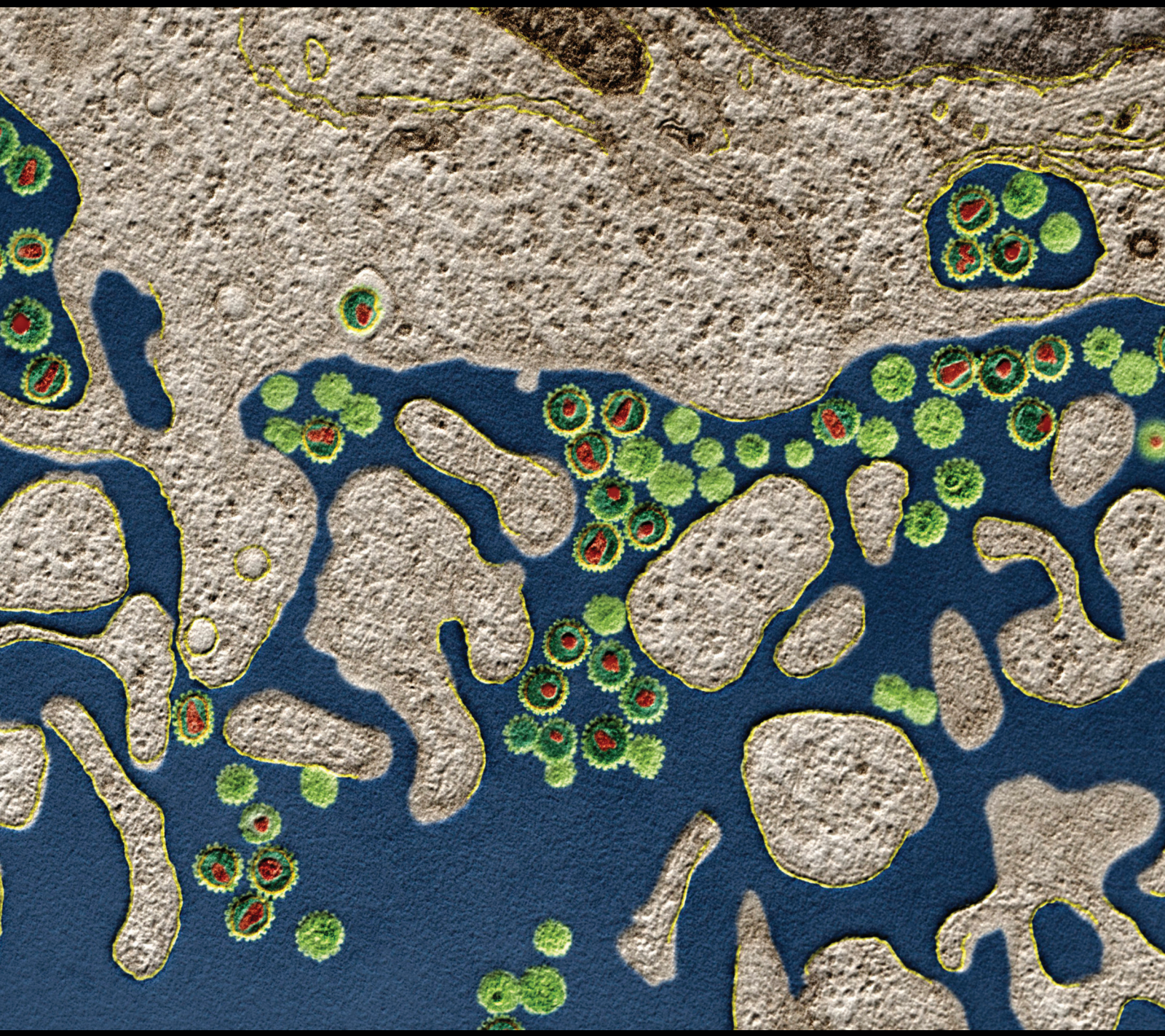
Journal of Immunology Research

T Cells Immunology in the Immunological Diseases

Guest Editors: Xiuli Wu, Grzegorz K. Przybylski, Qintai Yang, and Qifa Liu



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Editorial Board

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 690324, 2 pages
http://dx.doi.org/10.1155/2014/690324

Editorial

T Cells Immunology in the Immunological Diseases

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Received 8 July 2014; Accepted 8 July 2014; Published 1 September 2014

Immunological diseases, with the morbidity keeping increased, have become a major threat to human mental and physical health. However, the pathogenesis of these diseases is extremely complicated and remains unclear. Recent advances in biology have introduced new technologies to study the underlying mechanisms that contribute to the development of immunotherapy of immunological diseases. This special issue includes original research articles and review articles that do research on T cells immunology in the immunological diseases.

In the brief review "*The pathology of T cells in systemic lupus erythematosus*" by A. Mak and N. Y. Kow, the authors discuss a detailed account of the putative mechanisms by which the normal physiology of T cells are disturbed and why do the regulatory T cells fail to alleviate proinflammatory response in systemic lupus erythematosus (SLE) and introduce the current state of clinical trials evaluating therapeutic agents which target molecules expressing on and inside T cells for the treatment of SLE.

In the review "*The role of the γδ T cell in allergic diseases*" by R. Zheng and Q. Yang, γδ T cells have been considered to bridge the innate and adaptive immunity. γδ T cells may play crucial roles in the development and perpetuation of allergic inflammation as effector and immunoregulatory cells. This review focuses on the latest knowledge on characteristics and roles of γδ T cells in allergic diseases.

The review "*Improving cytomegalovirus-specific T cell reconstitution after haploidentical stem cell transplantation*" by X. Luo et al. summarizes the kinetics of cytomegalovirus- (CMV-) specific T cell recovery and its association with CMV infection after haploidentical stem cell transplantation and discusses the strategies to improve CMV-specific immune reconstitution.

In the research "*Alternative expression pattern of MALT1-A20-NF-κB in patients with rheumatoid arthritis*" by X. Wang et al., rheumatoid arthritis (RA) is a common, chronic, systemic, and inflammatory autoimmune disorder and abnormal T cell immunity plays a critical role in the development of RA. Recently, a negative regulator of nuclear factor kappa B (NF-κB), A20, was identified as a key regulator for T cell activation and inflammatory signaling and may be involved in RA pathogenesis. This study analyzed the expression level of A20, NF-κB, and the A20 regulatory factor mucosa-associated-lymphoid-tissue lymphoma-translocation gene 1 (MALT1) in patients with RA. The authors characterized the alternative expression pattern of MALT1, A20, and NF-κB in RA, which might be related to abnormal T cell activation. The lack of A20 and dysfunctional MALT1 are common features in Chinese patients with RA, and the results provide new data for the consideration of target regulation in RA inflammation.

In the research "*Rapamycin regulates iTreg function through CD39 and Runx1 pathways*" by Y. Lu et al., the induced regulatory T cells (iTreg) play important roles in treating various autoimmune diseases in mice including autoimmune diabetes, experimental arthritis and other immune-mediated inflammatory diseases. CD39 is a newly determined Treg marker that relates to cell suppression. Runx1, a regulator of FoxP3, controls the expression of adenosine deaminase gene, which is found recently in the downstream of CD39 pathway in trophoblast cells. The authors suggested that CD39 expression was involved in iTreg generation and the enhanced suppressive ability of

rapamycin induced Treg was partly due to Runx1 pathway. This study provides a novel insight into the mechanisms of iTreg generation enhanced by rapamycin.

In the research "*The feature of distribution and clonality of TCR γ/δ subfamilies T cells in patients with B-cell non-Hodgkin lymphoma*" by L. Wang et al., T-cell immunodeficiency is a common feature in cancer patients, which may contribute to the disease initiation and progression. This study provides a preliminary profile of distribution and clonality of T-cell receptor γ/δ subfamilies T cells in peripheral blood (PB), bone marrow (BM), and lymph node (LN) from B-cell non-Hodgkin lymphoma patients. The clonally expanded Vδ5, Vδ6, and Vδ8 subfamily T cells were detected only in PB but neither in BM nor in LN. While clonally expanded Vδ2 and Vδ3 T cells could be detected in both PB and BM or PB and LN. Similar clonally expanded Vδ subfamily T cells in PB and BM may be related to the same B-cell lymphoma-associated antigens, while the different reactive clonally expanded Vγ/Vδ T cells may be due to local immune response.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 963484, 8 pages
http://dx.doi.org/10.1155/2014/963484

Review Article

The Role of the $\gamma\delta$ T Cell in Allergic Diseases

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Received 30 March 2014; Accepted 11 May 2014; Published 3 June 2014

Academic Editor: Qifa Liu

The predominant distribution of $\gamma\delta$ T cells in the mucosal and epithelial tissues makes these unconventional lymphocytes the "guards" to contact external environment (like allergens) and to contribute to immune surveillance, as well as "vanguards" to participate in initiating mucosal inflammation. Therefore, $\gamma\delta$ T cells have been considered to bridge the innate and adaptive immunity. The role these cells play in allergy seems to be complicated and meaningful, so it makes sense to review the characteristics and role of $\gamma\delta$ T cells in allergic diseases.

1. Introduction

$\gamma\delta$ T cells are a minor population of lymphocytes expressing γ and δ T cell receptor (TCR) chains, which are often considered to bridge innate and adaptive immune responses. Recent studies have shown that $\gamma\delta$ T cells can comprise up to 50% of the T cells within epithelium or mucosa-rich tissues and less than 10% in peripheral blood [1]. The specific localization and abundance of these cells suggest that they might be markedly implicated in epithelial/mucosal immunity [2, 3]. In contrast to recognition of antigens by $\alpha\beta$ T cells, $\gamma\delta$ T cells recognize antigens directly without any requirement for antigen processing and presentation or major histocompatibility complex (MHC) molecules [4]. It has been indicated that $\gamma\delta$ T cells may play crucial roles in the development and perpetuation of allergic inflammation as effector and immunoregulatory cells, via production of T helper (Th)1-, Th2-, and Th17-associated cytokines [5], which not only induce the synthesis of IgE but also recruit effector cells like eosinophils and basophils into the site of allergic inflammation [6]. Besides, different subsets of $\gamma\delta$ T cells can show different functions, depending on what tissue they are found in and which specific TCRs they bear [7]. Even though there is a growing consensus about the importance of these cells in allergic immune responses, the specific mechanisms remain elusive. The present review focuses on the latest knowledge on characteristics and role of $\gamma\delta$ T cells in allergic diseases.

2. $\gamma\delta$ T Cells Have Diverse Subsets with Specific Locations and Functions

As research continues, it has been realized that $\gamma\delta$ T cells are not a homogeneous population of cells with a single physiological role, and their subset complexity is being characterized, both in mice and humans [7]. TCR Vγ- and Vδ-encoded chain pairs may interact with distinct ligands in different tissues and be expanded on that basis. Defined by the usage of either Vδ1 or Vδ2 TCR (Vδ3 and Vδ5 making up minor populations), human $\gamma\delta$ T cells fall into two major subsets: Vδ2 T cells account for the majority (50–95%) of circulating $\gamma\delta$ T cells, whereas Vδ1 T cells are rare in the blood but appear at increased frequencies in mucosal tissues and in the skin [7–10].

The Vγ9Vδ2 (also termed Vγ2Vδ2, collectively designated Vδ2) T cells in the peripheral blood can sometimes identify over 50% of leucocytes after certain bacterial or parasitic infections and rapidly get activated; therefore, such TCR-dependent activation of Vγ9Vδ2 T cells enables them to respond to a diverse range of pathogens [11]. According to the surface expression of CD45RA and

CD27, markers more commonly used to identify the naive, effector, or memory status of conventional $\gamma\delta$ T cells, human Vγ9Vδ2 T cells, are often subdivided into four subsets: "naive" (Tnaive) CD45RA$^+$CD27$^+$ cells; "central memory" (TCM) CD45RA$^-$CD27$^+$ cells; "effector memory" (TEM) CD45RA$^-$CD27$^-$ cells; "CD45RA$^+$ effector memory" (TEMRA) CD45RA$^+$CD27$^-$ cells [7, 12]. Tnaive and TCM cells express lymph node homing receptors, abound in lymph nodes, and lack immediate effector functions. Conversely, TEM and TEMRA cells, which express receptors for homing to inflamed tissues, are poorly represented in the lymph nodes while abounding in sites of inflammation and display immediate effector functions. It indicates a lineage differentiation pattern for human Vδ2 T cells that generates naive cells circulating in lymph nodes, effector/memory cells patrolling the blood, and terminally differentiated effector cells residing in inflamed tissues [12].

In contrast to Vδ2, the TCR-γ chain usage by the tissue-associated Vδ1 T cells varies at distinct anatomic locations. Vδ1 T cells in the periphery express a naive phenotype and may migrate preferentially to localized sites when they are activated [13]. For instance, Vγ2, Vγ3, Vγ5, Vγ6, and Vγ7 are used predominantly by $\gamma\delta$ T cells in peripheral lymphoid organs, skin, small intestine, tongue, and reproductive system, respectively [14, 15]. In contrast, Vγ1 and Vγ4 are preferentially expressed in the respiratory system like nasal mucosa and lung [16–18]. Recent studies have indicated that these tissue-associated Vδ1 T cells may play an important function not only in maintaining immune homeostasis in the local microenvironment [19] but also in wound healing, removing distressed or transformed epithelial cells and subduing excessive inflammation, in both mice and humans [20–22]. Besides, the role of these mucosa predominantly expressed $\gamma\delta$ T cells in allergic diseases has also been noticed. Our preliminary studies found that the infiltration of $\gamma\delta$ T cells significantly increases in the nasal mucosa of patients with perennial allergic rhinitis (AR) (data not shown). Moreover, Pawankar et al. [23] proved that the increased population of $\gamma\delta$ T cells in the perennial AR patients' nasal mucosa mainly comprises of Vγ1Vδ1 subsets. In mice sensitized and challenged with OVA, Cook et al. [24] observed that Vγ1$^+$ T cells spontaneously enhance airway hyperresponsiveness (AHR), whereas Vγ4$^+$ T cells, after being induced by allergen sensitization and challenge, suppress AHR. These data suggest that $\gamma\delta$ T cells of distinct phenotypes may play different, sometimes opposed, functions in airway allergic inflammation. However, it is still premature to speculate whether the Vγ4$^+$ subsets of $\gamma\delta$ T cells may exert an important role in maintaining immune homeostasis in local microenvironment of healthy humans; on the other hand, the Vγ1$^+$ subsets may take an essential part in the development and perpetuation of allergic inflammation as effectors in atopy patients. Besides, our another recent study showed that different subsets of $\gamma\delta$ T cells in peripheral blood of perennial AR patients before and after specific immunotherapy (SIT) appear with distinct expression patterns [25]. But whether $\gamma\delta$ T cells in peripheral blood and in mucosa function separately or synergistically remains an unsolved problem.

3. $\gamma\delta$ T Cells in Different Age and Gender Groups

With age, there comes the change from having fairly diverse pairs of $\gamma\delta$ T cells (of which Vδ1$^+$ subsets serve as the majority in cord blood at birth) to increasingly restricted pairings (with Vγ9Vδ2 T cells becoming the major subsets with very limited receptor diversity by adulthood). From birth to about 10 years of age, the absolute number of $\gamma\delta$ T cells in the periphery increases, with the Vγ9Vδ2 T cell subsets expanding from a minor population at birth to usually more than 75% of circulating $\gamma\delta$ T cells [13, 26]. Vγ9Vδ2 T cells are known to respond to many different phosphoantigens, so it is likely that the exposure to a variety of pathogens results in the selection of these cells in early life [27]. This clonal expansion has been seen as evidence of the vital role these T cells play in responding to environmental challenges in early life. However, it has not been demonstrated whether this phenomenon is in fact primary in response to environmental challenges but not, at least in part, endogenous stimuli, as an extension of the adaptive changes taking place within the newborn [10].

Previous longitudinal cohort studies have shown that most childhood asthma begins in infancy, and between 40% and 75% of children with asthma will have complete resolution of symptoms by adolescence or adulthood [28]. Respiratory syncytial virus (RSV) infection in lower respiratory tract in early childhood is a risk factor for the subsequent development of allergic sensitization such as wheezing up to age of 11 years [29]. Aoyagi et al. [30] reported that compared to age-matched controls, infants affected by RSV-bronchiolitis have lower frequencies of IFN-γ-producing $\gamma\delta$ T cells in peripheral blood. Moreover, they noticed normalization of this frequency during the convalescent phase, suggesting that the defective IFN-γ production by these cells may play an important role in the development of asthma. However, it is too early to conclude whether the expansion of Vδ2Vγ9 T cells with age is associated with childhood asthma spontaneous remission by adolescence.

In adulthood, studies have also found the possible great impact of age and gender on the $\gamma\delta$ T cell repertoires: in contrast to childhood, the absolute number of $\gamma\delta$ T cells decreases, as the result of reduction of Vδ2, but not Vδ1 T cells. Besides, the number of total $\gamma\delta$ T cells and Vδ2 T cells are both significantly higher in males than in females [31, 32]. It indicates that age- and gender-matched controls are essential for clinical studies of $\gamma\delta$ T cell repertoires in patients.

The term "allergic march" refers to the natural history of atopic manifestations, which is characterized by a typical sequence of IgE antibody responses and clinical symptoms that appear early in life, persist over years or decades, and often remit spontaneously with age. Several studies have shown that the "new" allergy can occur throughout life; generally, allergy prevalence and severity tend to decrease after young adult life [33], and Th2-type responses may weaken with age [34]. Hansen et al. [35] found that immunization dose, sex, and age are highly influential on allergy

outcomes in murine models. Nevertheless, further researches are required to make certain whether the change of $\gamma\delta$ T cell subsets is associated with the age and gender related allergic march.

4. The Antigen Recognition of $\gamma\delta$ T Cells in Allergy

Selective allergen recognition by TCR that binds specific regions of the antigen molecules is the priming and initiation of antigen-specific T cell immune responses. So far, more than 4000 substances in the environment, the vast majority of which are proteins, mostly enzymes, have been identified as allergens that elicit an IgE-mediated immune response in a genetically predisposed individual. Different from MHC-restricted recognition of bound peptides by $\alpha\beta$ TCRs, the antigen specificity of $\gamma\delta$ T cells involves the immunoglobulin-like structure of the $\gamma\delta$ TCR [36] with the recognition of unprocessed peptides, small organic phosphate molecules, or alkylamines derived from microbes and edible plants [37].

Studies have shown that the epithelial-associated $\gamma\delta$ T cells can recognize stress-induced self-antigens, which enables them to monitor multiple insults to the epithelium [38]. However, data from humans and mice seem to indicate the relevance of mucosa-associated $\gamma\delta$ T cells in allergen recognition and airway inflammation, perhaps mediated by interaction of foreign antigens with $CD1^+$ dendritic cells (DCs) [39]. The study by Russano et al. [40] showed that $CD1^+$ immature DCs expand in the respiratory mucosa of allergic subjects and are able to process both proteins and lipids, and CD1-restricted phospholipids (PL)-specific $\gamma\delta$ T cells represent the key mucosal regulatory subsets for the control of early host reactivity against tree pollens. These CD1-restricted $\gamma\delta$ T cells can respond promptly to lipid-antigen recognition by secreting a wide array of cytokines, including high amounts of IL-4, and expand at mucosal allergic inflammation sites [38]. In addition, $\gamma\delta$ T cells derived from nasal mucosa in allergic subjects could also recognize pollen derived PE in a CD1d-restricted fashion [40]. Therefore, it may be speculated from above that the early allergic response initiates with primary mucosal recognition of allergen by $CD1^+$ DCs and CD1-restricted $\gamma\delta$ T cells, which ensure rapid handling of the foreign inhaled grain.

5. The Cytokine Production of $\gamma\delta$ T Cells in Allergy

It is proposed that $\gamma\delta$ T cells may act as an extended arm of $\alpha\beta$T cells, by providing a rapid but weaker response before the $\alpha\beta$ T-cell response has fully developed. Substantial evidence has been accumulated to indicate that $\gamma\delta$ T cells have the potential to produce Th17-type cytokines (like IL-17) and Th2-type cytokines (IL-4, -5, and -13) and thus enhance airway allergic inflammation and AHR [41]. In contrast, Th1-type cytokines (IL-12 and IFN-γ) produced by $\gamma\delta$ T cells might be induced after special immunotherapy and inhibition of these allergic diseases [42, 43].

The study by Ribot et al. [44] showed that in the spleen and lymph nodes and in the peripheral tissues of mice, the presence or absence of the cytokine CD27 distinguishes two $\gamma\delta$ T cell subsets: the $CD27^+$ cells produce IFN-γ, whereas the $CD27^-$ cells (50% of $V\gamma4^+$ and 11% of $V\gamma1^+$) produce primarily IL-17. It suggests the existence of a differential requirement for optimum activation of these distinct $\gamma\delta$ T cell subsets in the peripheral immune compartment. In humans, 80% of circulating $V\gamma9V\delta2$ T cells are IFN-γ producers, while less than 1% produce IL-17 [45]. Caccamo et al. [46] observed that IFN-γ^+ $V\gamma9V\delta2$ T cells have a predominant TEM and at a lower extent TEMRA phenotype, while IL-17^+ $V\gamma9V\delta2$ T cells exhibit a TEMRA phenotype. However, IL-17^+ $V\gamma9V\delta2$ T cells significantly increase and secrete abundant IL-17 at sites of inflammation (perhaps primarily at epithelial surfaces), which may directly shape the inflammatory infiltrate, for example, by attracting neutrophils during bacterial infection [45]. Zhao et al. [47] also found that the IFN-γ^+/IL-17^+ ratio in $\gamma\delta$ T cells significantly decreases in patients with allergic asthma compared with healthy controls. What is more, several $\gamma\delta$ T cells are indicated to be a chief source of IL-17 in peripheral tissues such as lung, which share certain common features with Th17 cells [48–50]. With regard to humans, $\gamma\delta$ T cells are divided into two different phenotypes. An IL-4-producing phenotype, which possesses $V\gamma1/V\delta1$ segments, enhances allergic inflammation. $V\gamma9/V\delta2$ segments have an IFN-γ-producing phenotype and might thus have a partial ability to modulate allergen-specific Th2-skewed immunity [43].

In a word, after quick antigen recognition and full activation, diverse subsets of $\gamma\delta$ T cells in the circulating blood and various lymphoid compartments subsequently produce an array of different cytokines, performing proinflammation as well as pleiotropic immunoregulatory functions, as it will be further discussed.

6. $\gamma\delta$ T Cells Are Both Effector and Regulatory Cells in Allergic Inflammation

Lately, Kalyan and Kabelitz [10] provided a biographical sketch of $\gamma\delta$ T cells: in a continuum, innate natural killer (NK) cells and adaptive $\alpha\beta$ T cells respond to the "missing self" and the "dangerous nonself," respectively, while $\gamma\delta$ T cells respond to the "safe nonself" and deal with the inevitable "distressed self." What is more, NK cells could contribute to responding to the "distressed self," whereas $\alpha\beta$ T cells have some regulatory training to temper the response to the "safe nonself" [10] (Figure 1). This sketch supports the viewpoint that $\gamma\delta$ T cells serve as the bridge between innate and adaptive immunity. Allergy is primarily considered as a classic Th2-driven immune response against allergens (safe nonself), with important contributions to pathology by Th2-type cytokines IL-4, -5, and -13, which not only induce the synthesis of IgE but also recruit effector cells like eosinophils and basophils into the site of allergic inflammation [42]. However, inflammatory responses in allergic diseases are more complex than simple overexpression of Th2 cytokines. A recent hypothesis has been put forward to rely on

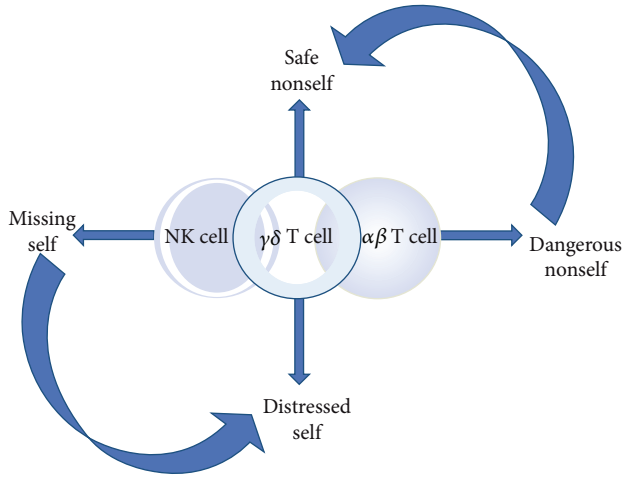

FIGURE 1: A simplified paradigm illustrating where in the continuum of immune protection and homeostasis $\gamma\delta$ T cells fall in relation to innate NK cells and the adaptive $\alpha\beta$T cells. Innate NK and adaptive $\alpha\beta$ T cells respond to the "missing self" and the "dangerous nonself," respectively, while, between these two extremes, $\gamma\delta$ T cells respond to the "safe nonself" and deal with the inevitable "distressed self." These different "selves" and the immune response(s) that they trigger exist in a continuum and are modulated by the context in which they are presented. Besides, NK cells could contribute to responding to the "distressed self," whereas $\alpha\beta$ T cells have some regulatory training to temper the response to the "safe nonself" (cited from [10]).

the genetically determined barrier deficiency and disruption by environmental and endogenous proteases in the epithelial barrier (distressed self), which might result in the allergen uptake as a primary defect in the pathogenesis of allergic reactions [51]. It seems that allergy is both an epithelial disease and a disease of the immune system. Using an adoptive cell transfer approach, Jin et al. [52] found that NK and $\gamma\delta$ T cells (only Vγ1Vδ5 subsets) are necessary for the acute stages of AHR in mice but not for the later airway eosinophilic inflammation. Another study on AHR demonstrated that NK cells secreted IL-4 and -13 to produce their effector function, but $\gamma\delta$ T cells did not have this effect [53]. In addition, $\gamma\delta$ T cells, similar to NK cells, express the NKG2D receptor that may contribute to effective stress-responses as well as immune surveillance, which may be relevant in the induction of food allergy [54]. These data suggest that the interaction of innate and adaptive immune cells and the impact of the inflammatory responses on this collaboration seem to be important and worthy of further research.

Abundant populations of $\gamma\delta$ T cells have been found in the epidermis of rodents [55]. The respiratory mucosa such as nasal mucosa, bronchial mucosa, and lung contain $\gamma\delta$ T cells as well [56]. This may be of importance in the remarkable resistance of the airway against environmental stimuli. Substantial evidence has been accumulated to indicate that $\gamma\delta$ T cells take part in Th2 immune responses. $\gamma\delta$ T cells themselves can not only take the function of follicular Th cells in certain responses but also can support responses that are dependent on classical help provided by $\alpha\beta$ T cells. An increase in $\gamma\delta$ T cells expressing Th2-type cytokines has been reported in bronchoalveolar lavage (BAL) fluids of allergen challenged asthmatic patients [57]. In addition to proinflammatory function, the $\gamma\delta$ T cells also engage as regulators of Th2 immunity [41], in particular regulating the IgE antibodies [3, 38, 40]. Svensson et al. [58] showed that $\gamma\delta$ T cell-deficient mice exhibited a diminished allergen specific IgE response compared with wild-type (WT) mice, indicating that $\gamma\delta$ T cells contribute to B cell secretion of allergen-specific IgE, either by promoting Ig class switch to IgE or by providing activation signals to differentiated IgE-producing cells. Likewise, Zuany-Amorim et al. [59] reported a low antigen specific IgE and IL-5 release and a decrease in T cell infiltration in the same mouse models. They further found that the response could be restored when IL-4 was administered, suggesting that $\gamma\delta$ T cells contribute to type 2-mediated airway inflammation by inducing IL-4 dependent IgE and IgG1 responses. In contrast, Lahn et al. [60] showed that $\gamma\delta$ T cells exert a suppressive role in the Th2 response to allergen challenge. Therefore, it is clear that $\gamma\delta$ T cells might have various, possibly opposing roles for $CD4^+$ T cells.

Studies have demonstrated that, in the airway, distinct subsets of $\gamma\delta$ T cells, defined by their expression of TCR-γ, seem to exhibit differential and sometimes opposed Th-like reactivities in allergen-induced allergic inflammation [7, 47]. In mouse models of allergic diseases, it has been shown that the $V\gamma1^+$ subsets can enhance AHR as well as levels of Th2 cytokines in the airways and eosinophilic infiltrates in the lungs [61, 62], and, in contrast, the $V\gamma4^+$ subset can be induced to inhibit AHR [63, 64]. Lahn et al. [64] selectively depleted either subset in the lungs (using aerosolized, inhaled anti-TCR Abs) following airway challenge and observed that AHR is altered in the predicted fashion; that is, depletion of $V\gamma1^+$ cells decreases and depletion of $V\gamma4^+$ cells increases AHR. After transferring few purified $V\gamma1^+$ cells into OVA/alum immunized TCR-$\delta^{-/-}$ mice, Huang et al. [65] observed the increase of the OVA-specific IgE responses, suggesting that individual enhancer cells are quite potent. However, the relative importance of $\gamma\delta$ T cells in human asthma remains to be determined.

It has been shown for some time that murine $\gamma\delta$ T cells become functionally competent in the thymus, particularly regarding the production of proinflammatory cytokines IFN-γ and IL-17 [60]. McMenamin et al. [66] found that $\gamma\delta$ T cells regulate IgE responsiveness to inhaled antigens by high production of IFN-γ. By using mice immunized with recombinant vaccine virus expressing RSV F protein and challenged with live RSV, Dodd et al. [63] reported that $V\gamma4^+$ subsets are recruited into the lungs and produce IFN-γ in a time-dependent manner. These studies suggest that antigen-specific $\gamma\delta$ T cells are able to suppress the pathogenic Th2 response in allergic asthma, whereas a recent study by Chen et al. [67] found that the serum levels of IL-4 and IL-13 in peripheral blood of children with AR and asthma markedly decrease while IFN-γ increases after receiving SIT, suggesting that IFN-$\gamma^+\gamma\delta$ T cells might exert their Th2 immunosuppression under certain conditions like

SIT. Our recent study showed that the serum levels of IL-17 and IL-23 in the AR patients were significantly higher than those in the healthy subjects, and positive correlations exist between the IL-17 and the IL-23 levels, as well as the IL-17 level and $\gamma\delta$ T frequencies [68]. Accordingly, we conjecture that the IL-23R$^+$ IL-17$^+$$\gamma\delta$ T cells may promote $\alpha\beta$ T cell-mediated traditional Th2 inflammation via producing abundant IL-17. In addition, IL-17-producing $\gamma\delta$ T cells could directly promote the development of other IL-17-producing T cells [69], and these innate IL-17-producing T cells are involved in sensing stress, injury, or pathogens and serve an immunoregulatory role at epithelial sites [63].

Gonçalves-Sousa et al. [70] reported that murine CD4$^+$ CD25$^+$Foxp3$^+$ regulatory T cells (Treg) abolish key effector functions and proliferation of $\gamma\delta$ T cells both in vitro and in vivo. They further showed that the suppression is dependent on cellular contact between Treg and $\gamma\delta$ T cells and is partially mediated by glucocorticoid-induced TNF receptor-related proteins. It reveals a novel mechanism, by which $\gamma\delta$ T-cell function is regulated, and suggests that endogenous Treg may prevent the desired effects of $\gamma\delta$ T cell-based immunotherapies. It has also been shown by Hahn et al. [71] that $\gamma\delta$ T cells affect the level of IL-10 in the airways and block their function resulting in an increase of Treg in the lung, which suggests that $\gamma\delta$ T cells might inhibit Treg function. While these data highlight the importance of understanding how the proinflammatory and immunoregulatory functions of $\gamma\delta$ T cells are regulated, the detailed processes remain poorly understood.

7. $\gamma\delta$ T Cells with the Prevention and Treatment of Allergic Diseases

$\gamma\delta$ T cells stimulated with bisphosphonate compounds, which are clinically well tolerated and used for $\gamma\delta$ T cell expanders in vitro and in vivo, have been considered to be good candidates for cancer immunotherapy, because of their IFN-γ production and cytotoxic effect [72]. Therefore, the adoptive transfer of autologous $\gamma\delta$ T cells expanded in vitro might also be an effective strategy for IL-4-mediated allergy.

It has been shown that oral tolerance, which refers to the active state of nonresponsiveness to food and food protein intake, is a unique feature of the (gut-associated) mucosal immune system. And the defects in this process result in allergic sensitization to food proteins [73]. Most intestinal epithelial lymphocytes (IELs) in the mouse consist of $\gamma\delta$ T cells, which are localized in the paracellular space between intestinal epithelial cells at the luminal site of the basement membrane [74]. Mengel et al. [75] showed that treating mice with a TCR-δ-specific antibody results in impaired oral tolerance induction and that oral tolerance could be transferred by means of $\gamma\delta$ T cells. It suggests that targeting intestinal $\gamma\delta$ T cells may provide preventing and therapeutic strategies for food allergy. However, currently IELs are among the least studied cells in the process of allergic sensitization.

Studies have shown that many phosphoantigens and fungal immunomodulators play an important role in $\gamma\delta$ T cell-mediated immunotherapy. For example, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate is characterized as a very potent agonist of Vγ9Vδ2 responses and could strengthen the principle of $\gamma\delta$ T cell based immunotherapy [76]. In contrast, Gonçalves-Sousa et al. [70] described that Treg could negatively modulate the $\gamma\delta$ T cell activities and stressed the importance of combining Treg inhibition with $\gamma\delta$ T cell activation for future immunotherapeutic strategies.

In animal models, chronic allergen challenge induces suppression of the Th2 response and reduces AHR and airway inflammation [77, 78]. Reductions of late-phase asthmatic responses to allergen after long-term allergen challenge have also been reported in clinical studies [79, 80]. Lahn et al. [64] indicated that the Vγ4$^+$ subsets appear to mediate such suppressive effect of long-term allergen challenge on AHR. In addition, $\gamma\delta$ T cells could also suppress Th2-dependent IgE responses without affecting parallel IgG responses to inhaled antigens [66].

Taken together, $\gamma\delta$ T cells may have the potential to help alter the Th2-skewed immunity in patients with allergic diseases. Further accumulated studies to clarify the ability of $\gamma\delta$ T cells as an allergic immunotherapy candidate are thus called for.

8. Concluding Remarks

There is ample evidence that $\gamma\delta$ T cells are involved in allergy. Recent studies in humans and mice suggest that they can both drive and regulate allergic immune responses through different mechanisms. However, many aspects of the characteristics and role of $\gamma\delta$ T cells in allergy remain to be fully elucidated in near future, for instance, the exact effects of various $\gamma\delta$ T cell subsets on allergic inflammation; the underlying relations between blood- and mucosa-associated $\gamma\delta$ T cells; how age and gender influence the population, distribution, and function of $\gamma\delta$ T cells in allergic diseases; the specific regulatory mechanisms of $\gamma\delta$ T cells in allergy; how $\gamma\delta$ T cells could be applied to prevent and treat allergic diseases, and so on.

Conflict of Interests

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

Acknowledgments

The study was supported by Grants from National Natural Science Foundation of China (no. 81371072) and Guangdong Natural Science Foundation (no. S2013010016386).

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 492872, 7 pages
http://dx.doi.org/10.1155/2014/492872

Research Article

Alternative Expression Pattern of MALT1-A20-NF-κB in Patients with Rheumatoid Arthritis

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Received 19 March 2014; Accepted 27 April 2014; Published 26 May 2014

Academic Editor: Qifa Liu

Rheumatoid arthritis (RA) is an inflammatory autoimmune disorder; abnormal T cell immunity plays a critical role in the development of RA. Recently, A20 was identified as a key negative regulator for T cell activation and inflammatory signaling and may be involved in RA pathogenesis. In this study, we analyzed the expression level of A20, NF-κB, and A20 regulatory factor mucosa-associated lymphoid tissue lymphoma translocation gene 1 (MALT1) in patients with RA. Real-time PCR was used to determine the expression level of MALT1, MALT-V1, A20, and NF-κB genes in RA and healthy individuals (HI). Significantly lower A20 expression was found in RA patients compared with those in the healthy group, while NF-κB overexpression could be detected in patients with RA. Moreover, the MALT1 and MALT1-V1 expression level was downregulated in RA patients. A positive correlation between MALT1 and A20 and MALT1-V1 and A20 was found in patients with RA, and a tendency towards a negative correlation was found between MALT1 and NF-κB, MALT1-V1 and NF-κB, and A20 and NF-κB. In conclusion, we first characterized the alternative expression pattern of MALT1, A20, and NF-κB in RA, which may be related to abnormal T cell activation.

1. Introduction

Rheumatoid arthritis (RA) is a common, chronic, systemic, and inflammatory autoimmune disorder that primarily affects the small diarthrodial joints of the hands and feet, affecting approximately 1% of the world's population [1–3]. The main characteristics of this disease are synovium hyperplasia, lymphocyte infiltration, and the abnormal proliferation of fibroblast-like synoviocytes (FLS) that can lead to the destruction of bone and cartilage and eventual disability [4]. Abnormal T cell immunity plays a critical role in the development of RA. Numerous factors that are involved in alternative T cell activation have been characterized, including the activation of inflammatory cells and expression of various cytokines. Inflammatory mediators, such as interleukin-6 (IL-6), interleukin-1 (IL-1), and tumor necrosis factor-α (TNF-α), are abundant in synovial tissues and fluid from patients with RA, and the overexpression of these cytokines promotes chronic inflammation and joint destruction. Many of the inflammatory mediators involved in the pathology of RA are regulated by nuclear factor kappa B (NF-κB) transcription factors [5, 6]. Abnormal NF-κB activation occurs during many pathological conditions including allergic and autoinflammatory diseases and malignancies [7].

Recently, A20, a negative regulator of NF-κB, was identified as a key regulator for inflammation signaling and may be involved in RA pathogenesis [8]. A20 has been reported to be ubiquitin-editing enzyme with several functions. A20 is also known as tumor necrosis factor-α- (TNF-α-) induced protein 3 (TNFAIP3), which was first discovered in 1990 by Dixit and colleagues as a cytokine-induced gene in human umbilical vein endothelial cells [9, 10]. Subsequent studies demonstrated that A20 overexpression inhibits NF-κB activation in response to different stimuli [11–13]. The cloning and

Table 1: Characteristics of RA samples.

Patient number	Gender	Age	Disease duration (mo)	RF (IU/mL)	ESR (mm/h)	CRP (mg/L)	DAS28 scores	CCP status
1	F	17	48	252.00	73	38.90	6.00	+
2	F	56	24	1940.00	111	7.40	6.78	+
3	F	51	2	17.50	77	37.70	7.09	+
4	F	43	60	74.30	37	5.34	5.53	+
5	F	62	72	38.30	69	17.00	6.88	+
6	F	32	120	19.90	85	9.06	7.51	ND
7	F	60	6	150.00	90	103.00	6.09	ND
8	F	26	9	31.00	32	1.84	4.31	+
9	F	53	6	368.00	89	31.90	7.41	−
10	F	54	9	9.19	64	33.25	7.39	−
11	F	45	12	102.00	82	68.98	6.26	+
12	F	53	240	65.30	76	27.08	7.91	+
13	F	71	12	58.90	110	71.35	6.79	+
14	F	63	12	153.00	32	4.84	5.23	+
15	F	33	84	299.00	42	18.70	5.61	+
16	F	60	6	10.10	41	0.57	6.63	−

Note: mo: months; F: female; +: positive; −: negative; ND: no detection.

characterization of the A20 promoter revealed two NF-κB DNA binding elements, which are recognition sequences for NF-κB transcription factors. It was also found that multiple NF-κB activating stimuli induce A20 expression via NF-κB sites in the A20 promoter [14]. Therefore, A20 has been demonstrated to downregulate its own expression, and it has been proposed that A20 participates in a negative feedback loop to attenuate TNF-α-induced inflammatory responses. A20 overexpression was subsequently demonstrated to block the NF-κB activation mediated by TNF-α, IL-1, LPS, phorbol esters, and hydrogen peroxide in different cell types [11, 12, 15–18]. This inhibition is most likely due to the inhibition of NF-κB activation in endothelial cells in response to proinflammatory stimuli, and an antiproliferative effect on smooth muscle cells has been observed upon A20 overexpression *in vitro*. All of these findings suggest that A20 attenuates the activity of proximal signaling complexes at proinflammatory receptors [19–21].

A20 is regulated by the CARMA1-Bcl-10-MALT1 (mucosa-associated lymphoid tissue lymphoma translocation gene 1) upstream signaling pathway complex, which bridges T cell antigen receptor (TCR) signaling with the canonical IκB kinase (IKK)/NF-κB pathway [20, 22–25]. TCR stimulation induces the recruitment of A20 and the Bcl-10 adaptor protein into the MALT1 complex, leading to MALT1-mediated A20 processing. Similarly, API2-MALT1 expression results in A20 cleavage. MALT1 cleaves A20 at arginine 439 and impairs its NF-κB inhibitory function. Therefore, A20 was identified as a MALT1 substrate, emphasizing the importance of the MALT1 proteolytic activity in "fine-tuning" T cell antigen receptor signaling [26].

A20 dysfunction by deletion or mutation was identified in numerous lymphocytic malignancies [27]. Recently, polymorphisms in the A20 region were reported in autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Crohn's disease, and psoriasis. Single nucleotide polymorphisms in the A20 region, including rs13192841, rs2230926, and rs6922466, have been independently associated with increased susceptibility for SLE [28, 29], and this finding provides a critical link between A20 and the etiology of SLE. More recently, it was shown that A20 deficiency in myeloid cells triggers erosive polyarthritis, resembling RA in a myeloid-specific, A20-deficient mice model [30]. There are three strongly associated genetic variants, including rs6920220, rs6927127, and rs6933404, which result in an A20 functional decrease in RA [8].

The etiology of RA remains to be understood; however, A20 deficiency may be a pivotal regulator for inflammation in RA. To characterize the role of A20 in RA, we analyzed the expression level of A20, NF-κB, and the A20 regulatory factor MALT1 in samples from Chinese patients with RA in this study.

2. Materials and Methods

2.1. Samples. This study included 16 patients with untreated RA (age: 26–71 years) and 20 healthy individuals (age: 22–70 years) who served as controls. The diagnosis of RA was based on the American College of Rheumatology criteria and expert opinion (1987 ACR criteria). All patients with RA were assessed for clinical disease activity by a trained rheumatologist using the disease activity score (DAS) [28]. The most recent erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and rheumatoid factor (RF) were collected (Table 1) [31].

All of the procedures were conducted according to the guidelines of the Medical Ethics Committee of the Health Bureau of Guangdong Province of China. Peripheral blood samples were collected by heparin anticoagulation, and peripheral blood mononuclear cells (PBMCs) were isolated using the Ficoll-Hypaque gradient centrifugation method. RNA extraction and cDNA synthesis were performed according to the manufacturer's instructions.

2.2. Quantitative Real-Time RT-PCR (qRT-PCR). The sequences of the primers for MALT1, A20, and NF-κB gene amplification are listed in Table 2. According to the structure of the MALT1 gene, there are two variants, that is, MALT1-V1 and MALT1-V2, and the latter contains a 33 bp deletion located between exons 6 and 8. To amplify the two MALT1 transcript variants, the MALT-V1-for and MALT-V1-rev primer pair was designed for MALT1-V1 amplification to cover the region that is missing in MALT1-V2, and the MALT1-for and MALT1-rev primer pair was designed to amplify the conserved region, which is contained by both variants.

The expression level of the A20, MALT1, MALT1-V1, NF-κB, and β2-microglobulin (β2M) genes was determined by SYBR Green I real-time PCR. Briefly, PCR in a 20 μL total volume was performed with approximately 1 μL of cDNA, 0.5 μM of each primer pair, 9 μL of 2.5 × Real Master Mix (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China), and 9 μL of dH_2O. After initial denaturation at 95°C for 15 minutes, 45 cycles of the following procedure were performed: 30 seconds at 95°C and 40 seconds at 60°C for the β2M, MALT1-V1, MALT1, A20, and NF-κB genes. The plate was read immediately after the 60°C step using an MJ Research DNA Engine Opticon 2 PCR cycler (Bio-Rad, Hercules, CA, USA) [32]. The relative amount of the genes of interest and β2M reference gene was measured in two independent assays. The specific, amplified PCR products were analyzed by melting curve analysis. The data are presented as the relative expression of the genes of interest compared with the internal control gene as determined by the $2^{(-\Delta CT)}$ method [33]. In addition, to analyze the MALT1-V1 expression characteristics, we calculated the MALT1-V1 expression ratio as MALT1-V1/MALT1 × 100%.

2.3. Statistical Analysis. Two independent-samples Wilcoxon tests were performed to compare the median expression level for each gene between patients with RA and the control group. Pearson correlation and linear regression analyses were used to determine the association between different genes in different groups. A $P < 0.05$ was considered statistically significant [34].

3. Results and Discussions

Abnormal T cell activation is a common feature of RA [35], and the upregulation of some positive regulating factors such as TNF-α, IL-6, and IL-2 was identified during the initiation of RA. In contrast, the downregulation of negative regulatory factors has the same effect on initiation of RA [16]. For example, T cell activation leads to the downregulation of A20 expression in mature thymocytes and peripheral T cells [9, 10]. Recently, abnormal A20 expression was described in patients with RA [8], and decreased A20 results in increased NF-κB expression and enhanced inflammation [25]. In this study, we analyzed the expression of A20 in 16 patients with RA in the active phase, and a significantly lower expression of A20 (median: 6.530) was found compared with those in the healthy group (median: 44.614, $P < 0.001$) (Figure 1(a)). These results are similar to findings of different reports examining mouse models or patients with RA [8, 36]. A20-deficient mice develop severe multiorgan inflammation. Moreover, it is well accepted that the activation of NF-κB-dependent gene expression plays a key role in the development of RA [30]; thus, decreased A20 expression may be a key reason for NF-κB overexpression in RA. Our study also demonstrated that NF-κB overexpression could be detected in patients with RA (median: 0.798) in comparison with healthy controls (median: 0.605, $P = 0.042$) (Figure 1(b)), indicating that decreased A20 resulting in NF-κB overexpression is also a common feature for Chinese patients with RA.

Lower A20 expression is associated with polymorphisms in the A20 genomic locus [29]; however, whether there is any dysregulation in A20 by upstream pathway factors is unknown. MALT1 is an upstream A20 pathway factor that cleaves A20 at arginine 439 and impairs its NF-κB inhibitory function. To characterize the relationship between MALT1 and A20 expression, we also examined the expression level of MALT1. Interestingly, the MALT1 expression level is downregulated in patients with RA (median: 0.541) compared with those in the healthy group (median: 1.638, $P < 0.001$) (Figure 2(a)). This result appears to be inconsistent with the lower A20 and higher NF-κB expression level results because MALT1 is also a positive regulatory factor of NF-κB. It is known that there are two MALT1 variants, MALT1-V1 and MATL1-V2, according to data in GenBank, and our previous study has found that these MALT1 variants could be identified by RT-PCR and sequencing (data not shown). In addition, we analyzed the expression level of the different variants, and similar results were found including the fact that a significantly lower MALT1-V1 expression level was detected in patients with RA (median: 0.062) compared with healthy controls (median: 0.140, $P < 0.001$) (Figure 2(b)).

Because we could not directly amplify MALT1-V2, which contains a 33 bp deletion, the expression level of MALT1-V2 could only be indirectly calculated by the relative expression of MALT1-V1/total MALT1 [37], and there are no significant differences in the ratio of MALT1-V1/total MALT1 between patients with RA and healthy controls (13.43 ± 7.98% versus 11.76 ± 6.66%), implying that the MALT1-V2 expression level was also downregulated in RA. Overall, either MALI1-V1 or MALT1-V2 was decreased in RA, unlike the finding in T cells from acute myeloid leukemia (AML), in which we found that the MALT1-V1 expression level was significantly higher in T cells from AML patients compared with healthy controls, while the MALT1-V2 expression level was downregulated [37]; this may indicate different expression pattern of these two MALT1 variants in RA. Little is known about

Table 2: List of primer information.

Primer	Sequences	Accession number	PCR products
A20-for	5′-CTGGGACCATGGCACAACTC-3′	NM_006290	182 bp
A20-rev	5′-CGGAAGGTTCCATGGGATTC-3′		
MALT1-V1-for	5′-AAGCCCTATTCCTCACTACCAG-3′	NM_006785.2	195 bp
MALT1-V1-rev	5′-CACTCCACTGCCTCATCTGTTC-3′		
MALT1-for	5′-TCTTGGCTGGACAGTTTGTGA-3′	NM_006785.2	230 bp
MALT1-rev	5′-GCTCTCTGGGATGTCGCAA-3′		
NF-κB-for	5′-CCACAAGACAGAAGCTGAAG-3′	NM_003998	149 bp
NF-κB-rev	5′-AGATACTATCTGTAAGTGAACC-3′		
β2M-for	5′-TACACTGAATTCACCCCCAC-3′	J00105	145 bp
β2M-rev	5′-CATCCAATCCAAATGCGGCA-3′		

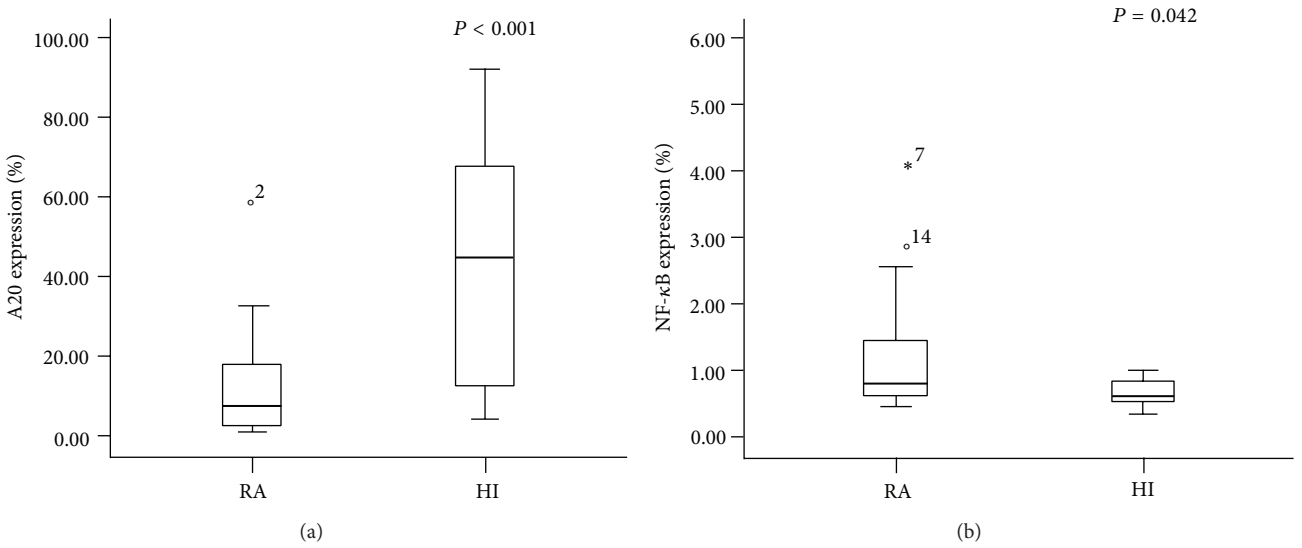

Figure 1: The expression level of A20 and NF-κB in patients with RA and healthy individuals.

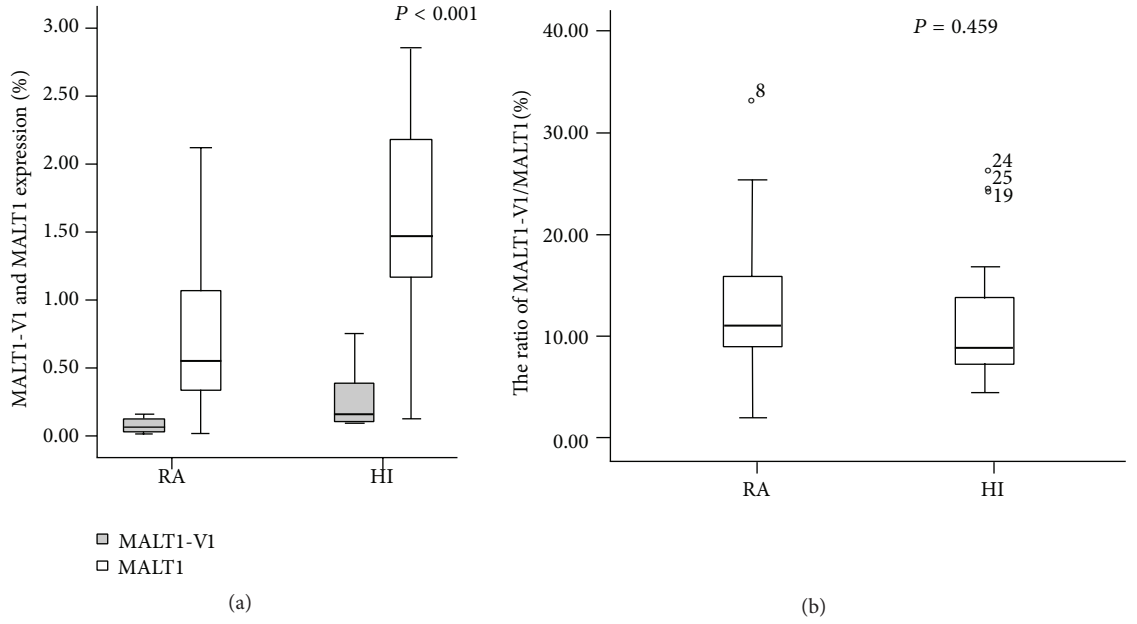

Figure 2: The expression level of MALT1-V1 and total MALT1 in patients with RA and healthy individuals.

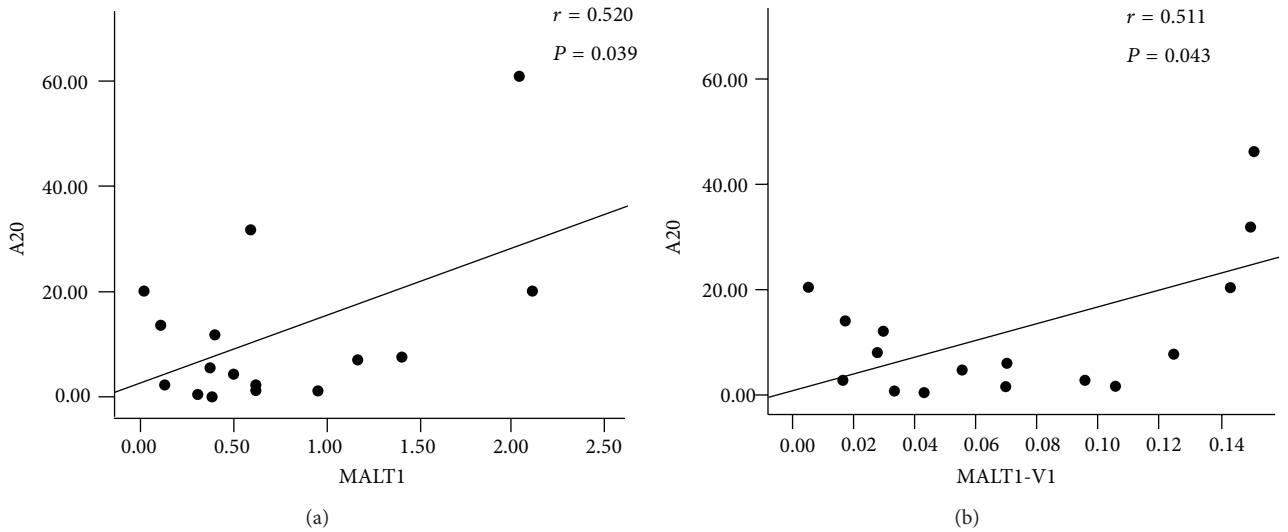

Figure 3: Correlation between the gene expression levels of MALT1 and A20 (a) and MALT1-V1 and A20 (b) in patients with RA.

the functional difference between the variants, and whether this is a feedback response from the expression pattern of A20 and NF-κB in RA is unknown because the impact of A20 cleavage by MALT1 on its capacity to regulate NF-κB has only been partially elucidated [24]. Further investigation is needed to characterize the upstream pathway regulators of A20 in addition to MALT1.

The role of MALT1 in the development of inflammation is largely unknown for RA and other autoimmune diseases, and only one study has reported that patients who had MALT-type lymphomas may also suffer from rheumatoid arthritis due to MALT1 dysfunction and continuous NF-κB activation [38]. A study has reported that MALT1 rearrangement in gastric MALT lymphoma is frequently associated with Sjögren's syndrome [39]. More recently, Brüstle A and coworkers indicated that MALT1 is a central cell intrinsic factor that determines the experimental autoimmune encephalitogenic potential of inflammatory Th17 cells *in vivo* [40]. It appears that MALT1 may play a role in the development of autoimmune diseases, and it may interfere with specific T cell subsets. Thus, our finding of lower MALT1 expression may imply a loss of the control of T cell activation in some T cell subsets in RA, which remains an open question. Further studies are needed to investigate the pathways upstream of MALT1 and TCR-CD3 signaling in different T cell subsets in RA.

In general, A20 is cleaved by MALT1; thus, the expression level of both genes should be negatively correlated with the expression pattern of A20 and MALT1 [24]. However, we found a positive correlation between MALT1 and A20 (r = 0.520, P = 0.039) (Figure 3(a)) and MALT1-V1 and A20 (r = 0.511, P = 0.043) (Figure 3(b)) in RA, and a tendency towards a negative correlation was found between MALT1 and NF-κB (r = −0.098, P = 0.718), MALT1-V1 and NF-κB (r = −0.204, P = 0.448), and A20 and NF-κB (r = −0.264, P = 0.322), indicating that the MALT1-A20-NF-κB expression pattern may be more complex in RA.

In conclusion, we characterized, for the first time, the alternative expression pattern of MALT1, A20, and NF-κB in RA, which may be related to abnormal T cell activation. Lacking A20 and MALT1 dysfunction are common characteristics of Chinese patients with RA, and our results provide new inflammation targets to consider for RA treatment. Moreover, further investigation is needed to follow up on patients with different MALT1-A20-NF-κB expression patterns and their association with cancer development.

Conflict of Interests

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

Authors' Contribution

Yangqiu Li contributed to the concept development and study design. Xu Wang, Lihua Zhu, Ziwei Liao, and Fan Zhang performed the real-time PCR. Ling Xu, Yan Xu, Shaohua Chen, and Lijian Yang performed the PBMC isolation, RNA extraction, and cDNA synthesis. Lihua Zhu and Yi Zhou were responsible for the collection of clinical data. Yangqiu Li, Xu Wang, Lihua Zhu, and Ziwei Liao coordinated the study and helped draft the paper. All authors read and approved the final paper. Xu Wang, Lihua Zhu, and Ziwei Liao contributed equally to this paper.

Acknowledgments

This study was supported by grants from the National Natural Science Foundation of China (no. 91129720) and a Collaborated Grant for HK-Macao-TW of the Ministry of Science and Technology (2012DFH30060).

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 241246, 6 pages
http://dx.doi.org/10.1155/2014/241246

Research Article

The Feature of Distribution and Clonality of TCR γ/δ Subfamilies T Cells in Patients with B-Cell Non-Hodgkin Lymphoma

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Received 30 March 2014; Accepted 3 May 2014; Published 21 May 2014

Academic Editor: Qintai Yang

Restricted T-cell receptor (TCR) Vα/Vβ repertoire expression and clonal expansion of $\alpha\beta$ T cells especially for putative tumor-associated antigens were observed in patients with hematological malignancies. To further characterize the $\gamma\delta$ T-cell immune status in B-cell non-Hodgkin lymphoma (B-NHL), we investigated the distribution and clonality of TCR Vγ/Vδ repertoire in peripheral blood (PB), bone marrow (BM), and lymph node (LN) from patients with B-NHL. Four newly diagnosed B-NHL cases, including three with diffuse large B-cell lymphoma (DLBCL) and one with small lymphocytic lymphoma (SLL), were enrolled. The restrictive expression of TCR Vγ/Vδ subfamilies with different distribution patterns could be detected in PB, BM, or LN from all of four patients, and partial subfamily T cells showed clonal proliferation. At least one clonally expanded Vδ subfamily member was found in PB from each patient. However, the expression pattern and clonality of TCR Vγ/Vδ changed in different immune organs and showed individual feature in different patients. The clonally expanded Vδ5, Vδ6, and Vδ8 were detected only in PB but neither in BM nor LN while clonally expanded Vδ2 and Vδ3 could be detected in both PB and BM/LN. In conclusion, the results provide a preliminary profile of distribution and clonality of TCR γ/δ subfamilies T cells in PB, BM, and LN from B-NHL; similar clonally expanded Vδ subfamily T cells in PB and BM may be related to the same B-cell lymphoma-associated antigens, while the different reactive clonally expanded Vγ/Vδ T cells may be due to local immune response.

1. Introduction

B-cell non-Hodgkin lymphoma (B-NHL) is a heterogeneous group of malignant lymphoproliferative disorders originating in B lymphocytes, which comprises approximately 80–85% of newly diagnosed cases with NHL. Although current therapeutic strategies, including standard chemotherapy, unlabeled or radiolabeled monoclonal antibodies, high-dose chemotherapy following autologous peripheral blood stem cell transplantation, or allogeneic hematopoietic stem cell transplantation, have significantly improved the outcome of this disease, the majority of patients relapse or become resistant to prior therapies. Therefore, novel strategies, such as cellular immunotherapy, are increasingly investigated [1].

Poor cellular immune function may relate to carcinogenic processes and to worse prognosis in tumor patients. Moreover, the progression of tumor might further induce the cellular immune suppression. In recent years, molecular analysis of the T-cell receptor (TCR) utilization feature, based on the principle of TCR α, β, γ, and δ gene rearrangement and deletion rearrangement, has proven to be an effective technique for studying the distribution of T cell repertoire, the diversity of TCR subfamilies [2, 3], the antigen specific expansion of T-cell clones, and the recent thymic output

function [4, 5]. This in turn can help to characterize the feature of host T-cell immune status and the identification of T-cell populations of interest in cancer, as well as the peripheral immune repertoire reconstitution after hematopoietic stem cell transplantation (HSCT).

T cells possessing a $\gamma\delta$ TCR are a small subset of human T cells (1–10% of all peripheral blood T cells). These cells share effector functions with $\alpha\beta$ T cells as well as with natural killer (NK) cells, particularly the capacity to interact with dendritic cells (DCs) [6, 7]. Mice deficient in $\gamma\delta$ T cells show a significantly increased incidence of tumors and provide clear evidence for an immune surveillance function of these unconventional lymphocytes [8]. Human Vγ9Vδ2 T cells can kill a broad spectrum of tumor cells with or without reduced MHC class I molecules expression in an MHC-unrestricted manner [9–14]. Moreover, $\gamma\delta$ T cells can migrate as infiltrating lymphocytes into solid tumors [9] and can recognize and eliminate cultured malignant cells (primary cells or cell lines) from myeloma [10, 11], non-Hodgkin lymphoma [12], prostate cancer [13], renal cell carcinoma [14], colon carcinoma [15], and squamous cell carcinoma [16]. Obviously, $\gamma\delta$ T cells play an important role in immunosurveillance and anticancer response and become more and more attractive for cell therapy strategies against cancer. However, little is known about $\gamma\delta$ T-cell immune status in B-NHL patients. Bartkowiak et al. have reported that the highly restricted TCR Vγ subfamily usage that is predominant for TCR VγII (Vγ9) was characterized in chronic lymphocytic leukemia (CLL) [17].

T cells recognize specific ligands by specific TCRs, which are glycoprotein heterodimers comprising either α/β or γ/δ chains. Rearrangement of the individual variable (V), diversity (D), joining (J), and constant (C) regions leads to the creation of the hypervariable complementarity determining region 3 (CDR3) of the functional TCR, which plays a pivotal role in the recognition of antigenic epitopes [18, 19]. $\gamma\delta$ T cells rearrange and express clonally diverse antigen receptors in a manner similar to $\alpha\beta$ T lymphocytes; however, the V, D, and J element repertoire in the TCR γ and TCR δ loci is limited in number. The TCR γ gene contains at least 14 functional variable (TCR Vγ) segments belonging to four subgroups (i.e., TCR VγI to IV), and the TCR δ gene contains at least eight functional TCR Vδ segments that are subdivided into eight Vδ subfamilies (i.e., Vδ1–Vδ8). Our previous study showed that restricted TCR Vα and Vβ repertoire expression and clonal expansion of $\alpha\beta$ T cells were observed in peripheral blood from patients with diffuse large B-cell lymphoma (DLBCL) [20]. To further characterize the $\gamma\delta$ T-cell immune status in B-NHL, we investigated the distribution and clonality of TCR Vγ and Vδ repertoire in peripheral blood (PB), bone marrow (BM), and lymph node (LN) from patients with B-NHL.

2. Materials and Methods

2.1. Samples. Four male patients with B-NHL diagnosed according to the World Health Organization (WHO) criteria were enrolled in the present study (designated as C1–C4). The clinical characteristics of these patients are described in Table 1. After the patient's consent, PB samples were obtained from all of the four patients, BM samples were obtained from 3 (C1, C2, and C3) of the four patients, and lymphoma-infiltrated LN samples were obtained from diagnosed biopsy in 3 patients (C1, C2, and C4), respectively. All procedures were conducted according to the guidelines of the medical ethics committees of the Health Bureau of Guangdong Province, China.

Table 1: Clinical characteristics of the included patients with B-NHL.

Patient number	Gender	Age (yrs)	Disease subtype	Ann Arbor staging
C1	Male	57	DLBCL	IVA
C2	Male	60	DLBCL	IVB
C3	Male	56	DLBCL	IVA
C4	Male	76	SLL	IIIB

DLBCL: diffuse large B-cell lymphoma; SLL: small lymphocytic lymphoma; yrs: years.

2.2. Mononuclear Cells Isolation, RNA Isolation, and cDNA Synthesis. Mononuclear cells of PB or BM samples (PBMCs or BMMCs) were isolated by Ficoll-Hypaque gradient centrifugation. RNA was extracted from PBMCs, BMMCs, or LN homogenate using a RNA extraction buffer according to the manufacturer's protocol (Trizol, Invitrogen, USA). The RNA quality was analyzed in 0.8% agarose gel stained with ethidium bromide. Two μg RNA was reversely transcribed into the first single-strand cDNA with random hexamer primers, using reverse transcriptase, Superscript II Kit (Gibco, USA). The cDNA quality was confirmed by RT-PCR for β2 microglobulin gene amplification.

2.3. RT-PCR for the TCR Vγ and TCR Vδ Subfamily Amplification. Three sense TCR Vγ primers and a single TCR Cγ reverse primer or eight TCR Vδ sense primers and a single TCR Cδ primer were used in unlabeled PCR for amplification of the TCR Vγ and Vδ subfamilies, respectively. Subsequently, a run-off PCR was performed with fluorescent primers labeled at 5′ end with the FAM fluorophore (Cγ-FAM or Cδ-FAM) (TIB MOLBIOL GmbH, Berlin, Germany). The sequences of primers are listed in Table 2. The PCR was performed as previously described [21]. Aliquots of the cDNA (1 μL) were amplified in 20 μL mixtures with one of the three Vγ primers and a Cγ primer or one of the eight Vδ primers and a Cδ primer. The final reaction mixture contained 0.5 μM of the sense and antisense primers, 0.1 mM dNTPs, 1.5 mM $MgCl_2$, 1 × PCR buffer, and 1.25 U Taq polymerase (Promega, USA). The amplification was performed in a DNA thermal cycler (BioMetra, Germany) with 3 min denaturation at 94°C and 40 PCR cycles. Each cycle consisted of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, respectively, and a final 7 min elongation at 72°C. The PCR products were stored at 4°C and ready for genescan analysis.

Table 2: List of primer sequences used in this study.

Primer	Sequence
VγI	5′-TACCTACACCAGGAGGGGAAG-3′
VγII	5′-GGCACTGTCAGAAAGGAATC-3′
VγIII	5′-TCGACGCAGCATGGGTAAGAC-3′
Cγ	5′-GTTGCTCTTCTTTTCTTGCC-3′
Cγ-FAM	5′-FAM-CATCTGCATCAAGTTGTTTATC-3′
Vδ1	5′-GTGGTCGCTATTCTGTCAACT-3′
Vδ2	5′-GCTCCATGAAAGGAGAAGCGA-3′
Vδ3	5′-CACTGTATATTCAAATCCAGA-3′
Vδ4	5′-TGACACCAGTGATCCAAGTTA-3′
Vδ5	5′-TCTGCACATTGTGCCCTCCCA-3′
Vδ6	5′-TATCATGGATTCCCAGCC-3′
Vδ7	5′-GAACATCACAGCCACCCAGACCG-3′
Vδ8	5′-ACTTCCAGAAAGCAGCCAAA-3′
Cδ	5′-AACAGCATTCGTAGCCCAAGCAC-3′
Cδ-FAM	5′-FAM-GTTTATGGCAGCTCTTTGAAGGT-3′

2.4. Genescan Analysis for TCR Vγ and TCR Vδ Subfamily Clonality. Aliquots of the unlabeled PCR products (2 μL) were subjected to a cycle of run-off reaction with fluorophore-labeled Cγ-FAM or Cδ-FAM primer, respectively. The labeled runoff PCR products (2 μL) were heat denatured at 94°C for 4 min with 9.5 μL formamide (Hi-Di Formamide, ABI, USA) and 0.5 μL of size standards (Genscan-500-LIZ, Perkin Elmer, ABI). The samples were then loaded into a 3100 POP-4 gel (Performance Optimized Polymer-4, ABI, USA) and resolved by electrophoresis in a 3100 DNA sequencer (ABI, Perkin Elmer) for size and fluorescence intensity determination using Genescan software [21]. Since the positions of the Vγ/Vδ and Cγ/Cδ primers are fixed, the length distribution observed in the PCR Vγ-Cγ/Vδ-Cδ products depends only on the size of the rearrangement of V-J (in TCRγ) or V-D, D-J (in TCRδ) gene segment and the randomly inserted nucleotides (VN(DN)J). After electrophoresis on an automated sequencer and subsequent computer analysis, the products of different size could be separated and expressed as different peaks.

3. Results

3.1. The Distribution and Clonally Expanded TCR Vγ and Vδ T Cells in Peripheral Blood from Patients with B-NHL. The expression of TCR Vγ and Vδ subfamilies was detected by RT-PCR, and no PCR products were scored as negative for the corresponding TCR subfamily by agarose gel electrophoresis. Only one or two of the three TCR Vγ subfamilies and four to six of the eight TCR Vδ subfamily members could be detected in PB samples. VγI, Vδ1, and Vδ2 were expressed in all samples, whereas VγII expression was absent in all of the four samples. In addition, VγIII (3/4), Vδ3 (2/4), Vδ4 (1/4), Vδ5 (1/4), Vδ6 (2/4), Vδ7 (2/4), and Vδ8 (2/4) could be detected in partial cases (Figure 1).

The clonality of TCR Vγ and Vδ subfamily T cells was identified by Genescan analysis, which was used to identify the CDR3 size and assess the spectratype pattern visually. Polyclonality of TCR Vγ and Vδ subfamily T cells displays a Gaussian or nearly Gaussian-like distribution consisting of multiple peaks (usually 6–8), and any deviation from the Gaussian profile (skewed repertoire) would indicate clonally expanded pattern. Oligoclonality and biclonality shows a skewed spectratype profile with a single dominant peak and double peaks, respectively. Oligoclonal trending is between polyclonal and oligoclonal. The PCR product analysis produces only one peak, which means that CDR3 lengths are identical, named as the monoclonal pattern [21, 22]. For the TCR Vγ and Vδ subfamily clonality analysis, only the clonally expanded VγIII subfamily was detected in samples from C2 and C4 cases, whereas clonally expanded Vδ T cells could be identified in almost all subfamilies (except for Vδ1) in different patients. Additional, at least one clonally expanded TCR Vδ subfamily member were found in PB in every patient. The distribution of the clonally expanded $\gamma\delta$ T cells in PB is shown in Figures 1 and 2.

3.2. Different Expression Patterns of TCR Vγ and Vδ in Peripheral Blood, Bone Marrow, or Lymph Node. The expressions of VγII/Vδ4/Vδ5/Vδ6/Vδ7/Vδ8 and VγII/Vδ5 were absent in BM and LN samples, respectively. Clonal expansion of TCR Vδ repertoire could be found in some TCR Vδ subfamilies in PB, BM, or LN, which displayed different patterns in different patients (Figure 3). In patient C1, the clonally expanded Vδ2, Vδ5, Vδ6, and Vδ8 subfamily T cells could be found in PB, whereas only the clonally expanded Vδ2 could be detected in BM, and there has been no clonal expansion of TCR Vγ/Vδ subfamilies in LN. In patient C2, the clonality of Vδ4, Vδ6, and Vδ8 changed from oligoclonality or oligoclonal trend in PB to polyclonality in LN, whereas the expression of those was absent in BM. In patient C3, the clonally expanded Vδ2/Vδ3 and Vδ1/Vδ2 T cells were found in PB and BM, respectively. In patient C4 with SLL, the clonally expanded Vδ3 and Vδ2/Vδ3 T cells were found in PB and LN, respectively. Interestingly, the oligoclonally expanded Vδ3 T cells could be found in PB from patients C3 and C4 and in LN from patient C4.

4. Discussion

Analysis of alterations in the TCR repertoire is an effective investigational approach that may help to understand involved immunological abnormalities and provide guidance for clinical applications using this information [23, 24]. Recent data indicated that T-cell immunodeficiency is a common feature in different hematological malignancies, including the absence of TCR Vα and Vβ subfamilies, decreased diversity of TCR repertoires, reduced thymic recent output function (naïve T cells), and lower frequencies of TCR subfamily naïve T cells [25]. Apart from $\alpha\beta$ T cells, $\gamma\delta$ T cells also play important roles in immunosurveillance and anticancer response. Different TCR Vγ and Vδ subfamily expression patterns have been reported in patients with leukemia, myelodysplastic syndrome (MDS), and immune thrombocytopenic purpura (ITP) [3, 17, 26, 27]. However,

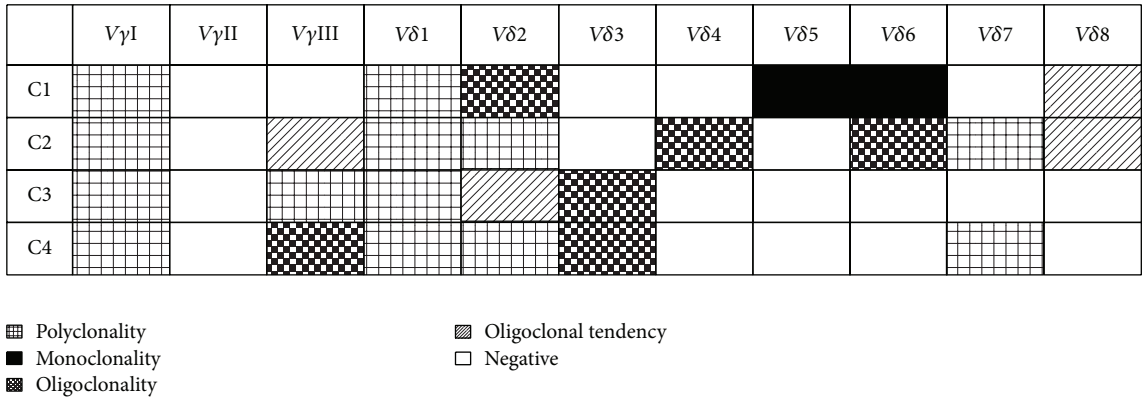

Figure 1: The distribution and clonality features of TCR Vγ and Vδ subfamilies in PBMCs from four patients with B-NHL (C1–C4).

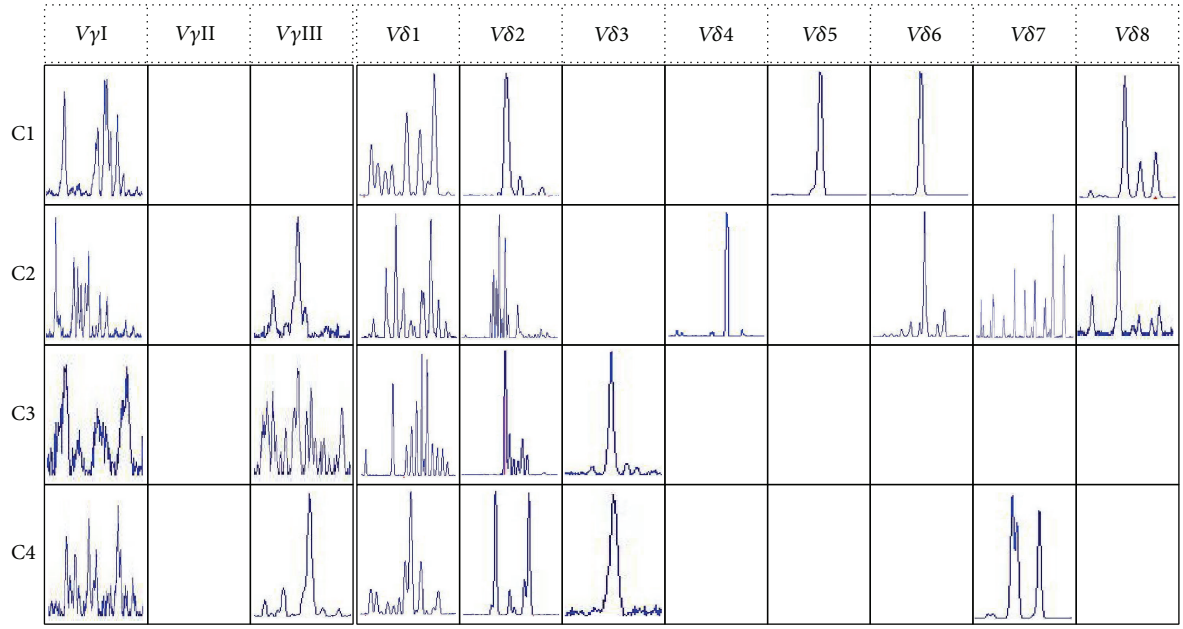

Figure 2: The results of Genescan of TCR Vγ and Vδ subfamilies in PBMCs from four patients with B-NHL (C1–C4).

little is known about the distribution and clonality of the TCR Vγ and Vδ subfamilies in B-cell lymphoma. In the present study, we analyzed the distribution and clonal expansion of TCR Vγ and Vδ T cells in four B-NHL patients and compared the different expression patterns of TCR Vγ and Vδ in peripheral blood, bone marrow, or lymph node in individual cases. In contrast to healthy individuals and patients with MDS or ITP previously reported [26, 27], the absence of TCR VγII subfamily was found in all blood, marrow, and lymph node samples from B-NHL patients, which imply a widespread restricted TCR Vγ repertoire expression pattern may be a feature in patients with B-NHL. Moreover, the distribution of Vγ and Vδ subfamilies was not identical in samples between peripheral blood, bone marrow, or lymph node, and this may be due to the distribution or expansion of $\gamma\delta$ T cells in different immune organs, however, further investigation is needed to summarize this feature in a large cohort samples and follow up the change of $\gamma\delta$ T-cell repertoire on the outcome of the patients.

Like the change of clonality of TCR subfamily in leukemia [17, 27], clonally expanded TCR Vδ subfamilies could be found in peripheral blood from all of four B-NHL patients, which is thought to be related to the tumor associated antigens [17, 20, 27]. In this study, we analyzed the distribution of clonally expanded Vδ T cells not only in peripheral blood, but also in bone marrow or lymph node, we were interested to find out the identical expanded Vδ T cell clones, similar clonal expanded Vδ2 subfamily T cells were detected in both peripheral blood and bone marrow samples in two cases with DLBCL, and similar clonality of Vδ3 subfamily T cells was identified in peripheral blood and lymph node in one case with SLL; these preliminary data suggested that these Vδ T-cell clones might respond to the same B-cell lymphoma-associated antigens. However,

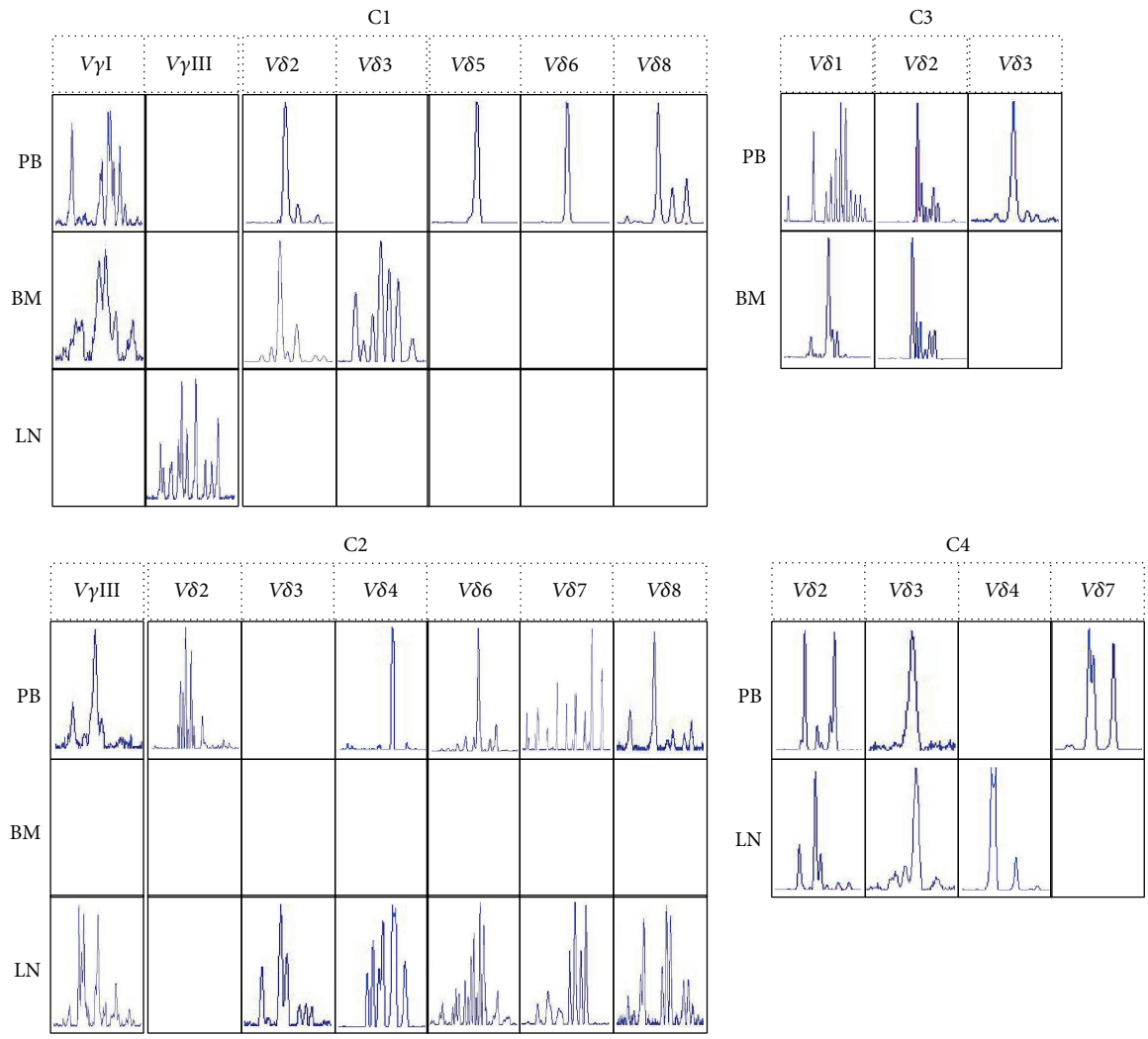

Figure 3: Different expression patterns of TCR Vγ and Vδ in peripheral blood (PB), bone marrow (BM), or lymph node (LN) from B-NHL cases (C1–C4).

different reactive clonally expanded Vδ T cells between peripheral blood, bone marrow, and lymph node may be due to local immune response. Further investigation is needed to determine whether these clonally expanded T cells are related to antilymphoma cells.

In addition, in contrast to the clonally expanded Vδ2, Vδ3, and Vδ4 T cells in lymph node from the SLL patient (C4), none clonal expansion of TCR Vγ or Vδ T cells was detected in lymphoma cell-infiltrated lymph node samples donated by the two cases with DLBCL (C1 and C2), suggesting that the deficiency of clonal expansion of $\gamma\delta$ T cells in lymphoma cell-infiltrated lymph node may be another feature in DLBCL. However, whether it is related to tumor microenvironments of lymph node remains an open question and needs to further explore.

5. Conclusion

In this study, we characterized the distribution and clonality of Vγ and Vδ repertoire in peripheral blood, bone marrow, and lymph node from B-NHL patients, we found obviously different features of restrictive usage and clonal proliferation of TCR Vγ and Vδ subfamilies in individual patients as well as in different immune organs; even if we found some identical clonally expanded Vδ subfamily T cells in peripheral blood and bone marrow, similar clonal expanded Vδ subfamily T cells in peripheral blood and bone marrow may be related to the same B-cell lymphoma-associated antigens, while the different reactive clonally expanded Vγ/Vδ T cells may be due to local immune response. However, whether it is related to different antigen stimulation and tumor microenvironments remains an open question and needs to further explore.

Conflict of Interests

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

Acknowledgments

This study was supported by Grants from the National Natural Science Foundation of China (no. 81270604),

the Guangdong Science & Technology Project (no. 2012B050600023), the Fundamental Research Funds for the Central Universities (no. 21614308), and the Medical Science Research Foundation of Guangdong Province (no. B2012195).

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 631951, 12 pages
http://dx.doi.org/10.1155/2014/631951

Review Article

Improving Cytomegalovirus-Specific T Cell Reconstitution after Haploidentical Stem Cell Transplantation

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Received 27 January 2014; Revised 13 March 2014; Accepted 19 March 2014; Published 24 April 2014

Academic Editor: Qifa Liu

Cytomegalovirus (CMV) infection and delayed immune reconstitution (IR) remain serious obstacles for successful haploidentical stem cell transplantation (haplo-SCT). CMV-specific IR varied according to whether patients received manipulated/unmanipulated grafts or myeloablative/reduced intensity conditioning. CMV infection commonly occurs following impaired IR of T cell and its subsets. Here, we discuss the factors that influence IR based on currently available evidence. Adoptive transfer of donor T cells to improve CMV-specific IR is discussed. One should choose grafts from CMV-positive donors for transplant into CMV-positive recipients (D+/R+) because this will result in better IR than would grafts from CMV-negative donors (D−/R+). Stem cell source and donor age are other important factors. Posttransplant complications, including graft-versus-host disease and CMV infection, as well as their associated treatments, should also be considered. The effects of varying degrees of HLA disparity and conditioning regimens are more controversial. As many of these factors and strategies are considered in the setting of haplo-SCT, it is anticipated that haplo-SCT will continue to advance, further expanding our understanding of IR and CMV infection.

1. Introduction

Haploidentical stem cell transplantation (haplo-SCT) is an alternative treatment for transplant candidates lacking a human leukocyte antigen- (HLA-) matched related or appropriate unrelated donor. After hematopoietic stem cell transplantation (HSCT), T cells are regenerated through thymic and peripheral pathways, with the thymus generating a more diverse T cell repertoire. Because thymic function is poor in adults, posttransplantation immune reconstitution (IR) in the months following transplant depends on the peripheral expansion of mature T lymphocytes in the allograft. Impaired recovery of adaptive immunity following haplo-SCT remains an outstanding issue and is associated with increased risk of infection, including bacterial, fungal, and cytomegalovirus (CMV) infections. CMV infection after haplo-SCT continues to adversely affect transplant outcomes [1–4] despite the use of prophylactic or preemptive treatment [5]. Lack of CMV-specific immune recovery has been reported as consistently associated with relapses of CMV infection and the development of CMV disease after allogeneic stem cell transplantation [6–9]. Therefore, this review summarizes the kinetics of CMV-specific T cell recovery and its association with CMV infection after haplo-SCT. Strategies to improve CMV-specific IR are also discussed.

2. Cytomegalovirus-Specific T Cell Immune Reconstitution after Haplo-SCT (Table 1)

2.1. Manipulated (T Cell Depleted, TCD) Haplo-SCT. Using a megadose of extensively T cell depleted, G-CSF-mobilized stem cells and a fludarabine-based conditioning protocol [10], the Perugia group demonstrated that haplo-SCT could be successful in patients with acute leukemia. Early results [2] showed a nonrelapse mortality rate of 40%, with infection as the leading cause of death, mainly CMV and *Aspergillus*. Additionally, improved IR and fewer deaths secondary to

Table 1: CMV-specific immune recovery after haploidentical stem cell transplantation.

Group/ Reference	Number	Disease	Graft manipulation	Conditioning	NRM or TRM	CMV infection	Immune reconstitution (IR)	Comments
TCD haplo-SCT								
Perugia; [2]	17	End-stage chemoresistant leukemia	Extensively TCD	TBI + ATG + Cy + Thio	40% NRM; mainly CMV and *Aspergillus* infection	NR	NR	
Perugia; [11]	43	Acute leukemia	Extensively TCD	TBI + ATG + Flu + Thio	The infection-related mortality rate 25–35%	NR	CD4+ $>0.1 \times 10^9$/L at day 60 and $>0.3 \times 10^9$/L at day 180	
Lilleri et al.; [12]	48	Malignant or nonmalignant hematological diseases	T cell-depleted peripheral blood CD34+ progenitor cells	ATG + TBI or chemotherapy	9% in R+ or 8% in R− (1-year)	4% in R− and 83% in R+	61% recipients reconstituting CMV-CTL within the first 3 months	Young patients
Chen et al.; [13]	22	Refractory hematological malignancies	Mobilized peripheral blood stem cells depleted of CD3+ cells	Flu + Thio + Mel + OKT3	NR	1/22 patients developed CMV infection	The median number of CD4+ and CD8+ T cells was about 0.2×10^9/L and above 0.1×10^9/L at 3 months	Pediatric recipients
Federmann et al.; [14]	28	Hematological malignancies	CD3/CD19-depleted grafts	Flu or (Clo) + Thio + Mel + OKT-3	NR	Eight of 28 patients had cytomegalovirus reactivation	A median of 205 CD3+ cells/μL, 70 CD3+ CD4+ cells/μL, and 66 CD3+ CD8+ cells/μL on day 100	
Pérez-Martínez et al.; [16]	30	Acute leukemia	CD3/CD19-depleted	Flu + Bu + Thio + mP	23% NRM (7/30)	Two of 30 patients have died because of CMV pneumonia	A median of 167 ± 64/μL CD4+ cells versus 364 ± 174/μL CD8+ cells on day 30, 155 ± 47/μL versus 410 ± 119/μL on day 60, and 217 ± 72/μL versus 537 ± 192/μL on day 90.	Children
Unmanipulated haplo-SCT								
Peking University; [21]	50	Hematological diseases	G-CSF-primed bone marrow and unmanipulated PBSCs	Ara-C + Bu + Cy + simustine + ATG	19.5 ± 6.0% NRM (2-year)	The cumulative incidence of CMV antigenemia in the early posttransplant phase was 49.9 ± 7.2%	CD4+ T cells at 152.91 (13.29–579.63)/μL on day 90, 163.28 (23.29–875.60)/μL on day 180, and 277.49 (16.91–579.48)/μL on day 365; CD8+ T cells at 672.79 (48.23–2,556.01) on day 90, 918.42 (115.00–4,047.91)/μL on day 180, and 884.16 (175.84–2,441.58)/μL on day 365	
Peking University; [22]	42	Malignant hematological disorders	G-CSF-primed bone marrow and unmanipulated PBSCs	Ara-C + Bu + Cy + simustine + ATG	24% NRM (10/42)	The cumulative incidence of CMV reactivation was 87.67% (75.70–95.48%); 5 of them had CMV disease (day 22–50).	The CD8+ T cell count equaled that of controls at day 60, and the median number of CMV-CTL cells was comparable to that of controls from day 30 to day 360	

Table 1: Continued.

Group/ Reference	Number	Disease	Graft manipulation	Conditioning	NRM or TRM	CMV infection	Immune reconstitution (IR)	Comments
Kurokawa et al.; [24]	66	Hematologic malignancies	Unmanipulated PBSCs and/or bone marrow	ATG + BU + Mel with TBI or Flu	11% NRM (7/66)	CMV antigenemia occurred in 45 of 57 evaluable patients	CD3+ >1600/μL at day 180 and CD8+ >1200/μL at day 180. The lowest numbers of CD3+, CD4+, and CD8+ T cells were seen at 1 month after transplantation but all continued to rise until 6 months after transplantation.	
Lee et al.; [25]	83	Acute leukemia and myelodysplastic syndrome	Unmanipulated PBSCs	BU + Flu + ATG	18% (95% CI, 12%–29%) TRM	Fifty-eight of 72 evaluated patients (81%) had CMV pp65 antigenemia.	CD8+ lymphocyte counts exceeded pretransplantation levels at 2 months, and >90% of patients maintained counts >200/μL at 3 months after transplantation. 12 months after transplantation, 24 of 27 patients (89%) had CD4+ lymphocyte counts more than 200/μL.	
Kanda et al.; [26]	12	Hematologic malignancies	Unmanipulated PBSCs	Alemtuzumab + TBI or Flu based	17% NRM (2/12)	Ten of the 12 patients experienced CMV reactivation, and CMV disease was observed in three patients	CD3+/CD4+ and CD3+/CD8+ T cells were strongly suppressed within 2 months after haploidentical peripheral blood SCT but recovered on day 90. CMV-CTLs were detected on day 90 at 0.03–0.25% of CD8+ T cells	
Rizzieri et al.; [27]	49	Hematologic malignancies or marrow failure	Unmanipulated PBSCs	Flu + Cy + Alemtuzumab	31% NRM (15/49)	Twenty-five percent of patients experienced a severe infection, whereas 86% experienced reactivated CMV	The median number of CD4+ and CD8+ T cells was about 100/μL and 400/μL at 3 months for transplant recipients without GVHD.	

TCD: T cell-depleted; PBSC: peripheral blood stem cell; NRM: nonrelapse mortality; TRM: treatment-related mortality; CMV: cytomegalovirus; R−: CMV-negative recipients; R+: CMV-positive recipients; CMV-CTL: cytomegalovirus-specific T cells; NR: not reported; TBI: total body irradiation; ATG: antithymocyte globulin; Cy: cyclophosphamide; Thio: thiotepa; Flu: fludarabine; Mel: melphalan; Clo: clofarabine; Bu: busulfan; mP: methylprednisolone; Ara-C: cytosine arabinoside.

infection occurred when G-CSF was eliminated from the regimen [11]. The results showed that in patients not treated with G-CSF, CD4+ cell counts were greater than 0.1×10^9/L 60 days after transplantation and greater than 0.3×10^9/L at 180 days. Subsequently, Lilleri et al. [12] performed a study with 48 young patients who received a TCD, allogeneic myeloablative HSCT from an HLA-disparate relative. The 1-year cumulative incidence of both CMV infection and specific T cell reconstitution was 83% among the 23 CMV-seropositive patients, and these incidences were 4% and 8%, respectively, among the 25 CMV-seronegative patients [12]. CMV-specific T cell (CMV-CTL) reconstitution was significantly delayed in patients receiving TCD grafts compared with patients receiving unmanipulated HSCTs [12].

Reduced intensity conditioning (RIC) is used to minimize toxicity while allowing rapid engraftment and expediting immune reconstitution during the early posttransplant period, thereby protecting the host from infection. Data showed that IR was rapid in 22 pediatric recipients after RIC and CD3-depleted haplo-SCT and was similar to, if not better than, outcomes obtained after myeloablative haploidentical transplantation [13]. CMV was detected in only one patient in this group, and no patient had died of viremia. In an attempt to reduce the risk of graft-versus-host disease (GVHD) and Epstein-Barr virus-related lymphoproliferative disease, Federmann et al. used CD3/CD19-depleted grafts with RIC and observed that T cell reconstitution after haplo-SCT was delayed with a median of 205 CD3+ cells/μL, 70 CD3+ CD4+ cells/ul, and 66 CD3+ CD8+ cells/μL on day 100, respectively [14]. Eight of the 28 patients had CMV reactivation, and no deaths due to infections were observed. Bader et al. reported their experience of CD3/CD19-depleted haplo-SCT for 22 children with acute leukemia [15]. Reconstitution with T cells can start on day 30 and the early T cell regeneration following transplantation results from the expansion of T precursor cells contained in the stem cell transplant. Thymus-dependent T cell regeneration only begins on day 100. In contrast to these published data, reports from Pérez-Martínez et al. using allogeneic CD3/CD19-depleted grafts showed that T cell recovery achieved normal values within the first 60 days after transplantation [16]. And up to 2 years, 2 of the 30 patients had died because of CMV pneumonia. Similar results were reported in patients with acquired severe aplastic anemia [17].

2.2. Unmanipulated Haplo-SCT. Although extensive depletion of T cells or selective depletion of alloreactive T cell subsets improves engraftment and reduces GVHD, this manipulation is associated with prolonged immune deficiencies and increased risk of infection. In an attempt to perform haplo-SCT without T cell depletion, Peking University researchers developed the GIAC protocol for haplo-SCT by combining G-CSF-primed bone marrow and unmanipulated PBSCs [18–22] (Figure 1). Using this protocol [23], we previously observed that patients undergoing haplo-SCT had a higher 100-day cumulative incidence of CMV antigenemia compared with a matched group (65% versus 39%), whereas the incidence of CMV-associated interstitial pneumonia was

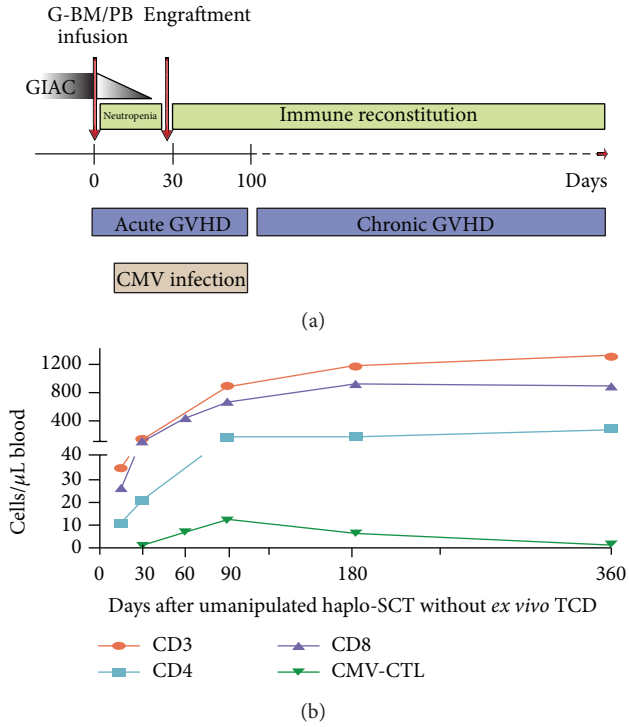

Figure 1: T cell immune reconstitution and CMV infection following unmanipulated haplo-SCT without *ex vivo* TCD (GIAC transplant protocol, Peking University Institute of Hematology). CMV, cytomegalovirus; GVHD, graft-versus-host disease; CMV-CTL, CMV-specific CTL; TCD, T cell depleted; G-BM/PB, combining G-CSF-primed bone marrow (G-BM) and peripheral blood (G-PB) harvests.

the same between the two groups (17% versus 17%). We investigated IR in patients with hematological malignancies after haploidentical transplantation and HLA-matched transplant [21]. Compared with those from HLA-matched recipients, T cell subset cell counts in the first 90 days after grafting were lower in haploidentical recipients. The difference was most striking for CD4+ and CD4+ naïve T cells. T cells appeared equally functional among patients without GVHD from both groups. Furthermore, we prospectively investigated CMV-CTL IR in 42 recipients after haplo-SCT [22]. CMV reactivation occurred in 36 of the 42 patients, but only 5 had CMV disease. The CD8+ T cell count in transplant recipients was equal to that of controls at day 60 after transplantation. The median number of CMV-specific T cells and the subsets to which they belonged was comparable to those of the controls from day 30 to day 360. Furthermore, CMV-CTLs from transplant recipients were found at high frequencies and demonstrated robust proliferation capacities and interferon-γ responses at 1 year after transplantation.

Recent reports showed that it is feasible to perform haplo-SCT without *ex vivo* TCD after RIC. Kurokawa et al. from Japan [24] conducted haplo-SCT on 66 adults with hematologic malignancies using RIC without TCD. CMV antigenemia occurred in 45 of 57 evaluable patients at a median of 19 days after transplantation. CMV-related diseases were diagnosed in 3 patients, and one patient died of CMV-colitis.

The lowest numbers of CD3+, CD4+, and CD8+ T cells were observed at 1 month after transplantation, but all values continued to increase until 6 months after transplantation and remained stable thereafter [24]. Data from a Korean study [25] showed a RIC therapy with busulfan, fludarabine, and antithymocyte globulin (ATG) for haplo-SCT in acute leukemia and myelodysplastic syndrome. Fifty-eight of 72 evaluated patients (81%) had at least 1 positive assay result for CMV pp65 antigenemia. Four patients developed CMV disease, and 3 of them died of CMV-colitis per se or of other causes. Despite the use of ATG, CD8+ lymphocyte counts exceeded pretransplantation levels at 2 months, whereas CD4+ lymphocyte counts recovered more slowly, with only approximately half of all patients showing CD4+ lymphocyte counts > 200/μL at 2 to 6 months after transplantation [25].

Alemtuzumab, which has a strong lympholytic effect, is usually used against GVHD in a reduced intensity conditioning regimen. Using an *in vivo* alemtuzumab-based regimen, Kanda et al. [26] reported that CD3+/CD4+ and CD3+/CD8+ T cells were strongly suppressed within 2 months after haploidentical peripheral blood SCT but recovered on day 90. CMV-specific cytotoxic T lymphocytes were detected on day 90 after transplantation in two patients and represented 0.03% and 0.25% of CD8+ T cells, respectively, for each patient. Ten of the 12 patients experienced CMV reactivation, and CMV disease was observed in three patients but was not fatal. Rizzieri et al. [27] extended the prior work and reported the large series assessing outcomes and immune reconstitution in nonmyeloablative haplo-SCT for 49 patients with alemtuzumab-based regimen. Twenty-five percent of the patients experienced a severe infection, whereas 86% experienced reactivated CMV with CMV disease in seven patients. Quantitative lymphocyte recovery through expansion of transplanted T cells was noted by 3 to 6 months [27]. Recently, Kanda et al. [28] updated their transplant data with continued use of *in vivo* T cell depletion with alemtuzumab. Nine patients experienced positive CMV antigenemia with CMV disease in three patients, none of which was fatal. The numbers of CD4+ and CD8+ T-cells remained low within one year after HSCT. The median quantities of CMV-specific CD8+ T lymphocytes as measured by the tetramer-based assay were 0.05%, 0.01%, and 1.83% at 90, 180, and 365 days after HSCT, respectively.

3. Cytomegalovirus Infection Associated with T Cell Immune Reconstitution

IR of the immune subsets is likely to have the greatest impact on clinical outcomes after haplo-SCT [29]. In healthy CMV-seropositive individuals, high frequencies of CMV-specific CD4+ and CD8+ T cells that mediate control of viral reactivation can be detected [30, 31]. Both the quantity and quality of CMV-specific T cell recovery are essential for immune control of CMV infection following HSCT. A strategy of deferred antiviral therapy based on the presence of a detectable functional CMV-specific T cell response at the time of documentation of CMV DNAemia was clinically administered and allowed for the sparing of antiviral treatment in transplant patients [32, 33]. A recent phase II study by Blyth et al. showed that donor-derived CMV-specific T cells reduce the requirement for CMV-directed pharmacotherapy without increased GVHD after allo-HSCT [34].

In immunocompromised HSCT recipients, few patients with levels of CMV-specific CD8+ lymphocytes > 2×10^6–10×10^6/L developed CMV disease [35–37]. Both CD4+ and CD8+ CMV-specific IR are required for protection from recurrent activation [38–40], and an absolute CD4+ and CD8+ T cell response at day 60 may confer protection against viremia in young patients [41]. Borchers et al. [42] reported that the presence of CMV-CTL before day 50 and their expansion after reactivation appear to protect against recurrent CMV reactivation. In patients with uncontrolled reactivation, differentiated CMV-specific T cells of the late differentiation phenotype CD45RA+CD27−CD28− did not develop [37]. Furthermore, Lilleri et al. [12] found that detection of CMV-specific T cells also correlated with control of CMV infection after T cell depleted haplo-SCT.

In our own analysis [43], high CMV-CTL with terminally differentiated effector CD45RO−CD62L− (T_{EMRA}) phenotype in the allografts was associated with reduced risk of CMV reactivation when sufficient CD45RO+CD62L− cells (T_{EM}) were provided by infusion ($\geq 0.208 \times 10^6$/kg). Early after transplantation, there was significant expansion of CMV-CTL with central memory CD45RO+CD62L+ (T_{CM}) phenotype when CMV was reactivated [23, 43]. We further investigated CMV-CTL in bone marrow (BM) after haplo-SCT. BM-resident CMV-CTLs displayed distinct phenotypes when CMV was reactivated [23], as there are more T_{EMRA} in the BM at day 360 after SCT and relatively higher T_{Naive} cells (CD45RO−CD62L+) in the BM at day 90 in patients with infection compared with those without infection. This result suggested that CTL in BM may play an important role in controlling CMV infection, as mature T cells in the BM play an essential role in maintaining normal peripheral T cell numbers, and CMV-CTL could therefore be more efficiently derived from the BM than from the PB [44, 45].

4. Factors Influencing CMV-Specific IR

The process of IR is influenced by patient- and transplant-related factors, such as donor and patient ages, primary disease, transplant type, conditioning regimen, stem cell source, HLA disparity, GVHD, and infection [46]. Not surprisingly, the intensity of immunosuppression and the degree of T cell depletion in transplant protocols, such as ATG or alemtuzumab, both critically affect the risk of CMV reactivation [47]. As for CMV-specific IR after haplo-SCT, there are several influences, except graft manipulation described above.

4.1. Donor and Recipient CMV Serostatus. CMV-negative recipients of grafts from CMV negative donors (D−/R−) rarely develop CMV infection and D− should be chosen when possible. Ljungman et al. reported that only acute GVHD grade II–IV and D−/R+ were significant risk factors for CMV disease after multivariate analysis [48]. D+/R+ transplants,

on average, generate higher levels of multifunctional CMV-specific T cells and require less antiviral therapy compared with D−/R+ transplants [49]. D+/R+ patients had a lower cumulative incidence of CMV reactivation, recurrent reactivation, CMV disease, and mortality compared with D−/R+ patients [50]. Pretransplant human CMV infection of the recipient is a major factor driving human CMV-specific immune reconstitution [12]. Our previous data also suggested protective immunity could be transferred by infusion of CMV-CTL within allografts [43]. Nevertheless, Pietersma et al. found that reactivation of CMV infection occurred more frequently in patients receiving a CMV-positive graft but was less severe than in patients receiving a CMV-negative graft [51]. These data suggest roles for both virus and CMV-specific immunity present in the graft. Based on current knowledge, the use of D+/R+ transplant is preferred for improved IR, and D−/R− is preferred for decreased risk of CMV infection. Other donor/recipient combinations remain to be confirmed in clinical trials. Determining CMV serostatus and levels of CMV-CTL in the donor grafts may help in controlling CMV reactivation, which is closely correlated with immune reconstitution and differentiation of CMV-CTL subsets.

4.2. Stem Cell Source and Graft Composition. Numerous studies have compared IR during SCT using different stem cell sources. IR after peripheral blood stem cell transplantation (PBSCT) is generally characterized by faster myeloid and lymphoid recovery versus BMT [52–54]. Along with accelerated and sustained naïve CD4+ recovery, improved *in vitro* proliferative responses have been measured following PBSCT [52–54]. Hakki et al. suggested that BM as the source of stem cells resulted in delayed recovery of functional T cell immunity at 3 months after transplantation [39]. In the setting of HLA-matched sibling transplantation, recipients receiving PBSCT had lower risks of documented bacterial, fungal, and viral infection, including CMV viremia [52]. These data clearly indicate rapid T cell reconstitution and a lower incidence of CMV infection when PBSCT is used.

Transplantation using PBSCs with *ex vivo* TCD is the most common HLA-mismatched/haploidentical transplant approach in Europe and the United States [55]. In China, Peking University researchers combined G-BM and G-PB harvests (G-BM/PB) without *ex vivo* TCD for the GIAC protocol and achieved encouraging results [18–21]. Recently, unmanipulated PBSCT [56] and unmanipulated G-BM [57] have been accomplished in haplo-SCT settings with very encouraging results. However, limited data are available concerning CMV-specific IR after haplo-SCT. Lilleri et al. reported that children receiving T cell depleted transplants exhibited significantly delayed CMV-specific T cell reconstitution, and only D− and BM as a stem cell source were found to significantly delay CMV-specific T cell reconstitution [12]. A small comparative series showed better survival among patients who received T cell-replete transplants, with less viral infections, including CMV reactivation, and better immune reconstitution of T cell subsets compared with T cell-depleted haplo-SCT [58]. Reconstitution of CMV-specific T cell immunity may have proceeded at a faster rate in patients treated with our GIAC protocol than in patients described in other reports of haplo-SCT [23]. A differential pattern of T cell reconstitution is expected after *in vivo* TCD and *ex vivo* TCD haplo-SCT. In TCD haplo-SCT, the time lapse during IR, even in the absence of GVHD, is most likely lengthened by extensive *ex vivo* T cell depletion itself, while greater numbers of donor T cells cotransfused with allografts are not immediately eradicated by *in vivo* TCD. The effect of *in vivo* T cell depletion could be balanced by graft infusion at the time of transplantation [43, 59]. Therefore, using PBSCT or G-BM/PB is preferred for IR to CMV.

4.3. Conditioning Regimens. Although limited, studies comparing IR following myeloablative and nonmyeloablative regimens have been insightful. Maris et al. compared IR for one year after transplantation in 51 patients receiving HLA-matched PBSCT following nonmyeloablative conditioning with a reference group of 67 myeloablative recipients [60]. Both regimens demonstrated similar levels of total and subset-specific lymphocyte recovery, lymphoproliferative responses to viral stimulants, and in total and pathogen-specific antibody levels. Overall infection rates were significantly lower in nonmyeloablated patients, who also had lower rates of CMV infection coinciding with greater numbers of CMV-specific T cells at days 30 and 90. Data from Nakamae and colleagues showed [61] that residual host cells after nonmyeloablative SCT reduce progression to higher CMV viral load in transplant recipients; however, this effect does not appear to protect against serious complications of CMV. Recent results [62] showed that CMV reactivation was less common in the RIC group during the midrecovery period, while there was no difference during the late-recovery period. CMV disease is as much of a problem following nonmyeloablative transplantation as it is following myeloablative transplantation [61, 63].

4.4. GVHD and Steroid Administration. The deleterious effects of acute GVHD on T cell function are well established. GVHD inhibits T cell recovery through T cell apoptosis via activation-induced cell death, immunosuppressive cytokine production by regulatory cell populations, and direct damage to thymic epithelium and stroma [64, 65]. GVHD appears to adversely affect all levels of T cell function, from delaying T cell ontogeny and limiting TCR diversity to impairing cytokine production in recovered T cells. Multivariable analysis showed that patients receiving methylprednisolone had a 1.5 times higher risk of infection, with acute GVHD being another independent risk factor for infections after transplantation [66]. Steroids can suppress immune function by promoting the development of high IL-10-producing regulatory T cells and inhibiting GATA-3 phosphorylation [67, 68]. High-dose steroid use (≥2 mg/kg/d) predicts delayed recovery of functional T cell immunity at 3 months after transplantation [39]. Özdemir et al. [69] showed that steroid administration resulted in a significant impairment in CD8+ tumor necrosis factor α (TNFα) production but not a decrease in the frequency of CMV-specific CD8+ T cells. Corticosteroid treatment may favor active viral replication even in patients

with CMV-specific T cells [12]. These findings have implications regarding the tapering of steroids in patients with active infections normally controlled by T cell responses, such as CMV disease.

4.5. Subclinical CMV Reactivation. It is known that CMV infection drives T cells to an effector phenotype in healthy individuals [70]. Subclinical CMV reactivation while on ganciclovir appears to be a potent stimulator of T-cell function [39]. Among patients who received ganciclovir at engraftment, those who had breakthrough antigenemia had significantly better recovery of T cell function at 3 months compared with patients who remained antigenemia negative [39]. In the setting of HSCT and the absence of high-dose steroids, low-level, short-term antigenemia may, in fact, have a protective effect by enhancing late immune function. CMV infection is required for the generation and/or maintenance of the CMV-specific T cell pool, and reactivation of latent virus was identified as the main factor leading to immune reconstitution [12, 41]. Our data also showed that CMV-CTLs with a central memory CD45RO+CD62L+ phenotype significantly expanded when CMV was reactivated [23, 43]. However, prolonged CMV reactivation may lead to exhaustion of T cells, as has been suggested for other antigens [71]. These studies suggested that subclinical CMV reactivation, but not persistent CMV reactivation, may be required for the reconstitution of CMV-specific T cell responses.

4.6. Age and Degree of HLA Disparity. Children may have a better capacity than adults to develop anti-CMV primary immune responses after HSC transplantation [41]. Patients <8 years of age demonstrate improved IR, with a probability ratio of 4.57, and this likely results in better reconstitution of CMV-specific CD4+ and CD8+ T cells [12]. Increased thymic function might be responsible for better immune reconstitution in younger children [72], especially when compared with adult patients in whom naive thymic emigrants have been reported to appear in the circulation only 6 months after receipt of a T cell depleted HSCT [73]. Recently, Azevedo et al. [74] investigated long-term IR after RIC based haplo-SCT with TCD, which followed by preemptive donor lymphocyte infusions (DLI). They found the proportion of naive and memory subsets in the recipients, both within CD8+ and CD4+ T cells, more closely resembled that observed in age-matched control subjects than in the donors. Their data [74] suggested that long-term IR was successfully achieved after haploidentical HSCT, a process that appears to have largely relied on de novo T cell production. IR after haplo-SCT is usually slower than that after matched-sibling donor or matched-unrelated donor transplants [75]; however, the impact of HLA disparity on CMV-specific IR after haplo-SCT remains uncertain.

5. Adoptive Immunotherapy to Accelerate CMV-Specific T Cell Immune Reconstitution

Any further reduction in CMV infection after haplo-SCT will only be achieved by hastening posttransplant IR. To improve posttransplant IR, various strategies of adoptive donor T cell immunotherapy have been investigated over the past years. However, T cell-based adoptive therapy is problematic in the adult haploidentical transplant setting, for alloreactivity still exists. Research is focusing on strategies to hasten IR by adding back broad-repertoire or pathogen-specific mature donor T lymphocytes after *ex vivo* depletion of antidonor alloreactivity [76, 77].

Amrolia et al. demonstrated an accelerated immune reconstitution in 16 patients who received adoptively transferred T cells that were allodepleted *in vitro* [78]. After 2 to 4 months, CMV-specific T cells and a broad Vβ T cell receptor repertoire could be observed, while the incidence of GVHD was low. Posttransplantation CD8-depleted DLI can also contribute to improved T cell recovery after haplo-SCT for the treatment of advanced hematologic malignancies, while reducing the incidence and severity of acute GVHD [79]. Despite the high incidence of CMV reactivation (82%), no patients developed CMV disease. Circulating CD3+/CD4+ T cells significantly increased at day 120 after DLI, while the expansion of CD3+/CD8+ was at a median value of 23/μL. Preliminary studies using gene engineering of donor lymphocytes to deplete alloreactive T cells appear to be promising [80, 81], but larger-scale investigations are warranted to confirm the results.

Given high degree of mismatching makes cell immunotherapy impossible, Perruccio et al. [76] improved the immune recovery after myeloablative haploidentical SCT by the infusion of nonalloreactive clones specific for CMV and *Aspergillus*. Within 3 weeks of the immunotherapy infusion, CMV-specific CD4+ T cell clones were 404 ± 124 per 10^6 cells, and IFN-γ-producing CMV-specific CD8+ cells were detected in normal frequencies. Of the 25 patients who received CMV-specific adoptive therapy, CMV reactivation was observed in only 7 patients, while thirty of the 33 control patients experienced repeated CMV reactivation. More recently, Perruccio et al. [82, 83] tested a photodynamic approach to purge DLI of alloreactive, but not pathogen-specific, donor T cells. Pathogen-specific responses to CMV were retained, although with a 19 ± 9.7 time reduction in frequency [83]. Not only did the researchers achieve the success of described prophylactic infusion, but Feuchtinger et al. [84] also treated 18 patients with refractory CMV infection after allo-SCT using polyclonal CMV-specific T cells. In 83% of cases, CMV infection was cleared or viral burden was significantly reduced. Viral control was associated with the *in vivo* expansion of CMV-specific T lymphocytes in 12 of 16 evaluable cases, without GVHD induction or acute side effects.

These manipulated DLI approaches are effective but expensive and labor intensive, and the effect on global IR is unclear. For a long time following transplant, allogeneic DLI can accelerate IR, treat infections, and provide a graft-versus-malignancy effect [85, 86]. Currently, we focus mainly on the infusion of G-CSF-mobilized peripheral blood progenitor cells. Previous studies have shown the multiple biological effects of G-CSF on peripheral blood stem cells, such as the ability to polarize T cells from Th1 to Th2 and the promotion of regulatory T cell and tolerogenic dendritic cell

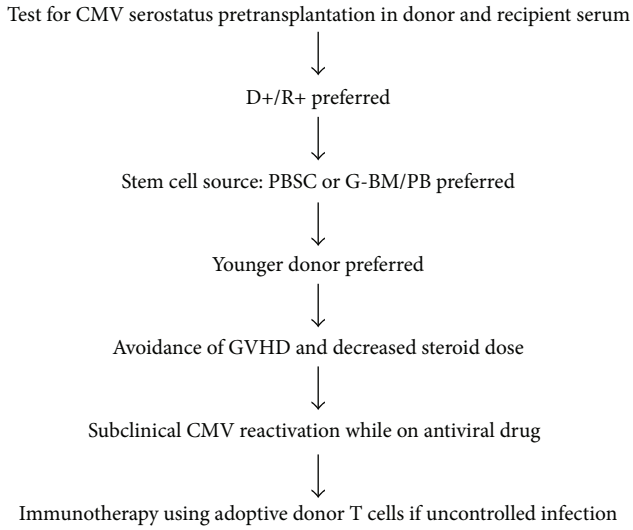

Figure 2: Proposed algorithm for improving CMV-specific IR following haplo-SCT. CMV, cytomegalovirus; D+/R+, CMV-positive recipients of grafts from CMV-positive donors; PBSC, peripheral blood stem cell; G-BM/PB, combining G-CSF-primed bone marrow (G-BM) and peripheral blood (G-PB) harvests; GVHD, graft-versus-host disease.

differentiation [87, 88]. Huang et al. [89] reported that G-CSF-mobilized peripheral blood progenitor cell infusion produces superior disease-free survival in patients who received unprimed lymphocytes for relapse after allo-HSCT, although the difference in the incidence of severe GVHD was not significant. We extended the use of DLI for the treatment of infections. Our preliminary data showed that DLI is an effective and safe therapy for EBV-associated PTLD after mismatched/haploidentical SCT [90]. Investigation of DLI for CMV infection and other opportunistic infections is underway. Until pathogen-specific T cells and/or alloreactive-depleted T cells are more readily available, unmanipulated, nonspecific DLI will continue to play a role in the treatment of uncontrolled infections and improvement of IR following haplo-SCT.

6. Conclusions

The current options for haplo-SCT present intrinsic challenges. In T cell depleted haplo-SCT, the minimal residual T lymphocytes in the grafts successfully prevent lethal GVHD without any posttransplantation immunosuppression, but the small number of T cells infused leads to delayed IR. In unmanipulated haplo-SCT, although the high T cell content of the graft potentially enhances the graft-versus-leukemia effect, recipients of unmanipulated grafts from alternative donors remain at risk of TRM for months/years after transplantation because of GVHD and its immunosuppressive treatments that antagonize T cell expansion and function. Delayed IR and increased risk of CMV infection remain critical problems early after transplantation, although long-term IR can successfully be achieved after haplo-SCT [74, 91].

To address these shortcomings, several factors identified to affect IR to CMV should be considered for better outcome (Figure 2). Our data indicate that selection of D+ for R+, young donor, stem cells derived from PBSC or G-BM/PB, subclinical CMV reactivation while on antiviral therapy, avoidance of GVHD, and decreased steroid dose can improve CMV-specific IR. The effects of varying degrees of HLA disparity and conditioning regimens are uncertain. Therefore, more in-depth preclinical and clinical studies in this area are needed, both in terms of reconstitution of normal immune cell function and their effectiveness in anti-CMV cell activity.

Conflict of Interests

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

Acknowledgment

This work was supported by the Key Program of the National Natural Science Foundation of China (Grant 81230013), the Chang Jiang Scholars Program, and the National Natural Science Foundation of China (Grant 81100388).

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 419029, 8 pages
http://dx.doi.org/10.1155/2014/419029

Review Article

The Pathology of T Cells in Systemic Lupus Erythematosus

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Received 19 February 2014; Revised 12 March 2014; Accepted 12 March 2014; Published 23 April 2014

Academic Editor: Xiuli Wu

Systemic lupus erythematosus (SLE) is characterized by the production of a wide array of autoantibodies. Thus, the condition was traditionally classified as a "B-cell disease". Compelling evidence has however shown that without the assistance of the helper T lymphocytes, it is indeed difficult for the "helpless" B cells to become functional enough to trigger SLE-related inflammation. T cells have been recognized to be crucial in the pathogenicity of SLE through their capabilities to communicate with and offer enormous help to B cells for driving autoantibody production. Recently, a number of phenotypic and functional alterations which increase the propensity to trigger lupus-related inflammation have been identified in lupus T cells. Here, potential mechanisms involving alterations in T-cell receptor expressions, postreceptor downstream signalling, epigenetics, and oxidative stress which favour activation of lupus T cells will be discussed. Additionally, how regulatory CD4+, CD8+, and $\gamma\delta$ T cells tune down lupus-related inflammation will be highlighted. Lastly, while currently available outcomes of clinical trials evaluating therapeutic agents which manipulate the T cells such as calcineurin inhibitors indicate that they are at least as efficacious and safe as conventional immunosuppressants in treating lupus glomerulonephritis, larger clinical trials are undoubtedly required to validate these as-yet favourable findings.

1. Introduction

Systemic lupus erythematosus is characterized by the production of plethora of autoantibodies which potentially drive immune-complex related inflammation in various tissues and organs [1]. Breakdown of immune tolerance is believed to be one of the major mechanisms which triggers the production of autoantibodies by B cells and antibody forming cells, leading to inflammation upon binding to autoantigens and consequent tissue damage [2]. As such, SLE was classically thought to be a B-cell driven disease. Recent compelling evidence has demonstrated that T cells are actually crucial in the pathogenesis of SLE in that they enhance the production of autoantibodies by offering substantial help to B cells through stimulating the latter to differentiate, proliferate, and mature, in addition to their support on class-switching of autoantibodies which B cells are expressing [3]. Therefore, SLE is currently believed to be a T cell-driven condition and, indeed, targeting molecules expressed on T cells and their signalling pathways can be one of the potential therapeutic strategies in SLE.

In comparison with healthy subjects, a number of studies have demonstrated that T cells isolated from patients with SLE are abnormal, with regard to their phenotypes and functions [4, 5]. Phenotypic and functional alterations in lupus T cells including expansion of the Th17 population, perturbations of the physiology of T-cell receptors (TCRs) and postreceptor downstream signalling, oxidative stress, and epigenetic changes result in exaggeration of TCR response to stimuli and the propensity of lupus T cells to get activated [6]. Additionally, the failure of the regulatory CD4+ and CD8+ T lymphocytes in alleviating the proinflammatory milieu occurring in SLE is contributory to the pathogenicity of the condition [7, 8]. In this brief review, a detailed account of the putative mechanisms by which the normal physiology of T cells are disturbed and why regulatory T cells fail to alleviate proinflammatory response in SLE will be discussed. The current state of clinical trials evaluating therapeutic agents

which target molecules expressing on and inside T cells for the treatment of SLE will be updated.

2. T Cells, Their Receptors and Signalling in Normal Situations, and SLE

2.1. T-Cell Receptors and CD3: A Brief Discussion of Their Normal Structures and Functions. T cells recognize antigens presented to them by the major histocompatibility complex of antigen-presenting cells via the TCRs expressed on their surface. Stimulation of TCRs upon antigen binding triggers downstream signalling pathways which enables various physiological functions of the T cells. The majority of TCRs (95%) are heterodimers which compose of an α and a β chain ($\alpha\beta$ receptors) and are anchored into the plasma membrane by a short cytoplasmic tail [9]. A minor group (15%) of TCRs comprise a γ and a δ chain ($\gamma\delta$ receptors) which are expressed in certain populations of thymic T cells and peripheral T cells in the epithelia [10, 11]. TCRs are associated with CD3 which is a series of polypeptides with consistent amino acid sequences and is responsible for signal transduction upon antigen recognition by the TCRs [9, 12]. CD3 consists of four invariant polypeptides, namely, γ, δ, ε, and ζ, and the CD3-TCR complex is arranged in such a way that the four TCR chains (two α and two β positively charged chains) are associated with two ε, two ζ, one γ, and one δ chain polypeptides of the CD3 which are all negatively charged [9, 12]. The CD3 has extracellular, transmembrane, and cytoplasmic tails whereby the 2ζ chains (or its variant—the η chain) are the longest cytoplasmic chains amongst the rest. The cytoplasmic portions of ζ and η chains are critically involved in TCR signal transduction for they possess the immunoreceptor tyrosine-based activation motifs (ITAMs) which are targets of phosphorylation by various specific protein kinases in the signal transduction processes [13]. Briefly, ITAMs become phosphorylated in a few minutes following TCR engagement. ITAMs and the subsequent pathways activated, such as the ζ-associated protein 70 (ZAP-70) pathway, are essential for T-cell activation [14].

Closely related to CD3ζ, FcγR also associates with the ITAMs. However, instead of stimulating the ZAP-70 pathway, the spleen tyrosine kinase (Syk) pathway is preferentially utilized [15, 16]. Syk stimulation characteristically results in higher calcium influx into cells than that involves the ZAP-70 pathway, probably regulated by transcription factors c-Jun and Ets2 [17]. Such "rewiring" of postreceptor downstream signalling mechanism has a strong pathological implication in lupus T lymphocytes (discussed in the next section).

2.2. Alterations in T-Cell Receptors and Their Signalling Pathways. CD3ζ subunits are suboptimally synthetized in T cells from patients with SLE [18]. Moreover, reduction of stability and increase in degradation of CD3ζ in lupus T cells are evident [19–21]. To replace the deficient CD3ζ subunits, FcRγ receptors are reciprocally activated and expressed on lupus T cells [16]. Instead of coupling with ZAP-70 for signalling by the CD3ζ subunits, FcRγ associates with the Syk pathway and such "rewired" downstream signalling confers stronger phosphorylation of signalling molecules and higher calcium influx which intensifies the TCR-derived signals in lupus T cells [17]. Increase in intracellular calcium activates calcineurin in the cytoplasm which enhances the action of the nuclear factor of activated T cells (NF-ATc2) through the dephosphorylating action of calcineurin [22]. (See Figure 1). Activated NF-ATc2 alters the expression of certain genes including the *CD40L* gene of lupus T cells by binding to the promoter of the *CD40L* gene [22]. CD40L is a costimulatory molecule expressed on T cells and its cognate interaction with CD40 expressed on B cells promotes differentiation, proliferation, and antibody production of the latter, as well as class switching, in conjunction with the action of IL-10 and IL-21 [23].

Another mechanism whereby lupus T cells exhibit a lower threshold of activation is the presence of preaggregated lipid rafts on their cell membrane [24]. The lipid rafts are lipid-rich areas on the cell surface where TCRs and the associated signalling molecules are concentrated [25, 26]. During inactivated state, lipid rafts are evenly distributed throughout the cell membrane but, in lupus T cells, clustering of lipid rafts has been demonstrated even when they are minimally stimulated [24, 27]. Clustered lipid rafts enhance lower threshold of signal transduction as molecules necessary for receptor activations are physically clustered in lupus T cells. To prove these potential mechanisms, intraperitoneal administration of pharmacologically active compounds which disrupt (MβCD) and enhance (cholera toxin B) lipid raft clustering demonstrated reduction and promotion of T-cell activation, respectively, in a murine lupus-prone model [28].

2.3. Other Abnormal Signalling Pathways in Lupus T Cells. Abnormalities in certain signalling pathways in lupus T cells which lead to defects in T-cell activation in patients with SLE have been increasingly identified. Impairment of cyclic adenosine monophosphate (cAMP-)dependent phosphorylation due to the reduction of protein kinase A levels has been reported [29, 30]. In addition, the activities of pathways involving protein kinase C and p56-lymphocyte-specific protein tyrosine kinase (p56lck) have also been found to be compromised [31, 32]. On the other hand, the activities of protein kinase PKR and phosphatidylinositol-3 kinase (PI3K) were found to be increased in a lupus-prone animal model [33, 34]. Pharmacological inhibition of PI3K can ameliorate glomerulonephritis and decrease mortality in the MRL/Fas^{lpr} murine lupus model [35]. The activity of the mitogen-activated protein (MAP) kinases, which is crucial in the proliferation and apoptosis of T cells, is reduced in T cells of patients with SLE [36]. Animals which are deficit in PKC (an activator of MAP kinase) have been shown to develop spontaneous lupus-like disease [37].

2.4. Alterations in Gene Expression Partly due to Reduced DNA Methylation. As described above, upregulation of CD40L in lupus T cells is evident as a result of the activation of NF-ATc2 secondary to high calcium flux [17, 22]. Increase in CD40L upregulates expressions of CD80 and other costimulatory

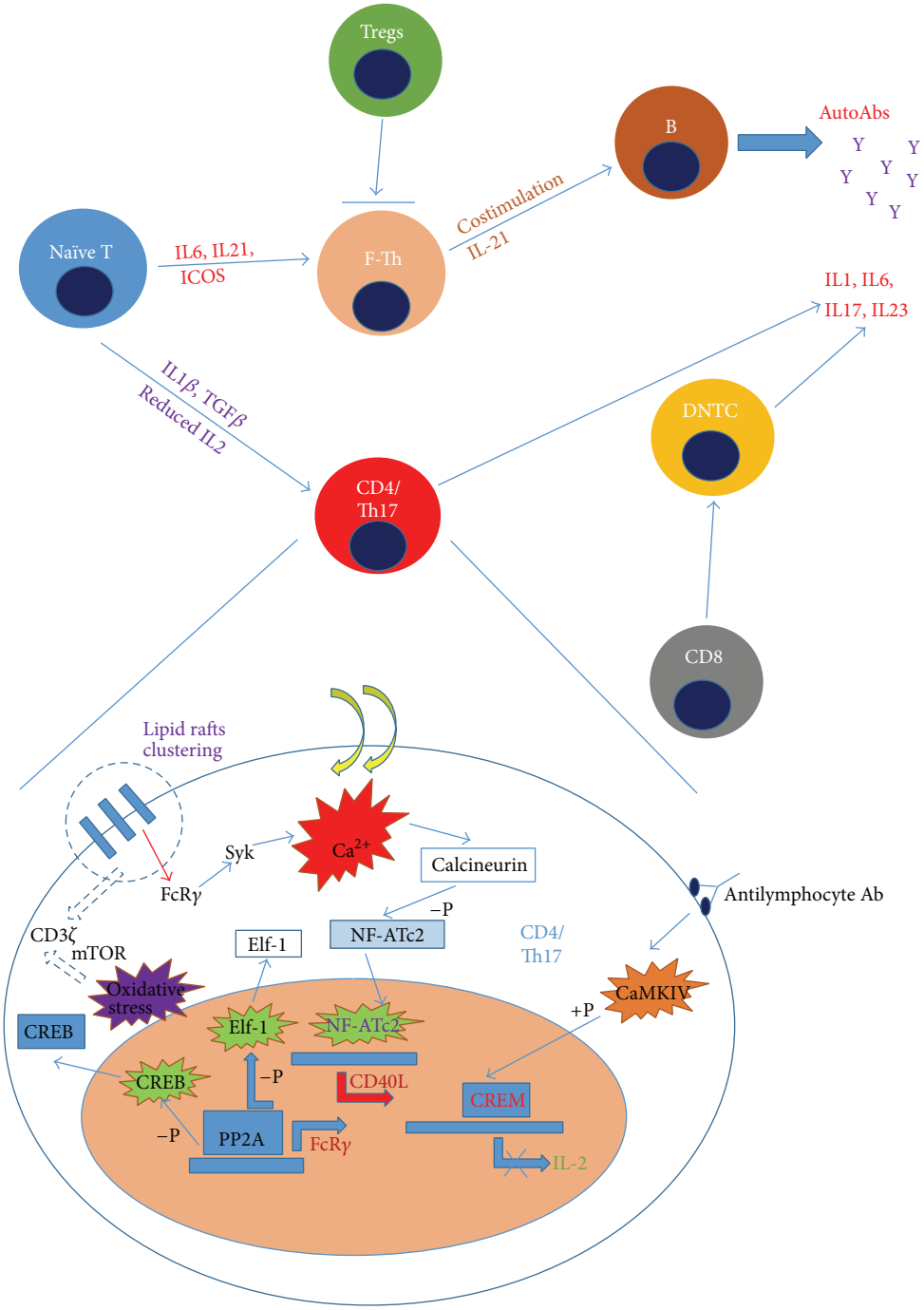

Figure 1: Development of lupus T cells, their interactions with T-regulatory cells and B cells, and alterations of the intracellular physiology of effector lupus T cells. Naïve T cells develop into follicular T-helper cells which cross-talk with B cells for autoantibody production under the stimulation of IL6, IL-21, and ICOS. Naïve T cells develop into effector CD4+ and Th17+ T cells which produce proinflammatory cytokines and exhibit altered intracellular physiology including clustering of CD3-TCR, oxidative-stress induced calcium flux, and consequent change in mRNA transcriptions of various important genes (see text for details). Abbreviations: Tregs, regulatory T cells; ICOS, inducible T-cell costimulator; F-Th, follicular T-helper cells; Syk, spleen tyrosine kinase; CaMKIV, calcium/calmodulin-dependent kinase IV; CREB/CREM, cAMP response element (CRE) binding protein (CREB)/CRE-modulator (CREM); NF-ATc2, nuclear factor of activated T cells; Elf-1, transcription factor Elf-1; Ca^{2+}, calcium ion; PP2A, protein phosphatase 2A; mTOR, mitochondrial transmembrane potential and mammalian target of rapamycin; +P, phosphorylation; −P, dephosphorylation.

molecules on the antigen-presenting cells which further intensify the stimulatory signals to the T cells [23]. Being a SLE susceptible gene, the *CD40L* gene is methylation sensitive. DNA methylation generally suppresses gene transcription and expression. In SLE, DNA methylation which has been shown to be reduced in T cells, is linked to T cell auto-reactivity [23]. Hypomethylation in one of the X-chromosomes which is inactive in female lupus patients induces overexpression of CD40L mRNA and hence CD40L expression on lupus T cells [23]. Altered MAP kinase and PKCδ activities are also caused by hypomethylation secondary to the deficiency of DNA methyltransferase 1 in lupus T cells [23, 38].

IL-2 is essential in reducing the polarization of naïve CD4+ cells towards the Th17 phenotype [39] (see Figure 1). Reduced production of IL-2 demonstrated in patients with SLE enhances the expansion of Th17 population which promotes local inflammation and recruitment of immunocytes in part due to the increased production of IL-17 [40]. Expression of IL-2 by T cells is in fact tightly regulated by the transcription factors cAMP response element (CRE) binding protein (CREB) and the CRE-modulator (CREM) [41]. CREB enhances the transcription of the *IL-2* gene while CREM suppresses it by competing for the CRE binding site with CREB [42]. The balance between CREB and CREM activity, which is important in determining whether IL-2 is upregulated or downregulated, is altered in lupus T cells [43]. The high CREM and CREB ratio in lupus T cells contributes to IL-2 deficiency [43]. There are at least 2 proposed mechanisms to explain the increased CREM and reduced CREB activities in lupus T cells. First, high levels of antilymphocytic antibodies in patients with SLE activate calcium/calmodulin-dependent kinase IV (CaMKIV) which enhances CREM activity through phosphorylation [44]. Second, the increased intranuclear level of protein phosphatase 2A (PP2A) in lupus T lymphocytes dephosphorylates and inactivates CREB [45, 46]. One point of note is that Elf-1, an important transcription factor of CD3ζ, is dephosphorylated by the increased level of intranuclear PP2A in lupus T cells [47]. Dephosphorylated Elf-1 fails to associate with the DNA and initiate transcription of CD3ζ transcription, leading to the increased FcRγ and CD3ζ ratio [47], favouring subsequent activation of the Syk instead of ZAP-70 pathways in lupus T cells [17] (see Figure 1).

Increase in oxidative stress has been demonstrated in lupus lymphocytes as evidenced by ultrastructural changes in the form of tubuloreticular structures of organelles in lymphocytes from patients with active lupus [48]. Oxidative stress induces nitric oxide activity and elevation of mitochondrial transmembrane potential which lead to activation of the protein kinase named mitochondrial transmembrane potential and mammalian target of rapamycin (mTOR) in lupus T cells [49]. Increase in mTOR activity causes RAB4A-mediated CD3ζ downregulation and results in high calcium flux when lupus T cells are stimulated [50]. Increase in intracellular calcium activates cAMP response element modulator (CREM) which inhibits IL-2 and enhances IL-17 expressions. These changes favour T_h1 to T_h17 polarization and inhibit CD8+ T cells [51]. mTOR activation also suppresses FoxP3 transcription by inhibiting DNA methyltransferase 1 (DNMT1) which results in hypomethylation of the FoxP3 promoter [51]. Rapamycin, an inhibitor of mTOR, was demonstrated in a small clinical study of nine lupus patients to be able to normalize T-cell activation-induced calcium influx and reduce overall lupus disease activity [50]. Other potential mechanisms of mTOR in immune response inhibition will be discussed in a subsequent section.

3. Alteration in the Number and Suppressor Activity of Regulatory T Cells in SLE

3.1. CD4+ T Regulatory Cells. CD4+ regulatory T cells (CD4+ Tregs) were shown to be reduced in the secondary lymphoid organs of the NZB/W F1 lupus-prone mouse model as compared with age-matched nonautoimmune mice [52]. Deficiency of CD4+ Tregs is linked to the development of lupus-like disease, while adoptive transfer of CD4+ Tregs slowed the progression of renal disease and reduced mortality in NZB/W F1 mice [52]. Besides thymic CD4+ Tregs, peripheral-induced CD4+ Tregs (CD4+ iTregs) conferred by the action of IL-2 and TGFβ were shown to be able to reduce serum anti-dsDNA levels and alleviate immune complex glomerulonephritis secondary to the reduction of T-cell help to B cells in NZB/W F1 mice [53].

In humans, the number of CD4+ Tregs was generally found to be lower in patients with active SLE as compared with those with inactive disease and healthy individuals [54]. Reduced levels of forkhead box P3 (FoxP3) in CD4+ Tregs in patients with active lupus are generally believed to be the reason why these patients have less Tregs-suppressive activity than their counterparts with inactive disease [55–57]. Interestingly, effective immunosuppressive therapies with glucocorticoids and rituximab have been shown to restore the number of functional Tregs in patients with SLE [58–60]. Despite the prevailing belief of the inferior quantity and functional quality of Tregs in patients with SLE, the lack of truly reliable markers which allow identification and isolation of the genuine Treg population renders reliability and reproducibility of Treg studies in SLE an issue [61]. Helios, which is a transcription factor that belongs to the Ikaros family, has recently been shown to be expressed by most of the FoxP3+ T-cells in humans and it has been demonstrated to be able to upregulate FoxP3 expression by binding to the FoxP3 promoter [62]. In contrast to the previous findings which advocated the lower quantity of Tregs in lupus patients with more active disease, the population of Foxp3+ Helios+ Tregs was indeed shown to be significantly expanded in patients with active SLE when compared with those with inactive disease and healthy controls [61, 62]. In addition, the FoxP3+ Helios+ T cells isolated from 20 lupus patients were shown to have lower IL-2 and IFNγ productions when compared with those from FoxP3+ Helios− T cells [62].

3.2. CD8+ T Regulatory Cells. In both the NZB/W F1 and human monoclonal anti-DNA-induced experimental mouse models, expansion of CD8+ Tregs by tolerogenic peptide

suppressed anti-dsDNA production, CD4+ T cell proliferation, and type-2 interferon production, probably as a result of TGFβ and FoxP3 produced by the CD8+ iTregs [63, 64].

Similar to the findings of CD4+ Tregs, studies addressing the number of circulating CD8+ Tregs in patients with SLE have yielded inconsistent results [65, 66]. CD8+ Tregs from patients with active SLE failed to suppress effector T cells, while CD8+ Tregs from patients with inactive SLE demonstrated comparable suppressive ability as those from healthy individuals [65]. Of particular note, since the data of CD8+ Tregs in SLE are based on a small number of clinical studies, more robust studies are required to further characterize the quantity and functional aspects of CD8+ Tregs in patients with SLE.

3.3. $\gamma\delta$ T Regulatory Cells. Recently, a group of rare $\gamma\delta$ T cells which express high levels of CD25 and CD27 and low level of CD45RA has been found to possess regulatory and suppressive activities ($CD27^+CD45RA^-\gamma\delta$ Treg cells), particularly the Vδ1 subset [67]. Enumeration of the peripheral blood mononuclear cell (PBMC) populations revealed a significantly lower number of circulating $CD27^+CD45RA^-\gamma\delta$ Treg cells in patients with SLE as compared to that of healthy controls [67]. Furthermore, a significant inverse correlation was found to exist between lupus disease activity and the level of circulating $CD27^+CD45RA^-\gamma\delta$ Treg cells [67]. *In vitro* experiment confirmed the ability of lupus $CD27^+CD45RA^-\gamma\delta$ Treg cells to express FoxP3 in a CD27-dependent fashion when the cells were cultured in the presence of TGFβ [67]. In addition, $CD27^+CD45RA^-\gamma\delta$ Treg cells were demonstrated to be able to suppress the proliferation of autologous effector CD4+ cells in coculture systems [67]. Though rare in the PBMC population, further experiments are required to fully characterize the phenotype and function of these $\gamma\delta$ Treg cells which may play an important immunopathogenic, as well as potential therapeutic, roles in suppressing the disease activity of SLE.

4. Therapeutic Trials Testing Drugs Which Manipulate T Cells in SLE

4.1. Calcineurin Inhibitors. The most commonly used calcineurin inhibitors including cyclosporin and tacrolimus have been proven in randomized controlled trials to be at least as efficacious and safe as conventional treatment for proliferative and membranous lupus glomerulonephritis [68–70]. A one-year quasirandomized trial revealed proteinuria remission rates of 83%, 60%, and 27% in patients who were in the cyclosporine, intravenous cyclophosphamide, and prednisolone groups, respectively, although the relapse rate of proteinuria was higher in patients receiving cyclosporine than those who received cyclophosphamide [68]. As an induction therapy, the combination of prednisolone and intravenous cyclophosphamide (a total of six 4-week pulses starting at 750/m^2 of body surface area) or tacrolimus (starting at 0.05 mg/kg/day and being titrated to a trough level of 5–10 ng/mL) has been shown to be equally efficacious in achieving complete renal remission [70]. Tacrolimus appeared to be safer as adverse events including leucopenia and gastrointestinal complaints were less frequent as compared to subjects in the cyclophosphamide group [70].

4.2. Anti-CD40L. As discussed previously, CD40L, which is overexpressed on lupus T cells, stimulates CD40 expressed on B cells to produce autoantibodies. Antagonization of CD40L is thus a potential therapeutic target for the treatment of SLE. Two main clinical trials testing the blockade of the CD40-CD40L pathway in the treatment of SLE are, however, disappointing [71, 72]. In addition to the failure of satisfying the predefined study end-points, the unfavourable side-effect profile of anti-CD40L unfortunately led to the premature termination of a multicentre phase II trial of BG9588 in SLE [72]. In a double-blind, placebo-controlled trial, 85 patients with mild to moderately active SLE were randomized to receive 6 infusions of anti-CD40L at doses of 2.5, 5, and 10 mg/kg and placebo at 0, 2, 4, 8, 12, and 16 weeks [71]. After 20 weeks of treatment, lupus disease activity improved in all groups from baseline but no statistical significance was detected amongst the different groups [71]. No difference in fatigue score and quality of life was noted either [71]. In the smaller phase II, open-label trial evaluating BG9588 in the treatment of 28 patients with proliferative lupus glomerulonephritis, the occurrence of 2 myocardial infarctions in the subjects led to premature termination of the trial although significant reduction of proteinuria, haematuria, and anti-dsDNA titre with increase in serum C3 levels were demonstrated [72].

4.3. Rapamycin. Being a safe and well-tolerated drug clinically used for preventing transplant rejection, rapamycin, a macrolide antibiotic which regulates mitochondrial transmembrane potential and calcium influx, was evaluated in a small uncontrolled trial for its effectiveness in patients with SLE [50]. In 9 lupus patients who were refractory to conventional treatment, rapamycin 2 mg daily reduced the disease activity and prednisolone requirement [50]. Mitochondrial calcium level and T-cell activation-induced calcium fluxing were normalized in rapamycin-treated patient [50]. In a recent prospective open-label study, rapamycin was shown to inhibit IL-4 production by and necrosis of double negative (DN) T cells in patients with SLE. In addition, rapamycin enhanced FoxP3 expression in CD25+/CD4+ T-cells and expansion of CD25+CD19+ B cells [73], signifying that mTOR can trigger IL-4 production by and necrosis of DN T cells in active SLE.

4.4. N-Acetylcysteine. Recently, N-acetylcysteine (NAC), the precursor of glutathione, was shown in a small clinical trial that at doses 2.4 gm and 4.8 gm daily it could reduce lupus disease activity and fatigue after 3 months of treatment as compared with placebo [74]. NAC reduced mTOR activity and enhanced apoptosis of T cells, accompanied by reversed expansion of the CD4/CD8 populations. Interestingly, NAC was shown to induce FoxP3 expression in CD4+ Treg cells and reduce serum anti-dsDNA levels [74]. Larger clinical trials are certainly required to validate the efficacy of this exciting therapeutic agent, especially it is anticipated that

adverse effects of NAC due to immunosuppression are very minimal.

5. Conclusion

In both murine system and human disease of SLE, T cells are found to be abnormal based on their alterations in the phenotype, receptor and signalling physiology, gene transcription, and perturbed suppressor activities of regulatory lymphocytes. The substantial involvement of T cells in the pathogenesis of SLE and the apparent success in therapeutics directing at T cells in patients with SLE lead to the firm belief that SLE is indeed a T-cell driven autoimmune disease. While manipulating the B cells and their families with the use of B-cell depleting therapy (BDT) appears very promising in the treatment of SLE and it is argued that B cells are relatively more important in the pathogenesis of SLE than other immunocytes, the discrepantly prolonged beneficial effects of BDT against the much shorter half-life of rituximab invariably explain the potential importance of the participation of T cells in the pathogenic process of SLE [58, 59].

Conflict of Interests

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

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2014, Article ID 989434, 8 pages
http://dx.doi.org/10.1155/2014/989434

Research Article

Rapamycin Regulates iTreg Function through CD39 and Runx1 Pathways

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Received 25 January 2014; Revised 25 February 2014; Accepted 5 March 2014; Published 31 March 2014

Academic Editor: Xiuli Wu

It has been shown that rapamycin is able to significantly increase the expression of FoxP3 and suppress activity in induced Treg (iTreg) cells *in vivo* and *in vitro*. CD39 is a newly determined Treg marker that relates to cell suppression. Runx1, a regulator of FoxP3, controls the expression of adenosine deaminase (ADA) gene, which is found recently in the downstream of CD39 pathway in trophoblast cells. Whether rapamycin would influence CD39 pathway and regulate the expression of Runx1 remains to be determined. The addition of rapamycin to human $CD4^+$ naïve cells in the presence of IL-2, TGF-β promotes the expression of FoxP3. In this paper, we found that CD39 positively correlated with the FoxP3 expression in iTreg cells. Rapamycin induced iTreg cells showed a stronger CD39/Runx1 expression with the enhanced suppressive function. These data suggested that CD39 expression was involved in iTreg generation and the enhanced suppressive ability of rapamycin induced Treg was partly due to Runx1 pathway. We conclude that rapamycin favors CD39/Runx1 expression in human iTreg and provides a novel insight into the mechanisms of iTreg generation enhanced by rapamycin.

1. Introduction

$CD4^+CD25^+FoxP3^+$ regulatory T cells (Treg) play an essential role in maintaining immunological homeostasis. Forkhead box P3 transcription factor (FoxP3) is responsible for the differentiation and function of Treg cells [1]. $CD4^+FoxP3^+$ T cells show a greater suppressive ability and immune function than normal T cells *in vivo* and *in vitro*. $CD4^+Foxp3^+$ T cells can be divided into two subsets, thymus-derived natural regulatory T cells (nTreg) and induced Treg cells (iTreg). In clinical trials, the major limitation of nTreg is the poor population of peripheral circulating $CD4^+$ T cells, while iTreg shows a good alternative to nTreg with a greater proliferative ability [2]. iTreg can be induced from naïve $CD4^+CD25^-$ T cells in both mice and human. Recently reports have shown that iTreg plays an important role in treating various autoimmune diseases in mice including autoimmune diabetes, experimental arthritis, and other immune-mediated inflammatory diseases [3, 4].

CD39 is an ectoenzyme that hydrolyzes ATP and adenosine diphosphate (ADP) into adenosine monophosphate (AMP) and is localized on the surface of endothelial cells and circulating platelets. Now it is determined as an activation marker of lymphoid cells and expressed in human $CD25^+FoxP3^+$ Treg cells [5]. CD39 has also been elucidated to be related to suppressive function of Treg [6, 7]. Researches show that the expansion of $CD39^+$ Treg inhibits IL-2 expression in activated T cells and correlates directly with immune activation in AIDS patients [8, 9]. Adenosine deaminase (ADA) which is able to catabolize adenosine plays an obbligato role in CD39-CD73-adenosine pathway [10]. ADA deficiency may result in a fatal severe combined immunodeficiency disease (SCID) [11].

Runt-related transcription factor 1 (Runx1) belongs to a small family of transcription factors, including Runx1, Runx2, and Runx3, and is composed of an NH2-terminal DNA-binding runt homology domain followed by a transcriptional activation domain and COOH-terminal negative regulatory

domain [12, 13]. The study with respect to Runx1 has focused largely on its indispensable effect on FoxP3 expression and Treg function [14]. Knockdown of Runx1 by siRNA eliminates the ability of Treg cells to suppress T effector cells *in vitro* [13]. A previous study demonstrates that the Runx1 transcription factor plays a significant role in regulating ADA gene expression in the trophoblast cells [15].

Rapamycin has been demonstrated to be an immune modulator which prevents graft rejection in transplant patients [16, 17]. Previous studies have shown that rapamycin improves the FoxP3 expression and selectively expands the functional Treg cells both *in vivo* and *in vitro* with the appropriate suppressive activity [18–21]. However, it is not clear that how rapamycin influences CD39 and Runx1 pathways in human iTreg cells.

2. Materials and Methods

2.1. Isolation of Naïve T Cells. Peripheral blood mononuclear cells (PBMCs) were prepared from heparinized venous blood of healthy adult volunteers by Ficoll-Hypaque density gradient centrifugation. All protocols that were involved in human blood donors were approved by Nanjing Medical University. $CD4^+CD45RA^+$ naïve T cells were isolated from PBMC with human naive $CD4^+$ T-Cell Isolation Kit II (Miltenyi Biotec) by MACS. The purity of selected cells was routinely more than 95% as determined by flow cytometry.

2.2. Generation of Human iTreg Cells In Vitro. Fresh naïve T cells from PBMC were stimulated with anti-CD3/CD28 beads (Life Technologies) at a bead: T-cell ratio of 3 : 1 in the presence of IL-2 (100 U/mL), TGF-β (10 ng/mL), and rapamycin (100 ng/mL) in different conditions. The concentration of naïve T cells was 0.5 million/mL at the beginning. All the cells were incubated at 37°C for 7 days. IL-2 (100 U/mL) was renewed every 2 days.

2.3. Flow Cytometric Assays. All the cells were analyzed by flow cytometry after staining with the following antibodies (all from BD-Biosciences): anti-human CD4, CD25, CD28, CD39, and CD127. For FoxP3 staining, cells were first stained with surface antibodies, then fixed/permeabilized in cytofix/permeabilization solution (Biolegend), and stained with anti-human FoxP3.

2.4. Real-Time PCR. Total RNA was extracted with RNA simple total RNA kit (Tiangen Biotech), and cDNA was obtained using RT-Master Mix (TaKaRa). mRNA levels were quantified with SYBR Premix Ex Taq (TaKaRa). Primer sequences were as follows (18S as internal control):

ADA, 5′-TTCCTTCCAAGAAGACCATGA-3′ and
5′-GGTTTCAGATTCAACCATGC-3′;
Runx1, 5′-GGACGCCAGAAGGAAGTCAA-3′ and
5′-TCGGACCACAGAGCACTTTC-3′;
18S, 5′-CTCTTAGCTGAGTGTCCCGC-3′and
5′-CTGATCGTCTTCGAACCTCC-3′.

2.5. Suppressive Assays of $CD4^+$ Treg Cells In Vitro. PBMC was isolated as described previously and labeled with CFSE (Invitrogen). Anti-CD3 mAb-coated beads (Dynal) were added at 1 : 1 (bead : PBMC), and washed iTreg cells were added at ratios from 1 : 2 to 1 : 32 (Treg : PBMC). Finally cultures were incubated at 37°C. On day 4, cells were stained with anti-CD8 APC. Data was acquired and analyzed using the proliferation platform in FlowJo, and suppression index was determined using division index.

2.6. Statistical Analysis. Statistical analysis was performed using GraphPad Prism 5.0 software. Data was presented as mean ± SEM. Evaluation of differences between two groups was evaluated using Student's t-test. $P < 0.05$ was considered as statistically significant difference.

3. Results

3.1. CD39 Expression in $CD4^+$ T Cells in Human Peripheral Blood. A recent report shows that CD39 is related to T-cell proliferation and FoxP3 function [22]. However, whether CD39 plays an important role in maintaining human immunologic homeostasis is still unknown. To answer this question, initially, we evaluated the frequencies of CD39 in the human $CD4^+$ T cells and Treg cells. Peripheral blood mononuclear cells (PBMCs) were isolated from 3 healthy donors, and the expression of CD4, CD25, CD39, and FoxP3 was analyzed by flow cytometry. Approximately 30% of $CD4^+$ T cells were CD39 positive and these $CD4^+CD39^+$ T cells revealed an enhanced FoxP3 expression compared to $CD4^+CD39^-$ T cells (Figure 1(a)). Next, we gated on the $CD4^+CD25^+FoxP3^+$ Treg cells to calculate the CD39 expression in $CD4^+FoxP3^+$ T cells. As depicted in Figure 1(b), more than 70% of Treg cells were CD39 positive. Furthermore, FoxP3 expression was significantly distinct in $CD39^+$ Treg cells compared to $CD39^-$ cells. Figure 1(c) demonstrated the different proportion of the cells mentioned above. Here we conclude that CD39 is highly expressed in human $FoxP3^+$ T cells and positively correlates with the FoxP3 expression in human peripheral blood.

3.2. The Addition of Rapamycin Improves the Expression of FoxP3 and Develops the Potent Suppressive Activity In Vitro. Naïve $CD4^+$ T cells were cultured with suboptimal anti-CD3/CD28 beads in the presence of IL-2, with or without TGF-β and rapamycin for 7 days. While IL-2 and TGF-β increased the percentage of $CD4^+CD25^+FoxP3^+$ T cells, the addition of rapamycin markedly enhanced this effect (Figures 2(a) and 2(b)). These data were consistent with previous studies, which showed that rapamycin favored Foxp3 expression and promoted the suppressive activity of iTreg cells [19–21, 23]. Then, we observed a distinct population of $FoxP3^{high}$ cells, which is defined as $FoxP3^{++}$ cells. The percentage of $FoxP3^{++}$ iTreg cells was obviously increased from 13.6% to 27.8% in the presence of rapamycin (Figure 2(b)). The expression of CD25 was shown as the mean fluorescence intensity (MFI) for each culture condition which showed that rapamycin also enhanced CD25 expression

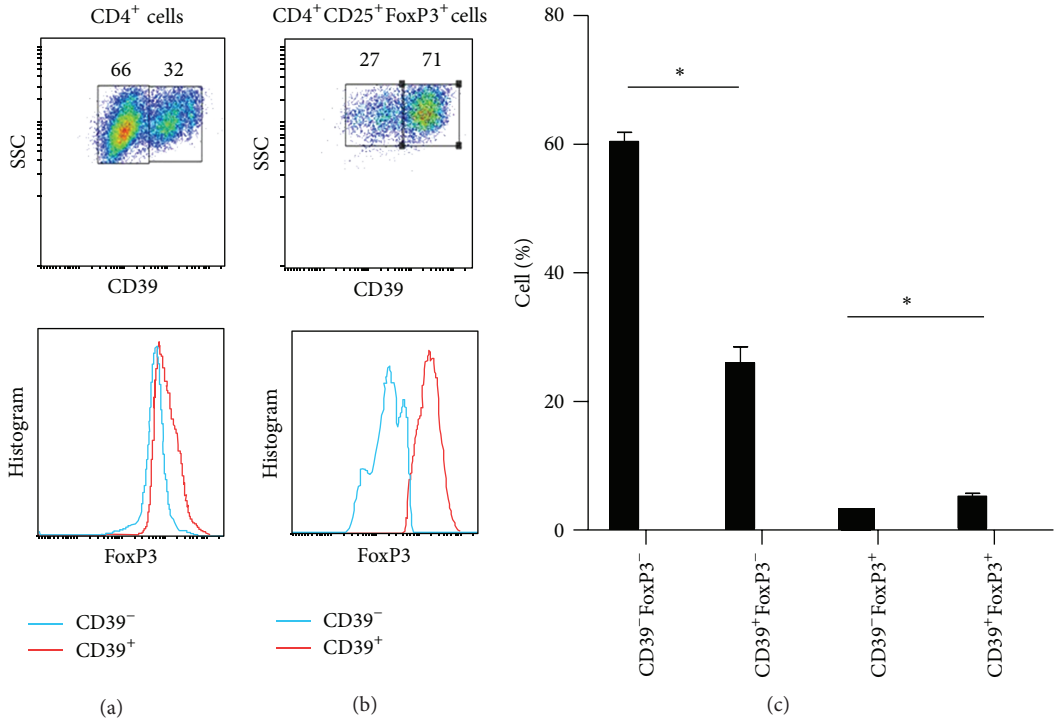

FIGURE 1: CD39 expression in human peripheral blood and nature FoxP3 cells. (a) Representative expression of CD39 gated in $CD4^+$ T cells. (b) Representative expression of CD39 gated in $CD4^+CD25^+FoxP3^+$ cells. (c) Different proportions of $CD39^+$ and $FoxP3^+$ cells in $CD4^+$ T cells. The values indicated the mean ± SEM of 3 separate experiments. $^*P < 0.05$.

in vitro (Figure 2(c)). Since the ability of cell suppression was important for the treating effect of iTreg cells, CFSE coculture assays were performed to estimate the suppressive ability of rapamycin-expanded Treg cells. Washed iTreg cells were coincubated with CFSE-labeled fresh PBMC in the presence of anti-CD3 beads. Although there was no significant difference between the iTreg cultured with or without rapamycin in low ratio (1 : 32), rapamycin did improve suppressive activity in high ratio (from 1 : 2 to 1 : 8) compared to IL-2 and TGF-β group (Figures 2(d) and 2(e)). On the whole, rapamycin improves the FoxP3 expression and enhances suppressive activity *in vitro*.

3.3. Rapamycin Improves CD39 Expression in iTreg Cells. As CD39 is important for the function of iTreg [24], next we evaluated the expression of CD39 in iTreg cultured in different conditions. TGF-β enhanced the expression of CD39 from 14% to 62% compared with the group with IL-2 alone, while the proportion ascended to nearly 70% with the addition of rapamycin (Figures 3(a) and 3(b)). CD39 MFI (Figure 3(c)) were also detected in these Treg cells which confirmed that the expression of CD39 and FoxP3 was positively correlated and enhanced by rapamycin. Therefore, we suggest that rapamycin improves CD39 expression in iTreg cells.

3.4. Rapamycin Upregulates ADA and Runx1 mRNA Level in iTreg Cells. ADA and Runx1 play essential roles in FoxP3 expression and Treg function [13, 14, 25]. A previous study reveals that Runx1 regulates ADA gene expression in the trophoblast cell line [15]. RT-PCR was performed to estimate the mRNA level of ADA; Figure 4(a) showed that the mRNA level of ADA was almost doubled in TGF-β group compared to IL-2 group and rapamycin slightly increased the ADA mRNA level. Next, we detected Runx1 expression in the mRNA level, Figure 4(b) demonstrated that Runx1 expression was increased approximately 3-fold by TGF-β, and rapamycin obviously increased Runx1 mRNA level. Since FoxP3 expression is controlled by Runx1 [14], here we found that Runx1 expression was significantly enhanced by rapamycin. This observation suggests that rapamycin upregulates ADA and Runx1 in human iTreg cell induction.

3.5. Phenotypic Characterization of Human $CD39^+$ iTreg Cells. We elucidated that rapamycin promotes CD39 expression and then assumed that $CD39^+$ iTreg cells might be a new subset of iTreg cells. To see phenotypic characterization of human $CD39^+$ iTreg cells, we calculated MFI for CD25, FoxP3 in different Treg phenotypes. As shown in Figure 5(a), a stronger CD25 and FoxP3 expression was detected in $CD39^+$ iTreg cells compared to $CD39^-$ iTreg in the same culture condition. In rapamycin group, the relative FoxP3 MFI in $CD39^+$ iTreg was about 20% higher than $CD39^-$ iTreg. Since CD127 could be an effective surface marker for $CD4^+CD25^+FoxP3^+$ Treg cells in flow sorting, we also determined the expression of CD39 in $CD4^+CD25^+CD127^-$ iTreg cells. $CD4^+CD25^+CD39^+CD127^-$

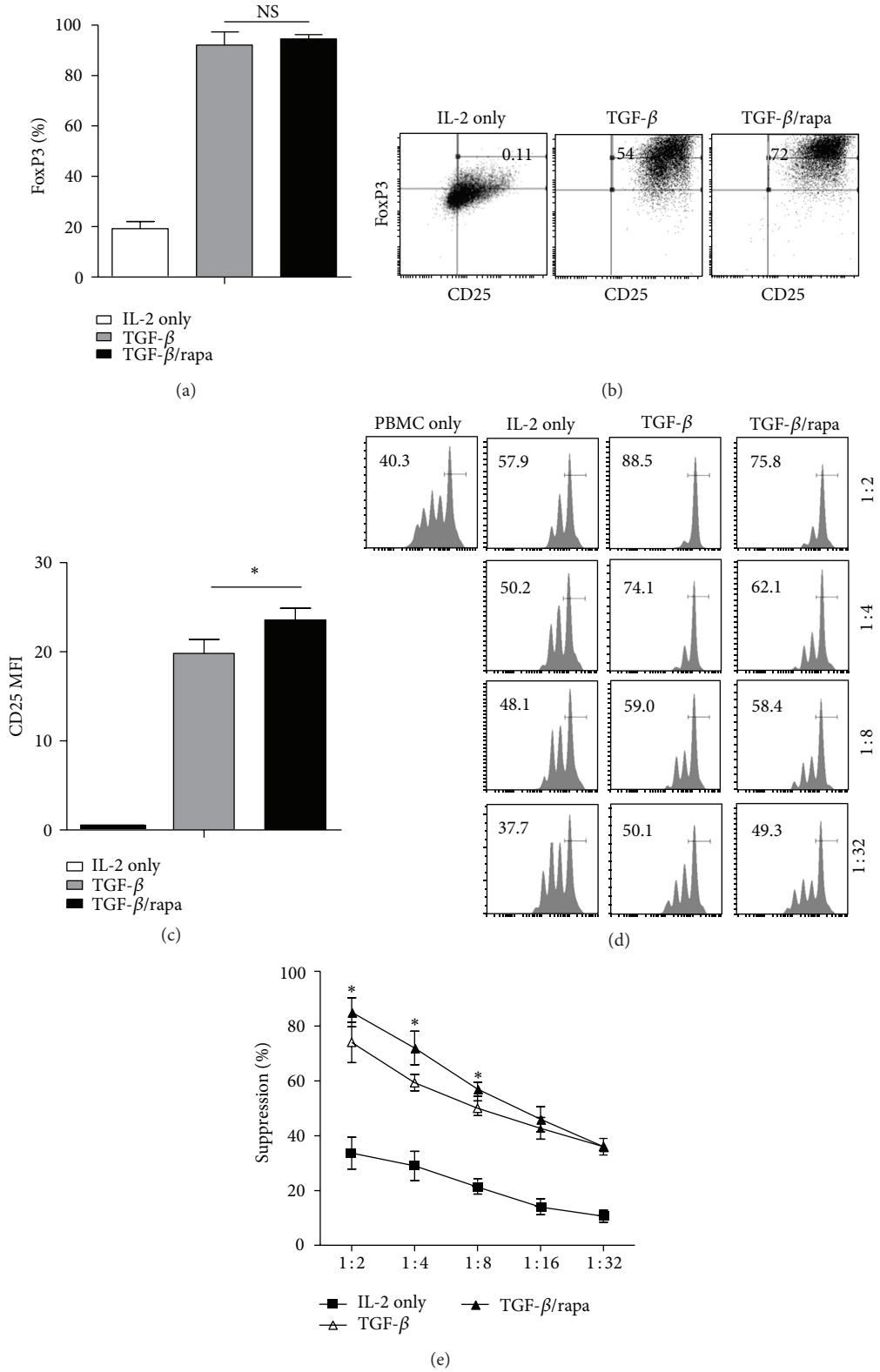

Figure 2: Rapamycin improved the expression of FoxP3 and developed the potent suppressive activity *in vitro*. (a) The proportion of $CD4^+CD25^+FoxP3^+$ iTreg induced from naïve T cells. (b) CD25 and FoxP3 coexpression in iTreg was assessed by flow cytometry on day 7. (c) Relative CD25 MFI in iTreg cells on day 7 with or without rapamycin. (d) In this representative experiment, the cells were stained for anti-CD8 and the suppressive activity of various primed $CD4^+$ cells subsets on CFSE-labeled $CD8^+$ at various T suppressor to T effector ratios was shown. (e) The mean ± SEM percent suppression of iTreg at the various ratios. The values indicated the mean ± SEM of 3 separate experiments. $^*P < 0.05$.

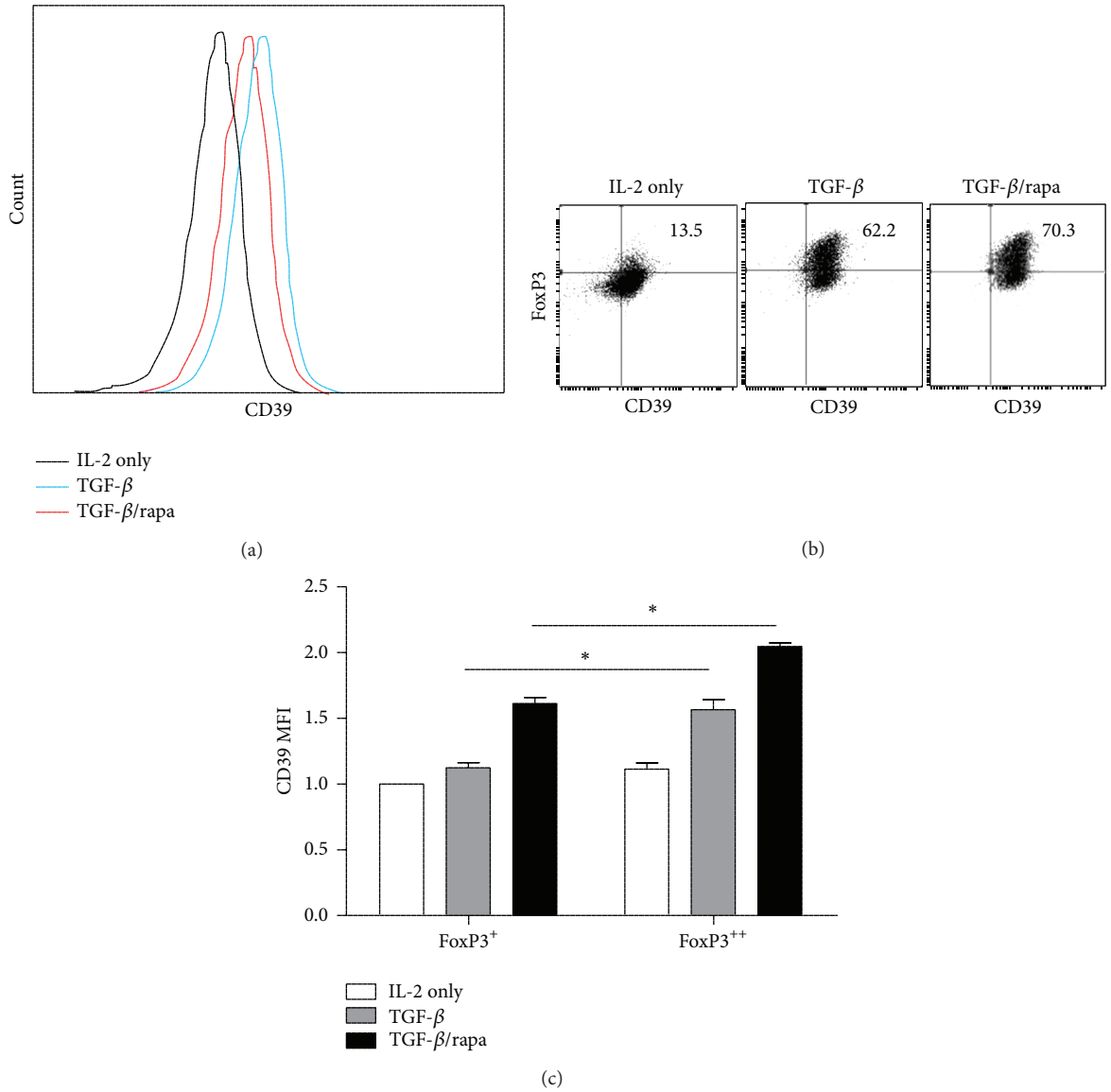

Figure 3: Rapamycin improved CD39 expression in iTreg cells. (a) Representative intensity of CD39 in iTreg cells. (b) FACS analysis of CD39 and FoxP3 expression with or without rapamycin was shown. (c) Relative CD39 MFI in $FoxP3^{+}$ and $FoxP3^{++}$ iTreg cells on day 7 with or without rapamycin. The values indicated the mean ± SEM of 3 separate experiments. $^{*}P < 0.05$.

iTreg also showed a significant difference of FoxP3 and CD25 expression compared to $CD4^{+}CD25^{+}CD39^{-}CD127^{-}$ iTreg cells (Figure 5(b)), and the relative FoxP3 MFI in $CD4^{+}CD25^{+}CD39^{+}CD127^{-}$ iTreg was also about 20% higher than $CD4^{+}CD25^{+}CD39^{-}CD127^{-}$ iTreg. Taken together, we demonstrate that $CD4^{+}CD25^{+}CD39^{+}CD127^{-}$ iTreg cells show a stronger FoxP3 expression and CD39 could be an additional marker for Treg cell sorting.

4. Discussion

CD39 is a newly determined Treg marker that relates to cell suppression [26]. $CD39^{+}$ Treg subset mediates a higher suppression compared to control HIV patients [8, 27]. However, CD39 is also expressed in activated T cells [28]. Herein we proved that more than 30% of $CD4^{+}$ T cells in human PBMC were CD39 positive, while $CD4^{+}CD39^{-}$ iTreg cells showed a low frequency of FoxP3 compared to $CD4^{+}CD39^{+}$ iTreg. Thus, we demonstrate that CD39 is involved in FoxP3 expression and Treg cells in human $CD4^{+}$ T cells.

ATP inhibits the generation and function of regulatory T cells [29]. CD39 which plays a crucial role in immunological system by generating adenosine and removing ATP becomes a promising therapeutic target in oncology [5]. ADA is involved in this pathway and converts adenosine and deoxyadenosine into inosine and deoxyinosine.

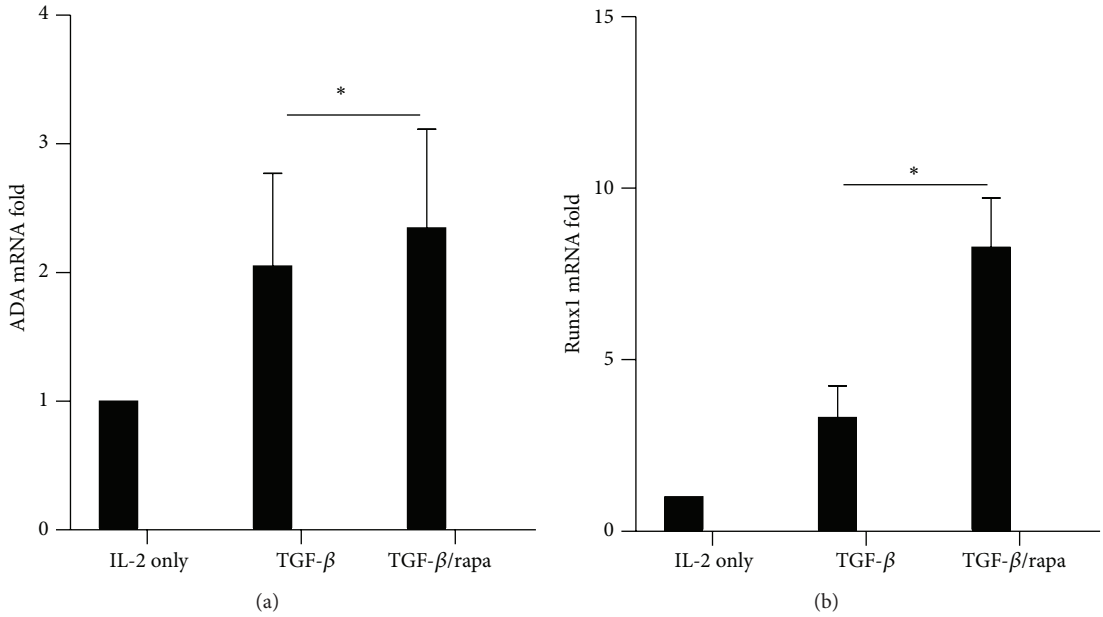

Figure 4: Rapamycin upregulated the mRNA level of ADA and Runx1 in Treg cells. The expression of (a) ADA mRNA and (b) Runx1 mRNA was determined by RT-PCR. Gene expression levels from the IL-2 group were set at 1. The values indicated the mean ± SEM of 3 separate experiments. $^{*}P < 0.05$.

$CD4^{+}CD25^{high}$ T cells express low level of ADA compared with effect T cells [30]. Adenosine and deoxyadenosine would accumulate in cells and then lead to ADA-SCID in the absence of ADA [25]. Thus, it might need a balanced metabolism of adenosine in Tregs because both overexpression and underexpression of ADA would cause Treg function unbalance. We demonstrate that rapamycin would obviously increase CD39 expression in iTreg cells and also enhance FoxP3 expression and suppressive function. A slightly enhanced ADA mRNA level was also observed in our study, which might be a positive feedback along with the increased CD39 expression. CD39/adenosine pathway is important to the balance of activation and regulation of effect immune responses. Since we found that $CD39^{+}CD127^{-}$ iTreg cells acquired a stronger FoxP3 expression compared to $CD39^{-}CD127^{-}$ iTreg cells, it provides us with a new marker for novel strategy of flow cell sorting.

Rapamycin is an inhibitor of mTOR pathway, which is able to favor the proliferation of Treg cells [31]. Here we conclude that iTreg induced from naïve T cells would acquire an enhanced CD25 and FoxP3 expression in the presence of rapamycin.

Rapamycin promotes the demethylation of Treg cells in the TSDR region and improves FoxP3 expression and suppressive activity [32]. Runx1 is also proved to be an unreplaceable gene which controls FoxP3 expression and Treg function [13, 33]. Recently, Strober W's group discovered that RORγt which is related to TH17 cells can also be induced by Runx1 [34]. Thus, Runx1 may have a complicated mechanism in balancing the generation of FoxP3 and IL-17 in human naïve cells. We found that the number of $FoxP3^{++}$ Treg was greater in rapamycin group and Runx1 expression was upregulated by rapamycin. It is consistent with the conclusion that rapamycin promotes FoxP3 expression and restrain IL-17 by altering expression of RORγt [35]. However, more researches are required to reveal the mechanism that how Runx1 regulates Treg and Th17 cells.

Human Treg shows a great suppressive effect and stability in treating autoimmune disease. However, because of the limited amount of these cells in the blood, there are technical difficulties to expand their clinical usage. The well-described protective effect of $CD39^{+}$ iTreg induced effectively by rapamycin provides us with a new strategy for Treg generation. Rapamycin greatly enhanced the expression of CD39 and Runx1. ADA, a promoter of CD39 pathway, can also be activated by rapamycin. Rapamycin treated $CD39^{+}$ iTreg may be an alternative choice which can be used for autoimmune disease treatment *in vivo*. Finally, we conclude that CD39 as a Treg function marker could provide us with new insights into clinical cell therapy in autoimmune diseases.

5. Conclusion

Our study indicated that the CD39 and Runx1 signaling pathways were involved in the induction of human iTreg by rapamycin. Rapamycin enhanced the FoxP3 expression and suppressive activity of iTreg with the elevated ADA and Runx1 expression; CD39 correlated positively with FoxP3 expression which proved to be a promising marker for human Treg cell sorting.

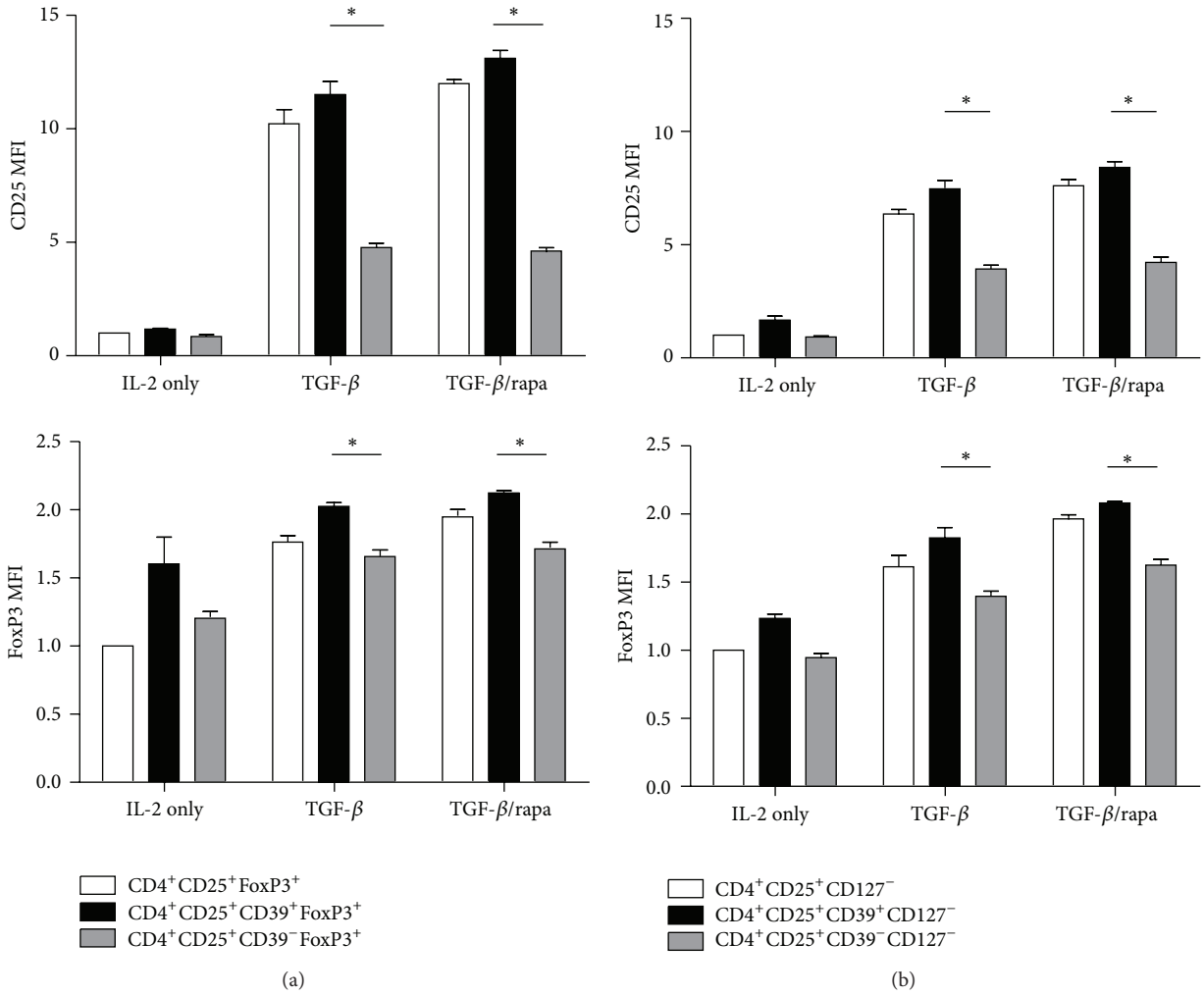

Figure 5: Rapamycin induced $CD39^+$ iTreg cells showed a greater CD25 and FoxP3 expression. Relative CD25 and FoxP3 MFI of $CD25^+CD39^+FoxP3^+$ and $CD25^+CD39^-FoxP3^+$ in (a) $CD4^+CD25^+FoxP3^+$ iTreg cells and (b) $CD4^+CD25^+CD127^-$ iTreg cells. The values indicated the mean ± SEM of 3 separate experiments. $^*P < 0.05$.

Conflict of Interests

All the authors declare that they do not have any commercial or associative interest that represents a conflict of interests in connection with the work submitted.

Authors' Contribution

Yunjie Lu and Jirong Wang contributed equally to this work.

Acknowledgments

This study was supported by the International Collaboration Foundation of Jiangsu Province (BZ2011041, BK2009439, ZX05 200904, and WS2011106), the special funds of Ministry of Health for health research (201302009), Program for Development of Innovative Research Team in the First Affiliated Hospital of NJMU, and the National Nature Science Foundation of China (81273262, 81210108017, 81100270, and 81070380).

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