from the inhibition of mTOR activation in the growth of synovial fibroblast cells [16].

However, the clinical application of sirolimus therapy in RA patients is few up to now, and the evaluation of therapeutic efficiency of mTOR inhibitors in active RA is very limited. Furthermore, a relatively higher dose of sirolimus that was used before has side effects [17]. In this study, we initiated firstly this prospective study to assess safety, tolerance, efficacy, and the status of immunological cells in patients with active RA treated with low-dose sirolimus combined with original therapy.

2. Methods

2.1. Study Design and Participants. To assess the efficacy and safety of sirolimus for patients with RA, we did a prospective, single-arm, open-label, phase 1/2 trial at the Department of Rheumatology, Second Hospital of Shanxi Medical University (Taiyuan, China), with approval from the Second Hospital of Shanxi Medical University Ethics Committee (ethics number: 2016-KY-014). This trial is registered at the Chinese Clinical Trial Registry (number ChiCTR-IPR-17010307).

All the patients fulfilled the 1987 and 2010 rheumatoid arthritis classification criteria [18, 19]. These patients enrolled in the study were aged between 18 and 65 years and had active disease (DAS28-ESR scores > 3.2). The patients were excluded from this study if they were allergic or intolerant to sirolimus, suffering malignant disease, had a history of malignancy, or had a recent clinically significant infection.

2.2. Procedures. The patients had a complete physical examination before enrolment and were randomly assigned (2 : 1) to the sirolimus group and the conventional group. All patients were freely receiving prednisone and other immunosuppressive medications to control disease activity to meet the treat-to-target (T2T) recommendations [20]. Patients in the sirolimus group received additional oral sirolimus (ordered from North China Pharmaceutical Co. Ltd.) at a dosage of 0.5 mg per other day.

Patients were treated with sirolimus for 6 months. The clinical and laboratory indicators were assessed on week 0 (before administration of the first sirolimus dose), and week 3, week 6, week 12, and week 24 after initiation of sirolimus treatment. Laboratory tests included complete blood counts, erythrocyte sedimentation rate (ESR), liver and kidney function tests, and urinalysis. Assessments of flow cytometry for peripheral blood lymphocyte subgroups and $CD4^+T$ subsets are described in the supplementary materials (supplementary figure 1). Treatment was discontinued if the patients developed infections, which could not be controlled within 5 days after intravenous antibiotics therapy.

2.3. Outcomes. The primary efficacy endpoints were a decrease in disease activity, defined as a decrease in DAS28-ESR scores at each visit during treatment compared with the baseline. Secondary endpoints were a decrease in doses of prednisone or disease-modifying antirheumatic drugs (DMARDs) required to control disease activity and changes in immunobiological biomarkers of clinical responsiveness compared to conventional groups.

Safety outcomes included tolerance as assessed by the occurrence of common side effects. The development of nonhealing oral ulcers or a new onset headache indicated intolerance to sirolimus. Thrombocytopenia, mucositis, oedema, and proteinuria, which have been observed in renal transplant patients, were also monitored as safety outcomes.

2.4. Statistical Analysis. The demographic parameters of the control and sirolimus-treated patients were compared using an unpaired t-test for parametric data (age, PB lymphocyte subpopulations, and $CD4^+T$ subsets as well as blood routine, and liver and renal function) and the χ^2 test for proportions (sex) and drug usage. Repeated measure mixed model logistic regression analysis was used to assess the effects of treatments on clinical indices and biomarkers recorded at weeks 3-24 compared with week 0. All p values reported herein are two-tailed. p value < 0.05 was taken as statistical significance. The software for the statistics was SPSS 22.0 and GraphPad Prism6.0.

3. Results

Between April 7, 2017, and February 9, 2018, 62 patients signed the informed consent form and were enrolled in this study. One of the consented patients was excluded for not meeting eligibility criteria after screening (patient number: 025, DAS28 < 3.2). Only one of them discontinued

sirolimus treatment because of intolerance (patient number: 028).The mean age was 50.3 ± 10.6 years in the sirolimus group and 51.8 ± 8.7 years in the conventional group ($t = 0.563$, $p > 0.05$), and 44 (77.2%) patients were female, and there was no difference in proportions of sex between the two groups ($\chi^2 = 0.152$, $p > 0.05$). Baseline clinical characteristics of all enrolled patients, including age, sex, disease duration, DAS28-ESR index score, prednisone dose, and usage of immunosuppressant medication, are shown in the Table 1 and supplementary table 1. Finally, 55 (88.7%) patients completed 6 months of treatment (sirolimus group $n = 37$; conventional group $n = 18$). 48 (87.3%) of 55 eligible patients donated their blood and tested their immunobiological biomarkers at week 12, and 39 (70.9%) of them tested at week 24 after the treatment (Figure 1).

3.1. Clinical Efficacy Outcomes. In the sirolimus group, the mean DAS28-ESR score decreased from 4.55 ± 0.98 at week 3 to 3.13 ± 0.94 at week 24 ($Z = -5.130$, $p < 0.001$; Figure 2(a)). Other diseases activity measures such as ESR, TJC, and SJC were all significantly reduced during 6 months of treatment with sirolimus ($p < 0.05$; Figure 2(b)–2(d)). There was also a significant decrease of disease activity in the conventional group with a lower level of TJC at 24 weeks; other disease activity indexes were comparable to that of the sirolimus group.

To control disease activity to meet the treat-to-target (T2T) recommendations, all patients were free to increase or decrease prednisone or DMARDs. When analyzing details in medication uses, no difference of the mean daily prednisone dose required to control disease activity was observed between the sirolimus and conventional groups ($p > 0.05$; Figure 3(a)). But compared with the conventional group, patients in the sirolimus group had a lower usage rate of DMARDs such as methotrexate, leflunomide, or hydroxychloroquine, which was more observable during the follow-up period (Figure 3(b)–3(d)).

3.2. Changes in Immunobiological Biomarkers. Patients treated with conventional immunosuppressants alone had a significant decrease of proinflammatory Th17 cells at week 12 ($Z = -2.722$, $p < 0.05$) and 24 ($Z = -2.762$, $p < 0.01$; Figures 4(a) and 4(b)), but meantime, anti-inflammatory Tregs were also significantly reduced from $32.2 \pm 12.1/\mu l$ at week 0 to $21.2 \pm 11.2/\mu l$ at week 12 ($Z = -2.102$, $p < 0.05$) and $23.1 \pm 6.4/\mu l$ at week 24 ($Z = -1.882$, $p < 0.05$; Figure 4(c)). In contrast, patients who received sirolimus combination treatments had a higher level ($31.0 \pm 2.1/\mu l$) of Tregs compared with patients ($23.1 \pm 1.8/\mu l$) who received immunosuppressive therapy alone at week 24 ($Z = -2.235$, $p < 0.05$; Figure 4(c)), indicating that low-dose sirolimus partly reversed the reduction of these cells.

We also assessed the mean proportions and absolute numbers of specific lymphocyte subsets, Th1 and Th2 cells during 24 weeks of treatment. The levels of peripheral blood lymphocyte subgroups and Th2 cells had no significant change, while patients with sirolimus treatment had higher proportion of Th1 cells than did the matched control group at week 12 (supplementary figure 2 and 3).

Table 1: Baseline characteristics of all enrolled patients.

	Sirolimus group	Conventional group
n	42	20
Sex (female/male)	34/8	17/3
Age (years), $\bar{x} \pm s$	50.3 ± 10.6	51.8 ± 8.7
Duration of disease (years), median (range)	5 (1-20)	6 (2-14)
DAS28, $\bar{x} \pm s$	4.5 ± 1.1	4.1 ± 0.6
TJC, $\bar{x} \pm s$	6.7 ± 5.9	4.0 ± 2.7
SJC, $\bar{x} \pm s$	3.0 ± 4.5	3.0 ± 3.0
ESR (mm/h), $\bar{x} \pm s$	43.3 ± 33.3	31.0 ± 20.4
Prednisone dose (mg/d), $\bar{x} \pm s$	6.2 ± 5.4	6.2 ± 5.7
Use of concomitant agents (no. of patients)		
Methotrexate	3	5
Leflunomide	23	11
Hydroxychloroquine	10	4
Thalidomide	1	1

3.3. Safety Outcomes. We measured the blood concentration of sirolimus at last visit and found that the concentration was 1.8 ± 0.4 ng/ml, which indeed was much lower than the reported therapeutic range of 6-15 ng/ml [17]. At this low dosage, blood routine tests showed no significant changes in RBC counts (Figure 5(a)) and hemoglobin concentration (Figure 5(b)) compared with the two groups at each time point ($p > 0.05$). Platelet counts in the conventional group were transiently decreased at week 3 compared with the baseline ($Z = -2.265$, $p < 0.05$), which was lower than that of the sirolimus group ($Z = -2.668$, $p < 0.05$), but no statistically significant differences were observed at weeks 6, 12, and 24 between the two groups (Figure 5(c)). Patients treated with conventional immunosuppressants had a decreased tendency of WBC counts (Figure 5(d)), which was slightly lower than that of sirolimus treatments at week 3 ($Z = -2.360$, $p < 0.05$) and week 24 ($Z = -2.498$, $p < 0.05$). Compared with the baseline, sirolimus-treated patients had a lower level of neutrophilic granulocyte percentage at week 3 ($Z = -1.092$, $p < 0.05$) and week 12 ($Z = -1.091$, $p < 0.05$; Figure 5(e)). At the same time, mixed model logistic regression analysis revealed a significant increase in the lymphocyte proportions in the sirolimus group at week 3 ($Z = -2.037$, $p < 0.01$) and week 6 ($Z = -2.172$, $p < 0.05$; Figure 5(f)). Liver function, assessed by aspartate aminotransferase and alanine aminotransferase concentrations, was not affected ($p > 0.05$; Figures 6(a) and 6(b)). Renal function, valued by blood urea nitrogen and serum creatinine, was not affected too ($p > 0.05$; Figures 6(c) and 6(d)). Except for one patient who developed limb oedema after one week of treatment with sirolimus and therefore withdrew from the study, no thrombocytopenia, mucositis, or proteinuria was observed.

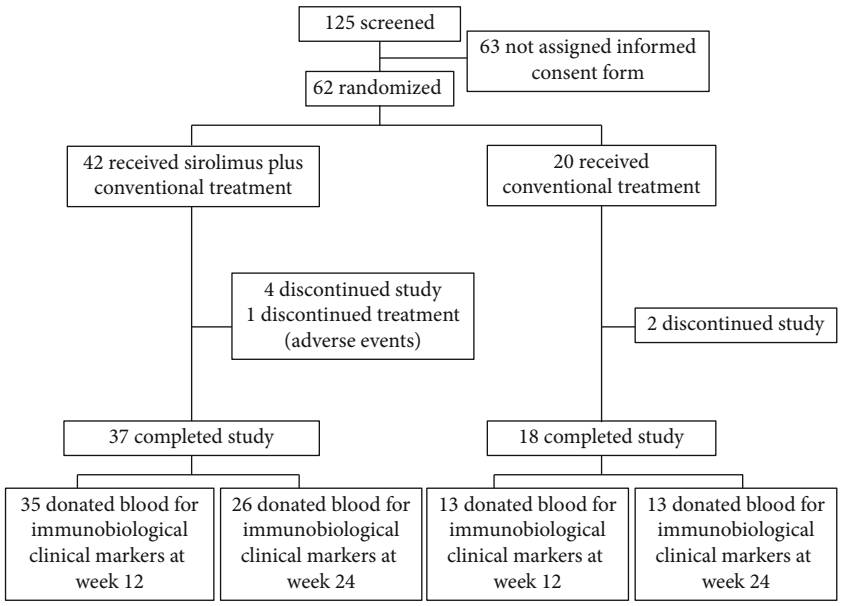

Figure 1: Disposition of patients in the trial.

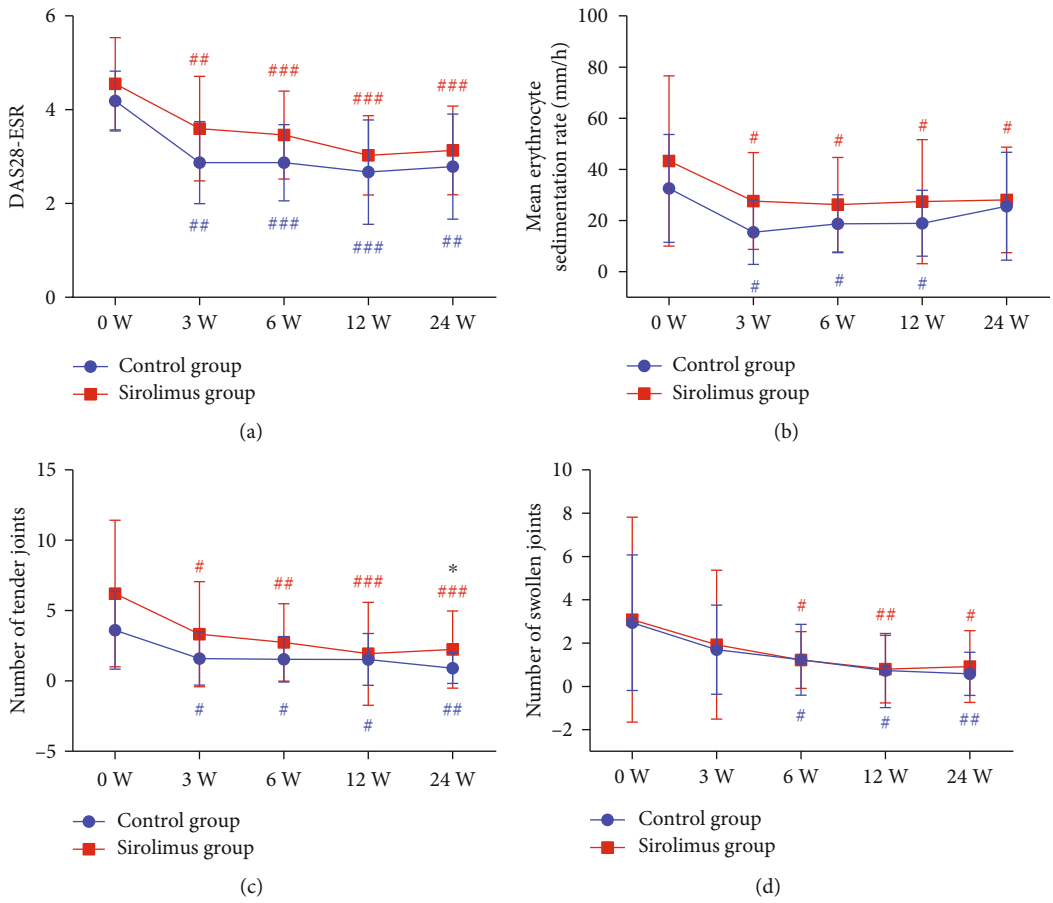

Figure 2: Efficacy of sirolimus in reducing disease activity. Mean DAS28-ESR score (a), ESR (b), mean number of tender joints (c), and swollen joints (d). Overall changes during treatment were assessed by repeated measure analysis using a mixed-effect model. A two-tailed unpaired t-test was used to compare the disease activity measures between sirolimus and conventional groups. Error bars show SD. DAS28: 28-joint disease activity score; ESR: erythrocyte sedimentation rate. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ relative to baseline (week 0) in the conventional group (blue); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ relative to baseline in the sirolimus group (red); $^{*}p < 0.05$ compared between groups.

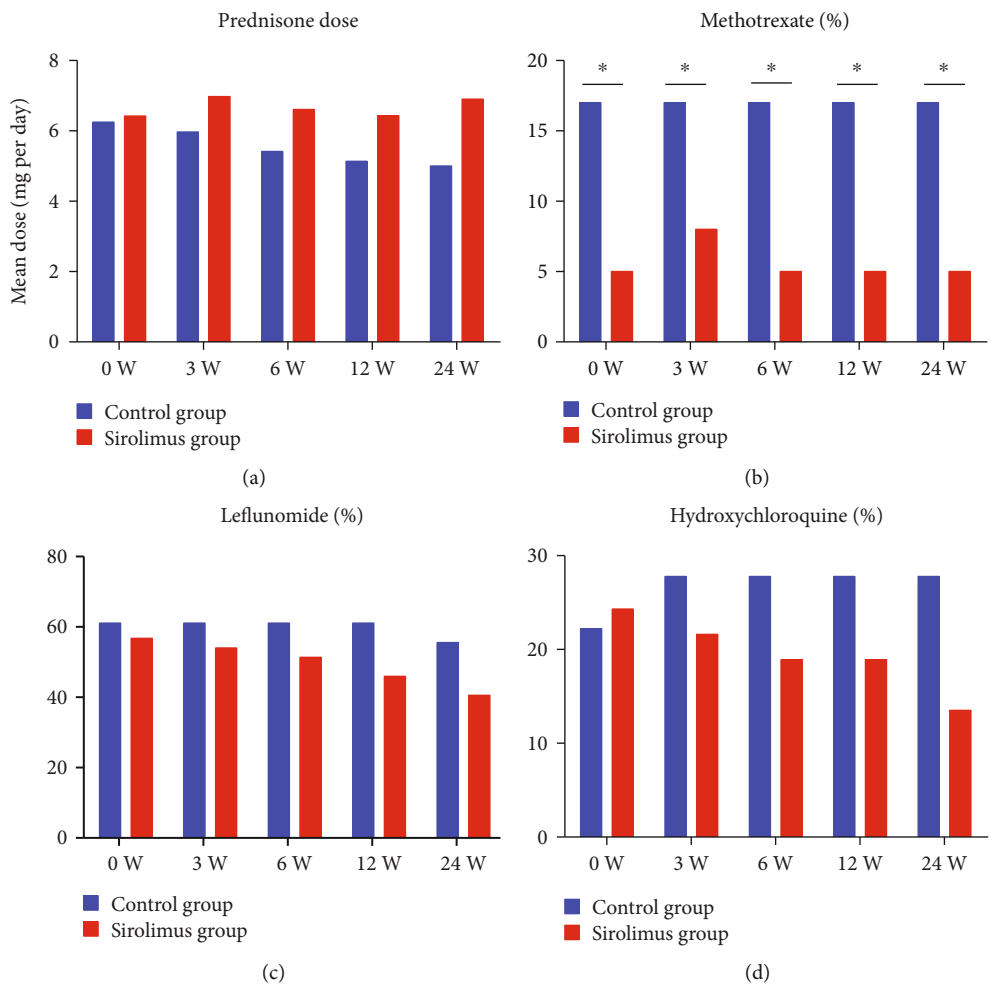

Figure 3: Reductions of drugs required to control disease activity. Mean daily prednisone dose (a), percentage of patients receiving treatment of methotrexate (b), leflunomide (c), and hydroxychloroquine (d) at baseline (week 0) and during treatment (weeks 3, 6, 12, and 24).

4. Discussion

mTOR serves as a regulator of growth, proliferation, and survival in eukaryotic cells. This pathway has been well known to play important roles in regulating adaptive and innate immune cell function [21] and is also critical for inflammatory bone destruction in RA [22]. Sirolimus, an mTOR inhibitor, exhibits immunosuppressive effects via inhibition of B cell and T cell proliferation. Therefore, it was initially developed as an immunosuppressant in solid organ transplant setting and as a growth suppressor in the treatment of tumors [23]. Subsequently, the potency of sirolimus in blocking T-cell activation was first found to be beneficial in the treatment of rheumatic diseases in the context of systemic lupus erythematosus (SLE), both in animal models [24] and patients [17, 25]. However, so far, clinical evidence for the use of mTOR inhibitors in RA is very limited. Only one clinical study has been published that showed a moderate effect on the signs and symptoms of disease over 12 weeks of treatment with an mTOR inhibitor in combination with methotrexate [12]. On the other hand, no other study has shown that the absolute number of peripheral Tregs is decreased in RA patients and that mTOR inhibitors affect the levels of Treg and other T cell subsets in RA patients.

Since the main adverse events of sirolimus are dose-dependent [26], our parallel-controlled study provides preliminary evidence that low-dose sirolimus (0.5 mg per other day) combined with conventional immunosuppressive drugs is safe, better tolerated, and clinically efficacious in patients with RA. Lai et al. [17] reported that active patients with systemic lupus erythematosus treated with sirolimus at a starting dosage of 2 mg daily (8 times higher than ours) had reduced hemoglobin and neutrophil counts or extensive oral ulcers. In contrast, except for one patient who suffered from oedema, our low-dose sirolimus therapy did not show evaluable side effects such as cytopenia or ulcer.

Sirolimus has been proven to have antirheumatic properties at the dosage of 2-6 mg once daily [17, 27], with the

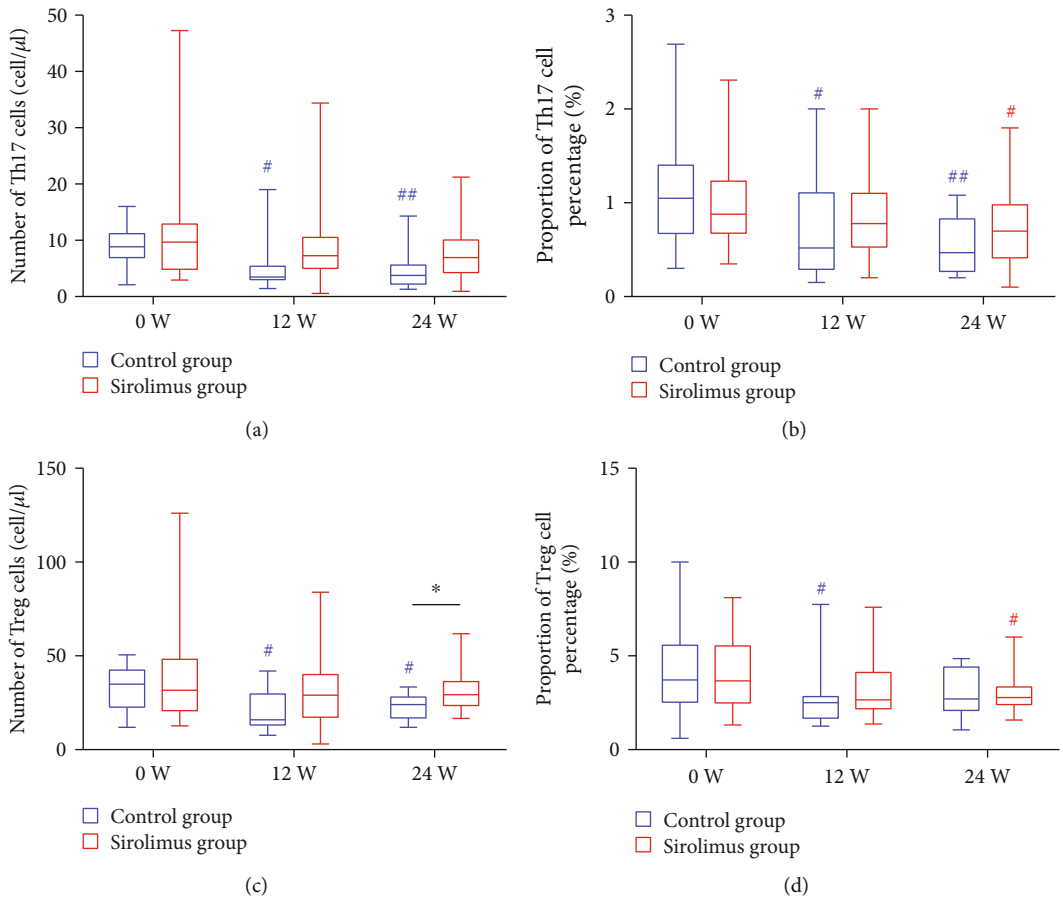

Figure 4: Changes of levels of Th17 (a, b) and Tregs (c, d) after different treatments. Effects of treatments were assessed by repeated measure analysis using a mixed-effect model. (a) and (c) represent the changes in absolute count (median, range) of Th17 and Treg cells, respectively, while (b) and (d) represent the changes in their percentages (median, range). A two-tailed unpaired t-test was used to compare the disease activity measures between the sirolimus and conventional groups. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ relative to baseline (week 0) in the conventional group (blue); $^{\#}p < 0.05$ relative to baseline in the sirolimus group (red); $^{*}p < 0.05$ compared between groups.

serum trough level maintained between 4.9 and 15 ng/ml [15, 27]. Our study found that low-dose sirolimus (0.5 mg per other day with the serum concentration of 1.8 ± 0.4 ng/ml) had an immunoregulatory property besides immunosuppression by the rebalancing of Th17 and Tregs. Conventional immunosuppressant medications can alleviate disease activity by inhibiting lymphocytes nonspecifically [28, 29]. Our results showed that conventional immunosuppressive strategy not only decreased the proinflammatory Th17 cells but also reduced anti-inflammatory Tregs, which may aggravate the disturbance of the immune balance. On the contrary, patients treated with sirolimus had higher levels of Tregs compared with those who received conventional treatments, which means low-dose sirolimus could reverse the reduction of Tregs not only by the disease itself but also by immunosuppressive agents. Similarly, Li et al. [30] reported that sirolimus promotes the expression of $FoxP3^{+}$ in $CD4^{+}T$ cell subsets and the proliferation of Tregs by inducing TGF-β secretion. Biswas et al. [31] found that mTOR inhibitors synergistically promote induction of antigen-specific Tregs via selective expansion of plasmacytoid dendritic cells. These evidences support that sirolimus participates in immunoregulation by augmenting Tregs. Our results indicate that sirolimus can be used to rebalance Th17 and Tregs as an immunoregulatory drug besides an immunosuppressant to treat RA patients. In this study, we defined low-dose sirolimus combined with immunosuppressants to treat RA as sirolimus immunoregulatory therapy.

Importantly, disease activity was significantly reduced by low-dose sirolimus immunoregulatory therapy. Though no differences of disease activity measures other than TJC at week 24 were observed in sirolimus-treated patients compared with those receiving conventional medications, the restoration of Tregs by sirolimus should have longer-term benefit for the remission of the disease and the withdrawal of conventional immunosuppressants. Interestingly, application of immunosuppressants required to control disease activity in sirolimus treatment patients was significantly reduced compared with that in the conventional group, indicating that sirolimus could effectively replace the usage of conventional immunosuppressants.

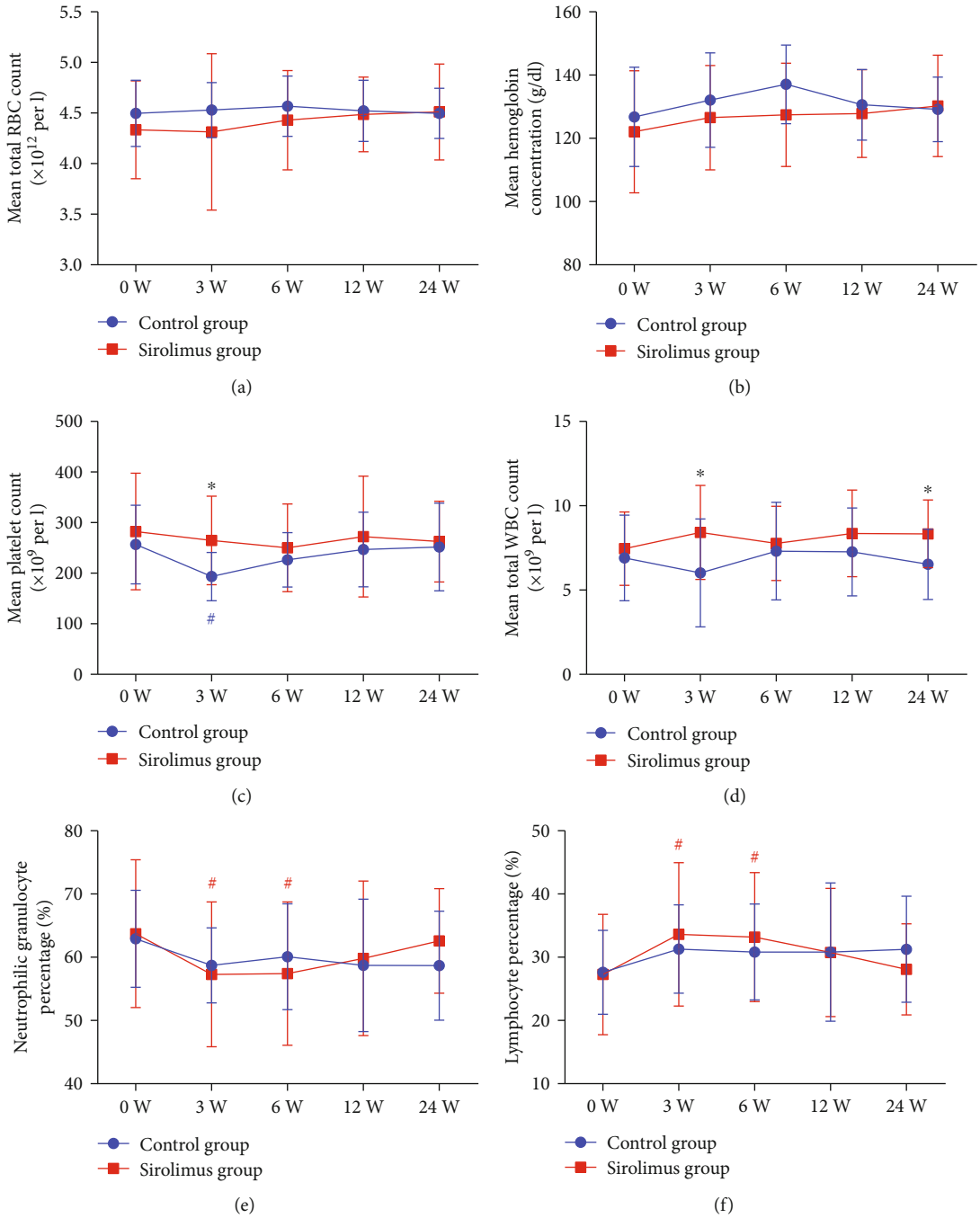

Figure 5: Safety outcomes of blood routine. Mean RBC counts (a), hemoglobin concentration (b), platelet counts (c), total WBC counts (d), and proportion of neutrophils (e) and lymphocytes (f) were measured before treatment (week 0) and after initiation of treatment at weeks 3, 6, 12, and 24 in the control and sirolimus groups. An unpaired t-test was used to compare the differences in blood routine measures between the sirolimus and conventional groups. Error bars show SD. RBC: red blood cell; WBC: white blood cell. $^{\#}p < 0.05$ compared to the baseline (week 0) in the conventional group (blue); $^{\#}p < 0.05$ compared to the baseline in the sirolimus group (red); $^{*}p < 0.05$ compared between groups.

Overall, this study was the first clinical trial on the effect of low-dose sirolimus on active RA. The sirolimus immunoregulatory therapy not only effectively reverses the reduced Tregs and inhibits effector T cells but also alleviates clinical symptoms and decreases the immunosuppressive applications in patients with active RA, which could avoid overtreatment and evaluable side effects of conventional therapy. More high quality trials with large samples and longer following-up are proposed to clarify the further benefits of sirolimus combination therapies.

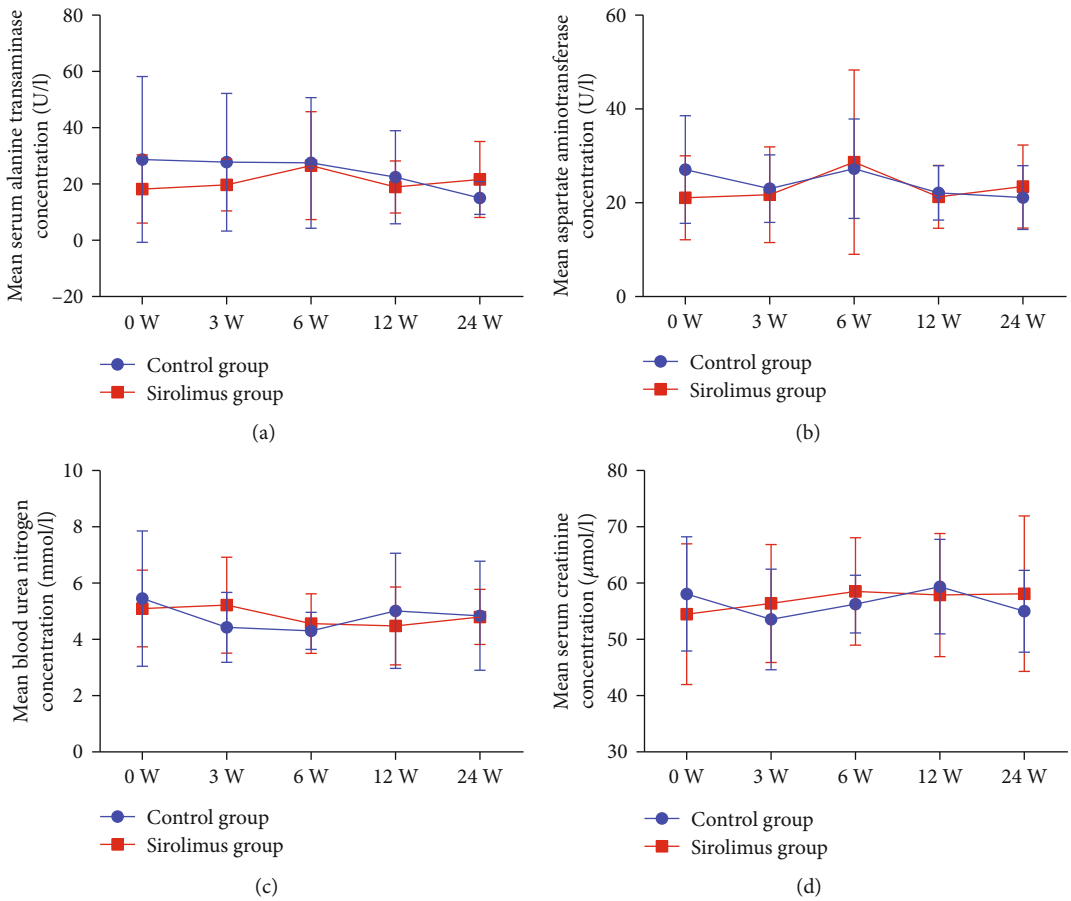

Figure 6: Safety outcomes of liver and renal function. Mean concentration of serum alanine transaminase (a), aspartate aminotransferase (b), blood urea nitrogen (c), and serum creatinine (d) are shown. Overall changes in safety endpoints during treatment were assessed to indicate for each safety outcome. An unpaired t-test was used to compare the blood routine measures between the sirolimus and conventional groups. Error bars show SD.

Data Availability

All data generated or analyzed during this study are included in this published article.

Ethical Approval

This trial is registered at the Chinese Clinical Trial Registry (number ChiCTR-IPR-17010307).

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

HW, JW, SZ, and XL were responsible for the study design and manuscript writing and the data extraction, quality assessment, analysis, and interpretation of the data. All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Li had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. HW and JW contributed to the work equally and should be regarded as co-first authors.

Supplementary Materials

Supplementary Table 1: clinical details of patients enrolled in the study. Supplementary Figure 1: phenotypic characterization of lymphocyte subpopulations by flow cytometry. (A) Representative flow cytometry analysis of peripheral lymphocytes. T: $CD45^+CD3^+$; B: $CD45^+CD3^-CD19^+$; NK: $CD45^+CD3^-CD16^+CD56^+$ NK; $CD4^+$T: $CD45^+CD3^+CD4^+$; $CD8^+$T: $CD45^+CD3^+CD8^+$. (B) Representative flow cytometry analysis of $CD4^+$T cell subsets. All dot plot analyses are of $CD4^+$ gated lymphocyte. Th1: $CD4^+$INF-γ^+; Th2: $CD4^+$IL-4^+; Th17: $CD4^+$IL-17^+; Treg: $CD4^+CD25^+Foxp3^+$. Supplementary Figure 2: comparison of lymphocyte subpopulations between the groups at visits 1, 4, and 5. (A) and (B) represent the T cell level, (C) and (D) the B cell level, (E) and (F) the $CD4^+$T cell level, (G) and (H) the $CD8^+$T cell level, and (K) and (L) the total lymphocyte cell level. Effects of treatments were assessed by repeated measure analysis using a mixed-effect model. Two-tailed unpaired t-test was used to compare

the disease activity measures between sirolimus and conventional groups. No significant differences were observed at each visit and between the groups. Supplementary Figure 3: comparison of $CD4^+$T cell subsets between the groups before treatment and weeks 6, 12, or 24 after the treatment. (A) and (B) represent the absolute number and percentage of the Th1 cells and (C) and (D) represent those of the Th2 cell levels, respectively. Two-tailed unpaired t-test was used to compare the disease activity measures between the sirolimus and conventional groups. $^{\#}p < 0.05$ relative to the baseline (week 0) in the sirolimus group (red); $^{*}p < 0.05$ compared between the groups. *(Supplementary Materials)*

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Review Article

The Dynamic Interplay between the Gut Microbiota and Autoimmune Diseases

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The human gut-resident commensal microbiota is a unique ecosystem associated with various bodily functions, especially immunity. Gut microbiota dysbiosis plays a crucial role in autoimmune disease pathogenesis as well as in bowel-related diseases. However, the role of the gut microbiota, which causes or influences systemic immunity in autoimmune diseases, remains elusive. Aryl hydrocarbon receptor, a ligand-activated transcription factor, is a master moderator of host-microbiota interactions because it shapes the immune system and impacts host metabolism. In addition, treatment optimization while minimizing potential adverse effects in autoimmune diseases remains essential, and modulation of the gut microbiota constitutes a potential clinical therapy. Here, we present evidence linking gut microbiota dysbiosis with autoimmune mechanisms involved in disease development to identify future effective approaches based on the gut microbiota for preventing autoimmune diseases.

1. The Gut Microbiota

All mammals, including humans, emerge into the world from a sterile environment; thereafter, microorganisms gradually colonize the skin, oral cavity, and nasal, genital, respiratory, and alimentary tract surfaces, which are covered by epithelia [1]. The human gut is colonized by various microorganisms collectively termed the gut microbiota, which has a mutualistic relationship with the host. The gut microbiota is the major source of microbes that may exert beneficial or pathogenic effects on host health. Moreover, the gut microbiota hosted in the gastrointestinal tract, which is the largest host interface exposed to the external environment, comprises approximately two-thirds of the human microbial commensal community [2]. The establishment and development of a beneficial microbiota composition occur during early infancy, influencing health and immune homeostasis in adulthood [3], and disturbing the establishment of this microbiota during early life may have negative effects [4]. Progression of the gut microbiome undergoes the following three phases in early life: the developmental (3-14 months), traditional (15-30 months), and stable (31-46 months) phases. In general, breastfeeding is the most significant factor associated with the development of the microbiome [5].

In addition to the expected role in maintaining gastrointestinal homeostasis, the microbiota is also fundamental for maintaining nutritional activities, metabolic functions in nutrient digestion, detoxification, vitamin synthesis, and immunologic homeostasis in the host. Although the gut microbiota includes viruses, fungi, protozoa, archaea, and

bacteria [6], the bacterial component is the most studied and maintains a symbiotic relationship with the host. The bacterial microbiota is divided into aerobic, facultative anaerobic, and obligate anaerobic bacteria according to the degree of aerobic tolerance, with most of the gut microbiota consisting of obligate anaerobic organisms.

The microbiota of the human body consists of more than 10^{14} microorganisms that inhabit different areas of the body, among which the intestine harbors the largest community [7]. The main groups of the gut microbiota in the human intestinal lumen include Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria. Due to the expansion of the application of high-throughput deep-sequencing technology in the past decade, it has gradually been revealed that the gut microbiome encodes 3.3 million genes, which is 100-fold more than the number of human genes [8]. Therefore, the gut microbiome is also termed the "human second genome." Gut microbiota constituents are divided into another three groups according to their functions, called commensal beneficial microorganisms, potentially sensitive pathogens, and pathogenic bacteria. The gut microbiota constituents classified as commensal "beneficial" microorganisms maintain a healthy host environment and offer benefits, also interacting with host tissues in a cooperative and nonpathogenic manner. An imbalance in "sensitive" microorganisms occurs during disease; "pathogenic" microorganisms cause disease, and "therapeutic" microorganisms can help rectify any alterations [9]. The highest species diversity and number are observed in the colon, and various factors affect the composition of the human gut microbiota, including but not limited to diet, age, sex, and geographical location [10, 11]. A change in the microbiota during individual ontogeny is mainly influenced by radical changes in diet, application of antibiotics, or probiotics, and diverse diseases [12].

2. The Gut Microbiota and Enteric Mucosal Immunology System

The human mucosa is the site in the human body that most frequently interacts with the complex external environment. The enteric mucosal immunology system relies mainly on gut-associated lymphoid tissue (GALT), which consists of Peyer's patch lymphocytes (PPLs), intestinal intraepithelial lymphocytes (IELs), lamina propria lymphocytes (LPL), and mesenteric lymph nodes (MLNs). IELs include most $CD3^+$ T cells, a few B cells, and natural killer (NK) cells; LPLs mainly comprise different subpopulations of T cells and B cells. Our immune system is responsible for the defense against microbial pathogens via recognition and removal. However, another significant role of our immune system is to balance the microbiota inhabiting our mucosal and skin surfaces. The enteric mucosal immune system partially maintains homeostasis by shaping the gut microbial community toward a beneficial effect, and it is essential not only for human health but also for the survival of trillions of microbial community members residing within the intestines. As the gut microbiota in the human body forms a barrier to resist invasion of pathogenic bacteria and synthesis of nutrients, such as proteins and vitamins, changes in the intestinal microbiota impair intestinal mucosal barrier function [13]. With regard to gut barrier homeostasis, the aryl hydrocarbon receptor AhR, which is a ligand-activated transcription factor that recognizes dietary compounds, microbial-derived secondary metabolites, and environmental pollutants to control transcriptional programs in the immune system, plays a key role in the immune response [14]. Because AhR(-/-) mice have insufficient gut barriers, AhR plays a crucial role in sustaining and developing gut barriers. AhR, which is highly expressed in epithelia, is also important for the host gut-microbiome interaction, and AhR expression is significantly decreased in germ-free mice.

The gut microbiota maintains the homeostasis of our immune system. Innate and adaptive immunity plays an important role in the containment and clearance of microbial pathogens. Consequently, subsets of innate and adaptive lymphocytes are considered to sequentially shape the gut microbiota in distinct ways [15]. As a component of one of the most important pathways by which the host recognizes microbial metabolites, AhR participates in the regulation of both the adaptive and innate immune systems, as well as in a variety of diseases and relieving inflammation [16, 17]. Secretory immunoglobulin A (IgA) secreted by the gut mucosa is a major contributor to intestinal mucosal immunity. IgA responses both clear pathogens and promote host-microbial symbiosis, which is driven by the microbiome. For example, *Bacteroides fragilis* regulates its surface to encourage binding of IgA in vivo, which facilitates bacterial adherence [18]. The repertoire of IgA bound to the gut microbiota is related to both T-cell-dependent and T-cell-independent pathways. AhR signaling can modulate the dynamics of the gut microbiota, which might be involved in the interaction between host metabolism and the gut microbiota [19]. Furthermore, activation or disruption of AhR can influence the microbiota [20], and specific dietary and microbial metabolites of tryptophan can serve as the ligand for AhR [21]. After tryptophan metabolite binding and activation of AhR, the transcription factor induces expression of downstream cytokines, including IL-22 and IL-17, to modulate intestinal homeostasis [22]. Ozcam et al. used whole-genome sequencing, comparative genomics, and targeted gene inactivation to show that *Lactobacillus reuteri* R2lc and 2010 harbor an orthologous polyketide synthase (PKS) gene cluster that serves as a novel pathway responsible for AhR activation [23].

Gut dysbiosis not only affects the expression level of Toll-like receptors (TLRs) of antigen-presenting cells but also contributes to Th17/Treg imbalance [24]. The composition and metabolites of the gut microbiota have major roles in producing antibodies, shaping the B cell repertoire, maintaining the Th17/Treg balance, regulating different subpopulations of Th17 cells, and regulating homeostasis in different populations of T helper cells [25]. The promotion of gut regulatory T cells via AhR signaling is important for gut immune homeostasis. In a T cell-transfer genetic mouse model of colitis, AhR-expressing Tregs exhibited increased suppressive activity compared with Tregs lacking AhR expression [26]. AhR regulates immune surveillance of the gut by retaining IELs and redistributing

the Th17/Treg cell balance. Studies have also shown that AhR expression plays a fundamental role in contributing to the establishment of the intestinal microbial community structure in mice [27].

In collagen-induced arthritis (CIA) rats, the intestinal mucosal immune response is enhanced and immune tolerance is disturbed. Moreover, madecassoside treatment can increase relative expression of forkhead box P3 (FoxP3) mRNA in the small intestine, downregulate concentrations of sIgA and IFN-γ in the small intestinal content and tissue, respectively, decrease the ratio of $CD4^+/CD8^+$ cells in the epithelium and laminar propria, and decrease relative expression of CD80, CD86, IL-6, and IL-12 mRNA in CIA rats to downregulate the intestinal mucosal immune response and restore normal immune tolerance [28]. Partial immunosuppressive activities of triptolide that lower arthritis scores and upregulate the TGF-β level might occur through an effect on enteric mucosal immune lymphocytes in Peyer's patches, IELs, and LPLs of DBA/1 with CIA [29]. Additionally, a Gui Zhi decoction was found to lower the number of slgA+ cells and $CD4^+$ and $CD8^+$ T cells in the lamina propria in CIA mice and downregulate IL-6 and TNF-α concentrations in serum, revealing that the mechanism of decreased incidence of CIA occurs in part through the regulation of enteric mucosal immunity in mice [30].

3. The Gut Microbiota and Host Health

Manipulation of the dense gut microbiota has broad implications for host health. The diverse human gut microbiota plays a fundamental role in the well-being of hosts and is closely correlated with the growth, development, substance metabolism, and immune function of the host [31]. Indeed, the gut microbiota is intimately connected to numerous facets of host biology, and beneficial microbiota organisms play an important role in the processes of food digestion, immune system homeostasis maintenance, anti-infection immunity support, lipid metabolism modulation, and others [32]. Specifically, the gut microbiota can influence intestinal functional integrity, barrier strength, and permeability regulation, thereby influencing the immune response, which has been linked to the development of inflammatory disease. Studies have shown that high levels of *Prevotella copri* correlate with low levels of the gut microbiota previously associated with immune-regulating properties [33]. Nutrient availability may also influence the gut microbiota and expand the possibility of cell-based therapeutic strategies in the gut. For example, Shepherd et al. demonstrated that the transfer of the porphyran utilization locus into an exogenous *Bacteroides* strain in the mouse gut can control strain abundance by multiple orders of magnitude [34]. Differences between microbiota profiling of AhR(+/+) and AhR(-/-) mice fed a diet enriched with a specific AhR ligand or a diet depleted of any known AhR ligands have been reported; thus, the AhR signaling pathway may influence microbiome composition in the small intestine. Microbiome metabolites, such as short-chain fatty acids (SCFAs), also regulate AhR and its target genes in the intestine [35].

An imbalance of the gut microbiota might underlie a broad spectrum of human illnesses, such as immune, metabolic, and cardiovascular diseases as well as neurological disorders, and alterations of the gut microbiome may be carried out in the future to improve clinical outcomes across diverse genetic backgrounds. The gut microbiome may also improve the prediction accuracy of human traits, including glucose measures and obesity [36]. Host genetic factors play a minor role in determining the microbiome composition compared with factors associated with drugs, diet, and anthropometric measurements [37]. Based on epidemiological data, the propensity for horizontal transmission of bacterial genera representing pathogenic genera in humans may be responsible for hospitalizations [38]. AhR is a pleiotropic factor that might influence the community structure of the gut microbiota. By utilizing C57BL6/J AhR(-/+) and AhR(-/-) cohoused littermates after 18 days of genotypic segregation, Murray et al. found significant changes in the phylum abundance of the gut microbiota, particularly for *Verrucomicrobia* and segmented filamentous bacteria (SFB). Transfer of the AhR(-/-) microbiota to wildtype germ-free mice indicated Ahr(-/-)-microbial dependence, which increased *Verrucomicrobia* abundance and inflammatory tone [27]. AhR is also a potential target in the treatment of antibiotic-associated gut barrier damage. Wang et al. showed the downregulation of AhR, IL-22, and phosphorylated Stat3 (p-Stat3) with decreasing antimicrobials but that antimicrobial molecule levels were significantly rescued after antibiotic treatment when given the exogenous AhR agonist 6-formylindolo (3,2-b) carbazole (Ficz) [39]. AhR is a promising therapeutic target in immune-mediated diseases because it can induce IL-22 and regulate anti-inflammatory signals as well as control the differentiation, proliferation, and activation of immune cells.

4. The Gut Microbiota and Autoimmune Diseases

Autoimmune diseases are characterized by aberrant production of autoantibodies. Genetic and/or environmental factors act on the immune system and cause abnormal generation of autoantibody-producing B cells and autoreactive T cells and anomalous production of proinflammatory cytokines. It has been hypothesized that the increasing incidence of autoimmune diseases is due to considerable shifts in the gut microbiota among multifactorial reasons following dietary changes and the widespread application of antibiotics (Table 1).

Both genetic and environmental factors contribute to autoimmune diseases, including complex genetic elements, geographical location, patient exposure, immunologic derangement, and viral infections. By binding diverse dietary, cellular, and microbe-derived ligands, AhR might be involved in autoimmune diseases via the transduction of extrinsic and intrinsic signals into cellular responses [40]. According to Qiu et al., reduced innate IL-22 levels in AhR-deficient mice allowed the expansion of commensal SFB (an immune activator), promoting Th17 cell proliferation. Innate expression of AhR plays a protective role in T-cell-mediated experimental colitis by suppressing pathogenic Th17 cells [41]. In addition, Stedtfeld et al. reported

Table 1: Autoimmune diseases and alteration of the gut microbiota composition.

Diseases	Species	Methods	Increasing microbiota species	Decreasing microbiota species	References
RA	Mouse	16S rRNA gene sequencing	*Desulfovibrio* *Mucispirillum* *Helicobacter* Lachnospiraceae *Rikenellaceae_RC9*	*S24-7* *Rikenella*	[60]
	Human	16S rRNA sequencing		Euryarchaeota	[54]
	Human	16S ribosomal DNA	*Collinsella*	Actinobacteria	[53]
	Human	Denaturing gradient gel electrophoresis	*Lactobacillus*		[57]
	Mouse	16S rRNA sequencing	Bacteroidaceae *Lachnospiraceae* S24-7		[47]
SLE	Mouse, human	16S rDNA sequencing	*Lactobacillus reuteri*		[62]
	Human	16S rRNA sequencing	Proteobacteria	Ruminococcaceae	[61]
	Human	16S ribosomal RNA gene sequencing	*Bacteroides*	Firmicutes/Bacteroidetes ratio	[112]
	Human	16S rRNA sequencing	*Streptococcus* *Lactobacillus* *S. anginosus* *L. mucosae* *Megasphaera*	*Faecalibacterium* *F. prausnitzii* *Cryptophyta* *Roseburia* *Bifidobacterium*	[66]
	Human	16S rRNA sequencing	*Ruminococcus gnavus*		[67]
	Human	Metagenomic analyses		Firmicutes/Bacteroidetes ratio	[69]
	Mouse	16S rDNA sequencing	Lachnospiraceae	*Lactobacillus* spp.	[47]
SpA	Human	16S rRNA gene- and ITS2-based DNA sequencing	*Escherichia-Shigella* *Veillonella* Lachnospiraceae NK4A136 group Dothideomycetes	*Prevotella* strain 9 *Megamonas* *Fusobacterium* Agaricales	[79]
	Human	16S ribosomal RNA amplicon sequencing	Dialister		[83]
	Human	16S ribosomal RNA gene sequencing	*Ruminococcus gnavus*		[84]
IBD	Human	Metagenomic sequencing	*Escherichia coli*	*Eubacterium rectale* *Faecalibacterium prausnitzii*	[101]
	Human	Tag-sequencing the 16S rRNA gene		*Roseburia* spp.	[93]
	Human	16S rRNA sequencing	Actinobacteria Proteobacteria (Enterobacteriaceae)	Firmicutes (Clostridiales)	[94]
	Human	16S rRNA sequencing	Proteobacteria	Firmicutes Actinobacteria	[95]
	Dog	16S rRNA gene next-generation sequencing	Paraprevotellaceae *Porphyromonas*		[91]

that compared with levels under a traditional gut microbiome, 2,3,7,8-tetrachloro dibenzo-*p*-dioxin (TCDD, an AhR ligand) induced shifts in the abundances of SFB and *Bacteroides fragilis* (an immune suppressor) in mice. Moreover, the TCDD-induced host response was significantly regulated by the presence of SFB in the gut microbiome, demonstrating therapeutic potential between AhR ligands and key commensals [42]. Dysbiosis of the gut microbiota has been identified as a potential factor that causes autoimmune diseases, which in humans are attributed to multiple factors, even though the relative contribution of the gut microbiota remains elusive. Posttranslational modification of autoantigens and cross-reactivity with autoantigens represent mechanisms by which the gut microbiota mediates autoimmunity at the molecular level. At the cellular level, translocation of live gut bacteria across a dysfunctional gut barrier provokes direct interactions with immune and tissue cells and subsequently instigates systemic autoimmunity [43].

The association between gut microbiota imbalance and autoimmune diseases may be due to several mechanisms that

may impact the human immune system and function. For instance, modulating the host immune response and activation of antigen-presenting cells (APCs), including dendritic cells (DCs), may evoke antigen presentation and cytokine production, subsequently affecting T cell differentiation and function. Furthermore, this impact disrupts the homeostasis between T helper 17 (Th17) cells and T regulatory cells (Tregs). There may also be similarities between foreign antigens and self-antigens attributed to antigenic mimicry; consequently, pathogen-derived autoreactive T and B cells are activated, thus promoting autoimmunity. However, the permeability of the intestinal mucosa is altered because expression of tight junction (TJ) proteins is modulated.

Abundant evidence suggests that the gut microbiota may be involved in the initiation and amplification of disease progression in patients with autoimmune diseases. The possible mechanisms include molecular mimicry, impacts on intestinal mucosa permeability, the host immune response caused by the microbiota, and antigenic mimicry. Alterations in gastrointestinal microbial communities have been linked to autoimmune diseases. The gut microbiota can influence or interfere with immune sensing in discriminating between self and nonself, which may contribute to autoimmune diseases. Patients with autoimmune diseases commonly display signs of impaired gut barriers, which may result in immune exposure to commensal gut bacteria. In addition, a breakdown in mucosal immune tolerance leads to aberrant and pathological immune responses toward the gut microbiota, which contributes to disease severity.

In recent years, an increasing number of studies have shown that several approaches, including antibiotics, prebiotics, antimicrobial interventions, fecal microbiota transplantation (FMT) [44], and selective probiotics, can regulate the gut microbiota [45, 46]. However, some inappropriate antibiotic use and even nonantibiotic (human-targeted) drugs have been correlated with changes in gut microbiota composition. Growing experimental and clinical evidence has suggested that the chronic inflammatory response induced by gut microbiota dysbiosis can strongly contribute to the development of autoimmune diseases. Overall, germ-free animal models are more suitable for exploring the influence of the host microbiome on the course and development of many disorders [47, 48]. In general, the microbiota may trigger autoimmunity in genetically susceptible individuals or prevent autoimmunity in others.

4.1. The Gut Microbiota and Rheumatoid Arthritis. Rheumatoid arthritis (RA) is a systemic, inflammatory, and autoimmune disease characterized by the destruction of cartilage and bone, ultimately progressing to functional disability. RA is one of the most common autoimmune diseases, affecting approximately 1% of the worldwide population and occurring twice as frequently in women than in men. It has been demonstrated that RA correlates with the inflammatory response mediated by $CD4^+$ Th1 and Th17 lymphocytes and the imbalance between Th17 lymphocytes and Tregs, and the complex interaction between genes and environmental factors is implicated in the etiology and progression of RA. Talbot et al. demonstrated that cigarette smoking induces experimental arthritis aggravation and increases the frequency of Th17 cells in vivo, and experiments in vitro have also shown that cigarette smoking directly promotes Th17 differentiation. Furthermore, the effects of cigarette smoking on inducing arthritis aggravation are AhR dependent, and environmental pollutants with AhR agonist activity exacerbate arthritis by directly enhancing Th17 cell differentiation [49]. Nevertheless, berberine (BBR) promotes the differentiation of $CD4^+$ $CD25^+$ Treg cells by inducing FoxP3 activation via AhR to regulate the Th17/Treg imbalance in RA [50]. The emerging field of microbiota research has raised awareness that the gut microbiota and its metabolites interact with the host immune system, with associations with the pathogenesis of RA. Moreover, changes in the gut microbiota have been implicated in the loss of tolerance of self-antigens and in increasing inflammatory events that promote joint damage.

The most commonly used experimental animal model is CIA. A recent experimental study revealed that the gut microbiome can influence arthritis susceptibility. Differences in the gut microbiota were consistently detected between CIA-susceptible and CIA-resistant or healthy mice. Before arthritis onset, the susceptible mice showed enriched operational taxonomic units (OTUs) related to *Lactobacillus* as the dominant genus. As arthritis developed, the abundances of families Bacteroidaceae, Lachnospiraceae, and S24-7 increased significantly in CIA-susceptible mice. When transplanted into germ-free mice, the microbiota showed a higher frequency of CIA induction in animals receiving the CIA-susceptible microbiota than in those receiving the microbiota from CIA-resistant mice [47]. In addition, Sato et al. found that CIA was aggravated in mice by oral administration of *Porphyromonas gingivalis*, significantly altering the gut microbiome, with concomitant elevation in interleukin- (IL-) 17 levels in sera, gut barrier function impairment, and increasing Th17 cell proportions among mesenteric lymphocytes [51]. Liu et al. investigated the effect of *Lactobacillus salivarius* isolated from RA patients on CIA mice by oral administration for 5 weeks starting at 2 weeks before arthritis induction, and the bacterium exhibited antiarthritic and anti-inflammatory effects. Furthermore, pretreatment with *L. salivarius* induced profound changes in the cellular adaptive immune response by reducing and increasing the Th17 and Treg cell fractions, respectively [52].

RA patients exhibit decreased gut microbiota diversity compared with controls, which correlates with RA duration and autoantibody levels. Studies of the gut microbiota have shown that RA is characterized by an increase and/or decrease in the abundance of microbe groups compared with that in healthy individuals. A previous taxon-level analysis suggested that *Actinobacteria* levels are decreased among abundant taxa in RA patients compared with that in controls. Based on a random forest algorithm, it has been suggested that *Collinsella*, *Eggerthella*, and *Faecalibacterium* are related to RA. The abundance of *Collinsella* was associated with increasing levels of alpha-aminoadipic acid and asparagine as well as production of IL-17A, and the role of *Collinsella* in altering gut permeability and RA severity in experimental arthritis has been confirmed [53]. The composition of the gut microbiota in RA patients free of therapy is

characterized by certain abnormalities compared to that in healthy controls [54]. A data-driven study suggested that human gut microbial metabolites directly interact with 18.1% of all 166 RA-associated genes, participate in 71.4% of 311 RA-associated genetic pathways, and affect 51.3% ofRA-related phenotypes, including immune system pathways and phenotypes; this in turn demonstrated that the gut microbiota and its metabolites contribute to RA at genetic, functional, and phenotypic levels [55]. Another study reported that the HLA-B27 and HLA-DRB1 alleles affect susceptibility to RA, with effects on the gut microbiome that partially cause or increase the risk of RA [56]. In addition, changes in fecal bacteria may represent the RA condition or the outcome of RA progression. Additional significantly affected bacterial species and increased bacterial diversity and abundance have been observed in patients with early RA based on denaturing gradient gel electrophoresis (DGGE); the fecal microbiota (FM) of patients with early RA contains significantly more *Lactobacillus* (*L. salivarius*, *L. iners*, and *L. ruminis* becoming the predominant bacteria) than a healthy control group, and the presence of *L. mucosae* was also observed [57]. Regardless, the same gut microbiota species might not play a concurrent role in the pathogenesis of RA, and different species may produce different effects on RA. For instance, the abundance of *Prevotella copri* is increased in some patients with early-stage RA, but *Prevotella histicola* from the human gut microbiota suppresses the development of RA [58]. Interestingly, Hablot et al. evaluated the impact of experimental colitis on the development of CIA in mice and observed that concomitant experimental colitis reduced severity in association with modification of the gut microbiota composition [59].

Once altered, the gut microbiota can be partially restored through disease-modifying antirheumatic drugs. Restoration-altered gut microbial ecosystems may have therapeutic effects, and this interaction has an impact on clinical outcome in RA. Xiao et al. have suggested that *Paederia scandens* extract has a protective effect on CIA mice by decreasing the relative abundance of inflammatory-related microorganisms, including *Desulfovibrio*, *Helicobacter*, *Mucispirillum*, and Lachnospiraceae [60]. Gut dysbiosis is a hallmark of RA with different serological and clinical parameters. The phylum Euryarchaeota correlates directly with the disease activity score on 28 joints (DAS-28), emerging as an independent risk factor. RA patients under treatment with immunosuppressive drugs, such as etanercept, present partial restoration of a beneficial microbiota [54]. Although RA patients may require antibiotic treatment, partial depletion of the natural gut microbiota by oral Baytril (enrofloxacin) aggravates arthritis symptom severity in CIA mice [32]. Therefore, precautions should be taken when RA patients are treated with drugs, as some of them may aggravate the disease.

4.2. The Gut Microbiota and Systemic Lupus Erythematosus. Systemic lupus erythematosus (SLE) is a complex, chronic, and inflammatory prototypical autoimmune disease characterized by persistent inflammation in multiple organs of the body. SLE most often affects women but is triggered by complicated interactions of different factors (genetic predisposition, hormonal alteration, and environmental factors, among others) that are not completely clear at present. Alterations in the composition of the microbiota lining the intestines are suspected to be involved in the etiopathogenesis of SLE, and a number of studies have been carried out to demonstrate that gut microbiota dysbiosis affects the onset and development of SLE. Specified genera of the microbiota can be used to distinguish SLE patients from healthy individuals. A recent study revealed a decrease in Ruminococcaceae and an increase in Proteobacteria in patients with SLE in northeastern China [61]. In addition, compared with control group subjects, SLE patients were characterized by a decrease in bacterial richness, a lower Firmicutes/Bacteroidetes ratio, and an increase in the relative abundance of *Bacteroides* species in fecal samples. In studies of human SLE and murine models, gut microbiota compositions have been shown to differ from those of healthy controls. Zegarra-Ruiz et al. found that *Lactobacillus* in feces is enriched in a subset of SLE patients, and using Toll-like receptor 7- (TLR7-) dependent mouse models of SLE, *Lactobacillus reuteri* was shown to exacerbate autoimmune manifestations, which was inhibited by dietary resistant starch (RS) via SCFAs [62]. Ligands of AhR involved in the pathogenesis and development of SLE have also been identified [63]. Previous data from a hospital-based case-controlled study showed that the expression level of AhR in PBMCs was significantly higher in SLE patients than in healthy controls and that the proportion of AhR-overexpressing cells to Th17 cells was significantly increased in SLE patients compared with that in the control group. In the high-AhR ratio group, there were more younger SLE patients with skin lesions and ultraviolet allergies and lower complement 3 levels than in the low-AhR ratio group, indicating AhR as a potential biomarker for predicting SLE skin injury [64]. Mohammadi et al. reported that the AhR agonist indole-3-carbinol (I3C) mediates AhR activation, contributing to immunoregulatory effects on macrophages of SLE patients by decreasing expression of proinflammatory cytokines and overexpression of anti-inflammatory cytokines [65].

Obvious dysbiosis of the gut microbiota is associated with active and remissive SLE patients and thus may be used to diagnose SLE and predict disease activity. SLE patients reportedly exhibit characteristic patterns of gut microbiota dysbiosis that directly parallel disease activity. Among SLE-related microbiota, the genera *Streptococcus*, *Campylobacter*, and *Veillonella* and species *S. anginosus* and *E. dispar* were found to be positively associated with lupus activity; in contrast, the genus *Bifidobacterium* correlated negatively with disease activity. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis showed that metabolic pathways are different not only between SLE patients and healthy controls but also between active and remissive SLE patients [66]. Fecal 16S rRNA analyses were performed in a cross-sectional discovery cohort, showing that patients with SLE had lower species richness diversity than did controls, with obvious reductions in taxonomic complexity in patients with a high SLE disease activity index. It was also found that SLE patients had an

overall 5-fold increase in the representation of *Ruminococcus gnavus* (Lachnospiraceae) and that intestinal expansions of *R. gnavus* were directly proportional to overall disease activity and most pronounced in patients with lupus nephritis [67]. SLE patients with active lupus disease possess an altered gut microbiota that differed in the genera *Odoribacter* and *Blautia* as well as an unnamed genus in the family Rikenellaceae and exhibit increased representation of gram-negative bacteria. Gut microbiota dysbiosis has also been reported in experimental murine lupus models, but differences between murine models and human subjects were found, including with regard to diversity. Nonetheless, the composition of the gut microbiota changed significantly from the predisease stage to the lupus disease stage in NZB/W F1 mice, with increased diversity and representation of some bacterial species. Using dexamethasone as an intervention to treat NZB/W F1 mice, an increased abundance of a group of lactobacilli in the gut microbiota might be correlated with enhanced disease severity [68].

Free fatty acids (FFAs) are upregulated in the serum of SLE patients, and the production of SCFAs plays an important role in the metabolism modulated by the gut microbiota. As an example, Rodriguez-Carrio et al. investigated potential links among total and specific serum FFA levels, fecal SCFA levels, and gut microbiota composition, and the findings revealed that altered gut microbiota composition in SLE patients is linked to altered SCFA production and increased FFA levels in serum [69]. Oxidative stress has been implicated in SLE, with the requirement of a balance in the gut microbiota and oxidant-antioxidant ratios. Gonzalez et al. compared levels of blood antioxidants and the gut microbiota with serum malondialdehyde (MDA) and C reactive protein (CRP) levels in 21 subjects with nonactive SLE; in this study, the serum copper content was positively associated with CRP levels, and CRP was also positively associated with the proportion of *Lentisphaerae*, Proteobacteria, and Verrucomicrobia in feces. In addition, MDA levels displayed negative associations with the proportions of *Cyanobacteria* and Firmicutes, though the proportion of *Actinobacteria* showed a positive correlation [70]. In general, the gut microbiome profiles of SLE patients are more influenced by disease than by ethnicity, and SLE patients from both China and Spain show depletion of Firmicutes and enrichment of *Bacteroidetes*. Additionally, numerous gut microbiota genera were identified as SLE-related microorganisms in Chinese subjects, with significant enrichment of the genera *Rhodococcus*, *Eggerthella*, *Klebsiella*, *Prevotella*, *Eubacterium*, *Flavonifractor*, and *Incertae sedis* and significant depletion of the genera *Dialister* and *Pseudobutyrivibrio* [71].

SLE is correlated with increased intestinal permeability that results in increased systemic microbial exposure. Reducing microbial exposure or improving barrier function may provide therapeutic approaches for SLE patients [72]. In fact, targeting the gut microbiota with drugs may be one of the mechanisms for treating SLE. For instance, treatment with a conjugate of tuftsin and phosphorylcholine (termed TPC) improved both immune and disease parameters of SLE, which was accompanied by a reduced abundance of Akkermansia and correlated with the clinical and serological parameters of lupus and an increased abundance of *Bifidobacterium*; Turicibacter; unclassified Mogibacteriaceae; unclassified *Clostridiaceae*; *Adlercreutzia*; *Allobaculum*; and Anaeroplasma [73]. Oral antibiotic administration during active SLE disease might reshape the gut microbiota, remove harmful Lachnospiraceae, increase the relative abundance of *Lactobacillus* spp., attenuate SLE-like disease in lupus-prone mice termed MRL/lpr mice, and ameliorate systemic autoimmunity and kidney histopathology when given after disease onset [47]. A study with in vitro cultures revealed that *Bifidobacterium bifidum* supplementation prevented $CD4^+$ lymphocyte overactivation, supporting a possible therapeutic method of probiotics containing Treg-inducer strains to restore the Treg/Th17/Th1 imbalance in SLE [74]. Because previous research demonstrated that the beneficial anti-inflammatory effects of *Lactobacillus* treatment were observed in female and castrated male mice but not in intact males, the gut microbiota may control lupus nephritis in a sex hormone-dependent manner [75].

4.3. The Gut Microbiota and Spondyloarthritis. Spondyloarthritis (SpA) comprises a group of several related diseases with distinct phenotypes, including ankylosing spondylitis (AS), reactive arthritis (ReA), psoriatic arthritis (PsA), arthritis related to inflammatory bowel disease (IBD), and a subgroup of juvenile idiopathic arthritis. Both environmental and genetic factors are responsible for the onset and development of SpA; however, the cause of SpA remains to be elucidated. The presence of HLA-B27 has a direct effect on SpA development [76]. Among nongenetic factors, it has been observed that the gut microbiota is distinct in early SpA subjects compared with that in controls, and an unbalanced gut microbiota possibly mediates activation of the inflammatory pathways observed in SpA. Previous studies have revealed that the proportions of certain gut bacterial families were increased in SpA patients, including Bacteroidaceae, Porphyromonadaceae, and Prevotellaceae [77]. Gill et al. demonstrated that the effects of HLA-B27 on the gut microbiota and dysbiosis in SpA are strongly associated with the host genetic background and/or environment, despite convergence of dysregulated immune pathways. Histologic analysis showed that both HLA-B27-transgenic Lewis rats and HLA-B27-transgenic Fischer rats developed gut inflammation but that DA rats were resistant to the effects of HLA-B27; HLA-B7-transgenic rats of the control group were not affected. Gut microbial changes in HLA-B27-transgenic rats were strikingly divergent among the 3 different host genetic backgrounds, including different patterns of dysbiosis in HLA-B27-transgenic Lewis and HLA-B27-transgenic Fischer rat strains, with some overlap. The resistance of DA rats to SpA might be due to the lack of SFB, which promotes $CD4^+$ Th17 cell development [78].

Colonization of particular intestinal bacteria was sufficient to induce a disparate phenotypical SpA disorder. The microbiota of AS patients was characterized by an increased abundance of Proteobacteria based on 16S rRNA gene- and ITS2-based DNA sequencing, possibly due to the enrichment of *Escherichia-Shigella*, *Veillonella*, and *Lachnospiraceae* NK4A136 group, and was also characterized by a reduction

in *Prevotella* strain 9, *Megamonas*, and *Fusobacterium*. Moreover, the gut microbiota of AS patients features increasing levels of Ascomycota, especially of the class Dothideomycetes, and a decreasing abundance of Basidiomycota, mainly owing to a reduction in Agaricales. Moreover, decreased ITS2/16S biodiversity ratios and altered bacterial-fungal interkingdom networks were observed in AS patients compared with those in healthy controls [79]. Additionally, significant increases in the abundance of *Erwinia* and *Pseudomonas* and an increased prevalence of typical enteropathogens associated with ReA were found in ReA patients. Subjects with ultrasound evidence of enthesitis display an enrichment of *Campylobacter*, but subjects with uveitis and radiographic sacroiliitis are enriched in *Erwinia* and *unclassified Ruminococcaceae*, with both having *Dialister* enrichment [80].

Gut inflammation is closely linked to chronic joint inflammation. DNA from several mucosal bacteria, such as *Prevotella* spp., can be found in other tissues in addition to the intestine of the host, including the spleen, liver, lung, serum, MLN, eyes, and ankle joints [81]. More than half of SpA patients experience microscopic gut inflammation, often resembling early Crohn's disease (CD) [82], and there is a significant difference in the intestinal microbial composition in SpA patients who have microscopic gut inflammation compared to those without microscopic signs. The abundance of the genus *Dialister* was found to be positively correlated with the AS disease activity score, and a reduced frequency of *Dialister* was further observed in noninflamed ileal and colonic biopsy tissues from SpA patients and healthy controls [83]. Breban et al. performed 16S ribosomal RNA gene sequencing on fecal DNA isolated from stool samples in two consecutive cross-sectional cohorts comprising three groups of SpA and RA patients and healthy controls. Compared with both RA patients and healthy controls, SpA patients exhibited a significant increase in the abundance of RG, which may act as a marker of disease activity in patients who have a history of IBD [84]. Viladomiu et al. also evaluated the fecal microbiome of IBD patients with or without peripheral SpA, revealing selective enrichment in IgA-coated *Escherichia coli* in those with Crohn's disease-associated SpA (CD-SpA) compared to that in those with CD alone. Colonization of germ-free mice with CD-SpA *E. coli* isolates induces Th17 mucosal immunity. Furthermore, when modeling the increase in mucosal and systemic Th17 immunity, colonization of IL-10-deficient or K/BxN mice with CD-SpA-derived *E. coli* led to more severe colitis or inflammatory arthritis, respectively [85].

The microbiota may be utilized as a biomarker in the future to predict clinical response in SpA. An exploratory study investigated an increased proportion of the Burkholderiales order in future responder patients with SpA before three months of anti-TNF-α treatment; modifications in microbiota composition were observed after treatment, but modification in a specific taxon was not found, regardless of the clinical response [86]. Microbiota-dependent intestinal inflammation drives the systemic inflammatory and osteoclastogenic potential of the monocyte compartment in the HLA-B27-transgenic (B27-Tg) rat model of SpA [87]. Treating HLA-B27/β2-macroglobulin- (β2m-) transgenic rats, which are a model of SpA, with the SCFA propionate for 10 weeks attenuated the development of HLA-B27-associated inflammatory disease, and this result may provide a novel therapy of microbial metabolites for HLA-B27-associated SpA [88].

4.4. The Gut Microbiota and IBDs. IBDs are subdivided into CD and ulcerative colitis (UC), which are chronic inflammatory diseases of the gastrointestinal tract resulting from an inappropriate immune response. Etiological factors of IBD may include personal genetic susceptibility, immune responses, the intestinal microbiota, and environmental stimuli. IBD is one of the most common diseases associated with gut microbiota dysbiosis, and colonization by a particular intestinal bacterium is sufficient to induce the onset of IBD as well as disease exacerbation. A recent study suggested that alteration of intestinal extracellular vesicle proteins might mediate the aberrant host-microbiota interactions in pediatric IBD [89]. IBD patients exhibit hallmarks of stool microbiome dysbiosis, with loss of a diversified core microbiome, depletion of specific beneficial bacteria, and/or enrichment of bacterial virulence factors compared with those in healthy controls [90]. Omori et al. analyzed fecal samples by 16S rRNA gene next-generation sequencing and revealed that the proportions of the family Paraprevotellaceae and the genus *Porphyromonas* were significantly increased in IBD dogs compared to those in healthy dogs [91]. IELs are T cells that most closely contact intestinal bacteria and may be influenced by the dissimilarity in microbiota constituents in distinct subtypes of IBD. A previous study indicated that IELs and IEL-produced cytokines correlated positively and negatively with the relative abundance of multiple bacterial taxa. Compared to those in healthy controls, IELs from subjects with UC secreted significantly greater amounts of IL-1β, and those from subjects with CD secreted significantly higher amounts of IL-17A, IFN-γ, and TNF-α [92]. In general, interaction between the host genome and the gut microbiota based on individual differences might influence health. Healthy individuals with a high genetic risk for IBD display a decrease in the genus *Roseburia*. Additionally, the site of disease is a major determinant of the gut microbiota, with patients with ileal CD exhibiting a decrease in α diversity compared to those with colonic CD [93].

Gut microbiota signatures have potential in IBD prognosis and diagnosis as well as in evaluating disease activity for IBD. Zhou et al. revealed by meta-analyses that gut microbial alteration patterns in IBD were similar among Chinese and Western populations. However, these authors found relatively increased levels of *Actinobacteria* and *Proteobacteria* (Enterobacteriaceae) and a relatively decreased level of *Firmicutes* (Clostridiales), which were strongly related to IBD severity [94]. Altomare et al. have suggested that the gut mucosal-associated microbiota (MM) better discriminates IBD differential patterns from those of healthy controls than does the FM. They found that FM profiles in IBD patients were more similar to those in healthy controls than were MM profiles. Some microbiota are apparently altered in the MM of IBD patients, showing a statistically significant

increase in Proteobacteria and a decrease in Firmicutes and Actinobacteria proportions compared to those of healthy controls [95]. *Clostridium difficile* infection (CDI), which is a common complication in IBD, may also cause poor IBD outcomes. Sokol et al. performed a study using 16S sequencing and analysis by the QIIME pipeline and found that IBD patients with CDI had more specific microbiota dysbiosis with higher levels of RG and Enterococcus OTUs and lower levels of *Blautia* and *Dorea* OTUs than did IBD patients without CDI [96]. Additionally, differences in the gut microbiota might distinguish patients with IBD from those with other intestinal-related diseases. By combining species-level profiles and strain-level profiles with bacterial growth rates, metabolic functions, antibiotic resistance, and virulence factor analyses, patients with irritable bowel syndrome (IBS) showed a distinct microbiota composition compared with that of IBD patients [97]. Changes in the microbiota and microbiota-stool bile acid also allow for good separation of patients with primary sclerosing cholangitis-associated inflammatory bowel disease (PSC-IBD) and those with IBD alone, and this situation might predispose patients to future colorectal neoplasia [98]. Both the activity and presence of the gut microbiota play an important role in IBD. Schirmer et al. detected organisms, such as *Dialister invisus*, that were metagenomically abundant but inactive or dormant in the gut with little or no expression. Certain disease-specific microbial characteristics were more pronounced or detectable at only the transcript level, such as pathways predominantly expressed by different organisms, in a host with IBD [99].

IBD patients exhibit lower diversity and richness of gut microbiota; thus, restoration of certain gut microbiota diversity is beneficial for treating and predicting clinical effectiveness in IBD patients. A significant increase in the relative abundance of Clostridiales was observed in response to infliximab treatment compared to that during relapse. Moreover, the relative abundance of Clostridiales was able to predict treatment effectiveness with 86.5% accuracy alone and 93.8% accuracy in combination with calprotectin levels and a CD activity index [94]. Kalenyak et al. evaluated the intestinal microbiota of dogs with IBD and showed that an unclassified genus of Neisseriaceae was abundant in the duodenum but that after treatment, *Bacteroides* reached significant abundance in the colon [100]. FMT is an emerging therapy and may be a potential treatment approach for IBD. Knoll et al. assessed the composition of the fecal microbiome by comparing pediatric CD and UC patients to their healthy siblings. Species richness and diversity were significantly reduced in UC children, with significant reductions in the abundance of *Eubacterium rectale* and *Faecalibacterium prausnitzii*. *Escherichia coli*, the abundance of which correlates positively with certain virulence genes, was enriched in UC children. Microbiota remodeling therapy from family donors will be a viable option for UC children [101]. Fasting-mimicking diet (FMD) cycles are also a potential therapy to ameliorate IBD, with a protective effect on the gut microbiota composition in DSS-induced mice [102].

By promoting clinical and endoscopic benefits, AhR expression may be a therapeutic target of interventions for IBD. AhR agonists are among the immunomodulators that can maintain immune tolerance. Lyer et al. identified that dietary- and microbial-derived oxazoles induced CD 1d-dependent intestinal inflammation via the action of AhR in the intestinal epithelium [103]. Caspase recruitment domain family member 9 (CARD9) is a susceptibility gene for IBD, and Card9 (-/-) mice are more susceptible than are wildtype mice to colitis. Intestinal inflammation of Card9 (-/-) mice was attenuated after inoculation with three *Lactobacillus* strains capable of metabolizing tryptophan or when treated with an AhR agonist [104]. Aoki et al. suggested that indole-3-pyruvic acid (IPA), which is a major precursor of microbiota-derived AhR agonists, potentially improves the severity of experimental chronic colitis in a mouse model by regulating the frequency of different T cells [105]. In dextran sodium sulfate- (DSS-) induced colitis, a deficiency in AhR in mouse intestinal epithelial cells exacerbates inflammation [106]. Marafini et al. showed the benefit of AhR-dependent regulatory effects in an experimental mouse model of colitis by testing the modulatory effect of the compounds NPD-0414-2 and NPD-0414-24, which are chemical ligands of AhR that can induce additional IL-22. Mice given NPD-0414-2 and NPD-0414-24 developed a significantly less severe form of TNBS colitis following decreased expression of IFN-γ and increased expression of IL-22. Moreover, the therapeutic effect of these two derivatives on ongoing colitis was abrogated in AhR-deficient mice [107]. Oh-Oka et al. found that mesalamine, a first-line drug for treating IBD, exhibited an anti-inflammatory effect by inducing Tregs in an AhR-dependent manner and an increased level of the active form of TGF-β [108].

It is worth noting that some types of drugs used to treat IBD may have distinct effects on symptoms and microbial ecosystems. Because patients with IBD are usually advised to take cobalamin, Zhu et al. investigated the influence of cyanocobalamin (CNCBL) or methylcobalamin (MECBL) ingestion on DSS-induced IBD mice by 16S rRNA analysis in fecal samples. These authors found that a high concentration of CNCBL but not MECBL supplementation markedly aggravated IBD, with an increasing proportion of *Escherichia/Shigella* and a decreasing abundance of *Lactobacillus*, *Blautia*, and *Clostridium XVIII* [109]. This finding may provide a novel reference for treating IBD patients based on the microbiota in the clinic.

5. Conclusion

In summary, increasing gut microbiota-associated approaches in healthy and disease states may contribute to the discovery of ways to prevent or repair perturbations in host autoimmune diseases. Therapies targeting the gut microbiota may be effective in the future prevention or treatment of autoimmune diseases. In general, a progressive understanding of the dynamic interplay between the gut microbiota and the host will help in establishing highly individualized management for autoimmune disease patients and achieve better efficacy in clinical outcomes or even in discovering new therapeutic targets for RA. Dietary interventions, FMT, and live biotherapeutics targeting the corresponding gut microbiota will possibly become therapeutic treatments for

autoimmune diseases. Metabolites derived from bacteria may also be used as potential therapies for both nonintestinal and intestinal autoimmune diseases, and correlations between the gut microbiota and its metabolic signatures may determine a predictive profile for autoimmune disease causation and progression.

Nonetheless, additional work is still needed, and whether changes in gut microbiota signatures are the cause or consequence of autoimmune diseases remains unclear. Furthermore, it is still uncertain which microbiota or metabolite alterations influence autoimmune diseases, and whether they can serve as biomarkers or therapeutic interventions requires further research. Although the current evidence supports that changes in the gut microbiota can affect the balance of Th17 cells and Tregs, which will influence pro-/anti-inflammatory cytokine levels [110], further work remains to be conducted to fully understand the mechanisms of the interaction between gut microbiota and the immune system. Moreover, different locations in the intestinal tract may differ in providing nutrient or physicochemical conditions for bacterial communities [111]. Thus, different microbial distributions along the gut may contribute to diverse changes in microbial communities in autoimmune diseases, but most studies have focused on fecal bacterial alterations. At present, the development of approaches to prevent autoimmune diseases by altering bacterial structure, including prebiotics, probiotics, and FMT, is still challenging. In addition, finding novel AhR agonists may be a logical next step to developing more effective probiotics to alleviate autoimmune diseases. Moreover, when using FMT therapies, it is crucial to explore whether there are influencing factors including but not limited to diet, sex, age, and geographical location. Administration of prebiotics or probiotics may have a beneficial effect on autoimmune diseases, but the mechanisms by which they interact with the host remain unresolved.

Conflicts of Interest

The authors declare that there is no conflict of interest.

Authors' Contributions

HX and ML contributed equally to this paper. HZ, DJ, and JL designed the paper and formulated the concept. HX, ML, JC, XL, DF, YX, and XL collected the literature. HX and ML drafted the paper. JC assisted in generating Table 1. HZ and DJ revised the paper and edited the language. All the authors have approved the final manuscript.

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Review Article

Gut Microbiota Modulation on Intestinal Mucosal Adaptive Immunity

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The mammalian intestine harbors a remarkable number of microbes and their components and metabolites, which are fundamental for the instigation and development of the host immune system. The intestinal innate and adaptive immunity coordinate and interact with the symbionts contributing to the intestinal homeostasis through establishment of a mutually beneficial relationship by tolerating to symbiotic microbiota and retaining the ability to exert proinflammatory response towards invasive pathogens. Imbalance between the intestinal immune system and commensal organisms disrupts the intestinal microbiological homeostasis, leading to microbiota dysbiosis, compromised integrity of the intestinal barrier, and proinflammatory immune responses towards symbionts. This, in turn, exacerbates the degree of the imbalance. Intestinal adaptive immunity plays a critical role in maintaining immune tolerance towards symbionts and the integrity of intestinal barrier, while the innate immune system regulates the adaptive immune responses to intestinal commensal bacteria. In this review, we will summarize recent findings on the effects and mechanisms of gut microbiota on intestinal adaptive immunity and the plasticity of several immune cells under diverse microenvironmental settings.

1. Introduction

The mammalian intestine harbors a vast microbiota, fundamental for the development and maintenance of the host immune system serving as an important epigenetic system [1]. A range of microbiota derivatives and metabolites can modulate host intestinal immune functions by influencing various cell types, including intestinal epithelial cells (IECs), mononuclear phagocytes, innate lymphoid cells (ILCs), and B and T lymphocytes [2]. The intestinal homeostasis requires a dexterously regulated network of the immune cells and their interplay with symbionts. The intestinal innate and adaptive immunities initiated by commensal microbiota coordinate protection of the host from invasion by foreign pathogens and intestine homeostasis [3, 4]. The intestinal adaptive immunity induced by intestinal resident microbiota, associated with differentiation of $CD4^+$ T cells and IgA-producing B cells in Peyer's patches (PPs) and lamina propria (LP), and intestinal epithelial lymphocytes (IELs) play a critical role in maintaining immune tolerance towards symbiotic bacteria, integrity of intestine barrier, and gut homeostasis [5]. A critical time window shortly after birth is regarded as vital for the development of gut-associated lymphoid tissue (GALT) in addition to differentiation and maturation of T cells and B cells. This phenomenon is of high consequence as the sequence and composition of colonized intestinal microbiota in infants influence the vaccine efficacy [6].

Microbiota dysbiosis increases host susceptibility to various immune, inflammatory, and allergy disorders of intestine and remote organs [7, 8]. It is shown that restoration of microbiota dysbiosis induced by antibiotics or other factors through administration of probiotics or fecal microbiota transplantation (FMT) of intestinal commensal bacterial species, such as *Escherichia coli* and *Lactobacillus johnsonii*, restores the suppressed adaptive immune functions in human and mice [9–11]. The microbiota and their derived

small-molecule metabolites regulate immune cells through direct and indirect effects at the cellular and molecular level. For example, short-chain fatty acids (SCFAs)—a gut microbiota metabolite—influence the fate of immune cells through direct epigenetic modification-induced alteration of metabolism via inhibition of histone deacetylase (HDAC) [12–14].

The fact that intestinal microbiota is a key orchestrator in cancer immunotherapy has recently gained acceptance [15, 16]. Intestinal dysbiosis contributes to primary resistance towards immune checkpoint inhibitors (ICIs) [17]. Studies have revealed that certain species exhibit evident effects on antitumor efficacy of ICIs via promoting dendritic cell (DC) and $CD8^+$ T cell functions and inhibiting regulatory T cells (Tregs) [18, 19]. For instance, melanoma does not respond to cytotoxic T lymphocyte antigen 4 (CTLA-4) blockade in antibiotic-treated or germ-free mice (GF), and this defect is rescued by administration of *Bacteroides fragilis*, *B. fragilis* polysaccharides, or *B. fragilis*-specific T cells [20].

The precise effect mechanisms underlying the effect of defined intestinal microbiota and metabolites on specific immune cell function still need to be elucidated. This review is aimed at highlighting the results of recent studies on the effects and mechanisms of intestinal microbiota and derived metabolites and signals on the development as well as differentiation of intestinal adaptive immune cells. This review also pays special emphasis on the induction of intestinal immune tolerance that is also associated with the intestinal innate immunity.

2. Composition of the Intestinal Adaptive Immunity

The intestinal mucosal immune system involves GALT that mainly includes aggregated lymphoid follicles, such as the PP, effector lymphocytes in LP, intraepithelial lymphocytes (IELs), and a series of molecules. The intestinal homeostasis requires a delicate balance between the effector T cells and the regulatory T cells, which mediate immune response to pathogens and confine excessive immune activation. Secretory immunoglobulin A (sIgA) that binds to commensal and invading microbes contributes to the homeostasis of intestinal mucosal immunity and barrier function [21]. In addition, intestinal innate immunity plays an important role in contributing immune tolerant features of adaptive immunity. Recent research has demonstrated that group 3 innate lymphoid cells (ILC3s) regulate interactions between T follicular helper (Tfh) cells and B cells, in a steady state, to limit mucosal IgA responses [22, 23]. Furthermore, and under physiological conditions, the IECs promote generation of T cell responses against the resident microbiota through endocytosis of antigens from commensal bacteria, such as proteins from segmented filamentous bacteria (SFB) [23].

3. Gut Microbiota and $CD4^+$ T Cell Differentiation

$CD4^+$ T cells are mainly located in the intestinal LP, most of which are effector or memory T cells. $CD4^+$ T cell responses vary greatly depending on the niche of colonization, antigen type, and metabolic property of gut microbiota, which results in the generation of distinct T cell subsets and the functional plasticity of certain T cell subsets [24]. Upon activation by microbiota, antigens are presented by antigen-presenting cells (APCs) such as dendritic cells (DCs), and $CD4^+$ T cells differentiate into Tregs and various T helper (Th1) cells such as IFN-γ, IL-4, B cell regulating, and IL-17 producing Th1, Th2, Tfh, and Th17 cells, respectively [25, 26]. Th17 cells and Treg cells are the extensively studied subsets of T cells constituting a large proportion of the effector cells. The imbalance between the cells of these two subsets results in various disorders ranging from inflammatory and autoimmune diseases to infection and cancer [27]. A recent study indicates that the transfer of inflammatory bowel disease (IBD) microbiota into GF mice increases the number of intestinal Th17 cells and Th2 cells and decreases retinoic acid-related orphan receptor γt (RORγt)$^+$ Treg cells as compared with transferred microbiota from healthy donors. Gut microbiota-derived SCFAs have been demonstrated to regulate T cell differentiation in a concentration and immunological milieu-dependent manner. For instance, Kespohl et al. have reported that although a lower butyrate concentration can facilitate Treg differentiation under steady-state conditions *in vitro* and *in vivo*, higher concentrations of butyrate induce the expression of the transcription factor T-bet in all the investigated T cell subsets resulting in the differentiation of IFN-γ-producing Tregs or conventional T cells [28]. In contrast, Chen et al. have shown that butyrate controls the T cell capacity in the induction of colitis through differential regulation of Th1 and Th17 cell differentiation and promotion of IL-10 production [14].

Proportions of Th17 and RORγt$^+$ Treg cells induced by different microbiota are predictive of human disease status and contribute to disease severity in the Rag1-/- colitis model suggesting a general mechanism that enlightens the contributions of microbiota in IBD pathogenesis [29]. Han et al. have demonstrated that in patients of acute graft-versus-host disease (aGVHD), the microbiota was depleted of Clostridia (e.g., the Lachnospiraceae and Ruminococcaceae families) and enriched for Gammaproteobacteria (e.g., the Enterobacteriaceae family) as compared with the non-aGVHD group. Moreover, the Treg/Th17 ratio positively correlates with the relative abundance of intestinal Lachnospiraceae and Ruminococcaceae. Furthermore, the Treg/Th17 ratio and Lachnospiraceae/Ruminococcaceae ratio are correlated with the level of acetylated H3 in $CD4^+$ T cells [30]. Though functions of Treg and Th17 are prominently diverse, they share several important features during differentiation and function [31, 32].

3.1. Effects of Gut Microbiota and Metabolites on Th17 Cell Plasticity. Th17 cells are critical in the defense against pathogens, especially in the case of extracellular bacterial and fungal infections. In response to SFB colonization, naïve $CD4^+$ T cells can migrate to LP of the small intestinal and differentiate into IL-17A producing Th17 cells. IL-17A, IL-17F, and IL-22 produced by Th17, in turn, stimulate IECs to produce antimicrobial peptides (AMPs) [33] and maintain integrity of the intestinal barrier in a noninflammatory manner [34]. In

addition, Th17 cells are crucial for the production of T cell-dependent (TD) high-affinity bacteria-specific IgA [35]. Excessive activation of Th17 cells can result in autoimmune diseases [36]. Microbiota, especially SFB, influences the differentiation of Th17 cells [37]. Transcription factor RORγt is essential in the differentiation of IL-17 producing CD4$^+$ Th17 and Th1/17 cells that coproduce IL-17 and IFN-γ. It has been demonstrated that T cell antigen receptors (TCR) specific for SFB-encoded peptides promote CD4$^+$ T cell differentiation into RORγt-expressing Th17 cells, even if the SFB-colonized mice also harbors in their intestine Listeria monocytogenes—a strong Th1 cell inducer [38]. Nevertheless, a recently published data shows that the SFB responding T cells comprise of a heterogeneous population including Th1, Th17, and Tfh cells proven by single cell RT-PCR analysis [39].

The dichotomous nature of Th17 cells also enables them to be pathogenic drivers, especially in IBD [40]. Intestinal Th1 and Th17 cells also play an indispensable role in microbiota-promoted colorectal cancer development. A study has revealed that proportions of Th1 and Th17 cells and cytokines, IL-17A, IL-22, and IL-23A, produced by Th17 cells are higher in conventional mice than in GF mice when fed with stool from patients with colorectal cancer [41]. Furthermore, the Th1/Th17 balance has turned out to be associated with the prognosis of patients with colorectal tumors. Th1 cell-mediated anticancer responses are associated with better outcomes, whereas Th17 cell-mediated responses are associated with worse outcomes implicating that specific members of the gut microbiota promote IL-17A production, thereby contributing to carcinogenesis [42].

The role of intestinal microbiota in modulating response efficacy to ICI therapy has drawn considerable attention in recent years. In patients with melanoma, relative abundance of the Ruminococcaceae family is increased in responders who receive anti-PD1 therapy, while the poor responders exhibit increased counts of RORγt$^+$ Th17 cells in tumors. FMT in GF mice has demonstrated that activation of Th17 cells and the polarization of IL-17A production in the tumor microenvironment are essentially mediated by components of the microbiota [43]. A study has shown that the number of pathogenic IL-17A$^+$ IFN-γ^+ and IL-22$^+$ IFN-γ^+ Th17 cell subsets is negatively regulated by intestinal HLA-DR-expressing-NKp44$^+$-ILC3s in IBD patients [44]. IL-6 has been shown to trigger Th17 cell differentiation via STAT3 activation, thus promoting inflammation in IBD patients, whereas leukemia inhibitory factor (LIF)—a cytokine of the IL-6 family—effectively inhibits Th17 accumulation and promotes damaged intestinal epithelium repair [45]. In addition, the role of IL-6 in Th17 lineage priming and differentiation is tissue specific [46] and involves reversible induction of Th1-to-Th17 cell transdifferentiation in the intestine [47]. Smad7 is an intracellular inhibitor of the transforming growth factor-beta (TGF-β) signaling, whose expression in colorectal cancer-infiltrating Th17 cells increases tumor necrosis factor-α (TNF-α) and interferon-γ (INF-γ) expression and decreases IL-17A expression that is responsible for effective killing of cancer cells [48]. Therefore, the plasticity of Th17 cells is dependent on the physiological or pathogenic cytokine milieu *in vivo* and interactions between different immune cells. However, precise driving factors for this shift remain less understood. Therefore, the development of therapeutic strategies targeting the IL-17 pathway needs to be carefully evaluated lest the potential side effects may hijack the beneficial functions of Th17 cells. The effects of SFB on Th17 cells in humans need further investigation, as it is not well known in human intestine.

3.2. Treg Cell Induction by Microbiota and Interactions with Other Immune Cells. Within the intestine, forkhead box P3 transcription factor- (Foxp3-) expressing Treg cells are primarily located in the LP [49] and have a fundamental role in immunological tolerance towards commensal microbiota. The Foxp3+ Treg repertoire is heavily influenced by the microbiota composition [50]. Upon migration to the epithelium, Tregs lose their Foxp3 expression and convert to effective CD4$^+$ T cell in a microbiota-dependent manner [49].

Gut-derived Foxp3$^+$ Treg cells are distinct from those in other organs and have gut-specific phenotypes and functions. Symbiotic bacteria, for instance, *Clostridium* species clusters [51, 52] and *Bacteroides fragilis* and its polysaccharide A [53], can facilitate the expansion and differentiation of intestinal Foxp3$^+$-Tregs in addition to the production of IL-10 and TGF-β that regulate the functions of intestinal myeloid cells [54]. Some probiotic strains, for instance, *Lactobacillus acidophilus*, improve intestinal inflammation by modulating the balance of Th17 and Treg cells [55, 56]. SCFAs, which are an energy source for gut epithelial cells, serve an anti-inflammatory function by inhibiting HDACs in Tregs through G-protein-coupled receptors (GPRs). SCFAs can also promote naïve T cell differentiation into both effector T cells and Treg cells depending on the cytokine milieu, which is dependent on direct suppression of HDACs, independent of GPR41 or GPR43. Plasticity features of Foxp3$^+$-Treg cells facilitate their acquisition of an effector T cell phenotype in highly virulent or inflammatory contexts, and their fates are controlled by pathogenic effector T cells [57].

RORγt$^+$ Tregs are a distinct Treg population in the colon. The expression of transcription factor RORγt is induced by gut microbiota. The proportion of RORγt$^+$ Tregs is much lower in GF mice than in their conventionally raised specific-pathogen-free (SPF) counterparts [58]. The context specificity of RORγ results in significantly different outcomes, even in closely related cell types, which are consistent with their involvement in a range of immunological and non-immunological processes. A recent study has demonstrated that weaning immune reaction to microbiota is associated with the generation of RORγt$^+$-Treg cells that decreases the susceptibility to allergic inflammation, colitis, and cancer later in life [59]. Moreover, in colorectal cancer (CAC) patients with IBD background, RORγt-expressing tumor-infiltrating Treg cells sustain tumor growth in a transcription factor FoxO3-dependent manner [60]. However, the frequency of RORγt$^+$ Tregs correlates with the colitis score when momocolonized with different microbes in trinitrobenzenesulfonic acid- (TNBS-) challenged GF mice [58]. Foxp3$^+$RORγt$^+$ T cells are an important subset of effector Treg cells of the intestinal immune system that displays

features of both Tregs and Th17 cells. They accumulate in the LP of IBD patients that have enhanced immunosuppressive capacity as compared with Foxp3$^+$RORγt$^-$ Tregs during gut-specific immune responses [61]. However, a contradictory finding indicates that inactivation of RORγt in Treg cells has a minor effect on the balance of bacteria-specific Treg and Th17 cells and does not lead to inflammation. In addition, the expression of the transcription factor c-Maf is required for the terminal differentiation and function of RORγt$^+$ Treg cells in inhibiting intestinal Th17 responses [62].

The close relationship between Th17 and Treg cells marks them important in modulating the immune responses and maintaining immune homeostasis. Molecular mediators that induce RORγt$^+$ Treg development remain obscure. It is likely that SCFA is one of the mediators, and RORγt$^+$ induction in Th17 and colonic Tregs may follow a different pathway [58]. Currently, availability of small-molecule inhibitors, such as TAK-828F, or activators of metabolic enzymes has made it possible to manipulate the metabolism of T cells and shift the Th17/Treg cell balance, providing a novel therapeutic option for the treatment of inflammatory and immune diseases [27, 63].

3.3. Regulation of Intestinal T Follicular Helper (Tfh) Cells by Microbiota. Tfh has been identified as the chief cell subpopulation regulating B cells in germinal centers (GCs) that promotes high-affinity antibody production. Tfh development is deficient in GF mice and can be restored when fed by toll-like receptor-2 (TLR2) agonists via activating intrinsic MyD88 signaling [64]. Ablation of Tfh cells results in reduced amount of PPs, IgG1, and GC B cells. And significantly changes the gut microbiome composition [26]. Thus, Tfh cell activity is important for the generation of a diverse microbiota community in the gut. IL-21 produced by Tfh cells in PPs is essential in driving the GC reaction and high-affinity sIgA production in the small intestine [26]. IL-21R-deficient mice exhibit a significant decrease in IgA$^+$ plasmablasts and plasma cells, in response to SFB in the small intestine [65]. SFB can also drive differentiation of PP Tfh cells and egress into systemic sites, boosting systemic Tfh cell responses and autoantibody production that exacerbates arthritis [66]. Microbiota-derived extracellular ATP (eATP) limits Tfh cell expansion and GC reaction in the PPs via P2X7, regulates Tfh cell abundance, and affects high-affinity sIgA response against intestinal colonizing bacteria that leads to enteropathogenic infection [67, 68]. Thus, microbiota-derived eATP is an important signaling molecule, which can be a further modulation target of the intestinal immunity against intestinal bacteria. Several transcription factors are involved in the divergent functions of Tfh. Activating transcription factor 3 (ATF3) can protect against colitis by regulating Tfhs in the gut [69]. Interferon regulatory factor 8-(IRF8-) regulated Tfhs can function as pathogenic mediators of colitis in IBD, which is independent of B cells [70]. Transcription factor c-Maf is expressed early in Tfh cell precursors and identified as a regulator in the differentiation of Tfh cells in a cell-autonomous fashion [71].

Antibodies produced in the GC need precisely targeting of foreign pathogens while limiting excessive inflammation and autoimmunity for maintaining normal intestinal circumstances. T follicular regulatory (Tfr) cells, a recently identified cell subset, can migrate to the GC and inhibit Tfh-mediated B cell activation and Ig production. Deep understanding of Tfr cell function has potential to provide new insights into developing more effective vaccine strategies and new methods to treat antibody-mediated diseases [72].

4. Induction of Antivirus and Anticancer Immunity of Intestinal CD8$^+$ T Cells by Gut Microbiota and Metabolites

The effects of intestinal microbiota and metabolites on intestinal CD8$^+$ T cells functions are relatively uncharacterized. Previous studies have shown that recolonization of VSL#3 and *L. johnsonii* results in restoration of CD4$^+$ and CD8$^+$ T cell population in the small and large intestinal LP and mesenteric lymph nodes (MLNs) or partial restoration upon FMT after broad-spectrum antibiotic therapy [8, 10]. SCFAs, especially butyrate, directly modulate IFN-γ and granzyme B gene expression of CD8$^+$ CTLs and IL-17-producing CD8$^+$ T cells (Tc17 cells) in MLNs, which is mediated by inhibition of HDACs, independent of GPR41 and GPR43. Moreover, this influence is similar to the effects exerted by the pan-HDAC inhibitors trichostatin A (TSA) and sodium valproate [73]. It is shown that a consortium of 11 bacterial strains obtained from healthy human donor feces is capable of robustly inducing IFN-γ-producing CD8$^+$ T cells in the intestine. Their colonization enhances both host resistance to *Listeria monocytogenes* infection and the therapeutic efficacy of ICIs in syngeneic tumor models [74].

IELs that distribute along the intestinal epithelium are a large and diverse population of lymphoid cells, which are classified into TCR$^+$ and TCR$^-$ subsets. The former is similar to conventional T cells, whereas the latter functions resembling ILCs. Natural TCR$^+$ IELs include TCR$\alpha\beta^+$ or TCR$\gamma\delta^+$ T cells. In the small intestine of mice, 50%–60% of the IELs are TCR$\gamma\delta^+$ cells, whereas in humans, CD8$\alpha\beta^+$ IELs are the predominant population. There exists an intricate interaction between IEL subsets and IECs in the intestine, and other immune cells outside IECs [75]. Colonization of commensal microbiota in early life is important for the development of diet-induced CD8$\alpha\beta^+$ IELs [76]. Intestinal microbiota, such as *Bacteroidales*, recruits IL-6-producing IELs promoting intestinal epithelial proliferation, contributing to barrier integrity [77]. In antibiotic-treated and GF mice, the proportion and absolute number of CD8$\alpha\beta^+$ IELs drop significantly. Microarray analysis has revealed that CD8$\alpha\beta^+$ IELs expressed a series of genes encoding potent AMPs, which is supported by an antimicrobial-activity assay [78]. $\gamma\delta^+$ IELs can promptly migrate to and remain localized near IEC lumen in direct contact with bacteria in the case of pathogen invasion within hours, suggesting their essential role in early host defense against pathogen invasion [79]. Their activation is dependent on microbe-activated secretion of intrinsic MyD88 by IECs [80]. Assay for Transposase-Accessible Chromatin with high-throughput sequencing (ATAC-seq) of intestinal $\alpha\beta^+$ and $\gamma\delta^+$ IELs has disclosed

that their enhancer-associated transcription factors, signaling networks, and metabolic pathways are affected by the microbiota [12].

In mice, the small intestine contains $CD4^+CD8\alpha\alpha^+$ double-positive IELs (DP IELs), which have regulatory functions originated from intestinal $CD4^+$ T cells through downstream of the transcriptional factor Thpok. Uncommon in human adults, the differentiation of DP IELs depends on microbial colonization because they are not present in GF mice [81]. *Lactobacillus reuteri* may reprogram intraepithelial $CD4^+$ T cells into DP IELs in both GF mice and conventionally raised counterparts lacking these cells. Though not shaping the DP-IEL-TCR repertoire, it generates indole derivatives of tryptophan activating the aryl-hydrocarbon receptor that downregulates Thpok and promoting $CD4^+$ T cell differentiation into DP IELs. Thus, *L. reuteri*, together with a tryptophan-rich diet, results in the reprograming of IELs into cells of regulatory phenotype [82].

5. Induction of sIgA Production by Gut Microbiota and Metabolites

In the gut lumen, sIgA serves as a first-line barrier protecting the epithelium from invasion of pathogens and toxins by promoting mutualistic microbe colonization and neutralization of invasive pathogens. SIgA coats bacteria and other targets, thus preventing them from direct contact with the host intestinal immune system. A high level of sIgA production results from the interactions of intestinal Tfhs and B cells in the GCs. Due to sustained microbial antigen exposure, intestinal lymphoid tissues generate flora-reactive IgA-producing B cells [83]. Maturation of isolated lymphoid follicles (ILFs) into large B cell clusters requires the detection of bacteria by pattern-recognition receptors (PRRs) [84]. Peptidoglycan from gram-negative bacteria is necessary for the induction of ILFs in mice through recognition by the nucleotide oligomerization domain 1 (NOD1) receptor in epithelial cells and other signaling through the chemokine receptor CCR6 [83].

Intestinal microbiota influences not only the accumulation of sIgA-producing plasma cells but also the diversity of IgA in intestine lymphocyte tissue which has been demonstrated in gnotobiotic mice colonized with defined microbial consortia [85]. IgA directly participate in shaping the microbial community landscape. During infection, sIgA-coated pathogens are cleared via a process called immune exclusion [86, 87]. The diversity of IgA on the mammalian intestinal surface is paralleled with the intestinal taxa diversity [88]. PPs are essential sites for the TD IgA generation, contributing not only to the generation of somatically mutated gut antigen-specific IgAs production but also to the diversification of nonspecific antigen of the B cell repertoire [89, 90]. Moreover, PP can be substituted as the inductive sites for IgA production with the stimulation of specific immunostimulatory or pathogenic bacteria, such as SFB [84]. B cell development in GALT is also driven by super-antigen-like molecules, such as spores of *Bacillus subtilis* and other species of *Bacillus* [91]. SCFAs can promote IgA production through regulating the metabolism and gene expression in B cells independent of T cells [92, 93].

SIgA in response to the commensal microbiota is produced through a T cell-independent (TI) mechanism which has been confirmed in TCR$\alpha\beta$ and TCR$\gamma\delta$ T cell-deficient mice. These processes may not require PP-organized B cell follicles, which are specific for particular bacterial species. The intestinal TI-IgAs are highly stable antibody repertoires that perform a dual role in the protection against invading pathogens and the regulation of the composition of nonpathogenic microbial communities [94, 95].

5.1. Interactions of sIgA with Other Immune Cells and Tolerance Induction. Plasmacytoid DCs (pDCs) are essential for the induction of plasma cells in response to oral immunization and noninfectious antibody responses with the production of TD or TI-IgA in the intestine [96, 97]. PP IgA production, affinity maturation, and class switch recombination require PP subepithelial dome DC–B cell interactions, which are dependent on the secretion of TGF-β by ILCs [89]. The bacteria-loaded DCs induce the differentiation of B cells into IgA^+ plasma cells from lymphoid structures to LP, dimeric IgA secretion, and restrict the mucosal immunity locally [98]. In turn, sIgA-coated commensal bacteria contribute mucosal DCs towards tolerogenic profiles [99] and Treg cell expansion and IL-10 production, which dampen mucosal and systemic autoimmunity [100].

Human gut bacterial taxa targeted by IgA in the setting of barrier dysfunction result in intestinal pathology that may be attributed to the adhesion of these taxa to intestinal microfold (M) cells, a subset of IECs residing in the region of the epithelium covering PPs. For instance, the sIgA-coated *Clostridium difficile* [101] and adherent-invasive *E. coli* [102], which are located in proximity to the intestinal epithelium, elicit a Th17-cell-dependent sIgA-mediated response. The precise link of sIgA diversification and specific antigenic functions in modulating the microbiota composition, location, and metabolism are yet to be elucidated [88]. A novel technology of 16S ribosomal RNA (rRNA) gene sequencing of immunoglobulin A- (IgA-) coated bacteria (IgA-SEQ) may be helpful in the isolation and identification of unique colitogenic intestinal bacteria [103].

In brief, commensal microbiota trains and regulates the development and maturation of the intestinal adaptive immune system. Under a steady state, the mucosal adaptive immune cells, especially Tregs and Tfh, and sIgA coordinate with IECs, DCs, and ILCs contributing to a tolerogenic state of intestinal ecological environment, maintaining intestinal homeostasis and mutualistic relationship between the host and the microbiota, thus preserving the ability of eliciting an efficient immune response to infectious agents. In the case of dysbiosis, the equilibrium is disturbed that leads to chronic inflammatory and autoimmune pathology. Mechanisms of intestinal microbiota modulation of intestinal adaptive immunity are depicted in Figure 1.

6. Conclusions and Perspectives

Under a steady state, commensal microbiota and intestinal adaptive immune cells and sIgA coordinate to contribute towards tolerance for symbiotic bacteria and mutualistic

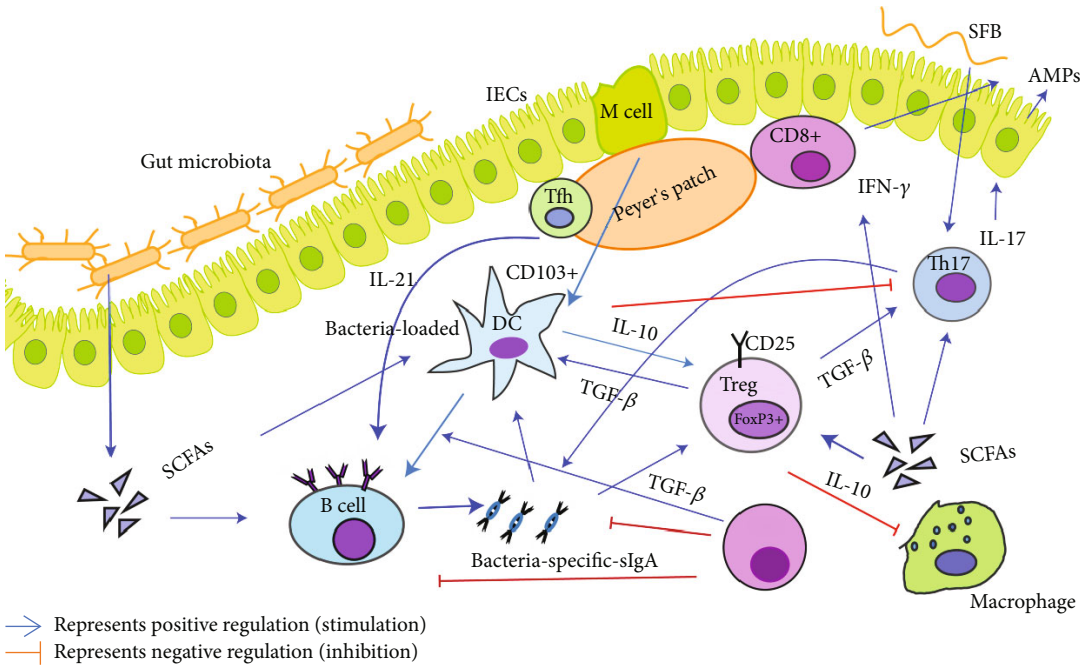

Figure 1: The intestinal microbiota modulate the intestinal mucosal adaptive immunity and interactions between immune cells. The intestinal microbiota, their components, and metabolites contribute to the regulation of innate and adaptive immune responses during homeostasis and dysbiosis states. Under homeostatic conditions, microbiota and microbial metabolites contribute to the intestinal immunological tolerance *via* Tregs and sIgA. Tolerogenic DCs contribute to the induction of Tregs and sIgA production by IL-10 secretion. Bacterial components such as SCFAs are potent inducers of Tregs and B cells and promote IFN-γ production from $CD8^+$ T cells. Microbiota, SCFAs, and IL-21 secreted from the Tfh in the PP contribute to the secretion of bacteria-specific sIgA. Segmented filamentous bacteria induce SFB-specific Th17 cell production. Tregs modulate DCs and Th17 cells mediated by TGF-β. DCs and sIgA negatively regulate the function of Th17 cells.

relationship between the host and the microbiota. This ultimately results in maintenance of intestinal homeostasis and the retention of the ability to elicit an efficient immune response to invading pathogenic agents. When this equilibrium relationship is disturbed, dysbiosis and intestinal immunological abnormalities develop leading to chronic local and systemic inflammatory and autoimmune disorders. Identifying effector microorganisms, derived molecules, and metabolites that causally affect an immune phenotype holds the key for deciphering the underlying mechanisms and the development of microbiota-based therapeutics [104–106]. The present-day widely applied molecular sequencing-based methods do not allow for the isolation of specific organisms, hindering the exploration of the association of their functional roles with host-specific immune parameters and definite disease phenotype. In the near future, database-centered predictions of strain-media, IgA-SEQ, engineered bacteria, high-throughput culturing, and microfluidic assays [106–108] may help to isolate uncultivated species of interest [109, 110]. These technical advances may thus provide utmost probabilities for exploring the mechanisms of microbiota-host interactions improving diagnostic and therapeutic applications.

Conflicts of Interest

The authors declare that they have no competing interests.

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Research Article

MHC Class I Molecules Exacerbate Viral Infection by Disrupting Type I Interferon Signaling

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MHC class I molecules are key in the presentation of antigen and initiation of adaptive CD8+ T cell responses. In addition to its classical activity, MHC I may possess nonclassical functions. We have previously identified a regulatory role of MHC I in TLR signaling and antibacterial immunity. However, its role in innate antiviral immunity remains unknown. In this study, we found a reduced viral load in MHC I-deficient macrophages that was independent of type I IFN production. Mechanically, MHC I mediated viral suppression by inhibiting the type I IFN signaling pathway, which depends on SHP2. Cross-linking MHC I at the membrane increased SHP2 activation and further suppressed STAT1 phosphorylation. Therefore, our data revealed an inhibitory role of MHC I in type I IFN response to viral infection and expanded our understanding of MHC I and antigen presentation.

1. Introduction

The innate immune system is the first line defense for viral infection. After recognition of certain pathogen-associated molecular patterns (PAMPs), diverse pattern recognition receptors (PRRs) trigger antiviral immune responses by inducing type I interferon (IFN) [1]. For RNA viruses, RIG-I and MDA5 are the main PRRs responsible for IFN production [2]. Type I IFN exerts its antiviral function by binding to its receptors and activating JAK-STAT signaling, which finally induces the expression of IFN-stimulated genes (ISGs) [3]. Both the production and downstream signaling of type I IFN are necessary for host innate antiviral immunity. Targeting type I IFN is the major mechanism employed by viruses to evade the host immune defense, and viruses have developed diverse strategies to circumvent the type I IFN system [4]. Although many regulators have been identified [5, 6], the details of fine-tuned IFN production and function remain unknown.

Major histocompatibility complex (MHC) class I molecules are among the primary two MHC molecules and are found on all nucleated cells. Their classical function is to display peptide fragments of endogenous antigens and present them to cytotoxic CD8 T cells [7, 8]. In vivo, MHC I is key for the selection of thymic CD8 T cells and is also involved in the education and tolerance of natural killer cells [9]. MHC I molecules are heterodimers composed of a heavy chain and a β2 microglobulin, and β_2m is essential for the stable expression of MHC I on a cell membrane. The heavy chain is composed of two extracellular Ig-like domains and an intracellular domain. In contrast to MHC class II molecules, MHC I molecules have a longer intracellular tail with approximately 40 amino acids, including a tyrosine site [10]. As tyrosine phosphorylation is a key posttranscriptional modification involved in signal transduction cascades [11], MHC I molecules are expected to have nonclassical functions in signal transduction.

Although MHC I molecules always function as ligands, reverse signaling was demonstrated two decades ago and plays important roles in cell apoptosis, activation, or function. Cross-linking MHC I on T cells triggers Lck, Zap70, and PLCγ1 activation, which leads to T cell activation [12, 13] or apoptosis [14]. Cross-linking MHC I on NK cells segregates MHC I from NK cell synapse, induces

intracellular phosphotyrosines, and inhibits NK cell function [15]. MHC I can also initiate intracellular signals in endothelial cells and smooth muscle cells, eliciting cell proliferation in synergy with growth factors [16]. In malignant tumor, anti-MHC I or anti-β_2m antibodies can specifically induce tumor cell apoptosis [17, 18]. We have previously demonstrated an inhibitory role of MHC I in TLR signaling in myeloid cells, which facilitated bacterial infection [19]. However, the role of MHC I on viral infection remains unknown.

Here, we reported that the lack of MHC I significantly suppressed viral replication in macrophages independent of IFN production, which depended on the disrupted IFN signaling pathway. Mechanically, after viral infection, MHC I enhanced SHP2 activation, which suppressed STAT1 phosphorylation and led to reduced ISG production. Our data revealed an inhibitory role of MHC I in type I IFN signaling during viral infection and expanded our understanding of MHC I function and antigen presentation.

2. Materials and Methods

2.1. Mice. C57BL/6 mice were from Joint Ventures Sipper BK Experimental Animals (Shanghai). Mice deficient in H-2Kb and H-2Db were from Taconic Farms and termed as MHC I-deficient mice. All animals were bred in specific pathogen-free conditions, and all animal experiments were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of the Second Military Medical University, Shanghai, China.

2.2. Cell Culture. Bone marrow-derived macrophages (BMDMs) were generated as previously described. Briefly, bone marrow cells were isolated from the femur and tibia and cultured in 10% RPMI1640 with 20% L929 cell-conditioned medium as a source of GM-CSF. Three to four days after seeding, the supernatants were removed and attached cells were further cultured with conditional medium for additional 3-5 days. The remaining cells were collected and used as macrophages.

2.3. Viral Infection and Viral Quantification. VSV virus was amplified in 293T cells, and mice were infected at 10^8 PFU per gram of body weight intraperitoneally. Cultured supernatants or tissue homogenates were serially diluted and added into monolayer BHK21 cells. At the end point, the amount of virus required to kill 50% of infected cells was determined as 50% Tissue Culture Infective Dose ($TCID_{50}$).

2.4. Bronchoalveolar Lavage Fluid (BALF) Collection. For BALF, the trachea was cannulated and lavaged with 1 ml PBS for two times. Collected withdrawing samples were centrifuged at 3,000 rpm for 5 min, and the cell pellets were resuspended in PBS and counted as infiltrating lymphocytes.

2.5. Histopathology. The lung tissues were fixed in 10% formalin, embedded in paraffin, cut, and stained with hematoxylin and eosin. The histopathologic inflammation score of lung tissues was evaluated by a pathologist blinded to the experimental design. Lung inflammatory changes were graded using a semiquantitative scoring system based on the following parameters: peribronchiolar and bronchial infiltrates, bronchiolar and bronchial luminal exudates, perivascular infiltrates, parenchymal pneumonia, and edema, as previously described [20]. Each parameter was graded on a scale of 0–4: 0, absent; 1, slight; 2, mild; 3, moderate; and 4, severe.

2.6. Real-Time PCR. RNA were extracted with an RNA purification kit (Fastagen, Shanghai) and reverse transcribed with a PrimeScript RT-PCR kit (Takara, Japan). The primers for Ifnb, Ifn4a Isg15, Isg54, Oas, and Mx1 were from the PrimerStar website. The following are the primers of VSV: forward 5′-ACG GCG TAC TTC CAG ATG G-3′ and reverse 5′-CTC GGT TCA AGA TCC AGG T-3′. The mRNA expression was done with an SYBR Premix Ex Taq qPCR kit (Takara) by LightCycler (Roche) and analyzed with the $\Delta\Delta$T method. Data were normalized with β-actin expression.

2.7. ELISA Assay. Cytokines in the supernatant of cell culture were collected and diluted as needed and analyzed using a mouse IFN-β ELISA kit (PBL Biomedical Laboratories) according to the manufacturer's instructions.

2.8. Flow Cytometry and Intracellular Staining. For intracellular cytokine staining, macrophages were stimulated *in vitro* with VSV for 8 hours, and protein transport inhibitor brefeldin A was added during the last 4 hours. Cells were collected and fixed with Fixation & Permeabilization Buffer (BioLegend). Then, cells were stained with intracellular IFN-β with anti-mouse IFN-β mAb-biotin (BioLegend), followed by secondary streptavidin-PE staining. Flow cytometry analyses were performed using FACSVantage (Becton Dickinson). Data were analyzed by FACSDiva.

2.9. Immunoprecipitation and Immunoblot. Cells were lysed with cell lysis buffer (CST, USA), supplemented with protease inhibitor cocktail (Calbiochem). Protein concentration was determined with BCA assay (Pierce), and equivalent proteins were loaded for western blotting or immunoprecipitation. Immunoblot was performed with anti-STAT1 (9172, CST), anti-p-SHP2 (3703, CST), anti-p-STAT1 (D4A7, CST), anti-p-JAK1 (3331, CST), and anti-p-Tyr (9416, CST) antibodies. And anti-H2Kb (MHC I, AF6-88.5) was from BioLegend.

2.10. Gene Overexpression and Silencing. MHC I molecule H-2Kb was transfected with JetPEI reagents (PolyPlus, France), and 24 hours later, overexpression was confirmed by western blot. The siRNA targeting Shp2 was from Dharmacon and transfected with an INTERFERin reagent (PolyPlus) according to a standard protocol. The silencing efficiency was confirmed with western blot analysis.

2.11. Statistical Analysis. The statistical significance between two groups was determined by Student's *t*-test. For the comparison of more than 2 groups, one-way ANOVA was

adopted, and Fisher's exact LSD test was used for the intergroup comparison. For two independent variables, two-way ANOVA was adopted for statistical analysis, and Tukey's multiple comparison method was used for the intergroup comparison. Probability values less than 0.05 were considered to be statistically significant.

3. Results

3.1. MHC I Promotes Viral Replication Independent of Suppressing Type I IFN Production. Our previous data revealed that MHC I molecules not only are key to adaptive CD8 T cell responses but are also involved in the fine tune of innate inflammatory cytokine production and antibacterial infection [19]. To examine whether MHC I is involved in innate antiviral immune responses, we first infected macrophages from MHC I-deficient mice and littermate control mice with VSV. Deficiency of MHC I caused significant decreased replication of VSV RNA in macrophages (Figure 1(a)). The VSV $TCID_{50}$ in the supernatants also confirmed a reduced VSV load in MHC I-deficient macrophages (Figure 1(b)). In addition, a VSV-GFP infection model was used to directly determine the viral load in infected cells. A fluorescence plot also confirmed a lower virus load in MHC I-deficient macrophages (Figure 1(c)). To quantify the data, the macrophages were further collected for flow cytometry analysis (Figure 1(d)). Both the percentage and the mean fluorescence intensity (MFI) of GFP-positive cells were decreased in MHC $I^{-/-}$ macrophages (Figure 1(e)). To further investigate the role of MHC I in viral infection, we overexpressed MHC I in macrophages. As expected, overexpression of MHC I promoted VSV replication (Figure 1(f)). These data demonstrate a promoting function of MHC I in viral infection.

Type I IFNs are the key antiviral innate cytokines. More type I IFN production would lead to reduced viral load in infected cells. To gain insight into the mechanism by which MHC I deficiency ameliorated viral load, type I IFN production was determined. Instead of upregulating these innate antiviral cytokines, MHC I deficiency reduced IFN-α and IFN-β mRNA levels in macrophages, (Figure 1(g)), which was confirmed by ELISA assay (Figure 1(h)). The cytokines in the supernatant by ELISA assay reflect the effect of cytokine secretion minus conception. To exclude reduced type I IFNs caused by more conception, we detected the IFN-β production by intracellular staining (Figure 1(i)). The flow cytometry data also revealed reduced intracellular IFN-β production in MHC $I^{-/-}$ macrophages. These data indicated that decreased viral load in MHC I-deficient macrophages cannot be attributed to the upregulation of type I IFN production. In contrast, decreased viral load may be the reason for the reduced type I IFN production.

3.2. MHC I Inhibited Type I IFN Signaling and ISG Induction. As type I IFNs bind receptors to exert its antiviral effect, we next examined whether MHC I deficiency influenced type I IFN downstream signaling. MHC I deficiency caused increased STAT1 phosphorylation in macrophages, without influencing STAT1 expression (Figures 2(a) and 2(b)). The antiviral effect of type I IFN mainly depends on ISG expression. We also found elevated ISG15, ISG54, OAS, and Mx1 expressions in MHC I-deficient macrophages (Figure 2(c)). These data strongly indicated that MHC I deficiency could promote type I IFN signaling and its antiviral activity.

To elucidate the role of MHC I in IFN signaling, we directly stimulated macrophages with IFN-β in vitro. However, we found no significant difference of STAT1 activation in MHC I-deficient macrophages compared with that in WT macrophages (Figures 2(d) and 2(e)). As MHC I intracellular tyrosine phosphorylation was necessary for its function in TLR signaling [19], we speculated that IFN stimulation alone may not induce MHC I phosphorylation. Figures 2(f) and 2(g) confirm that VSV infection induced significant phosphorylation of MHC I, while IFN stimulation did not have a similar effect. These data indicated a regulatory effect of MHC I in type I IFN signaling, which is dependent on its tyrosine phosphorylation.

3.3. MHC I Suppressed IFN Signaling through SHP2 Activation. After binding to the IFN receptor, the JAK-STAT pathway was activated and finally led to ISG production [21]. Though p-STAT1 was upregulated in MHC I-deficient macrophages, the activation of JAK1 was not significantly altered in the MHC I-deficient macrophages compared with that in WT cells (Figures 3(a) and 3(b)). These data suggested that MHC I may target STAT1 to regulate the type I IFN signaling pathway.

We previously revealed that during TLR stimulation phosphorylated MHC I sustained SHP2 activation. As MHC I was also phosphorylated post VSV infection, we wondered whether SHP2 was also involved in the regulation of IFN signaling during viral infection. We found obvious SHP2 activation at the indicated times post viral infection, which was suppressed in MHC I-deficient macrophages (Figures 3(a) and 3(b)). Knocking down SHP2 expression reduced the viral load in both the WT and MHC I-deficient macrophages (Figure 3(c)). In addition, SHP2 knockdown abrogated the reduction of viral load in MHC I-deficient macrophages compared with that in WT macrophages (Figure 3(c)). Silencing Shp2 also abrogated the difference in STAT1 activation between WT and MHC I-deficient cells (Figures 3(d) and 3(e)). In addition, SHP2 was found to interact with STAT1 after VSV infection (Figures 3(f) and 3(g)). These data strongly suggested that SHP2 is necessary for MHC I-mediated suppression of IFN signaling.

3.4. The Biological Relevance of MHC I Regulation of Type I IFN Signaling. We next wanted to reveal the biological and pathologic relevance of MHC I-mediated IFN signaling suppression. The main function of MHC I is presenting an antigen to TCRs to form immune synapse. In the immune synapse, pMHC-TCR aggregated into clusters and thus amplified the signaling. To mimic this cluster formation, we cross-linked MHC I molecules with anti-MHC I antibodies in vitro. The cross-linking increased SHP2 phosphorylation, inhibited STAT1 activation after VSV infection (Figures 4(a) and 4(b)) and exacerbated viral replication in macrophages (Figure 4(c)).

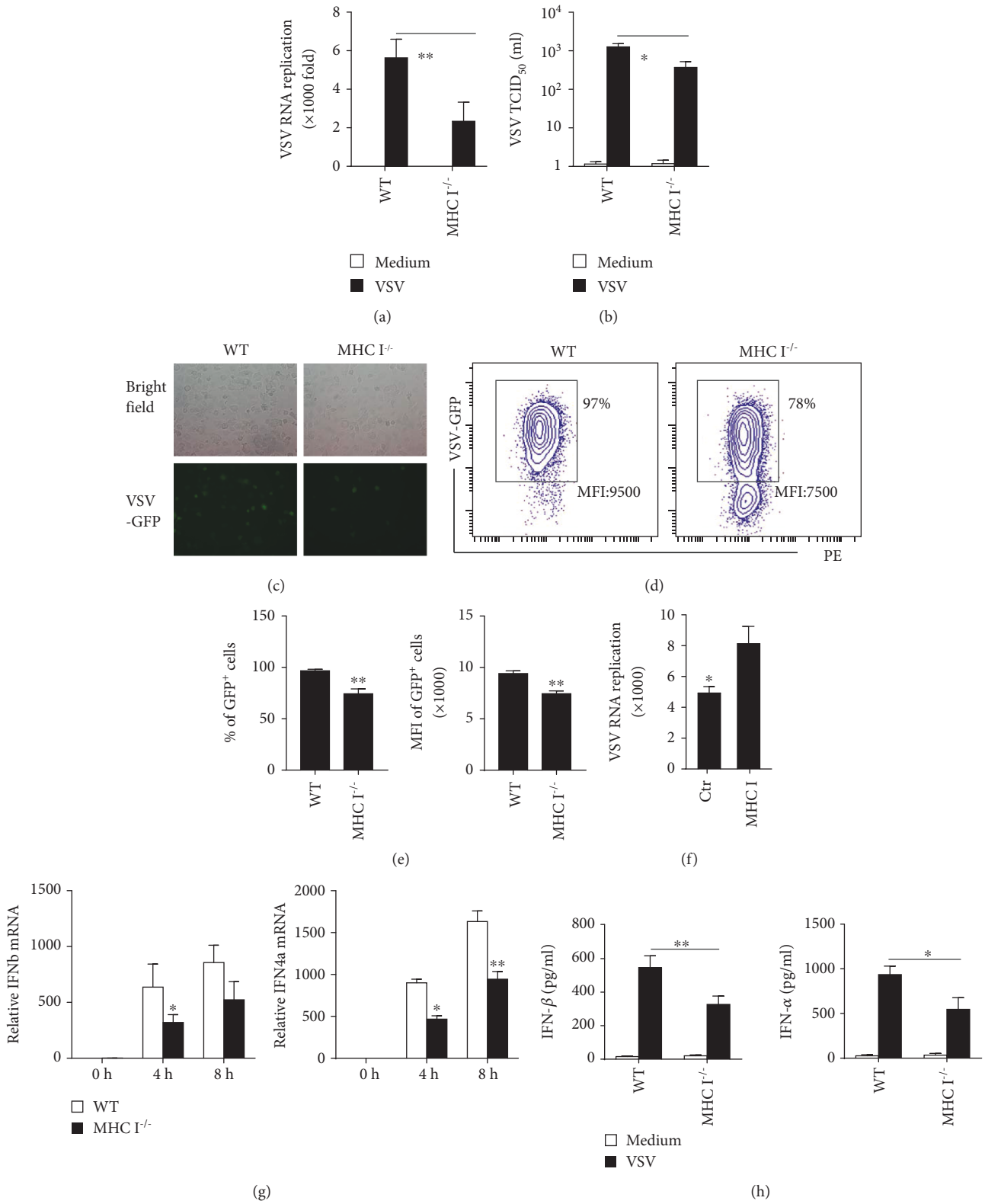
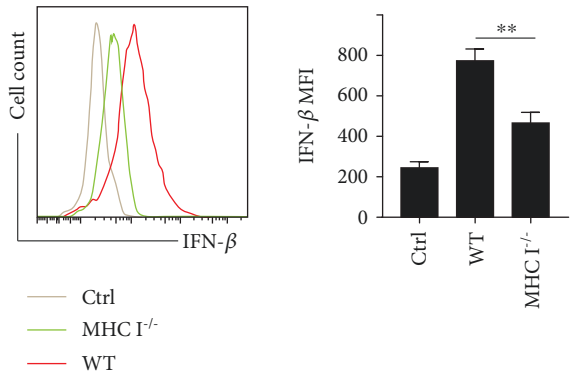


Figure 1: Continued.



(i)

Figure 1: MHC I promotes viral infection independent of type I IFN. (a) VSV RNA replication in WT and MHC $I^{-/-}$ macrophages stimulated with VSV for 6 h, as determined by real-time qPCR analysis. (b) Detection of VSV viral load by $TCID_{50}$ in the supernatant from WT and MHC $I^{-/-}$ macrophages. (c) VSV-GFP replication in WT or MHC $I^{-/-}$ macrophages after 12 h and visualized by fluorescence microscopy. (d) Flow cytometry analysis of WT and MHC $I^{-/-}$ macrophages infected with VSV-GFP after 16 h. (e) Percentages and FITC MFI of GFP^+ cells in (d). (f) VSV RNA replication in WT macrophages overexpressed with MHC I or control vectors. (g) Relative type I IFN mRNA expression in WT and MHC $I^{-/-}$ macrophages at indicated times post infection. (h) Type I IFN in the supernatant of VSV-infected WT and MHC $I^{-/-}$ macrophages 24 h later, analyzed by ELISA assay. (i) Intracellular IFN-β staining of WT and MHC $I^{-/-}$ macrophages post VSV infection (left) and statistical MFI of IFN-β (right). Data are the mean ± SD of at least three independent experiments. Two-way ANOVA was adopted for statistical analysis in (a), (g), and (h). One-way ANOVA was adopted for statistical analysis in (i). Student's t-test was adopted for analysis in (e) and (f). $^*p < 0.05$ and $^{**}p < 0.01$.

We further infected MHC I-deficient mice and littermate control mice with VSV. One day post infection, the viral titers in the lung were significantly lower in MHC I-deficient mice than they were in the littermate controls (Figure 4(d)). There were reduced infiltrating lymphocyte in BALF from MHC $I^{-/-}$ mice compared with that in WT mice (Figure 4(e)). H&E analysis of the infected lung also revealed less extensive lymphocyte infiltration in peribronchiolar and perivascular areas in MHC I-deficient mice (Figure 4(f)). A semiquantitative analysis of the inflammation score of the inflammatory lung showed less inflammation in MHC $I^{-/-}$ mice, compared with that in WT mice (Figure 4(g)). These data indicated the greater resistance of MHC I-deficient mice to viral infection during the early phase of infection. Thus, all these data have suggested a suppressive role of MHC I in type I IFN signaling, which was dependent on SHP2 activation and STAT1 dephosphorylation.

4. Discussion

MHC I belongs to the Ig superfamily, and most of the cell surface proteins in this family are engaged in cellular recognition and intercellular signaling. The primary function of MHC I molecules is to work as ligands, providing antigen signals for CD8 T cells. The nonclassical function of MHC I molecules was revealed more than two decades ago, and nonclassical function has been observed in T cells, B cells, NK cells, myeloid cells, endothelial cells, and tumor cells [13, 14, 22]. In T cells and B cells, cross-linking MHC I activated lck/zap70 and lyn/syk, respectively, and induced T/B cell activation, proliferation, or apoptosis, which depends on the specificity of antibody [12, 14, 23]. Specifically, in malignant tumors, especially in myeloma, anti-MHC I antibody selectively induced tumor cell apoptosis, by activating Lyn and PLCγ2 to upregulate proapoptotic Bad and Bax expression [18]. Here, we reported a nonclassical function of MHC I in macrophages: suppression of type I IFN signaling to impair innate antiviral immunity. In vivo data confirmed that MHC I-deficient mice were more resistant than WT mice in the very early time of viral infection.

Type I IFNs are the key innate antiviral cytokines and include IFN-α, IFN-β, IFN-κ, IFN-δ, IFN-ε, IFN-τ, IFN-ω, and IFN-ζ, with IFN-α and IFN-β as the most well defined types [24]. Type I IFN is induced when microbial products are sensed by PRRs and functions in an autocrine or paracrine manner. After binding to its receptors IFNAR1 and IFNAR2, type I IFN activates JAK1 and TYK2. Phosphorylation of IFNAR by these kinases recruits STAT proteins (STAT1 and STAT2), resulting in their phosphorylation, dimerization, and nuclear translocation [21]. These transcription factors bind to IFN-stimulated response element (ISRE) sequences to activate antiviral ISG transcription. The regulation of type I IFN production has been studied extensively [25], and there are also molecules which fine tune the downstream IFN signal pathway [21]. However, although MHC I is key to antiviral adaptive immunity, its role in innate antiviral immune regulation and type I IFN signaling remains undetermined, and our study may extend our understanding of MHC I.

MHC I deficiency reduced viral replication in macrophages, but did not increase type I IFN production. These data suggested that the reduced viral load cannot be attributed to more type I IFN production. In contrast, the reduced IFN secretion may be the result of reduced viral burden in MHC I-deficient macrophages. Increased STAT1 activation and ISG production in MHC I-deficient cells

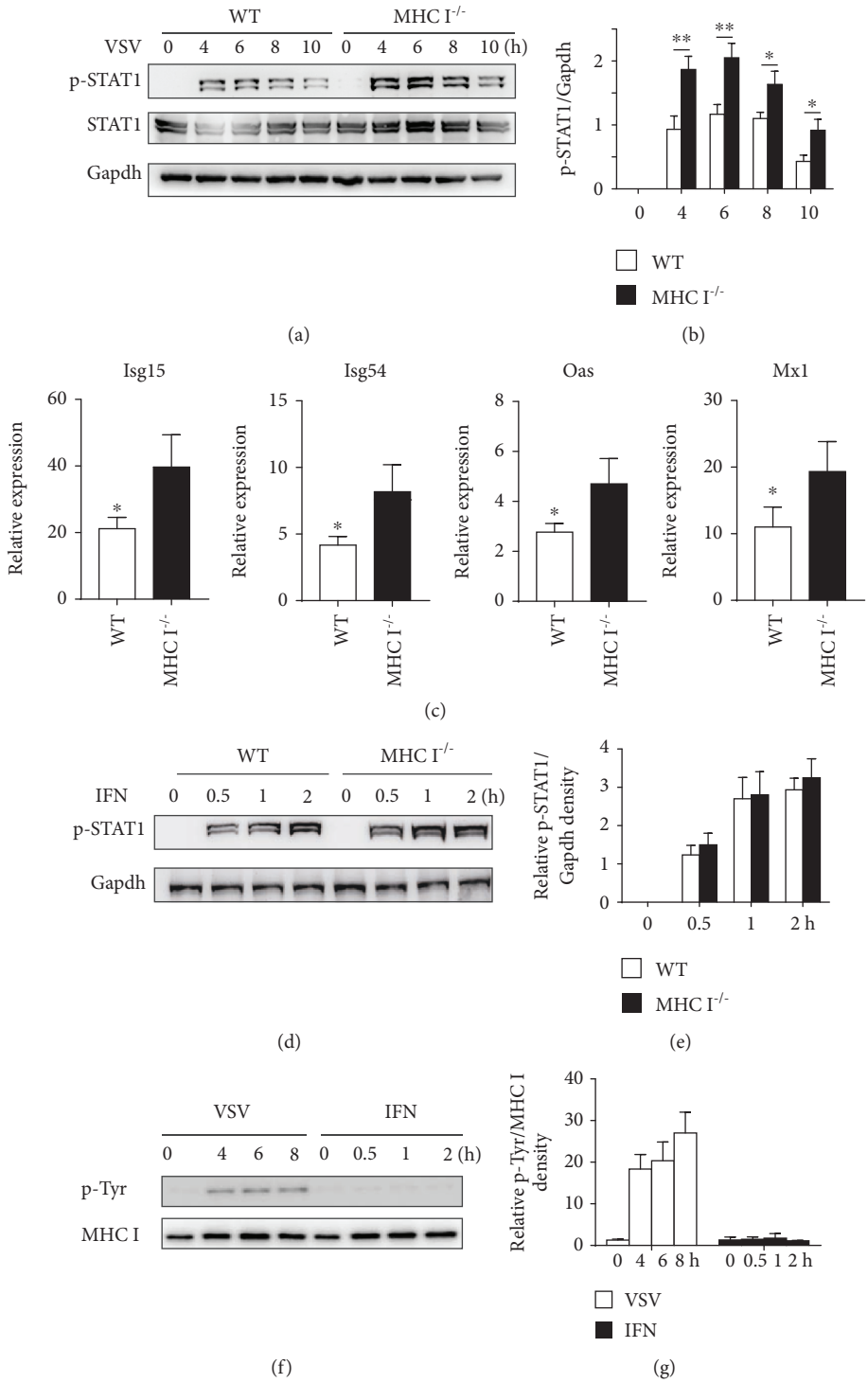


Figure 2: MHC I inhibited IFN downstream signaling. (a) Western blot analysis of STAT1 phosphorylation in WT and MHC I$^{-/-}$ macrophages post VSV infection. (b) Relative density of the blot in (a). (c) Representative ISG expression in WT and MHC I$^{-/-}$ macrophages post VSV infection. (d) Western blot analysis of STAT1 activation in WT and MHC I$^{-/-}$ macrophages treated with IFN-β for the indicated times. (e) Relative density of the blot in (d). (f) Phosphorylation of MHC I after coimmunoprecipitation with anti-H2Kb antibody visualized by immunoblot with anti-p-Tyr. (g) Relative density of the blot in (f). Data are the mean ± SD of at least three independent experiments. Two-way ANOVA was adopted for statistical analysis in (b), (e), and (g). Unpaired Student's t-test was adopted for statistical analysis in (c). $^{*}p < 0.05$.

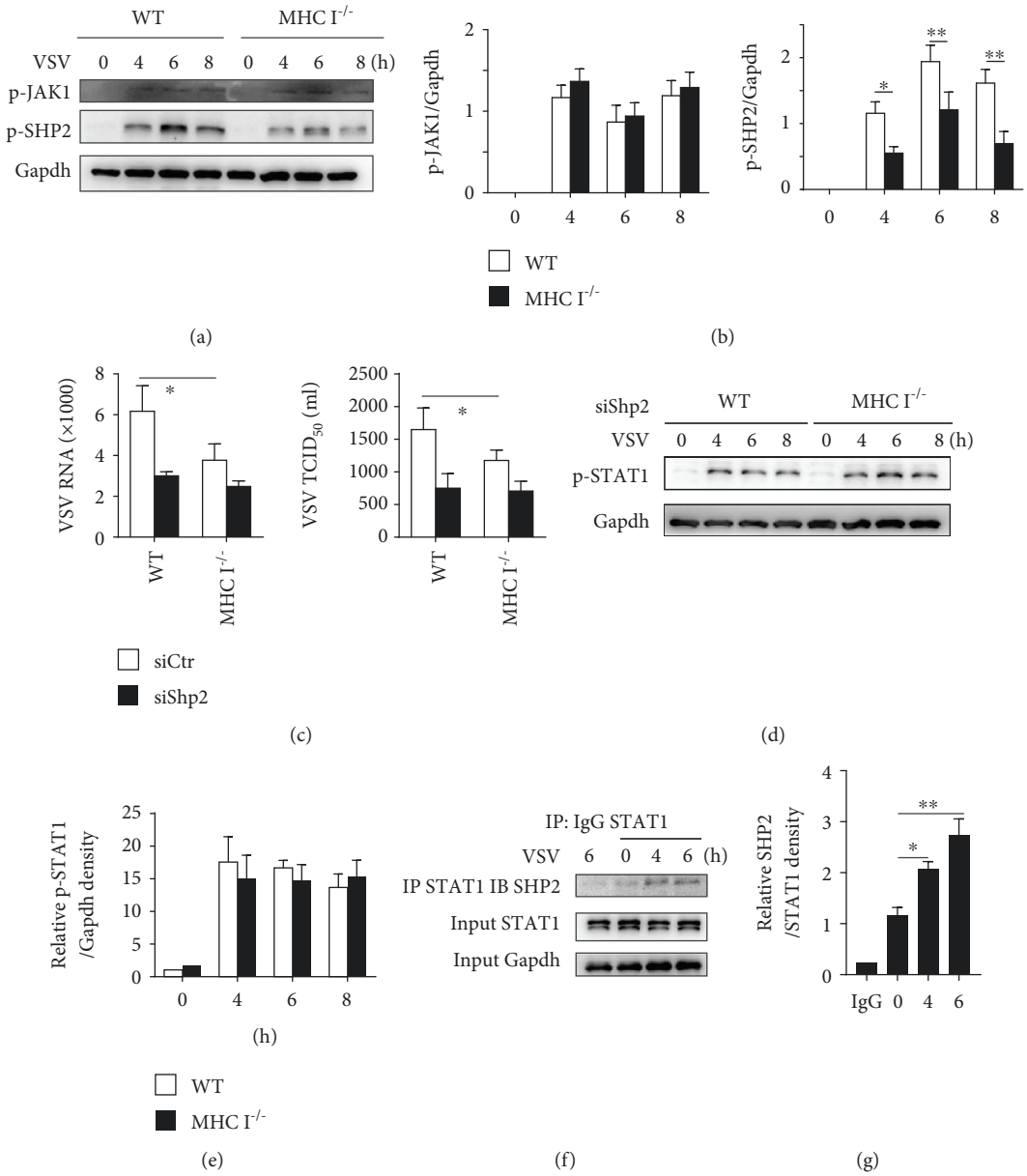


Figure 3: MHC I suppressed IFN signaling through SHP2. (a) Western blot analysis of JAK1 and SHP2 phosphorylation in WT and MHC $I^{-/-}$ macrophages at the indicated times post VSV infection. (b) Relative density of the blot in (a) from 3 independent experiments. (c) VSV RNA replication (left) and $TCID_{50}$ (right) in WT and MHC $I^{-/-}$ macrophages with Shp2 silenced before infection. (d) Western blot analysis of STAT1 activation in WT and MHC $I^{-/-}$ macrophages with Shp2 silenced before infection. (e) Relative density of the blot in (d). (f) Immunoblot assay of SHP2 and STAT1 post coimmunoprecipitation with anti-STAT1. (g) Relative density of the blot in (f). Data are the mean ± SD of at least three independent experiments. Two-way ANOVA was adopted for statistical analysis in (b), (c), and (e). One-way ANOVA was adopted for statistical analysis in (g). $^{*}p < 0.05$.

confirmed our speculation that MHC I impaired type I IFN downstream signal transduction. To determine the mechanism by which MHC I molecules inhibit type I IFN signaling, we first examined whether the interaction occurred at the STAT1 level or the upstream level (IFNAR and JAK1). Considering the activation of JAK1 was not different in WT and MHC I-deficient cells during viral infection, we concluded that STAT1 may be the target of MHC I.

Our previous study suggested that MHC I may recruit Fps and then activate SHP2 in myeloid cells [19]. Previous data have also revealed that SHP2 can regulate type I IFN signal transduction [26, 27]. Without SHP2, MHC I deficiency had no significance on viral replication, suggesting an indispensable role of SHP2 in MHC I function during VSV infection. Our study showed that MHC I inhibited IFN signaling through SHP2 activation and that SHP2 may bind directly to STAT1 to reduce STAT1 phosphorylation.

Considering the mechanism by which MHC I activates SHP2, the "open conformer" theory of MHC I molecules has been proposed [28]. A pool of MHC I at the membrane

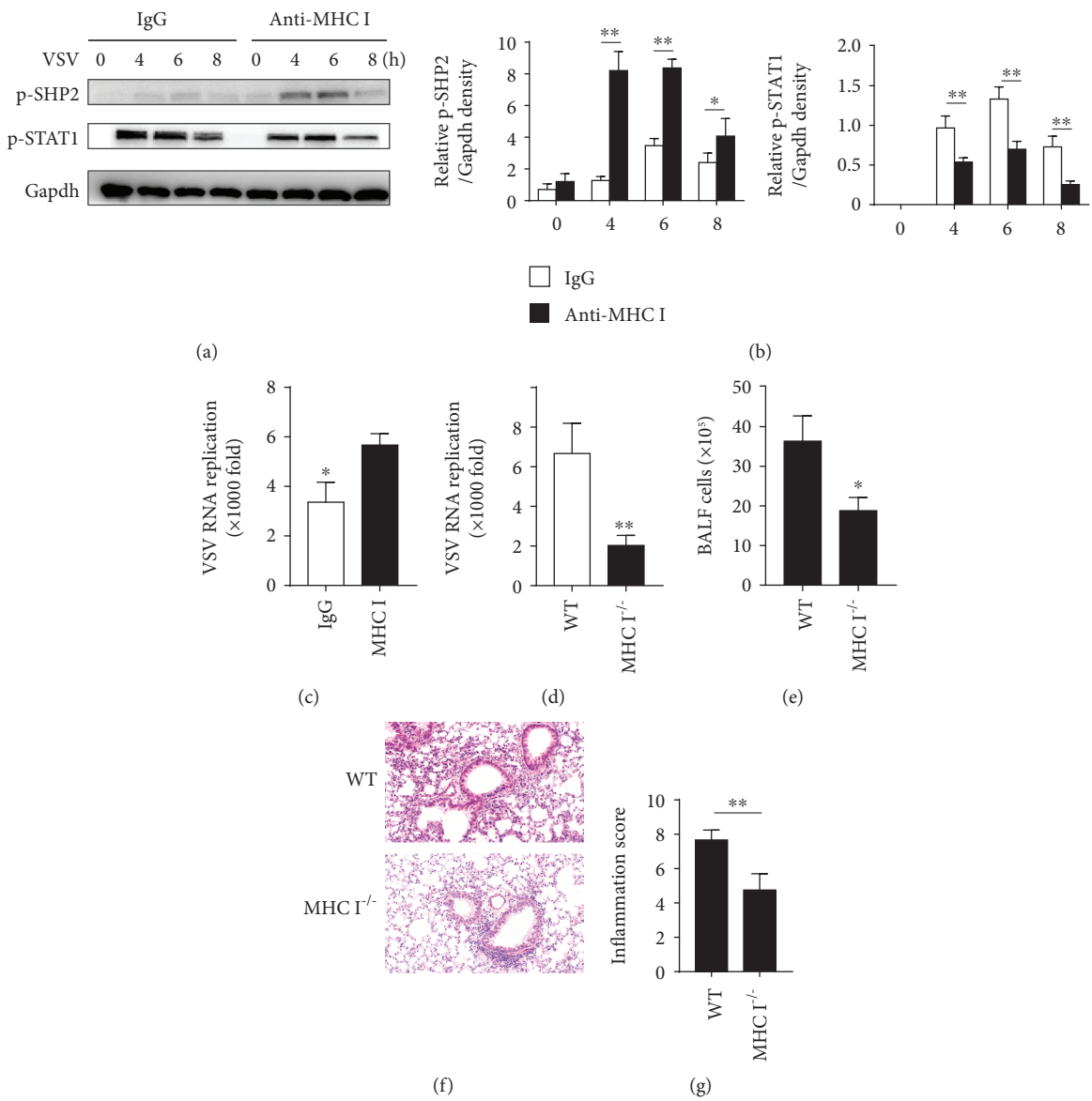


Figure 4: MHC I promotes viral replication during the innate phase of infection in vivo. (a) Macrophages were cross-linked with anti-MHC I antibodies, and the activation levels of SHP2 and STAT1 were determined by western blot assay at indicated times post VSV infection. (b) Relative density of the blot in (a). (c) Relative VSV replication in macrophages in (a). (d) WT and MHC $I^{-/-}$ mice were infected i.p. with VSV; viral load in the lung was calculated 18 h later. (e) BALF cell numbers in the lung from WT and MHC $I^{-/-}$ mice infected with VSV. (f) H&E analysis of the VSV-infected lung from mice infected with VSV (200x). (g) Inflammation score of the lung damage in (f). Data are the mean ± SD of at least three independent experiments. Two-way ANOVA was adopted for statistical analysis in (b). Unpaired Student's t test was adopted for statistical analysis in (c), (d), and (e). $^{*}p < 0.05$ and $^{**}p < 0.01$.

can dissociate from the antigen peptide, becoming the open MHC I conformers. These open conformers can associate with other receptors and possess hidden functions. The formation of open MHC I conformers depends on phosphorylation of its intracellular Tyr320 [29]. In our study, the suppression function of MHC I was also dependent on its tyrosine phosphorylation, suggesting that an open conformer may be needed for its inhibitory function. Sole type I IFN stimulation did not induce tyrosine phosphorylation, thus abrogating the suppressive function of MHC I.

In conclusion, our study has demonstrated a suppressive role of MHC I molecules in type I IFN signaling. Our findings provided new insight into the fine tune of antiviral type I IFN immune responses and indicated a nonclassical function of MHC I in antiviral responses.

Data Availability

The data used to support the findings of this study are available from the corresponding authors upon request.

Conflicts of Interest

The authors declare no competing financial interests.

Authors' Contributions

Simo Xia and Yijie Tao contributed equally to this work.

Acknowledgments

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