Clinical and Developmental Immunology

Lymphocyte Differentiation and Effector Functions

Guest Editors: Niels Olsen Saraiva Camara, Ana Paula Lepique, Nicola Jane Rogers, Howard T. Petrie, and Alexandre S. Basso

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Editorial Board

Contents

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Editorial

Lymphocyte Differentiation and Effector Functions

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Lymphocytes are essential in combating infections; they display powerful effector mechanisms and their activity must be regulated at all times to avoid self-tissue or cells destruction. These immunological lineages are detected in protochordates and vertebrates. Long-term hematopoietic cells originate, in the bone marrow, all hematopoietic lineages, including lymphocytes. B and T lymphocytes are responsible for adaptive immune responses. Natural killer cells, NK, are also considered a lymphocytic lineage; however, its development is completely different from that of lymphocytes. NK cells display a different set of receptors from those expressed by B and T lymphocytes and are responsible for innate, not adaptive responses against virus infected and tumor cells. Finally, NKT cells are also lymphocytes generated in the thymus through contact with glycolipid loaded CD1d-presenting cells with a diverse function in modulating immune responses against self- and foreign antigens.

Function of lymphocytes and its products range from the neutralization of pathogens with specific antibodies to the activation of macrophages and to direct cytotoxic activity. Lymphocytes differentiate in primary lymphoid organs where they commit a lymphocytic lineage, express B or T cell receptors (BCR and TCR, resp.,), which are essential for cell survival and further maturation as well as function, and are selected according to their capacity of antigen recognition. Virtually all antigens present or presented in primary lymphoid organs are self-antigens. Lymphocytes that express receptors with high affinity to self-antigens either trigger programmed cell death or differentiate into regulatory cells (natural regulatory T cells). Lymphocytes that succeed in expressing a functional receptor with low-to-moderate affinity to self-antigens emigrate to secondary lymphoid organs, where they are exposed to foreign antigens and may be activated to generate effector responses (Figure 1). While B cells develop, in mammals, in the bone marrow, T cell progenitors migrate to the thymus to develop to mature TCRalpha/beta CD4 and CD8 T cells, as well as TCRgamma/delta T cells. During development in primary lymphoid organs, lymphocytes depend on a series of signals to pass through the checkpoints necessary to generate mature cells. In all progenitor stages, interaction with the organ stroma is important, but soluble factors as cytokines are also important for the survival of progenitor cells, mainly before B or T receptor expression. Y. Wanget al. review the expression control and role of Bcl-xL, a protein that promotes cell survival, in T cell development in the thymus as well as in T cell activation in the periphery.

An interesting feature of lymphocyte progenitors that migrate to the thymus is their potential to originate other lineages. Besides T cells, these progenitors have the potential to originate NK cells, dendritic cells, and B cells. Notch signalling is necessary for T cell fate determination. M. Braunstein and M. k. Anderson bring HEB (HeLa E box binding factor) to the spotlight in the review about its role in T cell commitment and transition through $CD4^{-}CD8^{-}$ stages of differentiation. Interestingly, $HEB^{-/-}$ DN3 thymocytes can originate NK cells in the thymus. In the mouse embryo, mature NK cells are found in the thymus, but immature, therefore potential NK progenitors, are found in the bone marrow, spleen, and liver. X. Wu et al. compare the development of NK cells in the spleen and liver in the mouse embryo. Their data show that the expression of adhesion molecules as CD11c and CD73 in liver NK cells may account for the higher frequency of these cells in this tissue

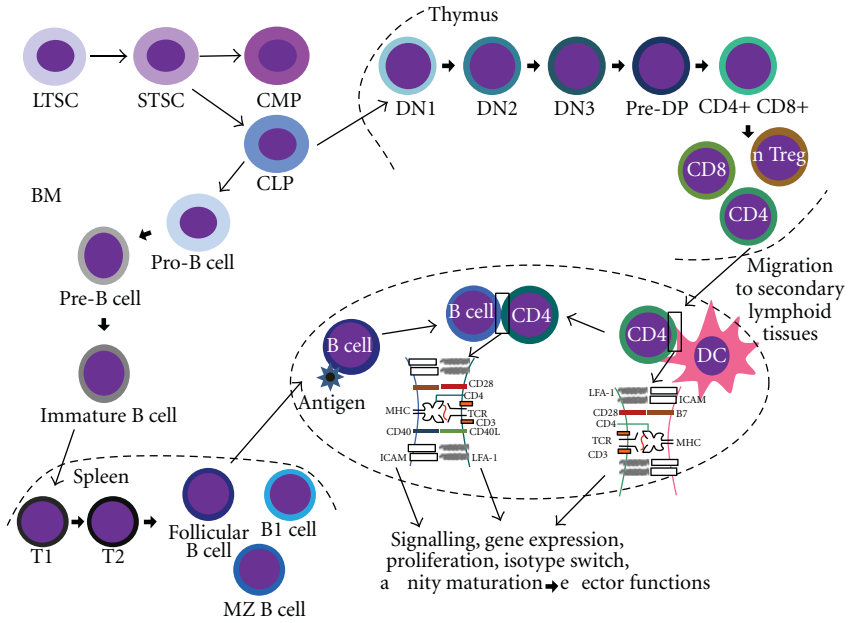

Figure 1: Schematic representation of lymphocyte development and activation. Development of lymphocytes takes place in primary lymphoid organs as the bone marrow (BM) and the thymus. Long-term hematopoietic stem cells (LTSCs) generate short-term hematopoietic stem cells (STSCs), which in turn generate common myeloid and lymphoid progenitors (CMP and CLP, resp.,). T cell progenitor, migrate to the thymus, where they undergo maturation through stages known as double negative (DN), representing $CD4^{-}CD8^{-}$ cells, double positive, and finally mature $CD4^{+}$ or $CD8^{+}$ T cells. Immature B cells leave the bone marrow to finish their development in the spleen, where they progress through transitional stages 1 and 2 (T1 and T2) to generate mantle-zone B cells (MZ B cells), follicular B cells, or B1 cells. All mature lymphocytes circulate through secondary lymphoid organs, where they are exposed to antigens, directly or through antigen presenting cells. After the first stimulation by antigens, B and T cells migrate towards each other to interact in a process that will determine B cell antibody production and T cell proliferation and further activation. The immunological synapses are represented between a dendritic cell and a naive T cell and between primed B and T cells. In detail, one may observe molecules present in immunological synapses.

compared to others and that the liver microenvironment has a role in NK differentiation. Focusing on the cell membrane, instead of cytoplasmic and nuclear factors, as Bcl-xL and HEB, B. Jin et al. review the role of the Toll like receptors in T cell differentiation and activation. This review brings information on the effect of different TLR ligands on T cell development and the effect of activation of different TLRs in antigen presentation, tolerance control, and T cell activation. Regarding interaction with stroma of primary lymphoid organs, R. Romano et al. review the role of FOXN1 in T cell development and primary immunodeficiencies caused by its altered expression in stromal cells in the thymus.

Once mature lymphocytes are generated, they migrate to secondary lymphoid organs where they may encounter antigens and depending on the conditions of this encounter, they may be activated to generate effector responses. Activation of B and T lymphocytes displays some common and some different aspects. Both must recognize the antigen through its B or T cell receptor. However, while BCR binds directly to the antigen, the T cell receptor only binds to antigen presented by antigen presenting cells through the Major Histocompatibility Complex (MHC). This binding takes place in a super structure called Immunological Synapse, where adhesion molecules, costimulatory molecules, and receptors, besides TCR and MHC, are present. Both types of lymphocytes need more than the antigen to mount an efficient effector response. For example, B cells may respond to ligands of TLR besides BCR, and T cells have receptors for costimulatory molecules presented by antigen presenting cells, which are expressed upon proinflammatory signals, as ligands for TLRs. Absence of a second stimulus besides activation of BCR or TCR promotes the induction of anergy or regulatory responses. Upon activation and depending on signals presented to lymphocytes during activation, these cells will differentiate into subtypes with specific functions. CD4 T cells undergo differentiation into CD4 helper phenotypes, as discussed in several articles in this special issue. Already primed B cells may encounter primed T cells and the communication between these two lineages in the lymph nodes will promote isotype switching, affinity maturation, and proliferation in B cells, as well as proliferation and further activation in T cells.

A. Visekruna et al. review the role of the transcription factor NFκB in the T cell activation and effector functions. R. V. Luckheeram et al. discuss stimuli that promote CD4 T cell differentiation in the known T cell Th subtypes, activation, and plasticity and effector functions. With a very different approach, R. von Essen et al. discuss, in a broad review, the concept of avidity maturation in T lymphocytes and signals involved in such mechanism. As mentioned before, for activation lymphocytes need at least two signals, in this review, cytokines are considered the "third" signal

to naive and primed T cell activation and differentiation. Cell polarity in different steps of T cell activation and differentiation is discussed in the review by I. Fung et al., with focus on GTPases, which are involved in cell migration in several immune lineages, and the DOCK8 protein a Rho-Rac guanine exchange factor.

During lymphocyte activation, different stimuli will influence the differentiation of memory cells, which are important for the control of new infections by the same pathogen. M. N. Norazmiet al. discuss the expression and role of Peroxisome proliferator-activated receptor γ 1 and 2 (PPARγ) in human naive and memory T cells upon TCR activation. Data presented by the authors suggest that the two PPARγ isoforms may have different roles during the activation of naive and memory T cells.

Research on lymphocyte biology has a strong bias towards clinical aspects and mechanism of several diseases, from cancer to graft transplantation rejection to pathogen immune responses. This special issue brings one article and one review regarding inhibition of allograft rejection. Antigen presentation and the role of B7 costimulatory molecule in allograft rejection are explored in the article by Y. F. Yao et al. Using an antisense B7 peptide, the authors were able to inhibit T cell alloactivation and inhibit arterial allograft intimal hyperplasia in a murine allogeneic carotid transplant model. R. Wang et al. discuss a population that has been actively studied in cancer, where it plays a protumoral role, but that may be beneficial for the survival of allotransplants. The myeloid derived suppressor cells are described and their potential role in inhibiting cardiac allograft.

C. Wickenhauser et al. describe different thresholds of the activation of B cells by antigen and hapten in patients with leukocyte adhesion-deficiency 1 (LAD1). Contrary to immunodeficiency, patients with chronic C hepatitis frequently develop thyroid disorders during IFNα therapy. Y. Kajiyama et al. show that female patients with a higher serum concentration of BAFF (B-cell-activating factor) display significantly a higher risk of developing B cell dependent thyroid disorders, as Graves disease and the production of thyroid auto antibodies.

Finally, C. Schlimperet al. related their experience in the generation of CIK (Cytokine Induced Killer cells) engineering lymphocytes from colorectal carcinoma patients, using the CAR chimera, chimeric antigen receptor, which binds to the carcinoembryogenic antigen. Their data indicate that this approach was successful in inducing patient T cell proliferation and IFNγ production in an antigen dependent manner.

This special issue covers several important aspects of lymphocyte development, differentiation, and function bringing relevant and up-to-date information in this area.

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Review Article

The Effects of TLR Activation on T-Cell Development and Differentiation

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Invading pathogens have unique molecular signatures that are recognized by Toll-like receptors (TLRs) resulting in either activation of antigen-presenting cells (APCs) and/or costimulation of T cells inducing both innate and adaptive immunity. TLRs are also involved in T-cell development and can reprogram Treg cells to become helper cells. T cells consist of various subsets, that is, Th1, Th2, Th17, T follicular helper (Tfh), cytotoxic T lymphocytes (CTLs), regulatory T cells (Treg) and these originate from thymic progenitor thymocytes. T-cell receptor (TCR) activation in distinct T-cell subsets with different TLRs results in differing outcomes, for example, activation of TLR4 expressed in T cells promotes suppressive function of regulatory T cells (Treg), while activation of TLR6 expressed in T cells abrogates Treg function. The current state of knowledge of regarding TLR-mediated T-cell development and differentiation is reviewed.

1. Introduction

Innate immunity protects the host from pathogenic infectious agents. Every infectious microorganism possesses conserved molecular structures, for example, lipopolysaccharide, peptidoglycan, flagellin, microbial nucleic acids and these are collectively referred to as pathogen-associated molecular patterns (PAMPs) [1]. PAMPs are recognized by corresponding germline-encoded pattern recognition receptor (PRR) expressed on innate immune cells of the host, for example, dendritic cells (DCs), macrophages and neutrophils [2, 3]. This triggers various signal pathways to produce inflammatory responses and adaptive immunity [4, 5].

At least 5 classes of PRRs have been characterized: Toll-like receptors (TLRs), retinoic-acid-inducible gene-I-(RIG-I-) like receptors (RLRs), nucleotide-binding domain and leucine-rich repeat containing gene family (alternatively named NOD-like receptors, NLRs), C-type lectin receptors (CLRs) and cytosolic DNA receptors (CDRs) [4, 6]. TLRs are membrane-bound receptors that sense PAMPs on the cell surface or in endosomes [7], while RLRs and NLRs recognize microbial molecules in the host cytosol [8]. CLRs are primarily expressed in myeloid cells and recognize polysaccharide structures of pathogens inducing immune responses [6, 9]. With the exception of TLR9, CDRs are a new family composed of at least 6 members that also trigger innate immunity upon detecting cytosolic DNA [10, 11]. TLRs were initially discovered in 1997 [12] and represent a canonical family of PRRs that govern adaptive immune response by inducing a Th1-skewed response, immunoglobulin G2c production and antigen-specific cytotoxic T lymphocyte (CTL) response [13–15].

Upon recognition of foreign antigen for DCs via the TLR-PAMP interaction [4, 16], immature DCs resident in tissues mature into professional antigen-presenting cells (APCs) to induce effector and memory T-cell responses in lymphoid organs. Additionally, DCs are capable of inducing antigen-specific T-cell tolerance immunosuppression (Figure 1) [16]. T cells are divided into different subsets based on their phenotypes, intracellular molecules expression, cytokine production, the lengths of telomeres and state of immunity [17]. The current knowledge of TLRs activation in relation to T-cell activation and differentiation is presented here.

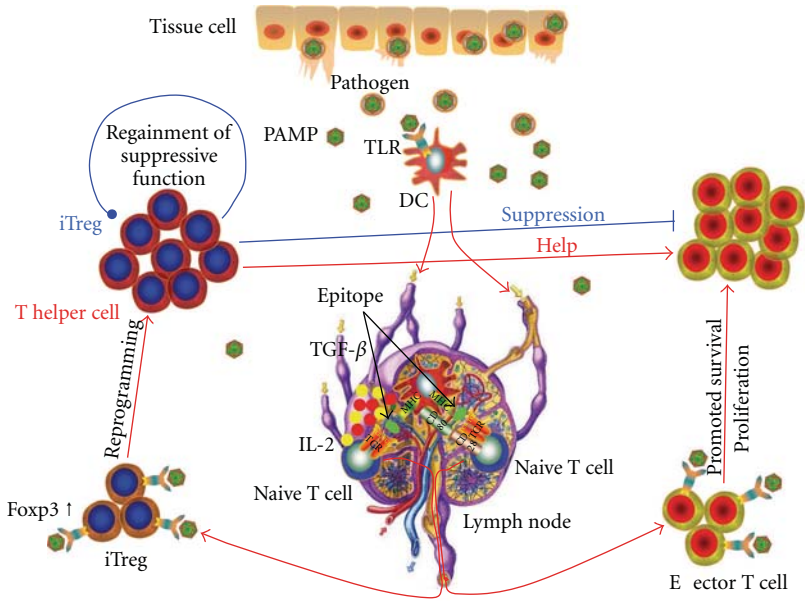

Figure 1: The effects of TLR on T-cell activation. PAMPs from invading pathogens bind with TLRs expressed in DCs, which causes DC activation. Activated DCs migrate to the draining lymph nodes where, in the presence of co-stimulatory signals and instructing cytokines, they present the antigen epitope with MHC molecules to activate naive T cells. DCs also induce iTreg in the presence of TGF-β and IL-2. These activated T cells move to the site of infection to fight against the invading pathogen. Activation of TLRs in activated T cells induces their survival and clonal expansion. Direct engagement of TLR in iTreg cells promotes their expansion with reduced suppressive function and reprograms them to differentiate into T helper cells, which in turn provide help to effector cells. When the infected pathogen is eliminated, the clearance of TLR ligands results in the suppressive function of the expanded iTreg cells being restored. This serves to regulate the expanded effector T-cell population.

2. T Lymphocyte Development and Subsets Differentiation

2.1. T-Cell Development in Thymus (Figure 2). Thymic T-cell progenitors are believed to come from circulating hematopoietic stem cells originating from bone marrow. All peripheral T cells are developed from these progenitor cells [18–20]. The entry of T-lymphoid progenitor cells at an early embryonic developmental stage before vascularization of thymus, or at later embryonic and postnatal stages after vascularization, initiates development of T cells in the thymus [21, 22]. Thus, T progenitor cells can travel to and reside in thymus via either a nonvascular route at an early embryonic developmental stage or via a vascular way at late embryonic and postnatal stages. Chemokines such as C-C chemokine receptor type 7 (CCR7) and CCR9 play a role in the prevascular colonization of T-cell progenitors into the thymus primordium [23], while the combination of P-selectin and P-selectin glycoprotein ligand-1 is involved in postnatal thymus seeding [22]. These cells initially express neither CD4 nor CD8 and are referred to CD4/CD8 double-negative (DN) thymocytes [24]. Such DN thymocytes migrate from the corticomedullary junction to the subcapsular region of the cortex and sequentially transform into DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$) and DN4 ($CD44^-CD25^-$) [25–27] cells with weak expression of CD4, CD8, CD25 and CD44. These are the direct precursors of CD4/CD8 double-positive (DP) thymocytes [28]. DP thymocytes develop in thymus cortex from pre-DP where son of sevenless gene 1 (Sos1), a guanine nucleotide exchange factor for Ras, plays a pivotal role during this transition [29]. DP thymocytes express TCR$\alpha\beta$ on the cell surface and these interact with self-peptide-MHC complexes presented by cortical thymic epithelial cells (cTECs) for positive selection (i.e., survival) or negative selection (clonal deletion, i.e. death). The process is determined by avidity and aggregation of TCR with the ligand interacting with one another [30]. Development of single positive (SP) lineages of $CD4^+CD8^-$ or $CD4^-CD8^+$ thymocytes is determined during positive selection [20] and the properties of protein degradation and self-peptide presentation of cTEC may play a role in SP lineages positive selection [30, 31].

Positively selected thymocytes migrate to the medulla via CCR7-mediated chemotaxis [30]. The medullary TECs (mTEC) ectopically express multifarious "tissue-specific" antigens (TSAs)/peripheral tissue-restricted antigens (PTAs), that is, promiscuous gene expression representing peripheral tissues [32, 33]. This expression is partially controlled by the transcription factor autoimmune regulator (AIRE) [34]. Antigens from either apoptotic mature mTECs or peripheral tissues are taken up by thymic DCs and cross-presented to developing thymocytes to induce negative selection of self-reactive thymocytes establishing self-tolerance [30]. It is suggested that circulating DCs bearing peripheral tissue antigens are also recruited intrathymically for cross-presentation and

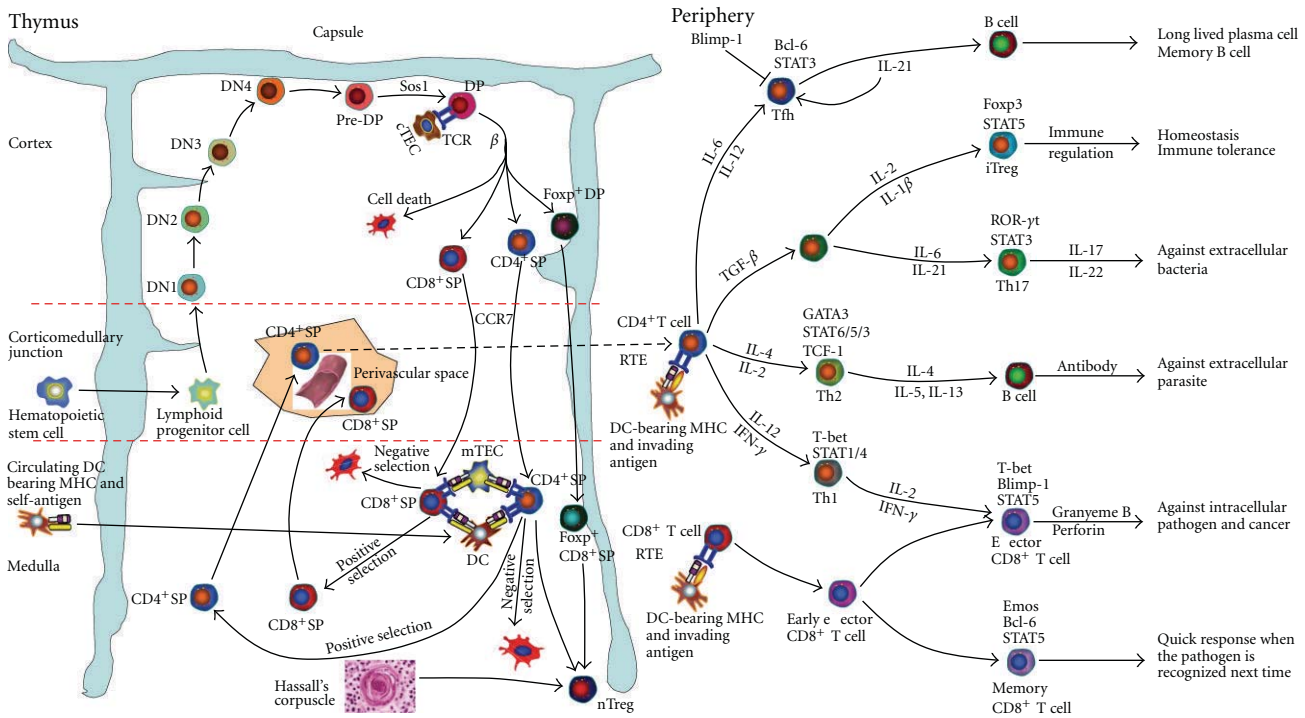

Figure 2: T-cell development and differentiation. It is believed that thymic lymphoid progenitor cells are derived from circulating hematopoietic stem cells originating from the bone marrow. The initial CD4/CD8 double-negative (DN) thymocytes migrate from the corticomedullary junction to the subcapsular region of the cortex and sequentially transform into DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$), DN4 ($CD44^-CD25^-$) and pre-DP cells, which weakly express CD4, CD8, CD25 and CD44. Then CD4/CD8 double-positive (DP) thymocytes under the influence of a guanine nucleotide exchange factor for Ras, Sos1 develop TCR$\alpha\beta$ surface expression. cTECs present self-peptide-MHC complexes to TCR$\alpha\beta$ to induce clonal deletion or thymocytes developing into CD4 or CD8 SP cell lineage. nTreg cell development possibly begins at the DP stage. $Foxp3^+$ DP thymocytes with a functional IL-7 receptor and upregulated expression of Bcl-2 protect themselves from being negative selected. $Foxp3^+$ DP thymocytes with CD103 expression are possible precursors of $Foxp3^+$ $CD8^+$ SP cells and finally differentiate into nTreg cells. SP cells move to the medulla through CCR7-mediated chemotaxis and interact with mTECs, which promiscuously express multifarious "tissue-specific" antigens. These antigens are taken up by DCs and cross-presented to developing thymocytes to induce negative selection establishing self-tolerance or nTreg lineage development. Circulating DCs bearing peripheral tissue antigens are also recruited intrathymically for cross-presentation. mTECs are also able to serve as APCs to induce nTreg lineage development and negative selection. Hassall's corpuscles are required to support nascent nTreg cell development. Positively selected mature thymocytes migrate through perivascular space in the corticomedullary junction and medulla and become peripheral naive T lymphocytes. When infection occurs, APCs process antigen and present epitope in combination with MHC molecules to TCR on the T-cell membrane in the presence of co-stimulatory molecules and with the help of specific cytokines to induce T-cell differentiation. IL-12 and IFN-γ are essential for the induction of Th1 cell. IL-4 and IL-2 are required for naive $CD4^+$ T-cell differentiation into IL-4-producing Th2 cells. TGF-β stimulates naive $CD4^+$ T cell to differentiate into Th17 cells in the presence of IL-6 or induces iTreg cell in the presence of IL-2. Th17 cells can also be induced by an alternative pathway through the cooperation of TGF-β and IL-21 without the participation of IL-6. Tfh cells are induced with the help of IL-6 (mice) or IL-12 (human) to produce IL-21, which backfeeds to promote Tfh cell differentiation. As a major transcription factor, T-bet along with STAT4 and STAT1 is essential for Th1 cell differentiation. Activated Th1 cell can produce IFN-γ and IL-2 to help $CD8^+$ effector T-cell functioning. GATA3 is the Th2 master regulator. STAT6 and STAT5 are essential in Th2 cell differentiation and expansion. STAT3 cooperates with STAT6 in promoting Th2 cell development. TCF-1 participates in GATA3 activation and promotes STAT6-independent IL-4-producing Th2 cell differentiation. Th2 cells secrete IL-4, IL-5 and IL-13 to boost antibody production in B cells against extracellular parasites. Production of IL-17 by Th17 cells is ROR-γt and ROR-α dependent. STAT3 is involved in Th17 cell differentiation, expansion and maintenance. Th17 cells participate in the immune response against extracellular bacteria by production of IL-17. Treg cell development is controlled by Foxp3 that is required for Treg lineage commitment, differentiation, expansion and function. STAT5 promotes Treg cell development by enhanced expression of Foxp3. Treg cells play a critical role in maintaining homeostasis and immune tolerance by suppression of effector cell in a cell-contact or cytokine-mediated pattern. Lineage commitment of Tfh cell is controlled by Bcl-6, while Blimp-1 plays an inhibitory effect on Tfh cell generation and function. STAT3 is necessary for Tfh cell development. Tfh cells interact with B cells in germinal center to induce generation of long-lived plasma cells and memory B cells. Naive $CD8^+$ T cell primed by signals from TCR and co-stimulatory molecules differentiate into early effector cell expressing transcription factor T-bet and cytotoxic cytokines, for example, IFN-γ, TNF-α to acquire partial cytolytic abilities. The early effector cell further differentiates into late effector cell or memory cell, and this is determined by multiple factors such as the strength of IL-2R and the presence of IL-12, the presence of distinct amounts of intracellular components such as proteasome, T-bet, CD8 and IL-7Rα, or the potency of TCR signals. T-bet and Blimp-1 are responsible for IFN-γ expression and participate in the cytolytic gene expression, for example, Granyeme B, Perforin to induce short-lived effector $CD8^+$ T cells. STAT5 plays a critical role in maintenance of phenotype of effector $CD8^+$ T cells. Eomes and Bcl-6 expressions favor memory $CD8^+$ T-cells differentiation. STAT5 activation also promotes memory $CD8^+$ T-cell survival.

therefore involved in clonal deletion [35]. Mature thymocytes that have completed T-cell development emigrate from thymus through perivascular space in the corticomedullary junction and medulla [36] to peripheral lymphoid organs. T-cell emigration is regulated by sphingosine-1-phosphate receptor 1 [37, 38]. Different subsets of T cells may have different affinities for blood/lymphatic vessels and these determine the routes of emigration [32]. A new subset phenotypically and functionally distinct from peripheral naive T cell that emigrates from the thymus referred to recent thymic emigrants (RTEs) requires further maturation in secondary lymphoid organ to become functionally competent peripheral T cells [39].

Self-tolerance is induced in thymus either by negative selection or by natural regulatory T cells (nTreg) development. Most of the nTreg cells are derived from $CD4^+$ SP thymocytes residing in the medullary compartment of the thymus [40, 41]. It is hypothesized that tolerance of uncommon self-antigens such as myosin usually presents after muscle injury is preferentially recognized by TCR and mediated by nTreg cells. By contrast, cells that are involved in chronic engagement of TCR/CD28 signaling by recognizing ubiquitous antigen, for example, albumin, the 5th component of complement, insulin, are negatively selected [40, 42, 43]. Decreased presentation of cognate antigens on mTECs or DCs can induce nTreg cell development [44]. Distinct APC subsets may preserve different TCR specificities and their ability to mediate negative selection [40, 45–47]. It has been suggested that forkhead box P3 (Foxp3) negative nTreg cell precursors, induced by TCR signaling, can use interleukin-2 (IL-2), IL-15, or IL-7 to activate Foxp3 expression without the need for additional TCR signals [40]. It is believed that nTreg cell development begins early at the DP stage in pediatric thymus. $Foxp3^+$ DP thymocytes with a functional IL-7 receptor and upregulated expression of Bcl-2 protect themselves from being negative selected. $Foxp3^+$ DP thymocytes that express CD103 are possible precursor of $Foxp3^+$ $CD8^+$ SP cells [48]. Hassall's corpuscles, groups of epithelial cells in the thymic medulla, may serve as specialized small niches required to support nascent nTreg cell development [49].

2.2. Development of T-Cell Peripheral Tolerance (Figure 3). In addition to the tolerance induced in thymus, autoreactive T cells that have escaped from negative selection in thymus due to low avidity of TCR to self-peptide-MHC complex [50] or insufficiently expressed TSA in mTECs will be deleted (cell death) or inactivated (anergy) in periphery, that is, peripheral tolerance [42].

Lymph nodes are a primary location where peripheral tolerance takes place. It has been demonstrated that lymph node stromal cells (LNSCs), similar to mTECs in thymus, are able to express a variety of TSAs to induce immune tolerance of T cells [51]. The peripheral expression of TSAs is either AIRE dependent [52, 53] or independent [54]. Another regulating factor, deformed epidermal autoregulatory factor 1 (Deaf1) is also involved in PTAs expression [55]. Deaf1 variant isoforms inhibit the transcriptional activity of canonical Deaf1 and this suppresses PTA expression [55]. Deaf1 transcript has been detected in every subset of LNSCs [56]. All the subsets of LNSCs can express TSAs and present TSAs to activate antigen-specific $CD8^+$ T cells under both steady-state and inflammatory conditions [56]. By contrast, cross-presentation of TSAs produced from LNSCs by lymph node resident DCs does not seem to play an important role [57]. Although TSA proteins expressed by LNSCs might be functional [51, 58], the expression of TSA protein by LNSCs is different from its expression in peripheral tissue. This is evidenced by the fact that even the products from a single type of differentiated peripheral cell can be produced separately from distinct subsets of LNSCs, for example, both the protein of mlana gene expression and tyrosinase are products of terminally differentiated melanocytes, their mRNA expression as PTAs in lymph node is segregated in fibroblastic reticular cells (FRCs) [56] and lymphatic endothelial cells (LECs) [54], respectively. In addition to TSAs expressed by LNSCs, lymph can also serve as a source of self-antigens to induce peripheral tolerance in lymph node [59]. Compared with plasma, lymph contains more processed protein fragments and peptides from draining organs or tissues [60] and thus a significant pool of self-antigen for the induction of peripheral tolerance [59].

LNSCs are reported capable of upregulating co-stimulatory molecules to induce T-cell lineage deletion rather than activation [56]. The role of LNSCs in the induction of Treg cell is unknown [51]. It has been suggested that autoimmunity is promoted by induction of self-antigen specific effector-memory T cells when their TCR is continuously engaged at sites of high TSA expression under conditions of tissue injury, infection and/or inflammation [42]. Without inflammation, DCs resident in peripheral lymph organs would induce tolerance in naive T cells bearing TCR with high avidity for self-antigen and incomplete maturation of DC also serves tolerance induction [42]. The peripheral deletion of autoreactive T-cell lineage is mediated by an apoptosis involving activation of Fas receptor by Fas ligand and inactivation of survival protein B cell lymphoma 2 (Bcl-2) by its antagonist Bcl-2-interacting mediator of cell death (Bim) [42, 61]. A nonapoptotic mechanism of peripheral deletion was recently identified in which autoreactive $CD8^+$ T cells actively invade hepatocytes in liver and are degraded in the endosome/lysosome of the hepatocytes [62]. This process is known as emperipolesis [63] and has been described as early as the 1920s [64]. The invasion of T cells into hepatocyte is dependent on T-cell activation, filamentous actin reorganization, myosin light chain kinase, as well as other kinases like PI3K. Inhibition of this suicide emperipolesis by wortmannin, a kinase inhibitor capable of inhibiting T-cell invasion into hepatocytes *in vivo*, is associated with accumulation of autoreactive $CD8^+$ T cells in the liver, and breach of tolerance results in the development of autoimmune hepatitis [62]. By interrupting costimulation, functional tolerance of T cell, that is, anergy can be developed and maintained by counter-regulatory receptors such as cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) that shares structural similarity with CD28 capable of binding CD80 and CD86 and blocking CD28 costimulation [42, 65]. Another counter-regulatory molecule, programmed

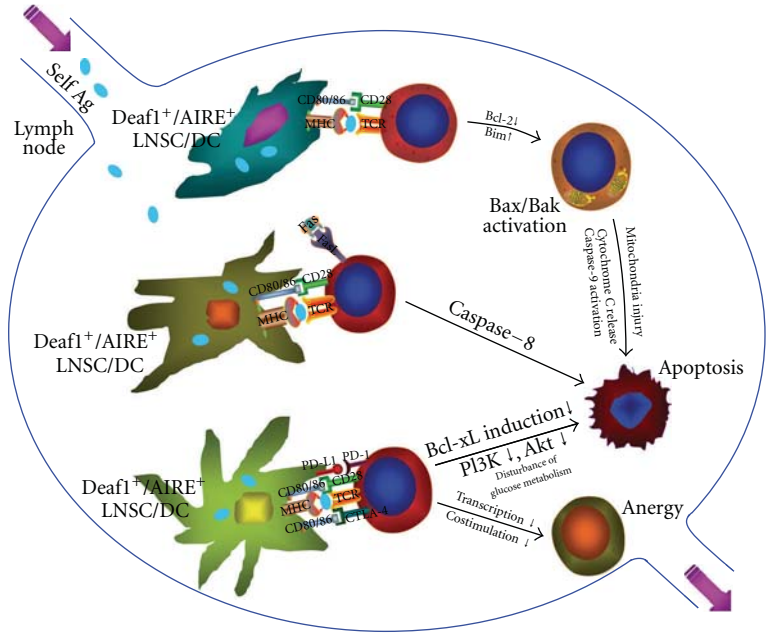

Figure 3: Peripheral T-cell tolerance in lymph node. All the subsets of LNSC can express PTA. AIRE and Deaf1 are involved in the regulation of this expression. Both the LNSC and follicular DC in lymph node can serve as APC to present or cross-present self-epitopes to T cells. Lymph contains abundant-processed protein fragments and peptides from draining organs or tissues and serves as a significant pool of self-antigen for the induction of peripheral tolerance. LNSC can upregulate co-stimulatory molecules to induce T-cell lineage deletion. The autoreactive T-cell lineage deletion is mediated by apoptosis mediated by Fas or Bim signals when inflammation is absent. The engagement of Fas ligand with Fas on T-cell surface triggers the apoptosis of activated T cell through caspase-dependent pathway. T-cell stimulation causes downregulation of Bcl-2 and a transient slight upregulation of Bim and this results in increased uncomplex Bim which is combined with Bcl-2 in resting status. This then activates Bcl-2 homologous antagonist/killer (Bak) and Bcl-2–associated X protein (Bax). Consequently, the integrity of mitochondria is damaged and this culminates in cell death. The tolerogenic DCs induce T-cell functional tolerance, that is, anergy by upregulation of either CTLA-4 or PD-1 expression in T cells. Augmented expression of CTLA-4 can block co-stimulatory signals by binding to CD80/86 in competition with CD28 to induce T-cell anergy. In recognition of self-antigen, PD-L1 on tolerogenic DCs interacts with PD-1 on T cells to limit T-cell activity in peripheral tissues and maintain T cell in unresponsiveness. PD-1 suppresses the PI3K induction and Akt activation. This disturbs cellular glucose metabolism and impairs T-cell survival. PD-1 activation also inhibits the cell-survival factor Bcl-xL production. CTLA-4 engagement blocks Akt phosphorylation by activation of protein phosphatase 2. Engagement of both PD-1 and CTLA-4 can significantly decrease gene transcriptions of T cell being activated.

cell death-1 (PD-1) is also crucial for the maintenance of peripheral tolerance [65].

2.3. Development of Mucosal Tolerance. Mucosa discussed here are those that line the gastrointestinal system and the respiratory system including nasal passages. The largest immune organ of the body is the gut-associated lymphoid tissue (GALT) consisting of Peyer's patches and isolated lymphoid follicles [66] located within the small intestine. Each meter of human intestine has approximately 10^{12} lymphoid cells [67]. GALT processes dietary antigens and is responsible for immunotolerance toward intestinal commensal flora.

Intestinal commensal microbiota is essential for adaptive and innate immunity. In germ-free mice, the absence of these bacteria results in impaired local and systemic immune responses. This is evidenced by a reduced number and smaller sized Peyer's patches, a reduced number of mesenteric lymph nodes and diminished IgA and IgG production [66, 68–70].

Metabolites of intestinal microbiota, for example, in mice with dextran-sulfate-sodium- (DSS-) induced colitis, short-chain fatty acids such as acetate, a fermented product of *Bifidobacterium* when it acts on dietary fiber, interact with G-protein-coupled receptor 43 and stop the differentiation of IL-17-producing cells in the lamina propria [71]. Metabolites from food and food proteins also determine susceptibility to systemic infection, immunoreactivity and immune tolerance [72–75]. A unique property of mucosa when exposed to ingested antigens is suppression of immune responses to subsequent parenteral challenges with the same antigen [76, 77]. This physiologically induced tolerance is referred to as oral tolerance [66, 67, 78, 79]. Mucosal DCs can produce TGF-β, IL-10 and induce $CD103^+$ DCs to promote Tregs induction [80, 81]. Resident lamina propria $CD103^+$ DCs can promote $Foxp3^+$ Treg cell differentiation and induce gut-homing receptors, for example, CCR9 and $\alpha 4\beta 7$ integrin expression in T cells [82].

The orally ingested antigen can be taken up by a variety of mechanisms. Microfold cells (M cells) are specialized epithelial cells without microvilli and thick glycocalyx in the small intestine overlying Peyer's patches and lymphoid follicles and are responsible for transcytosis [69]. These

cells express TLR4, platelet-activating factor receptor, $\alpha 5\beta 1$ integrin and galectin-9 on cell surfaces that enable M cells to sense and transport intestinal antigens into intraepithelial pockets to be processed by APCs [83]. Intestinal columnar epithelial cells are also capable of transporting luminal antigens through these PRRs [83] or the epithelial-associated neonatal Fc receptor to secrete and combine IgG or IgG-antigen complexes to cross mucosal epithelial cells [84]. DCs by their cellular processes which traverse the epithelium without disrupting tight junctions can sense luminal antigens [85, 86].

A variety of regulatory mechanisms are involved in oral tolerance. The amount of ingested antigen is a major factor that determines the mechanism of oral tolerance. Generally, low amounts of antigen result in Treg induction while higher doses lead to immune cell anergy or clonal deletion [67]. Activation of mesenteric lymph node $CD103^+$ DCs preferentially induces $Foxp3^+$ Treg cells differentiation from $Foxp3^-$ naive conventional $CD4^+$ T cells in the presence of TGF-β and the dietary vitamin A metabolite, retinoic acid [81, 87]. $CD103^+$ DCs express a retinal dehydrogenase, aldehyde dehydrogenase family 1 subfamily A2 that can convert retinal or vitamin A into retinoic acid. This facilitates $Foxp3^+$ iTreg cell induction [81]. Even in the absence of thymus-derived nTregs, the development of antigen-specific $CD4^+CD25^+Foxp3^+CD45RB^{low}$ cells that are anergic and suppressive can occur [76]. Gut $CD103^+$ DCs also expresses indoleamine 2, 3-dioxygenase (IDO) involved in the activation of $Foxp3^+$ iTreg cells and hence oral tolerance [88]. TGF-β can transform IDO^- DCs into IDO^+ DCs in mice and prostaglandin E2 plays similar role in human [67]. This process involves intracellular signaling for the self-amplification and maintenance of a stable regulatory phenotype in pDCs [89].

All major types of regulatory T cells are involved in oral tolerance, including thymic-derived nTreg, mucosally induced iTreg, IL-10 secreting $CD4^+CD25^{low}CD45RB^{low}$ type 1 regulatory T cell (Tr1 cell), TGF-β-dependent latency-associated peptide $(LAP)^+$ Th3 type Treg and $CD8^+$ Treg [67]. LAP is a propeptide capable of combining TGF-β to constitute a latent TGF-β complex [90]. It has been suggested that after exposure of oral antigen, $CD4^+CD25^-Foxp3^-LAP^+$ Th3 cells produce TGF-β to support $CD4^+CD25^+Foxp3^+$ nTreg cells, induce $CD4^+Foxp3^-$ T-cells differentiation into $Foxp3^+CD25^+LAP^-$iTreg cells and suppress Th1 and Th2 responses [67]. iTreg cells may modulate DCs to produce IL-27 which induces IL-10-producing Tr1 cells [91]. $Foxp3^+$ iTreg cells are essential for mucosal tolerance development [92]. Oral tolerance can also be elicited by oral administration of anti-CD3 monoclonal antibody instead of application of cognate antigen to activate TCR and induce Th3 type $CD4^+CD25^-LAP^+$ Tregs in mesenteric lymph nodes [93]. Oral exposure to ligands of aryl hydrocarbon receptor is also capable of inducing $Foxp3^+$ Treg and Tr1 cells by acting on both T cells and DCs producing IL-27, retinoic acid and IL-10 in the gut [94, 95].

Nasal administration of antigen preferentially induces IL-10-dependent Treg cell development, for example, Tr1 cell and $CD4^+CD25^-LAP^+$ Treg cell [67, 96, 97]. As the antigen exposed to respiratory mucosa does not exert digestion that occurred in the gut, the antigen dosage required to induce nasal tolerance is smaller than that needed in the induction of oral tolerance [98]. DCs that produce IL-10 in the lungs are critical in the induction of IL-10-secreting Tr1 cell development which elicits nasal tolerance [99]. The $CD4^+Foxp3^+$ Treg cells expressing membrane-bound TGF-β also participates in nasal tolerance [100]. CCR7-dependent migration of $CD103^+$ and $CD103^-$ pulmonary dendritic cells to the bronchial lymph node is indispensable for nasal tolerance induction [101]. $CD11b^+$ and $CD103^+$ DCs are the major DC subsets in the lung. In contrast to the actions in the gut, pulmonary $CD103^+$ DCs appears to prime Th2 responses to the inhaled antigen while $CD11b^{hi}$ DCs elicit Th1 responses [102].

2.4. T-Cell Subsets Development and Differentiation in Periphery (Figure 2). $CD4^+$ T cells play critical roles in the functioning of the host immune system. Upon stimulation, peripheral $CD4^+$ T cells can differentiate into T helper (Th) cells or inducible Treg cells (iTreg). Currently, at least 4 Th cell subsets have been identified, Th1, Th2, Th17 and iTreg [103]. T follicular helper (Tfh) has been suggested as a new subset of Th family [104–106]. There is debate whether new subsets such as Th9, Th22 [107–109] are separate lineages [103, 110].

APCs take up antigen and digest it in the cytosol to process the epitope. The epitope is then presented together with MHC molecules to TCR on the T-cell surface. Simultaneously, APCs also secrete co-stimulatory molecules for example, CD80, CD86 that bind the co-stimulatory receptor of T cells, for example, CD28. Thus all 3 elements are required for T-cell activation, that is, epitope, MHC molecules and costimulation signals. Upon TCR activation, T cells produce CD154 (alternatively named CD40L) to bind CD40 on the cell surface of APCs to further activate APCs. The lineage commitment of Th cells is determined by the cytokine milieu, transcription factors and co-stimulatory molecules such as CD28, CD154. The transcription factors involved in this process are activated by TCR signaling [16, 103]. IL-12 [111] and interferon (IFN) γ [112] are essential for the induction of the Th1 cells. When cognate antigen stimulation is present, IL-4 and IL-2 are required by the naive $CD4^+$ T cells to differentiate into IL-4-producing Th2 cells [113, 114]. Transforming growth factor- (TGF-) β stimulates naive $CD4^+$ T cells either to differentiate into Th17 cells in the presence of IL-6 or alternatively differentiate into iTreg cells in the presence of IL-2 (or IL-1β in human) [115–117]. In the absence of IL-6 and in the presence of TGF-β and IL-21, Th17 cells can also be induced [118]. Primed $CD4^+$ T cells are also able to differentiate into Tfh cells in the presence of IL-6 (mice) or IL-12 (human) expressing IL-21 [119–122]. IL-21 can promote Tfh cell differentiation by feedback. Therefore, it has been proposed that major products of the differentiated cells, for example, IFN-γ from Th1, IL-4 from Th2, IL-17 from Th17, IL-21 from Tfh, play critical roles in its self-induction [103].

Newly primed $CD4^+$ T cells are programmed by various cytokines and other factors from DCs to produce

transcription factors. T box expressed in T cells (T-bet) is a major factor for Th1 cell differentiation and IFN-γ production [123]. It can induce chromatin remodeling of IFN-γ alleles and IL-12 receptor (IL-12R) β2 expression and this promotes IFN-γ production as well as Th1 cell expansion induced by IL-12 [124]. However, in mature Th1 cells, reiteration of IFN-γ expression and stable chromatin remodeling are relatively independent of T-bet activity [125]. Signal transducer and activator of transcription (STAT) protein 4 and STAT1 are involved in Th1 cell differentiation. STAT4 is activated by IL-12 leading to Th1 and Th17 cells differentiation. IFN-γ production also occurs with nuclear factor κB (NF-κB) with multiple cis elements being involved [126, 127]. STAT1 can be activated by IFN-γ and serves as a regulator of T-bet activation and subsequent IL-12R expression *in vitro* [128]. The role of IFN-γ/STAT1 autocrine pathway in $CD4^+$ T-cell differentiation *in vivo* is not fully understood [103].

GATA3, a member of GATA transcription factor family capable of binding to the DNA sequence "GATA," is the master regulator of Th2 [129]. Without GATA3, Th2 cell differentiation is completely abolished both *in vivo* and *in vitro* [130, 131]. GATA3 can bind to 1279 genes in Th2 cells and 17 genes in 26 highly Th2-specific STAT6-dependent inducible genes. Among the 26 Th2-specific genes, 10 showed GATA3-dependent transcription while the remaining 16 genes were STAT6 dependent [132]. Production of Th2 cytokines is also promoted by GATA3 binding to promoters of IL-5 [133], IL-13 [134], and enhancers of IL-4 [135]. GATA3 has the ability to instruct Th2 commitment, promote Th2 cell expansion, suppress Th1 cell differentiation, thus facilitating Th2 differentiation [103].

STAT6 and STAT5 are essential in Th2 cell differentiation and expansion [136–139]. *In vitro* studies showed that activation of STAT6 is necessary and sufficient for Th2 cell differentiation with expansion triggered by IL-4 [140]. However, Th2 lineage commitment can still be induced by activation of GATA3 in a STAT6-independent manner *in vivo* [141]. Thus, it is possible that other transcription factors beside STAT6 may be involved in GATA3 activation. A recent report suggested that T-cell factor 1 (TCF-1) participated in GATA3 activation and promoted STAT6-independent IL-4-producing Th2 cell differentiation [142]. However, TCF-1 expression can be suppressed by IL-4 mediated by STAT6. Thus, the fine-tuning mechanism of Th cell polarization has a multichannel pattern [143]. STAT6 is also involved in the expression of Th2-specific cytokines, for example, IL-24 is mediated by the coordinate action of STAT6 and c-Jun transcription factors at the transcriptional level [144]. Recently, it was reported that STAT3 cooperates with STAT6 in promoting Th2 cell development [145]. A strong STAT5 signaling, correlated with higher expression of CD25, is required for Th2 and iTreg cell differentiation. By contrast, weak STAT5 signaling causes cell proliferation and survival of Th1 and Th17 cells [103]. *In vivo*, promiscuous expression of an activated form of STAT5 suppresses the production of both Th1 and Th17 cytokines and promotes the development of Th2 lineage cells [137].

The master regulator of Th17 cell is retinoic acid receptor related orphan receptor-γt (ROR-γt) [146, 147]. ROR-γt deficiency results in significant reduction in IL-17 production. The residual IL-17 production in ROR-γt-deficient cells appears to be attributed to ROR-α. Dual deficiency of ROR-γt and ROR-α completely abolished IL-17 production [147]. SR1001, a high-affinity synthetic ligand binding to the ligand-binding domains of both ROR-γt and ROR-α that induces a conformational change within the ligand-binding domain, is capable of reducing affinity for coactivators and increasing affinity for corepressors. This results in suppression of the receptors' transcriptional activity. Blocking the activities of ROR-γt and ROR-α with SR1001 can inhibit Th17 cell differentiation and function and suppress cytokines expression in mature Th17 cells [148]. STAT3 is involved in Th17 cell differentiation, expansion and maintenance [103, 149]. Stimulation of the common precursor cell of Treg/Th17 by IL-6 activates STAT3 signaling and induces IL-21 expression [150]. IL-21 induces Th17 differentiation, suppresses Foxp3 expression and maintains a sustained STAT3 activation in a self-service autocrine pattern, that is, Th17 cells secrete IL-21, which in turn causes Th17 cells to induce cell differentiation [151]. STAT3 can also be activated by IL-23 and is responsible for the induction of ROR-γt and IL-23R allowing the persistence of Th17 cells [103, 150, 152].

Treg cell development is controlled by the transcription factor Foxp3 [153, 154]. Mutation of Foxp3 gene results in fatal autoimmune disorders in human, for example, immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome or in mice, for example, lymphoproliferative disorder and stable expression of Foxp3 is essential for immune homeostasis [155, 156]. Foxp3 is required for Treg lineage commitment, differentiation, expansion and function [153, 154, 157]. Sustained expression of Foxp3 in the mature Treg cell is essential to maintain the existing phenotype status and to execute the immunosuppressive function of Treg cell. Reduced or abolished Foxp3 production in Treg cells results in acquisition of effector T-cell properties to produce inflammatory cytokines [158–160]. Foxp3 is probably a major but not the master regulator of Treg cell [161] and indeed, it is not necessary for Treg cell development or functioning under certain conditions, for example, the lineage commitment of Treg cells in murine thymus does not require the expression of functional Foxp3 protein [162]. Activated purified naive $CD4^+$ T cells transduced with a retroviral vector encoding Foxp3 and a Thy1.1 reporter produce a >95% $Foxp3^+$ cell population but reproduce only a fraction of the Treg cell signature transcript [163]. Instead, other transcriptional regulators, for example, the combination of IL-2–STAT5 signaling and TGF-β or CD103 responding to Foxp3 play complementary and synergistic roles in controlling Treg cell signature gene expression [161]. Cytokines such as IL-2, TGF-β induce Foxp3 expression and also activate STAT5. The latter directly binds the promoter and the first intron of Foxp3 gene to promote Treg cell development. The loss of STAT5 activation abolished Treg cell differentiation [164–168]. However, Foxp3 can be induced in the absence of STAT5 in developing thymocytes, and the maintenance of Foxp3 expression in

Treg cells is STAT5 independent [158]. Perhaps cytokine-induced STAT5 activation is not required in the development of $CD4^+CD25^+CD122^+GITR^{hi}Foxp3^-$ Treg cell progenitor. Nevertheless, activated STAT5 plays a critical role in converting Treg cell progenitors into mature Treg cells [40, 137, 169]. Treg cell suppresses Th1 cell function through inhibition of IFN-γ transcription during Th1 priming without disrupting T-bet expression and Th1 programming. This suppression is either IL-10 dependent or independent depending on the target T-cell stage of activation and its tissue location [170].

Lineage commitment of Tfh cells is controlled by transcriptional factor Bcl-6, identified by the transcriptional profiles obtained from microarray analysis in Tfh cells that was Bcl-6 upregulated [171]. Bcl-6-deficient T cells were unable to differentiate into Tfh cells and could not sustain germinal center responses [172, 173]. Enhanced expression of Bcl-6 in $CD4^+$ T cells promoted expression of Tfh cell signature molecules CXCR5, CXCR4, PD-1, and downregulated IFN-γ and IL-17 production [172] inhibited other Th lineage cell differentiation [173]. A transcriptional repressor, B lymphocyte-induced maturation protein 1 (Blimp-1) inhibits Tfh cell generation and function, indicating reciprocal regulation of Bcl-6 and Blimp-1 during Tfh cell differentiation [174]. STAT3 is necessary for Tfh cell development [104, 175]. Deletion of STAT3 in $CD4^+$ thymocytes resulted in a greatly reduced number of differentiated Tfh cells after immunization. STAT3 deficiency in T cells also led to defective germinal-center B cell generation and antibodies production [104, 175]. Without STAT3, for example, blockage by a STAT3 inhibitor, even after being activated by IL-6, Tfh cells did not signal B cells [175, 176].

When exposed to foreign antigens, peripheral naive $CD8^+$ T cells differentiate into two reciprocal subsets: short-lived effector T cells, that is, CTLs and long-lived memory T cells [177–179]. Memory T cells can be subdivided into central (Tcm) or effector memory T cells (Tem). Tcm cells express high levels of CCR7 and CD62L and lack immediate effector function but efficiently stimulate DCs in secondary lymphoid organs inducing a new wave of effector cells when secondary challenge occurs. Tem cells express low levels of CCR7 and CD62L, migrate to the infection site and produce cytokines and cytolytic molecules [177, 180]. Tem cells possess most features of CTL. However, Tem cells persist after the elimination of the invading pathogen [177]. A new memory T-cell subset with stem-cell-like properties has recently been identified and termed memory stem T cell (Tscm). This cell is present in humans [181] and mice [182]. Phenotypically within the naive T-cell compartment, for example, $CD45RO^-$, $CCR7^+$, $CD45RA^+$, $CD62L^+$, $CD27^+$, $CD28^+$ and IL-7Rα^+, human Tscm cells highly express CD95, CXCR3, Bcl-2, the β chain of the IL-2 and IL-15 receptor (IL-2Rβ) and lymphocyte function-associated antigen 1 (LFA-1). These cells possess the characteristics of memory T cells such as the ability to rapidly acquire effector functions upon antigen rechallenge. They also can secrete inflammatory cytokines in response to α-CD3/CD2/CD28 stimulation. Such Tscm cells represent the least differentiated T memory cell subset [181]. Wnt/β-catenin signaling may play a role in the induction of this subset [183] but there is conflicting evidence [184].

It has been suggested that the $CD8^+$ effector and memory T cell develops from a single precursor cell when instructed by distinct TCR signals, cytokines [185–189] and not by the APC or when priming of T cell takes place [188]. Naive $CD8^+$ T cells when primed by signals from TCR and co-stimulatory molecules differentiate into precursor cells or early effector cells expressing transcription factor T-bet and cytotoxic cytokines, for example, IFN-γ, tumor necrosis factor (TNF) α to acquire partial cytolytic abilities [177]. Whether the precursor cell further differentiates into late effector cell or memory cell is determined by a variety of factors such as the amount of IL-2R and IL-12 [190, 191], varying amounts of intracellular components, for example, T-bet, CD8, CD69, CD43, CD25, CD44, different expression of IFN-γ, Granzyme B, IL-7Rα, and distinct granularity due to asymmetric division [187, 192]. Point mutations in the TCR β transmembrane domain block the development and function of $CD8^+$ memory T cells. Yet primary effector $CD8^+$ T-cell response is not affected by this mutation. Mutant T cells are unable to induce polarized TCR and intact NF-κB signals in the immunological synapse (the interface between an APC and a lymphocyte). Therefore, distinct TCR signals trigger different programs for $CD8^+$ T-cell differentiation toward either effector or memory pathways [186].

Transcriptional factors, T-bet, eomesodermin (Eomes), Bcl-6 and Blimp-1 are involved in $CD8^+$ T-cell differentiation. T-bet is the master regulator of $CD8^+$ T cells [178]. Its expression is responsible for IFN-γ production and it participates in the activation of cytolytic genes, for example, Granyeme B, Perforin expression of $CD8^+$ T cell [193]. The presence of T-bet with a low level of IL-2 signaling is sufficient to induce $CD8^+$ T cells to develop effector functions but other factors may also participate in terminal differentiation [194, 195]. Eomes, another member of the T-box family of transcriptional factors, is a key transcriptional factor for $CD8^+$ T-cell differentiation [196]. T-bet and Eomes cooperate redundantly to induce effector $CD8^+$ T-cell differentiation and can also act reciprocally to induce memory $CD8^+$ T-cell development [197]. T-bet promotes the differentiation of short-lived effector $CD8^+$ T cells at the expense of central memory cells and Eomes expression favors memory $CD8^+$ T-cells differentiation [198, 199]. The differing quantities of T-bet in diverse T-cell lineages may be attributed to the asymmetric degradation [192]. Proteasomes are unequally distributed during asymmetric cell division and this is responsible for the imbalanced degradation of T-bet in the daughter cells resulting in differing allocation of T-bet to various cell lines [192].

Bcl-6 and Blimp-1 are transcriptional repressors. Blimp-1 expression is required for the terminal differentiation of effector $CD8^+$ T cells, that is, the short-lived $CD8^+$ CTLs [200–202]. Bcl-6 probably works as a reciprocal regulator of Blimp-1 in the process of $CD8^+$ T-cell differentiation [203]. In general, lymphocytes with higher expression of Bcl-6 exhibit greater proliferative capacity, less secretory capacity and promote memory T-cell development. Lymphocytes with higher expression of Blimp-1 exhibit lower proliferative

capacity and greater secretory capacity and they are more conducive to CTL development [203]. Blimp-1 is also highly expressed in exhausted $CD8^+$ T cells [204]. T-bet can induce Blimp-1 transcription via enhanced IL-2R signaling [194].

STAT5 plays a critical role in the maintenance of phenotype of effector $CD8^+$ T cells. It is also required in the induction of the anti-apoptotic molecule Bcl-2 expression by IL-7 and IL-15 and the maintenance of Bcl-2 expression in effector $CD8^+$ T cells [205]. Constitutive STAT5 activation can promote effector and memory $CD8^+$ T-cell survival and Bcl-2 expression [206].

3. TLRs Signaling

3.1. The TLR Family. Toll was initially identified as an essential protein that plays a central role in the establishment of dorsoventral polarity in the embryo of Drosophila [207, 208]. Later, it was recognized as a key modulator for the immune response against fungi in adult Drosophila [209]. Toll-receptor homologues have also been found to be capable of activating adaptive immune response through NF-κB signal [12, 210]. As these receptors are evolutionally and functionally homologous with Drosophila Toll, collectively they are referred to as Toll-like receptors [210, 211].

Thirteen TLRs have been currently identified, TLR1 to TLR13, of which TLR1 to TLR9 are conserved both in human and mice. TLR10 is not functional in mice while TLR11, TLR12 and TLR13 are absent from human genome [212]. TLRs are type-1 transmembrane glycoproteins with a trimodular structure consisting of an N-terminal extracellular ectodomain characterized by inclusion of 16–28 leucine-rich repeats (LRRs), a transmembrane portion containing a single α-helix and an intracellular cytoplasmic portion with Toll/IL-1 receptor (TIR) domain [213, 214]. Each LRR region is composed of 24 amino acids with the conserved motif XLXXLXXLXLXXNXLXXLPXXXFX in sequence, an α-helix and a β-sheet connected by a loop in conformation [214, 215]. The LRRs of the ectodomain combine to display horseshoe-like shape. However, the LRR regions of TLR1, TLR2 and TLR4 do not have the typical conformation in that the conserved asparagine ladder in the central region of LRRs is absent. Consequently, this allows them to adjust their conformation to bind a variety of ligands and coreceptors for signaling [215]. The TIR domain is composed of a five-stranded β-sheet encircled by 5 α-helices. The B-B loop that connects β-strand B with α-helix B in the TIR domain is considered the essential structure for TIR dimerization and subsequent recruitment of TIR domain-containing adaptors [215].

TLRs can be classified as cell-surface TLRs or intracellular TLRs. The former group consists of TLR1, TLR2, TLR4, TLR5, TLR6, TLR10, TLR11 and TLR12, and it is largely expressed on the cell surface and recognizes molecules mainly from microbial membrane, for example, lipid, lipoprotein, or lipopeptide and protein. The latter group is composed of TLR3, TLR7, TLR8, TLR9, and perhaps TLR13 in mice localized in intracellular compartments like endoplasmic reticulum (ER), endosomes, lysosomes, and endolysosomes to detect microbial nucleic acids [212, 216]. The distinct ligand-sensing functions of the individual TLRs may explain their different localization. TLRs on cell surfaces mainly recognize molecules on the surface of the pathogenic microorganisms while those localized intracellularly sense nucleic acids which are released by intracellular degradation of the invading pathogen [217]. An advantage of the intracellular localization of nucleic-acid-sensing TLRs may be the avoidance of TLRs activation by the host homogeneous nucleic acid. Such nucleic acids released from the dying cells can be readily degraded by serum or cytoplasmic nucleotidases before their arrival to the endosome. As nucleic acid-sensing TLRs reside intracellularly, this prevents the occurrence of autoimmunity. However, viral nucleic acid is protected by the viral capsid proteins and is capable of staying in the endolysosome, being recognized by nucleic-acid-sensing TLRs to trigger antiviral immunity [217, 218].

3.2. TLR Signaling Pathway. Intracellular TLRs are present in the ER in resting cells and move to endosomes upon stimulation of the cells (Figure 4). Their residence in ER is maintained by retention signals, for example, the cytoplasmic and ectodomains of TLR9 [219], a 23-amino acid sequence [Glu(727) to Asp(749)] present in the linker region between the transmembrane domain and TIR domain of TLR3 and the transmembrane region of TLR7 [220]. These TLRs can only be activated after being transported to endolysosome [217]. The trafficking of intracellular TLR9 from ER to endolysosomes is through traditional secretory pathways, and Golgi export is required for optimal TLR9 signaling [218, 221, 222]. Trafficking of TLR9 and TLR7 involves a cleavage by lysosomal cysteine proteases within their ectodomains. Without proteolytic modification, their association with myeloid differentiation protein 88 (MyD88) and subsequent signaling is disabled although the capacity of ligand-binding is preserved [216, 218, 221]. Proteolysis is not required for TLR3 signaling during its intracellular trafficking.

Chaperone proteins are required for maintaining the retention of these TLRs in ER in resting cells and their intracellular trafficking. UNC93B1, a highly conserved multiple membrane-spanning protein in ER, is involved in trafficking of nucleotide-sensing TLRs (Figure 4) [223]. A point mutation of UNC93B1 abolishes signaling of TLR3, 7, 9 and 13 as binding to their transmembrane domains is prevented [224]. Association with UNC93B1 promotes TLR9 signaling and represses TLR7-mediated response and mutation of the N-terminal D34A amino acid that suppresses TLR7 signaling enhances TLR7 trafficking and downregulates TLR9 trafficking in DCs. This suggests UNC93B1 favors DNA sensing but not RNA sensing. TLR3 signaling is promoted by overexpression of UNC93B1 and not affected by the N-terminal mutation [225]. However, a recessive N-ethyl-N-nitrosourea-induced mutation (triple D or 3d mutation) that is a missense allele of UNC93B1 disrupts exogenous antigen presentation and signaling via TLR3, TLR7 and TLR9 [226]. Therefore, UNC93B1 is essential for intracellular TLRs signaling and determines the trafficking efficiency of each

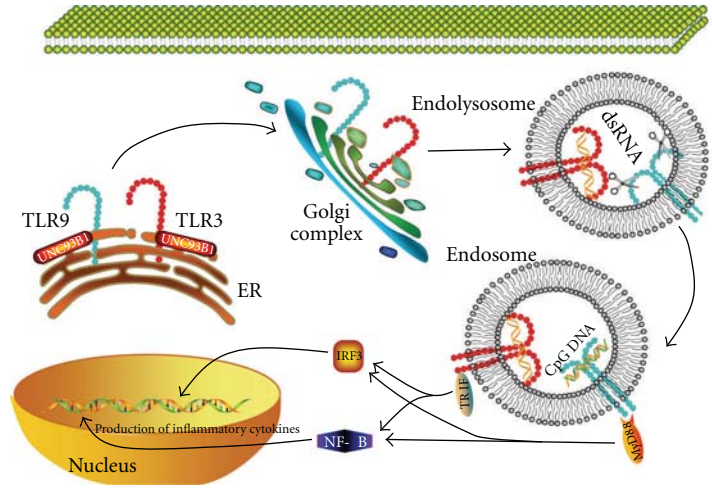

Figure 4: Intracellular TLRs traffic. Intracellular TLRs are present in the ER in resting cells and migrate to endosomes upon stimulation. Chaperone proteins, for example, UNC93B1 are required for their residence in ER and for their intracellular trafficking. When the ligands are taken into the cell, TLRs exit the ER through Golgi complex by conventional secretory pathways and reach the endolysosome where they interact with the ligands. TLR9 is cleaved by lysosomal cysteine proteases within their ectodomains in the endolysosome. TLR3 does not appear to be required for proteolysis during intracellular trafficking.

individual TLR from ER to endolysosome to recognize the ligand and trigger subsequent response [216].

Upon binding ligands, TLRs dimerize to form homodimer or heterodimer (e.g., TLR2/TLR1, TLR2/TLR6 and perhaps TLR2/TLR10) and recruit adaptor molecules through the interaction of their intracellular TIR domain and the TIR domain of adaptor molecules [227]. Four adaptor molecules have been characterized. MyD88 [228] and TIR domain-containing adaptor inducing interferon-β (TRIF)/TIR domain containing adaptor molecule-1 (TICAM-1) [229, 230] are the two major adaptors for TLRs signaling. The remaining two adaptors, that is, TIR domain-containing adapter protein (TIRAP)/MyD88-adapter-like (Mal) [231, 232] and TRIF-related adaptor molecule (TRAM) [233], bridge the TIR domains between some TLRs and MyD88 or TRIF, respectively. MyD88 is a universal adaptor for all TLRs except for TLR3 and activates NF-κB signal pathway to induce inflammatory cytokines. TLR3 and TLR4 use TRIF as their adaptor to activate interferon regulatory factor 3 (IRF3) and NF-κB to promote the productions of type-I IFN and inflammatory cytokines. TIRAP/Mal is required for TLR4 and TLR2 signal transduction by bridging the TIR domain of TLR4 or TLR2 and MyD88 [215, 234]. Similarly, TRAM also acts as a bridging adaptor for TLR4 and TRIF [215].

MyD88 is the essential adaptor for most TLRs. Upon ligand recognition, TLR recruits MyD88 to its cytoplasmic TIR domain by association with the TIR domain of the adaptor molecule (Figure 5). MyD88 possesses an N-terminal death domain (DD) that associates with DD of IL-1R-associated kinase-4 (IRAK4) [235]. IRAK1 and IRAK2 are phosphorylated by IRAK4 and then activate TNF receptor associated factor-6 (TRAF6) [236, 237]. TRAF6 acts as an E3 ubiquitin protein ligase to ubiquitinate itself and NF-κB essential modulator (NEMO) by the formation of polyubiquitin chains. Both TRAF6 and NEMO are connected with IRAK1 by the chains. These chains also connect NEMO with the transforming growth factor β-activated kinase-1-(TAK1-) binding proteins (TABs) including TAB2, 3 and 4 which promote phosphorylation of TAK1-TAB1 resulting in TAK1 activation [238–241]. The activated TAK1 induces phosphorylation of IκB kinase-related kinase (IKK) β. This causes IκB phosphorylation and its dissociation with NF-κB. Consequently, the nuclear translocation of NF-κB is induced and this culminates in the transcription of proinflammatory cytokines, for example, TNF and IL-6. The TAK1/TABs complex also phosphorylates and activates c-Jun N-terminal kinase (JNK) and p38 resulting in activation of activator protein 1 (AP1) [216, 227]. IRF5 can be activated by both MyD88 and TRAF6, and it promotes the transcription of proinflammatory cytokines [242]. This can be inhibited by the competition by IRF4 [243]. TRAF6 also induces TRAF3 triggering noncanonical TRAF3 self-ubiquitination [244] and this complex associates with TRAF family-member-associated NF-κB activator-binding kinase 1 (TBK1). It then acts with IRF3 to induce IFN-β production. Ubiquitinated TRAF3 also induces the anti-inflammatory cytokine IL-10 [245, 246]. In plasmacytoid DCs (pDCs), MyD88 signaling elicited by TLR7 and TLR9 is different from that in myeloid DCs (mDCs). Through phosphatidylinositol 3-kinase (PI3K), MyD88 signaling in pDCs ultimately activates IRF7 to induce production of enormous quantities of IFN-α [247–249]. In humans, TLR3 is predominantly expressed in mDCs whereas TLR7 and TLR9 are exclusively expressed in pDCs [250–255]. TLR expressions in murine DCs are not restricted as seen in human DCs. In mice, mDCs (alternatively named conventional DCs, cDCs) express all TLRs except TLR7 which is not expressed by CD8α^+ mDCs [250, 256]. Indeed, murine pDCs highly express TLR7 and TLR9 along with mRNAs of all the remaining identified TLRs.

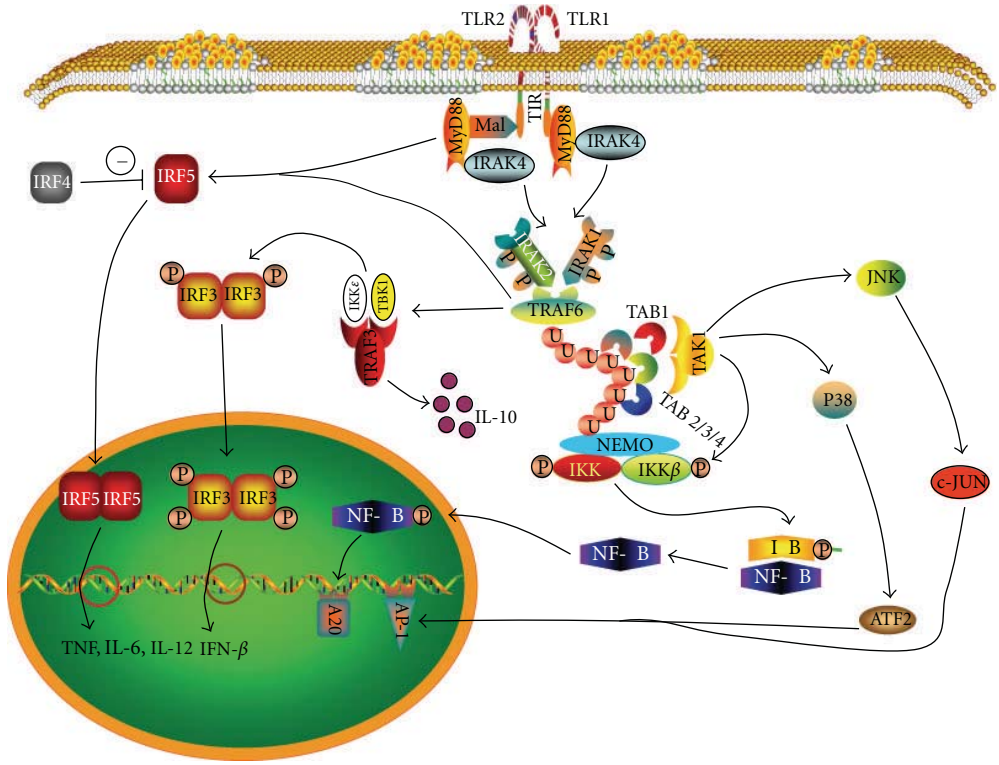

Figure 5: MyD88 signal pathway. MyD88 is the universal adaptor of all the identified TLRs except TLR3. In this figure, TLR1/TLR2 is used to illustrate the MyD88 signal pathway. TLR1/TLR2 uses triacryl lipopeptide as the ligand to recruit MyD88 via its cytoplasmic TIR domain. MyD88 interacts with DD to associate with IRAK4. IRAK4 then phosphorates IRAK1 and IRAK2 activates TRAF6. TRAF6 induces the synthesis of polyubiquitin chains that links TRAF6, NEMO, IRAK1 and TAB2, 3, 4. The ubiquitination of TAB2/3/4 in association with TAB1 activates TAK1. This induces phosphorylation of IKK complex resulting in the dissociation of IκB and NF-κB. NF-κB then translocates into nucleus to induce the gene transcription of proinflammatory cytokines. TAK1 also activates JNK and p38 which induce AP1 activation. MyD88 and TRAF6 both activate IRF5 and induce proinflammatory cytokines. This activation is inhibited by IRF4. TRAF6 also interacts with TRAF3 and then recruits TBK1 to activate IRF3 and IFN-β production. TRAF3 alternatively induces the anti-inflammatory cytokine IL-10.

TLR3 is preferentially expressed in CD8α^+ mDCs and possibly not expressed in pDCs [250, 256]. Therefore, effective antitumor immunity elicited by CpG DNA in mouse is not seen in humans [257].

TRIF is the sole adaptor of TLR3 and the adjunctive adaptor of TLR4. After sensing dsRNA, the TIR domain of TLR3 associates TRIF TIR, then TRIF interacts with receptor-interacting protein 1 (RIP1) through the RIP homotypic interaction motif (RHIM) present in both proteins (Figure 6). TRAF6 is also recruited to the N-terminal domain of TRIF followed by polyubiquitination of RIP1. Peli1, a member of Pellino family of RING-like domain-containing E3 ubiquitin ligases, also participates in RIP1 polyubiquitination along with TRAF6 [258]. The polyubiquitinated RIP1 recruits the ubiquitin receptor proteins TAB2 and TAB3, which in turn activate TAK1 [259]. TAK1 then phosphorylates IKKα and IKKβ leading to degradation of IκB which results in the translocation of NF-κB to cell nucleus to stimulate proinflammatory cytokine production [260]. Similar to MyD88 signaling, TAK1 activates AP1 through JNK and p38. TRIF also associates its adaptor protein NF-κB activating kinase- (NAK-) associated protein 1 (NAP1) to activate TBK1 and IKK resulting in the phosphorylation and nuclear translocation of IRF3, inducing the expression of IFN-β [261]. TRAF3 combines with the TBK1/IKK complex and is also involved in the TRIF-mediated IRF3 activation [245]. It is a unique signal pathway of TRIF that interacts with Fas-associated cell death domain (FADD) protein through RIP1 which in turn activates procaspase-8 to initiate cell apoptosis [262, 263]. Recently, a TIR-less splice variant of TRIF (designated as TRIS) was found capable of activating IRF3 through the interaction with TBK1 and/or activating NF-κB via RIP1 [264]. TLR3 itself is also involved in signaling, for example, the phosphorylation of Tyr759 and Tyr858 in the TLR3 TIR domain. Phosphorylated Tyr759 recruits PI3K to activate kinase Akt which in turn activates IRF3 in nucleus [265]. Additionally, the phosphorylation of Tyr759 and Tyr858 induces degradation of IκB to release and partially activate NF-κB by phosphorylation [266]. Tyrosine kinase c-Src also involves Akt activation [267].

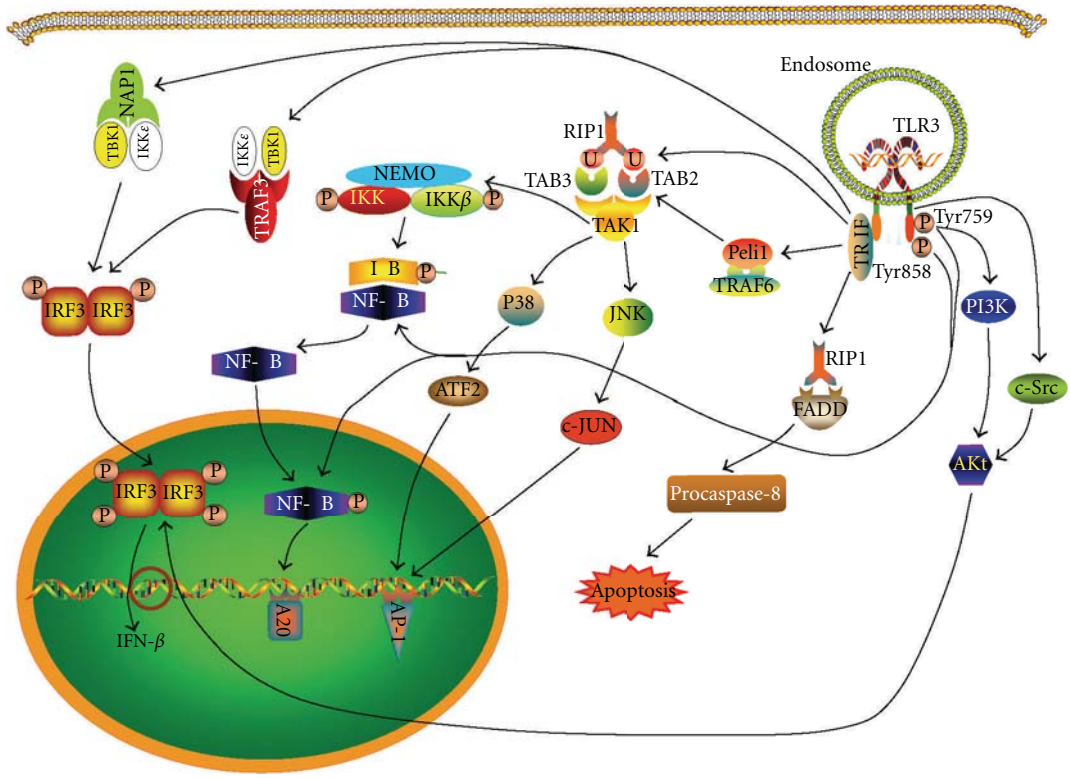

Figure 6: TRIF signal pathway. In TLR1-TLR13, TRIF is the sole adaptor of TLR3 and also an adjunct adaptor of TLR4. Here, the TLR3-TRIF signal is illustrated as an example of TRIF pathway. dsRNA that is internalized in endosome binds to TLR3, which possesses two dsRNA binding sites near the N-terminus and C-terminus, respectively. When combined with dsRNA, a sole dsRNA molecule associates two TLR3 molecules through four dsRNA binding sites in an "m" shape. TLR3 TIR domain combines with the TIR domain of TRIF. The interaction of TRIF with RIP1 or TRAF6 and Peli1 results in polyubiquitination of RIP1, the latter binds ubiquitin receptors TAB2 and TAB3 which activates TAK1. Activated TAK1 induces phosphorylation of IKK complex composed of IKKα and IKKβ and NEMO. This results in the degradation of IκB which ultimately causes the nuclear translocation of NF-κB to activate the specific gene promoter A20. TAK1 also interacts with JNK and p38 to activate c-JUN and ATF2. This results in the activation of the AP-1 transcription factors family. TRIF also activates TBK1 and IKK through NAP1 inducing phosphorylation and nuclear translocation of IRF3 culminating in IFN-β production. TRAF3 binds with the TBK1/IKK complex inducing IRF3 activation. Combination of TRIF results in phosphorylation of Tyr759 and Tyr858 in the TLR3 TIR domain which subsequently induces the phosphorylation and degradation of IκB leading to NF-κB release. Phosphorylated Tyr759 recruits PI3K and phosphorylates kinase Akt and activates nucleic IRF3. Tyrosine kinase c-Src also plays a role in Akt activation. The unique signaling of TRIF is that it interacts with FADD through RIP1 and activates procaspase-8 to initiate cell apoptosis.

4. Effects of TLR Activation on T Lymphocyte Subsets Differentiation

4.1. TLR Signals Affect Thymocytes Differentiation. Various viral infections through TLR interaction can induce type I IFN production. TLR3 recognizes ssRNA virus (West Nile virus), dsRNA virus (reovirus), respiratory syncytial virus, mouse cytomegalovirus (MCMV); TLR7 recognizes ssRNA viruses (vesicular stomatitis virus, influenza virus); TLR8 recognizes ssRNA from RNA virus; TLR9 recognizes dsDNA viruses (Herpes simplex virus, MCMV), CpG motifs from bacteria and viruses [268, 269]. Treatment of newborn mice with an active IFN-α2/α1 hybrid molecule reduced thymus cellularity by 85%. Phenotypic analysis revealed that the quantity of $CD44^+CD25^-$ DN1 cells increased while that of $CD44^-CD25^-$ DN4 cells decreased suggesting that the IFN-α2/α1 inhibition of T-cell development begins at an early progenitor stage [270]. There are deleterious effects of IFN-α on T-cell development mediated by upregulation of cyclin-dependent kinase inhibitor $p27^{Kip1}$ [271]. The TLR3 ligand polyinosinic-polycytidylic acid (poly(I:C)) and TLR7 ligand loxoribine are capable of inducing type I IFN expression resulting in a decrease in $CD44^-CD25^+$ DN3 population [272]. Poly(I:C) can block the DN1-DN2 transition, diminish the DN3-DN4 cell proliferation, promote apoptosis of DP thymocytes, which culminate in a reduced thymic output [273]. As poly(I:C) can activate the cytoplasmic helicases RIG-I and melanoma differentiation-associated gene 5 (MDA-5) pathways [260], the inhibitory effects of poly(I:C) on T-cell development may be not solely mediated by TLR3. Activation of MDA-5 causes a reduction in thymus size while TLR9 ligand CpG DNA and TLR4 ligand lipopolysaccharide (LPS) did not reduce thymus size [274]. Upon stimulation by LPS, the gene expression of downstream signals of TLR3 and TLR4, that is, TRIF signal, is the most differentially affected pathway in murine

thymocytes, suggesting a direct influence of altered TLR signaling on thymus involution [275].

4.2. Effects on T-Cell Differentiation through TLR Activation in APC. TLRs activation has been shown to bridge the innate and adaptive immunity [212, 276–278]. Beside its expression in professional APCs such as DCs and macrophages [276], TLRs can be expressed in T cells [254, 279, 280] and serve as co-stimulatory signals in T-cell activation [268, 277, 278, 281]. Traditionally, activation of TLRs in APCs would lead to the production of IFN-α, proinflammatory cytokines such as TNF-α, IL-1 and IL-6, and the cytokines IL-12 and IL-18 that instruct Th1 to differentiate, whereas an increased Th2 response was observed in MyD88 deficient mice with impaired TLRs signaling [282–284]. The IL-12 and IL-23 secretions of DCs induced by TLRs activation are enhanced by chemokine CCL17 in an autocrine manner. The productions of these cytokines are significantly reduced in CCL17-deficient DCs [285]. It has been demonstrated that the dose of antigen plays an important role in directing Th1/Th2 differentiation driving by DCs. A lower concentration of ovalbumin (OVA) peptide (1 and 10 ng/mL) induced Th2 commitment while higher concentrations (1 μg/mL and 100 ng/mL) failed to elicit Th2 development. Stimulation of $CD4^+$ T cells with DCs along with TLR2 or TLR9 agonists in the presence of the 10 ng/mL of OVA peptide, the optimal antigen concentration for Th2 development resulted in suppression of IL-4 production and Th2 development. This suggests that TLR-activated DCs can block Th2 lineage commitment independent of antigen dosage [286]. A lower dose of LPS (0.1 μg), through TLR4 signaling, induced a Th2 response to inhaled antigens in a murine allergic sensitization model. In contrast, high doses of LPS (100 μg) with antigen resulted in a Th1 response [287]. However, repeated administration of TLR2 ligand Pam_3CSK_4 or TLR4 ligand LPS leads to tolerance of TLR2 [288] or TLR4 [289] with reduced cytokine release and expression of IRAK-1 and IRAK-4 proteins [288]. Additionally, activation of TLR4 resulted in a MyD88-dependent Th17 response in memory $CD4^+$ T cells in the absence of TRIF molecule [284]. Activation of DCs via TLR2-MyD88 also induced Th1 and Th17 cell differentiation [290]. Still, signaling of TLR2 can inhibit DCs to produce IL-12p70 by dampening the type 1 IFN amplification loop. This signaling also drives the immune response induced by synergistic combination of TLR4 and TLR7/8 agonists (both are potent inducers of Th1 responses) toward Th2 and Th17 responses in the naive and memory T-cell subpopulations [291]. Murine DCs activated by LPS or CpG oligodeoxynucleotide (ODN) overcame Treg-mediated suppression by inducing IL-6 signals [292]. IL-6 also mediates the downregulation of Foxp3 expression in T cells induced by TLR7-activated DCs [293]. However, activation of TLR7 by resiquimod in OVA-induced experimental model of murine allergic asthma resulted in expansion of Treg cell through a TGF-β-dependent pathway [294]. Thus, it seems that T-cell subsets activated by TLR signals from APCs vary depending on the type and the status of APC involved, the cytokine milieu, as well as the amount of the antigen present [295–297].

On the other hand, a recent report indicated that signals from Th cells can govern the formation and function of specialized DC subsets, for example, Th1 and Th17 cells cause monocytes differentiation into Th1- or Th17-promoting DC subsets in psoriasis lesion, and Th2 cells induce the production of Th2-promoting DC subset in acute atopic dermatitis [298]. The phenotype of these polarized DC subsets cannot be altered even after subsequent stimulation of TLR ligands. With stimulation by ligands of TLR1-TLR9, the quantities of cytokine secreted by the specialized DC subset were changed but the overall cytokine secretion profile remained the same [298]. The TLR signaling in DCs is negatively regulated by adapters containing immunoreceptor tyrosine-based activation motif (ITAM) sequences to suppress activation of DCs [299], for example, DNAX-activating protein of molecular mass 12 kilodaltons (DAP12) in mDCs [300] and Fc receptors for IgG in pDCs [301]. The triggering receptor expressed on myeloid cell-2 (TREM-2) associates DAP12 to suppress TLR signaling in bone-marrow-derived DCs [302]. The ligand of TREM-2 is also detected on the surface of these DCs. Thus, it seems that the preexisting polarized immunity dictates that the subsequent immune response and this polarization will not be altered even if stimulated by PRR.

4.3. Direct Activation of TLR in $CD4^+$ T Effector Cells Induces Costimulation. The expression and the activity of TLRs in T cells are related to the functional status, for example, effector or memory cells and central memory or effector memory cells as well as the activation status of T cells by TCR signals (Table 1) [268, 277, 303]. Murine naive T cells can express TLR1-TLR9 although there is a considerable variation in expression levels [303]. TLR1, TLR4 and TLR6 were among those maximally expressed in $CD4^+$ and $CD8^+$ T cells [277]. Although naive human $CD4^+$ T cells express significant levels of intracellular TLR2 and TLR4 protein, cell surface expression of TLR2 and TLR4 was found only in activated $CD4^+$ T cells [281]. Cell surface expression of TLR2 in $CD4^+CD45RO^+$ (memory) T cells is significantly higher than that of $CD4^+CD45RA^+$ (naive) T cells. However, TLR2 expression by naive T cells can be significantly increased by anti-CD3 activating TCR. This is enhanced by TLR2 ligand. An activation marker, HLA-DR antigen, was found coexpressed with TLR2 in parallel suggesting that TLR2 expression is associated with T-cell activation [281]. Similar results were also obtained in $CD8^+$ T cells with transcript copies of TLR2 mRNA in CTLs 7–10 times higher than that in naive $CD8^+$ T cells [304]. However, TLR expression in T cells is controversial. When poly(I:C) and CpG DNA were added to murine $CD4^+$ T-cell cultures that were TCR activated by anti-CD3 antibody, TLR3 and TLR9 expression was upregulated with enhanced survival. By contrast, levels of TLR2, TLR4 were undetectable when peptidoglycan and LPS were used [305]. Activated murine $CD4^+CD25^-$ effector T cells can functionally express TLR2 [306]. The discrepancy may be attributed in part to the different protocols used for T-cell purification and the different ligands used for TLR activation. A study compared the differences in purity, activation requirements, specifically, the response to TLR

Table 1: TLR expression and direct effects on T cells [268, 277, 278, 329, 333].

TLR	Location	Typical ligand	Expression in T-cell subsets			Direct effect on T cells
			naive	Activated/Memory	iTreg	
TLR1	Cell surface	Triacryl lipopeptide	±	++	+	Increased effector T-cell proliferation and survival; abrogate the suppressive function of Treg cells
TLR2	Cell surface	Peptidoglycan	±	++	+	Increased cell proliferation and survival; promote cytotoxic activity of CTL; generate efficient memory T cells; augment Treg cell proliferation with temporal loss of suppression
TLR3	Endosome	dsRNA	+	++	−	Promote activated $CD4^+$ T-cell survival
TLR4	Cell surface	Lipopolysaccharide	±	++	+	Induce Treg cell activation; enhance the suppressive function of Treg cells
TLR5	Cell surface	Flagellin	+	+	+	Augment the suppressive capacity of Treg cells
TLR6	Cell surface	Diacryl lipopeptide	+	+	+	Block the suppressive function of Treg cells
TLR7	Endosome	ssRNA	+	+	−	Augment activation/function of T cells; block the suppressive function of Treg cells
TLR8	Endosome	ssRNA	+	+	+	Augment activation/function of T cells; block the suppressive function of Treg cells
TLR9	Endosome	CpG DNA	+	++	−	Promote activated $CD4^+$ T-cell survival; inhibit Treg cell suppression

++: enhanced expression; +: normal expression; ±: weak or low expression; −: expression not detectable.

ligands of human $CD4^+$ T cells isolated by immunomagnetic cell sorting (IMACS-$CD4^+$) or by IMACS followed by fluorescence-activated cell sorting (FACS, IMACS/FACS-$CD4^+$) [307]. It showed that the IMACS/FACS-$CD4^+$ T cells were highly purified (99.7%) and when stimulated by TLR4 ligand LPS, in the absence of TCR activation by anti-CD3 and costimulation from anti-CD28 did not elicit a response. On the other hand, a less pure sample of IMACS-$CD4^+$ T cells (92.5%) showed IL-2 and IFN-γ secretion responding to anti-CD3 without anti-CD28. Stimulation with anti-CD3, anti-CD28, and LPS significantly increased proliferation and cytokine production of IMACS-$CD4^+$ but not IMACS/FACS-$CD4^+$ T cells. The expression of TLR4 was also significantly higher in IMACS-$CD4^+$ cells than in IMACS/FACS-$CD4^+$ cells. This difference is likely to be the result of contaminating accessory cells in IMACS-$CD4^+$ population [307]. Another report using LPS derived from *Salmonella enteritidis*, *Salmonella minnesota* and *Salmonella typhimurium* demonstrated that only LPS from *Salmonella typhimurium* can induce proliferation and IFN-γ secretion in murine $CD4^+$ T cells [306].

TLRs expressed in T cells have been suggested to act as co-stimulatory molecules involved in T-cell activation [268, 277]. Application of Pam_3CysSK_4, the ligand of TLR1/TLR2 complex, in activated TCR transgenic mice $CD8^+$ T cells resulted in increased cell proliferation and survival. This was associated with a sustained CD25 expression and an enhanced expression of Bcl-xL, an antiapoptotic molecule. TLR2 engagement also enhances production of IFN-γ and granzyme B, promotes cytotoxic activity of antigen-activated $CD8^+$ T cells, reduces the activation requirements for co-stimulatory signals from APC and TCR signal strength, and generates efficient memory T cells in response to a weak TCR signal [308, 309]. TLR2 engagement on $CD8^+$ memory T cells is also involved in the direct control of memory cell proliferation and IFN-γ production [310]. The co-stimulatory role of TLR2 ligation on $CD8^+$ T cell is believed to be due to the intrinsic TLR2-MyD88 signaling and PI3K-Akt pathway activation in $CD8^+$ T cells [308, 311]. PI3K signal activated by MyD88 adaptor is indispensable to the costimulation of $CD4^+$ T cells by TLR9 ligand CpG ODN [312]. Costimulation by poly(I:C) of naive $CD4^+$ T cells through TLR3 in the presence of anti-CD3 and anti-CD28 can induce synthesis of IL-17A and IL-21, this being dependent on activation of the NF-κB pathway. IL-17A and IL-21 cause naive $CD4^+$ T-cell differentiation toward an IL-21 phenotype. These cells do not have the transcription factors T-bet, GATA-3 and ROR-c that represent the induction of Th1, Th2 and Th17 subsets, respectively [313] and consequently such cells are absent. TLR ligands can act directly on highly purified T cells in the absence of CD28 engagement [303] but is unable to induce functional responses in naive T cells without concurrent TCR stimulation [308]. Therefore, TLR-induced signals in T cells are strictly co-stimulatory [303] (Figure 7).

4.4. Effects of Direct Activation of TLR on Treg Cells. TLR2 agonist Pam_3Cys acts directly on purified Treg cells resulting in an augmented Treg cells proliferation. This is accompanied by a temporal loss of the suppressive Treg phenotype in the presence of TCR stimulation [314] and a transient suppression of Foxp3 expression [306]. The effects of a reversal of suppression on responder T cells by human $CD4^+CD25^+Foxp3^+$ Treg cells influenced by the TLR2 ligand were Akt being phosphorylated and $p27^{Kip1}$ (The cyclin-dependent kinase inhibitor which is highly expressed in Tregs and capable of arresting cell-cycle in the G1 phase, and can be reduced by IL-2) being downregulated. There was no alteration in Foxp3 expression [315]. On the other hand,

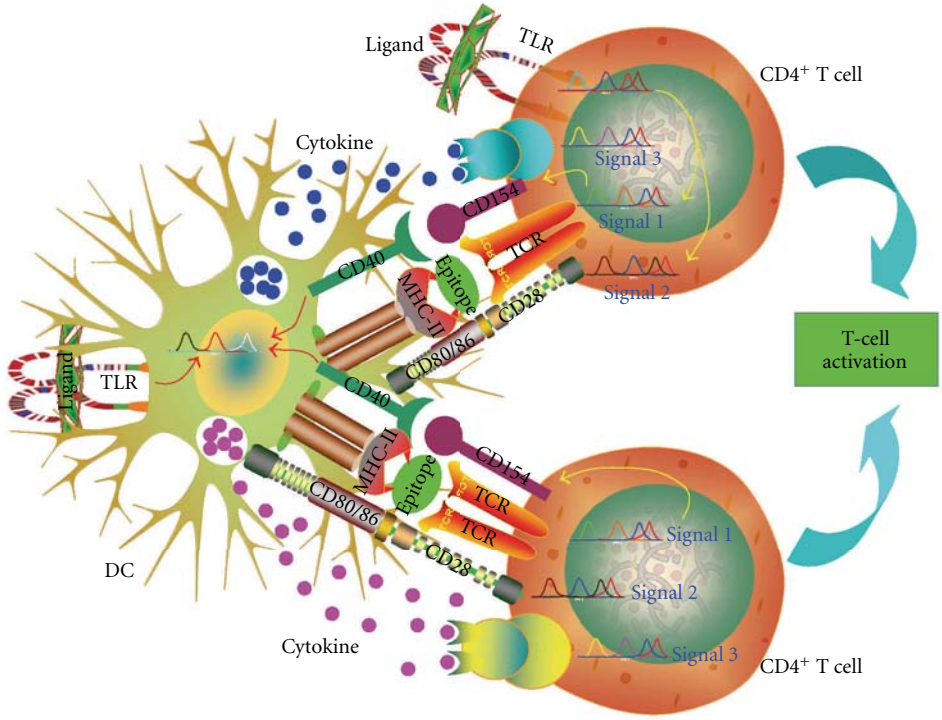

Figure 7: Costimulation of T cells. Antigen uptake by DCs is followed by epitope presentation by MHC complex molecules to TCR expressed on T-cells surface (signal 1). Upon TCR-activation signal, T cells produce CD154 to bind CD40 on the cell surfaces of DCs to further activate DCs. After interacting with TLRs, DCs express CD80 and CD86 which combine with CD28 in T cells for costimulation of T cells (signal 2). Activated DCs also produce cytokines to instruct T cells for polarized differentiation (signal 3). TLRs expressed in T cells act as co-stimulatory molecules in T-cell activation by reducing the activation requirements for signals 1 and 2 and generating efficient memory T cell in response to a weak signal 1. Some TLR ligands even can induce signal 2 in the absence of CD28 via activation of TLR expressed on T cells.

engagement of TLR2 resulted in human $CD8^+CD25^+Foxp3^+$ Treg cells expansion that directly suppressed $CD4^+$ T-cells proliferation by cell-contact inhibition and triggered $CD4^+CD45RO^+$ memory T-cell apoptosis inhibiting allergen induced Th2 immune responses [316]. Treg cells are able to regain their suppressive property in the presence of IL-2 once the TLR2 ligand is removed [306, 314]. Although TLR2-stimulated Treg cells readily lost their ability to suppress proliferation of effector T cells, cytokine production by effector T cells was still repressed. This suggests that the activity of Treg cells was cytokines independent [317]. Treg and Th17 cells are considered divergent and mutually inhibitory. It has been reported that when naive $CD4^+$ T cells were stimulated with TLR2 agonists Th17 differentiation *in vitro* and Th17 cytokine production occurred [318]. Thus, the reduced suppressive function of Treg cells induced by TLR2 stimulation may be a result of imbalanced phenotype and function between Treg and Th17 [315]. The suppression seen in both $CD4^+CD25^{hi}Foxp3^{low}CD45RA^+$ naive and $CD4^+CD25^{hi}Foxp3^{hi}CD45RA^-$ memory or effector Treg cells on $CD4^+CD25^-Foxp3^-CD45RA^+$ naive responder T cells can be reversed by activated TLR1/2. This is accompanied by increased production of IL-6 and IL-17, upregulation of ROR-c and downregulation of Foxp3 expression [319]. Pam_3Cys-mediated reduction of Treg suppressive function can be abrogated by neutralization of IL-6 or IL-17 [319]. All together, in a bacterial infection, the TLR2 ligand augments the functional activities and the clonal expansion of effector T cells as well as temporarily attenuating the suppressive function of Treg cells against the invading pathogen. The TLR2 signal also promotes the expansion of Treg cells that have reduced suppressive function. As the TLR9 ligand can reprogram Treg population toward Th17 differentiation [320, 321], it is conceivable that TLR2 may play a role in Treg cell reprogramming. The proinflammatory cytokines IL-6 and IL-1β are crucial reprogramming cytokines of Treg cells toward Th17 differentiation [322, 323]. When a pathogen is eliminated, the expanded clusters of Treg cells recover their suppressive activity preventing autoimmunity that may result from over activated effectors (Figure 1) [303, 306, 324]. However, it is not known whether the changes observed in reprogrammed Treg cells can be reversed.

Pam_3CSK_4, a TLR1/TLR2 ligand can induce tumor remission in severe combined immunodeficiency (SCID) mice by diminishing the suppressive function of $Foxp3^+$ Treg cells and enhancing the cytotoxicity of tumor-specific CTLs. Adoptive transfer of CTLs and Treg cells pretreated with Pam_3CSK_4 from wild-type mice into tumor-bearing SCID

mice can restore antitumor immunity in SCID mice by reciprocal downregulation of Treg cells and upregulation of CTL function [325]. However, treatment of $CD4^+CD25^+$ Treg cells with intrinsic TLR2 agonist, heat shock protein (HSP) 60, before anti-CD3 activation significantly enhanced the suppressive ability of the Treg cells to inhibit $CD4^+CD25^-$ or $CD8^+$ T-cell proliferation, IFN-γ and TNF-α secretion [326]. Nevertheless, the purity of $CD4^+CD25^+$ Treg cells used being >90% implies possible contamination of other cell types. Not all the $CD4^+CD25^+Foxp3^+$ cells from peripheral blood activated by HSP60 are Treg cells. Activated $CD4^+$ effector T cells can also transiently express Foxp3. It should be noted that only cells with $CD4^+CD25^+Foxp3^+CD30^+$ phenotype possess suppressive function. This induction of Treg cells by HSP60 is enhanced by signaling via TLR4 on APCs [327]. Thus, contaminated APCs within the Treg cell population may promote the suppressive function of Treg cells by TLR4 signaling triggered by HSP60 in APC rather than by TLR2 signaling in Treg possibly accounting for this discrepancy. Indeed, TLR2 expression in human $CD4^+CD25^+CD127^-$ Treg cells isolated from peripheral blood mononuclear cells is not present [328].

Activation of TLR4 in $CD4^+CD25^+$ Treg cells by LPS, in the absence of APC, can directly induce Treg cells activation. This activation involves the upregulation of activation markers, for example, CD69, CD44, CD38, as well as B7-1 and promotes cellular survival and proliferation [329]. TLR4 expression can be detected in peripheral human $CD4^+CD25^+$ Treg cells. Co-culture of these Treg cells with LPS induced activation of Treg cells with decreased expression of Foxp3. These cells repressed neutrophils in an IL-10- and TGF-β-dependent manner [330]. However, the enhancement of Treg cell function by LPS was not reproduced by other investigators [306, 314, 331]. It is possible that potential contamination of commercial LPS preparations with TLR2 ligands [314] or the presence of impurities of the cells [332] may create discrepant results [306, 314, 331]. Application of TLR5 agonist flagellin augments the suppressive capacity of $CD4^+CD25^+$ Treg cells with enhanced expression of Foxp3. $CD4^+CD25^+$ Treg cells can suppress effector T cells in a ratio of 1 : 81 and this inhibition was increased to 1 : 243 with the addition of flagellin [331]. TLR8 is exclusively expressed in human Treg cells, and triggering of TLR8-MyD88-IRAK4 signaling pathway can reverse the suppressive function of Treg cells [333]. A co-stimulatory effect of CpG DNA on $CD4^+CD25^-$ effector T cells is to abrogate the suppression by Treg cells [334]. CpG DNA can also directly act on $CD4^+CD25^+$ Treg cells to inhibit its suppressive effects [334]. Thus, the direct effect of individual TLR ligand on Treg cell is completely different although almost all of the TLR signals share a common pathway (Table 1).

Treg cells' phenotypic plasticity is seen by their expression of proinflammatory cytokines such as IL-17, IFN-γ, or IL-2 under certain conditions and their reprogramming into Th-like cells [321, 322]. Mice systemically administering high doses of CpG ODN at 50–100 μg/mouse show activation of naive Treg cells in the spleen to acquire potent suppressor activity. This was mediated by the immunoregulatory enzyme IDO in pDCs. When IDO was blocked, CpG treatment stimulated pDCs to express IL-6 which in turn reprogrammed Foxp3 lineage Tregs to express IL-17 to become Th17-like effector T cells [335, 336]. The converted Treg cells play a helper role essential for initial priming of $CD8^+$ T cells to a new cross-presented antigen. This was CD40L dependent. This process, unlike the help from conventional non-Treg $CD4^+$ cells, did not require preactivation or prior exposure to antigen [320]. $CD4^+Foxp3^+$ Treg cells can also be reprogrammed into Tfh lineage in mouse Peyer's patches under the interaction with B cells and loss of Foxp3 expression [337]. Although the reprogramming of Treg cell has been recognized to play a critical role in the initiation of certain innate immune responses by vaccination with a TLR agonist adjuvant, that is, CpG ODN [320, 321, 338, 339], the effects of the activation of other TLRs besides TLR9 on reprogramming of T cells especially Treg cells are not known.

4.5. Modulation of $CD8^+$ T-Cell Response by TLR Activation. Viral antigen taken up by APCs are processed into epitopes, loaded onto MHC-I molecules and cross-presented to $CD8^+$ T cells eliciting an anti-virus $CD8^+$ T-cell response. However, not all the potential epitopes can be equally cross-presented to $CD8^+$ T cells. The epitopes recognized by the most abundant cognate T-cell populations are referred to as being immunodominant, while those recognized by less abundant T-cell populations are named as subdominant determinants. Thus, the immunodominant and subdominant determinants constitute a hierarchy (α-, β-, etc.) in an antiviral immune response [340]. This can be altered by TLR signals. Combined activation of TLR2 and TLR3 by Pam_3cysk_4 and poly(I:C) at the infection site of lymphocytic choriomeningitis virus (LCMV) in mice reduced antigen uptake and cross-presentation of an immunodominant determinant of LCMV, NP396 and shifted it becoming a subdominant determinant. However, administration of TLR4 ligand LPS did not induce this shift [341]. Therefore, combined activation of multiple TLRs could possibly induce a complex response instead of being merely synergistic or antagonistic.

4.6. Effects of TLR Activation on Peripheral T-Cell Tolerance. The outcome of presentation by DCs depends on its activation status. DCs activated by PAMPs, for example, TLR ligands from invading pathogen will be capable of producing co-stimulatory molecules and proinflammatory cytokines immunogenic. On the other hand, self-antigen from apoptotic self-cells lack TLR ligands and cannot induce maturation of DCs and this eventually results in tolerance [342, 343]. However, a tumor-associate antigen NY-ESO-1 was able to induce T-cell dependent antibody response through activation of TLR4 on DCs [344]. In addition, mature DCs induced by distinct stimulation may function differently. A recent study suggested that LPS matured DCs produced IL-12 to promote $CD8^+$ T-cell trafficking and inflammation, whereas poly(I:C) matured DCs facilitate $CD8^+$ T-cell infiltration and autoimmunity in an IFN-α-dependent manner [345]. Mesenchymal stem cells can inhibit DCs activation induced by LPS, block DCs migration to draining lymph node and impair its capacities to prime $CD4^+$ T cells and

cross-presentation to $CD8^+$ T cells [346]. The cross-talk between different DC subsets is also important. The cDCs are indispensable for cross-presentation of cancer antigens in eliciting potent anticancer immunity. The efficacy of CpG in anticancer immunotherapy is dependent on activation of TLR9 in pDCs. CpG-activated pDCs induce upregulation of co-stimulatory molecule CD80 in cDCs, thus providing an adjuvant effect in anticancer immunotherapy [347]. Some specific DC subsets may be primarily tolerogenic even if activated. For example, a prototypic DC subset, Langerhans cells is found precommitted tolerogenic and unable to translocate RelB, an NF-κB family member, to the nucleus [348]. However, although Langerhans cells are tolerogenic to bacteria without cell surface expression of TLRs, they can effectively sense virus and poly(I:C) to induce naive $CD8^+$ T-cells expansion and differentiation into effector cells that are dependent on high expression of CD70 rather than mediated by IL-12 [349]. Therefore, mature DCs are not a homogenous population and instead a cell family with increasing new subset member being discovered [350]. They may function divergently depending on its activation status [351] and other factors such as the quality of stimulation, the communication between different DC subsets and the nature of DC subset.

Human monocytes, when cultured with Wnt5a and subsequently stimulated by TLR ligands, can differentiate into DCs. Enhanced production of inhibitory ligands PD-L1 and PD-L2 rather than upregulation of CD83, HLA-DR, CD40, CD86, CD80 and CCR7 molecules would also occur [352]. Additionally, these cells secrete low levels of IL-12p70 and TNF-α, however, there is an increased production of regulatory cytokine IL-10 with a reduced capacity of Th1 response. This tolerogenic DC induction by enhanced Wnt signaling is β-catenin independent but is dependent on non-canonical Ca^{2+}/calmodulin-dependent protein kinase II/NF-κB signaling [352]. Lymph node cells that have precommitted tolerant of self-antigen proteolipid protein, when stimulated by both CpG ODN and this protein, divided and differentiated into Th1 cell lineage. This is IL-12 dependent and these cells are capable of inducing autoimmune encephalomyelitis when they are transferred into naive mice [343]. The break of this cross-tolerance depends on the specific $CD4^+$ T-cell help and stimulation by sole TLR ligands without the help from $CD4^+$ T cell is insufficient to overcome this tolerance [353]. By contrast, induction of TLR signaling in T cells may increase tolerance. T-cell intrinsic TRAF6 is essential in the maintenance of peripheral tolerance. Deletion of TRAF6 in T cells leads to hyperactivation of PI3K-Akt pathway and increased resistance of T effector cells to the suppression by $CD4^+CD25^+$ Treg cells. This finally results in multiorgan inflammatory disease [354]. As TRAF6 is an important adaptor in TLR signaling, it is conceivable that activation of TLRs expressed in T cells may involve in maintenance of T-cell susceptibility to Treg cells via TRAF6.

Administration of TLR3 ligand poly(I:C) results in a strong expression of PD-1 ligand (PD-L1) in all subsets of LNSCs [56]. This may prevent the tolerized T cells in lymph nodes regaining their effector function. However, this also implies that a virus infection in LNSCs such as FRCs would not be eliminated hence becoming a persistent infection [355]. Activation of TLR3 by poly(I:C) also induces upregulation of MHC-I and co-stimulatory molecules in LNSCs, for example, CD80 and CD86 in FRCs, CD80 in LECs [56]. The net result of promoting immune response by enhanced expression of MHC-I and co-stimulatory molecules and promoting tolerance by augmented expression of PD-L1 is a decreased ability of FRCs to stimulate T-cell division in the presence of poly(I:C). However, the phenotypic alterations of these FRCs in PD-L1, MHC-I and co-stimulatory molecules such as CD80 and CD86 are similar to the DCs being treated by poly(I:C) [56]. The decreased stimulatory ability of these FRCs is considered to be the consequence of deduced production of specific antigen by FRCs [56]. Alternatively, this varying stimulatory capacity between FRCs and DCs may be due to the altered TLR signaling cascades in FRCs being tolerogenic cells [356].

The discrimination of self or nonself antigen by DCs is also TLR dependent [342]. TLRs control the TCR ligand generation in phagosome autonomously. With the conjugation of TLR ligand, the phagocytosed antigen by DCs can be selectively loaded on MHC-II molecules and preferentially presented in the context of costimulation [342]. Activation of TLRs is helpful to break tolerance in immunocompromised individuals. Blockade of CTLA4 or PD-1 in combination of TLR9 agonist CpG ODN treatment overcomes immune tolerance in tumor bearing mice with improved long-term survival, increased tumor-specific effector T-cell population and decreased Treg cell levels [357].

4.7. Effects of TLR Activation on Mucosal Tolerance. TLRs are directly involved in mucosal tolerance development. PAMPs from nonpathogenic commensal microorganisms in mucosa are also termed microbe-associated molecular patterns (MAMPs) [358].

TLR1, TLR2, TLR3, TLR4 and TLR5 as well as TLR9 proteins have been found expressed both in human small intestines and colon [359]. However, their expression and action in enterocytes are different even within the same cell. Activation of TLR9 through apical and basolateral surface domains of intestinal epithelial cell (iEC) results in distinct transcriptional responses. Basolateral activation of TLR9 induces IκBα degradation and activation of the canonical NF-κB signal pathway. Apical TLR9 stimulation elicits a unique response with accumulation of ubiquitinated IκBα in cytoplasm-suppressing NF-κB activation. This results in intracellular tolerance to subsequent TLR9 basolateral challenge. It also blocks apical TLR2 and basolateral TLR3 or TLR5 stimulation [360]. However, apical engagement of TLR3 or TLR5 is unable to induce tolerance to subsequent basolateral TLR stimulation [360]. Nasal vaccination of OVA adjuvanted by CpG overcame the nasal tolerance and induced strong Th1 and Th2 responses through activation of TLR9 [361]. This contrasts with the responses of commensal bacteria that suppress Th17 response via TLR pathway to create an immune tolerance niche for colonization. TLR2 on $CD4^+$ T cells can be activated by polysaccharide A from

Bacteroides fragilis but not other TLR2 ligands to induce IL-10 production in the absence of APCs. Specifically, polysaccharide A treated $CD4^+Foxp3^+$ Treg cells display a more potent TLR2-dependent suppressive capacity than those treated by other TLR2 ligands [362].

The mechanism of TLR in maintaining intestinal homeostasis is not fully understood. TLR hyporesponsiveness to commensal microbiota has been suggested to play an important role in keeping homeostasis in the gut. Several mechanisms to account for this hyporesponsiveness include downregulating TLR surface expression and upregulated inhibitory Toll interacting protein with reduced phosphorylation of IRAK [363]. The hyporesponsiveness of intestinal DCs to TLR ligand engagement appears limited to TLR4 [364]. Activation of TLR3 by poly(I:C) in iECs induced retinoic acid early inducible-1 production breaks self-tolerance [365]. Thus, without commensal microbiota, the engagement of TLR in gut epithelial cells from fetal or germ-free animals can induce an inflammatory response. iECs develop TLR tolerance immediately after commensal microbial colonization [366, 367]. It has been suggested that microRNA-146a-mediated translational repression and degradation of IRAK1 are responsible for the induction of neonatal innate immune tolerance in intestinal epithelium [368]. The activation of TLR3, TLR4, TLR5 and TLR9 in iECs induces mitogen-activated protein kinase phosphatase-1 (MKP-1) mediated by NF-κB signaling. MKP-1 plays an important role in the development of tolerance to TLR engagement [369]. Immunity to bacterial infection is tampered in TLR adaptor MyD88 deficient mice [370–372]. The absence of TLRs or MyD88 increased susceptibility to DSS-induced experimental colitis [360]. Administration of TLR ligands in these animals prevents the development of colitis [373]. Therefore, a base level of TLR signaling from the luminal commensal microbiota is required to maintain intestinal homeostasis [370].

A variety of DCs have been identified in intestine [374]. pDCs play an important role in the development of oral tolerance. Orally ingested antigen is presented to T cells in liver by pDCs to induce T-cell anergy or lineage deletion through a $CD4^+$ T-cell-independent mechanism [375, 376]. The output of DCs from lamina propria can be increased 20–30 fold by oral administration of TLR7/8 ligand resiquimod [377]. The activation of TLR in iECs also augmented the DCs sampling of antigen through their extension into gut lumen [86]. Stimulation of human monocyte-derived macrophages with a Gram-positive commensal *Lactobacillus rhamnosus* GG or a Gram-positive pathogenic *Streptococcus pyogenes* demonstrated that both the bacteria can promote TLR2 expression in macrophages. However, only pathogenic bacteria are capable of augmenting IFN-α/β-dependent TLR3 and TLR7 gene expression. Thus, it suggested that human macrophages can discriminate the presence between commensal and pathogenic bacteria by IFN-mediated TLR gene regulation [378, 379]. Intestinal DCs also play a similar discriminative role in identification of commensal or pathogenic agents and the subsequent decision between tolerance and immunity in intestines [380].

5. Conclusive and Perspective Remarks

T cells play a central role in the cell-mediated immunity of the host. All subsets of T cells originate from thymocytes in thymus where they acquire their surface TCR repertoires and develop the primary phenotypic markers then migrate to peripheral lymphatic organ. Upon detection of infectious agents, T cells are activated and differentiate into effector T cells or Treg cells. TLRs are canonical members of PRRs capable of inducing T-cell activation through cross-presentation of APCs or directly acting on T cells. Activation of all the identified TLRs except TLR3 results in signaling through the MyD88-NF-κB pathway. It is not known why activation of TLRs by different ligands results in different outcomes although they act via a common pathway.

The lymph node is the major peripheral lymph organ where antigen-specific responses or tolerance is triggered. As inflammation is a prerequisite to induce immune responses rather than tolerance, it is conceivable that delivery of inflammatory cytokines such as IL-12, IFN-γ to the tumor or its draining lymph node would be helpful to overcome the immunocompromised status in some patients, for example, in cancer patients. Thus, the immunity against cancer which has been suppressed would be reestablished in the cancer-bearing host. Indeed, intrinsic IL-12 is capable of converting $Foxp3^+$ Treg cells into IFN-γ^+ Th1, IL-17^+ Th17, or $Foxp3^+$IFN-γ^+/$Foxp3^+$IL-17^+/$Foxp3^+$IFN-γ^+IL-17^+ transitional cells. The transitional $Foxp3^+$IFN-γ^+ cells further differentiate into IFN-γ^+ Th1 cells but not $Foxp3^+$ Treg cells although they still retain their regulatory functions at this stage [381]. Intratumoral delivery of IL-12 and granulocyte macrophage colony-stimulating factor (GM-CSF) recruits immunogenic DCs to tumors and later migrates to the local draining lymph nodes. However, these cells have a short half life and become IDO-positive tolerogenic DCs after a few days. Interestingly, the initial recruitment and activation of DCs as well as the subsequent switch to tolerogenic activity are both under the influence of IFN-γ [382]. It would be of interest to note whether the delivery of IL-12 to the lymph node would maintain or restore these DCs immunogenic.

Current studies support the concept of reprogramming of TLR ligands, for example, CpG ODN on Treg cells. This raises the question of whether it might be possible to overcome the immunosuppressive effects of Treg cells, for example, in patients with disordered immunity. Indeed should the Th cell be reprogrammable, the roadmap of autoimmunity therapy and/or other types of therapy would have to be reevaluated. Some disorders of immunity requiring enhanced immunosuppression can occur in the context of liver transplantation [383], kidney transplantation [384], or stem cell transplantation [385] to name a few examples. Exploiting such pathways could lead to the development of new therapeutic agents against immune disorders.

Abbreviations

AIRE: Autoimmune regulator
AP1: Activator protein 1
APC: Antigen-presenting cell

Bcl-6: B cell lymphoma 6
Bim: Bcl-2-interacting mediator of cell death
Blimp-1: B lymphocyte-induced maturation protein 1
CCR: C-C chemokine receptor
cDC: Conventional DC
CLR: C-type lectin receptor
CpG: Unmethylated cytosine preceding guanosine motif
cTEC: Cortical thymic epithelial cell
CTL: Cytotoxic T lymphocyte
CTLA-4: Cytotoxic T lymphocyte-associated antigen-4
CXCR: C-X-C chemokine receptor
MCMV: Mouse cytomegalovirus
DAP12: DNAX-activating protein of molecular mass 12 kilodaltons
DC: Dendritic cell
DD: Death domain
Deaf1: Deformed epidermal autoregulatory factor 1
DN: CD4/CD8 double-negative
DP: CD4/CD8 double-positive
dsRNA: Double-stranded RNA
DSS: Dextran sulfate sodium
Eomes: Eomesodermin
ER: Endoplasmic reticulum
FACS: Fluorescence-activated cell sorting
FADD: Fas-associated cell death domain
Foxp3: Forkhead box P3
GALT: Gut-associated lymphoid tissue
GATA transcription factors: A family of transcription factors capable of binding to the DNA sequence "GATA"
GM-CSF: Granulocyte macrophage colony-stimulating factor
HSP: Heat shock protein
IDO: Indoleamine 2, 3-dioxygenase
iEC: Intestinal epithelial cells
IFN: Interferon
IKK: Inhibitor of κB kinase
IL: Interleukin
IL-12R: IL-12 receptor
IMACS: Immunomagnetic cell sorting
IRAK: IL-1R-associated kinase
IRF: Interferon regulatory factor
iTreg: Inducible regulatory T cell
IκB: Inhibitor of κB
ITAM: Immunoreceptor tyrosine-based activation motif
JNK: c-Jun N-terminal kinase
LAP: Latency-associated peptide
LCMV: Lymphocytic choriomeningitis virus
LEC: Lymphatic endothelial cell
LNSC: Lymph node stromal cell
LPS: Lipopolysaccharide
LRR: Leucine-rich repeat
Mal: MyD88-adapter-like
MAMP: Microbe-associated molecular patterns
MCMV: Mouse cytomegalovirus
MDA-5: Melanoma differentiation-associated gene 5
mDC: Myeloid DC
MKP-1: Mitogen-activated protein kinase phosphatase-1
mTEC: Medullary thymic epithelial cell
MyD88: Myeloid differentiation factor 88
NAK: NF-κB activating kinase
NAP: NF-κB activating kinase-associated protein
NEMO: NF-κB essential modulator
NF-κB: Nuclear factor κB
NLR: Nucleotide binding domain and leucine-rich repeat containing gene family
nTreg: Natural regulatory T cell
ODN: Oligodeoxynucleotide
OVA: Ovalbumin
PAMP: Pathogen-associated molecular patterns
PD-1: Programmed death 1
pDC: Plasmacytoid DC
PI3K: Phosphatidylinositol 3-kinase
poly(I:C): Polyinosinic-polycytidylic acid
PRR: Pattern recognition receptor
PTA: Peripheral tissue-restricted antigen
RHIM: Receptor-interacting protein homotypic interaction motif
RIG-I: Retinoic acid-inducible gene-I
RIP: Receptor-interacting protein
RLR: Retinoic acid-inducible gene-I-like receptor
ROR: Retinoic acid receptor related orphan receptor
RTE: Recent thymic emigrants
SCID: Severe combined immunodeficiency
Sos1: Son of sevenless gene 1
SP: CD4/CD8 single-positive
ssRNA: Single-stranded RNA
STAT: Signal transducer and activator of transcription
TAB: Transforming growth factor β-activated kinase-1 binding protein
TAK: Transforming growth factor β-activated kinase
T-bet: T box expressed in T cell
TBK: TRAF family member-associated NF-κB activator-binding kinase
TCF-1: T-cell factor 1
Tcm: Central memory T cell
TCR: T-cell receptor
Tem: Effector memory T cell

Tfh:	T follicular helper cell
TGF:	Transforming growth factor
Th:	T helper cell
TICAM-1:	TIR domain containing adaptor molecule-1
TIR:	Toll/IL-1 receptor domain
TIRAP:	TIR domain-containing adapter protein
TLR:	Toll-like receptor
TNF:	Tumor necrosis factor
TRAF:	Tumor necrosis factor receptor associated factor
TRAM:	TRIF-related adaptor molecule
TREM-2:	Triggering receptor expressed on myeloid cell-2
TRIF:	TIR domain-containing adaptor inducing interferon-β
TSA:	"Tissue-specific" antigen
Tscm:	Memory stem T cells.

Conflict of Interests

The authors declare no conflict of interests.

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Review Article

Mechanisms behind Functional Avidity Maturation in T Cells

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During an immune response antigen-primed B-cells increase their antigen responsiveness by affinity maturation mediated by somatic hypermutation of the genes encoding the antigen-specific B-cell receptor (BCR) and by selection of higher-affinity B cell clones. Unlike the BCR, the T-cell receptor (TCR) cannot undergo affinity maturation. Nevertheless, antigen-primed T cells significantly increase their antigen responsiveness compared to antigen-inexperienced (naïve) T cells in a process called functional avidity maturation. This paper covers studies that describe differences in T-cell antigen responsiveness during T-cell differentiation along with examples of the mechanisms behind functional avidity maturation in T cells.

1. Introduction

T lymphocytes are very potent cells that play key roles in our immune system; without T cells we would quickly die from infection. The T cells patrol our organism to guard us against pathogenic microorganisms as part of adaptive immunity. In secondary lymphoid organs, such as lymph nodes and the spleen, small peptide fragments (antigens) of the pathogens are presented to antigen-inexperienced (naïve) T cells by professional antigen presenting cells (APC). This encounter induces proliferation and differentiation of the naive T-cell into an armed T-cell population that migrates to the site of infection. Here, reencounter with the same pathogen rapidly triggers the effector function of the armed T cells resulting in elimination of the pathogen. Following antigen clearance, most of the effector T cells die leaving only a small population of memory T cells. In case of re-infection with the same pathogen, memory T cells will mount a prompt response by immediately producing effector cytokines and by rapidly proliferating into a large number of secondary effectors [1–4]. This substantial increase in antigen-responsiveness of both effector and memory T cells upon reencounter with the pathogen is a fundamental property of adaptive immunity.

2. The Concept of Functional Avidity Maturation

Lymphocytes recognize antigens through specialized antigen receptors. These include the B-cell receptor (BCR) on B cells and the T-cell receptors (TCR) on T cells. During the cause of an immune response, a high number of point mutations take place in the BCR genes of the dividing B cells. This result in a panel of B cells expressing BCR with varying affinities against the antigen, and the B cells carrying BCR with the highest affinity are selectively expanded. As a consequence, high-efficiency B cells are selected during the immune response in a process known as affinity maturation [5]. Unlike B cells, T cells lack the capacity to mutate their TCR genes after T-cell activation, and thus classical affinity maturation does not take place in T cells. Still, T-cell sensitivity to antigens can be extensively enhanced in antigen-experienced (primed) T cells compared to naïve T cells in a process called "functional avidity maturation" [6–13].

3. T-Cell Activation Signals: The Basis of Functional Avidity Maturation

3.1. Early Studies That Indicated the Existence of Functional Avidity Maturation. The observation that fundamental differences exist in antigen sensitivity between naïve and primed T cells was first described in the late 80′s by Cooper and coworkers. They found that only primed T cells produced IL-2 and proliferated *in vitro* in response to TCR triggering induced by anti-CD3 antibodies and monocytes [14]. Similar observations were later reported by others [7, 9–13, 15]. Cooper and co-workers also introduced the idea that signals in addition to TCR signals, here exemplified by IL-2 receptor signals, were required for activation of naïve T cells [14]. Along this line, Mark Davis' group demonstrated that in addition to TCR signals naïve T cells require costimulatory signals through CD28 to become fully activated [16]. This finding was supported in a subsequent study, where Croft et al. showed that activation of both effector and memory T cells were considerably less dependent on co-stimulatory signals than naïve T cells [9]. Several *in vivo* and *ex vivo* studies have confirmed the early observations that effector and memory T cells have a lower threshold of activation and respond more robustly than naïve T cells [12, 13, 17]. As an example, Slifka and Whitton demonstrated a 50 fold increase in T-cell responsiveness to antigen during a LCMV infection. Furthermore, they found that coengagement of the coreceptor CD8 with the TCR was required for naïve T-cell activation, whereas activation of effector T cells was relatively CD8-independent [17]. In an equivalent study also examining T-cell responses to infection, Pihlgren et al. demonstrated a similar 50-fold increase in antigen responsiveness of both effector and memory cell populations as compared to naïve cells [12]. Interestingly, a study by Mescher and co-workers suggested that memory T cells were intrinsically more sensitive to TCR stimulation than their naïve counterparts [13], adding TCR signaling to the growing list of differences between naïve and primed T cells. An overview of studies indicating the existence of functional avidity maturation is given in Table 1.

Today, it is widely accepted that T-cell activation should not be considered as a single signal process, but as a sum of interdependent signals. The current model for T-cell activation, referred to as the 3-signal model, predicts that in addition to antigen-induced TCR-triggering optimal activation of naïve T cells requires at least two additional signals. These signals are delivered through co-stimulatory receptors predominantly CD28 [18, 19] and receptors for cytokines like IL-2, IL-12, IFN-α, and IL-1 [20–25].

3.2. TCR Signal Initiation in Naïve versus Primed T Cells: The Immunological Synapse and CD28. TCR signaling takes place at the interface between the T-cell and the antigen presenting cell. At this contact zone, often referred to as the immunological synapse (IS), TCR-signaling components including the TCR itself as well as intracellular-signaling molecules are continuously accumulated during antigen contact [26]. Although somewhat controversial [26, 27], formation of an IS correlates with generation of a robust immune response, and is considered a prerequisite for T-cell activation [28, 29]. Even so, new insight into the biology of immunological synapses has revealed that TCR signaling is already initiated in TCR microclusters prior to IS formation. In a ligand-dependent manner, CD28 localizes to preformed TCR microclusters counting 11–17 TCRs [30] together with key signaling molecules [31]. Formation of the mature IS includes accumulation of hundreds of such TCR microclusters [31]. At the IS, CD28 signaling both induces structural stabilization and enlargement of the area itself [32, 33]. Formation of the IS is a mechanism shared by naïve and primed T cells; however, a mature IS is formed more quickly in primed T cells and only naïve T cells require CD28 co-stimulatory signals to form the IS [34, 35]. These observations are consistent with reports indicating that primed T cells are less dependent on CD28-costimulation than naïve T cells [9, 36–38]. Eventhough the exact implication of CD28 signaling in T-cell activation is still elusive, it is generally agreed that CD28 amplifies intracellular signaling induced by antigen-triggering of the TCR through modulation of morphological features and TCR signals [32, 33]. In addition to CD28, signaling other differences between naïve and primed T cells exists at the IS. A study by Watson and Lee illustrated that the phosphatase CD45 is a more integral component of the IS in primed T cells as compared to naïve cells [35]. CD45 is a transmembrane tyrosine phosphatase that maintains Lck activity by promoting dephosphorylation of an inhibitory carboxy-terminal tyrosine residue of Lck. Lck activity is a necessity for initiation of TCR signal transduction [39]. Interestingly, Watson and Lee also showed that CD45 is already associated with TCR microdomains in the plasma membrane prior to synapse formation in resting memory T cells in contrast to their naïve counterparts [35]. This finding parallels the study of Kersh et al. who showed that a higher basal level of phosphorylation (activation) was seen in membrane associated signaling molecules in resting primed T cells [40]. It, therefore, appears that primed T cells are in a higher "state of alert" prior to antigen encounter, correlating with the higher sensitivity of primed T cells to antigen stimulation.

3.3. TCR Signaling in Naïve versus Primed T Cells. In addition to differences in the organization of signaling molecules, the actual TCR signaling events induced in naïve and primed T cells following TCR triggering differs. The current model for TCR signaling postulates that following TCR triggering the tyrosine kinase Lck is activated resulting in phosphorylation of the CD3 and zeta chains of the TCR in addition to activation of Zap70 [41, 42]. Activated Zap70 phosphorylates LAT that subsequently recruits and activates several proteins including PLC-γ1. Activation of PLC-γ1 results in the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP2) to inositol 3,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 regulates intracellular calcium mobilization, and DAG regulates the activation of PKC and contributes to Ras and mitogen-activated protein kinase

Table 1: Studies describing differences in antigen sensitivity between naïve and primed T cells. Differences listed are in comparison to naïve T cells.

Study	Species	T-cell phenotype	Effector T cell	Memory T-cell	Mode of (re)-stimulation
Slifka and Whitton [17], 2001	Mouse	CD8	>50 fold ↑ Ag responsiveness	>50 fold ↑ Ag responsiveness	Peptide antigen
Pihlgren et al. [12], 1996	Mouse	CD8	50 fold ↑ Ag responsiveness (proliferation)	50 fold ↑ Ag responsiveness (proliferation)	*In vivo* or peptide-pulsed splenocytes
Curtsinger et al. [13], 1998	Mouse	CD8		↑ Ag responsiveness (e.g., proliferation)	Beads coated with MHC/peptide
Robinson et al. [10], 1993	Human	CD3		↑ Responsiveness to TCR triggering (e.g., proliferation)	Soluble anti-CD3 Ab
Sanders et al. [7], 1989	Human	CD3		↑ Responsiveness to TCR triggering (e.g., proliferation)	Soluble anti-CD3 Ab
Schwinzer et al. [11], 1994	Human	CD3		↑ Proliferation	Anti-CD3 Ab + APC
Byrne et al. [14], 1988	Human	CD4		↑ Proliferation	Anti-CD3 Ab + APC
Croft et al. [9], 1994	Mouse	CD4	↑ Proliferation	↑ Proliferation	Anti-CD3 Ab + APC lacking co-stimulation
Luqman and Bottomly [8], 1992	Mouse	CD4		↑ Proliferation	Anti-CD3 Ab + APC lacking co-stimulation

(MAPK) cascade activation [41, 42]. The vast majority of studies contributing to the current model for TCR signaling were performed using immortal T-cell lines or primed T cells propagated *in vitro*. However, as significant differences in gene and protein expression exist between naïve and primed T cells [43], significant differences in TCR signaling in primed and naïve T cells could be imagined. By studying naïve human T cells isolated from freshly drawn blood samples, we have recently shown that the classical model for TCR signaling must be revised as naïve T cells only express PLC-γ1 at very low levels compared to primed (effector) T cells. Following *in vitro* priming, PLC-γ1 was upregulated approximately 75 fold, an upregulation that correlated with greater TCR responsiveness [44]. One of the striking signaling differences that we and others have observed between naïve and primed T cells is a strongly diminished ability of naïve T cells to flux calcium in response to TCR triggering [10, 44, 45]. The very low expression of PLC-γ1 in naïve T cells could explain the impaired calcium flux in these cells [44]. Based on previous studies demonstrating that vitamin D can up-regulate PLC-γ1 in other cell types [46, 47], we investigated if vitamin D via the vitamin D receptor (VDR) was responsible for PLC-γ1 up-regulation during T-cell priming. Indeed, we found that VDR was quickly up-regulated following TCR triggering and that induction of VDR was required for PLC-γ1 up-regulation. As PLC-γ1 is a central molecule in the classical TCR signaling pathway and is weakly expressed in naïve human T cells, we wondered which signaling events could be responsible for the activation-induced VDR up-regulation. We found that the nonclassical TCR signaling pathway in which Zap70 directly activates p38-induced VDR expression. We further found that whereas activation of Zap70 and p38 was at least as efficient in naïve T cells as in primed T cells following TCR triggering, activation of Erk was significantly reduced in naïve T cells. Thus, our study demonstrated that fundamental differences exist in the signaling pathways between naïve and primed T cells.

Adachi and Davis also compared TCR signaling in human naïve and primed (memory) T cells. In contrast to us, they found a stronger Erk activation along with lower activation of Zap70 and p38 in naïve T cells as compared to primed cells. They proposed that the strong Erk activation observed in naïve T cells disrupted early TCR signaling events as part of a negative feedback mechanism [48]. The discrepancy between the two human studies might be due to two different primed T-cell populations studied (effector and memory cells, resp.); however, it might also be explained by the different modes of TCR triggering used. In our study, purified naïve human T cells were stimulated using beads coated with anti-CD3 and anti-CD28 antibodies. Adachi and Davis used high concentrations of soluble anti-CD3 and anti-CD28 antibodies cross-linked by secondary antibodies to stimulate the T cells. By using cross-linked antibodies for stimulation, a very strong receptor signaling is achieved. As illustrated in a series of mouse virus studies, the strength of TCR signaling determines the requirement for additional activation signals like CD28 signaling and also results in

Table 2: Studies describing differences in the TCR signaling machinery of naïve and primed T cells. Green cells indicate the investigated T cell populations. Arrows indicate an increase. P denotes phosphorylation of the given enzyme following TCR triggering.

Study	Species	T cell phenotype	Naïve T cell	E ector T cell	Memory T cell	Mode of (re)-stimulation
von Essen et al. [44], 2010	Human	CD3	↑ Zap70-P, LAT-P, p38-P	↑ PLCγ1/VDR, Erk-P, Ca^{2+} flux		Beads coated with anti-CD3 + anti-CD28 Ab
Robinson et al. [10], 1993	Human	CD3			↑ Ca^{2+} flux, PKC activity ↑ Basal level of DAG	Soluble anti-CD3 Ab
Adachi and Davisa [48], 2011	Human	CD4 + CD8	↑ Erk-P, Ca^{2+} flux (CD4 cells only)		↑ p38-P, LAT-P, Ca^{2+} flux (CD4 + CD8)	Anti-CD3 + anti-CD28 Ab cross-linked with a secondary Ab
Ericsson et al. [45],1996	Mouse	CD4	PLCγ1-P absent	PLCγ1-P, Ca^{2+} flux ↑ MAPK-P, RasGAP-P		Peptide-pulsed fibroblasts
Kersh et al. [40], 2003	Mouse	CD8		↑ Zap70-P ↑ Basal phosphoprotein level in membrane microdomains ↑ Microdomain size	↑ Erk-P, p38-P, LAT-P ↑ Basal phosphoprotein level in membrane microdomains ↑ Microdomain size	Peptide-pulsed splenocytes or macrofages
Watson and Lee [35], 2004	Mouse	CD4			↑ CD45 association with microdomains + IS ↑ Formation and maintenance of IS	Peptide-pulsed splenocytes

somewhat different responses [19]. In line with this, Adachi and Davis found that naïve CD4 T cells could flux calcium when their stimulation protocol was used, implying very strong signaling and the need for a fast negative feedback mechanism. Both scenarios could be relevant for human immunity where a wide range of pathogens with different origins is encountered.

A few studies investigating TCR signaling events in naïve versus primed T cells have also been conducted in mice [40]. Unfortunately, mouse and man seem to differ when it comes to some of the signaling molecules involved in TCR signaling. In contrast to human T cells, naïve and primed mouse T cells seem to express similar levels of both VDR and PLC-γ1 [45, 49]. Even so, studies on mice T cells have found that it is only in primed T cells that TCR triggering induces phosphorylation of PLC-γ1 and subsequent calcium flux [45] as found for human T cells. It is, therefore, likely that despite a different "route of action" the outcome are the same concerning the ability to flux calcium in T cells from man and mice.

Collectively, these studies illustrate fundamental differences in TCR signaling pathways between naïve and primed T cells, differences based in particular on the lack of naïve T-cell signaling molecules used by the primed T cells. A detailed overview of the published differences in the signaling machinery in naïve versus effector and memory T cells is given in Table 2.

3.4. Cytokines as the "Third" Activation Signal in Naïve versus Primed T Cells. Within the last years, the importance of cytokine receptor signaling as a "third-signal" in activation of naïve T cells has been acknowledged. The requirement for a "signal 3" mediated by inflammatory cytokines is considered a mean for T cells to determine if "danger" is present [50]. Although both naïve CD4 and CD8 T cells are dependent on these "danger signals" for full activation, they differ in their requirement for specific cytokines. Early studies describing a need for a third-signal cytokine came from a series of *in vitro* and *in vivo* experiments performed by Mesher and co-workers. They found that IL-12 and IFN-α provided a signal that along with antigen and CD28 signaling was crucial for naïve CD8 T-cell expansion and differentiation [51–53], findings that were validated by other groups [23, 54–57]. Eventhough IL-12 has a role in skewing the CD4 T-cell response, it has no effect on CD4 T-cell proliferation and differentiation in response to antigen. In contrast, IL-1 enhances *in vivo* expansion and differentiation of naïve CD4 T cells [58], both by acting directly on the CD4 T cells [24] and through APC modifications [25]. No studies have described a need for "the third-signal" in activation of primed T cells, but a role for IFN-α in homeostatic proliferation and maintenance of memory CD8 T cells has been demonstrated [59]. Thus, even though primed T cells to some extent rely on both IFN-α [59] and CD28 [19] for their continuous survival and antigen recognition, primed T cells clearly do not have the same prerequisite for cytokine and CD28 signaling as naïve T cells to be activated. The present literature, therefore, clearly states that the demand for the "3 signals" in T-cell activation greatly differs between naïve and primed T cells.

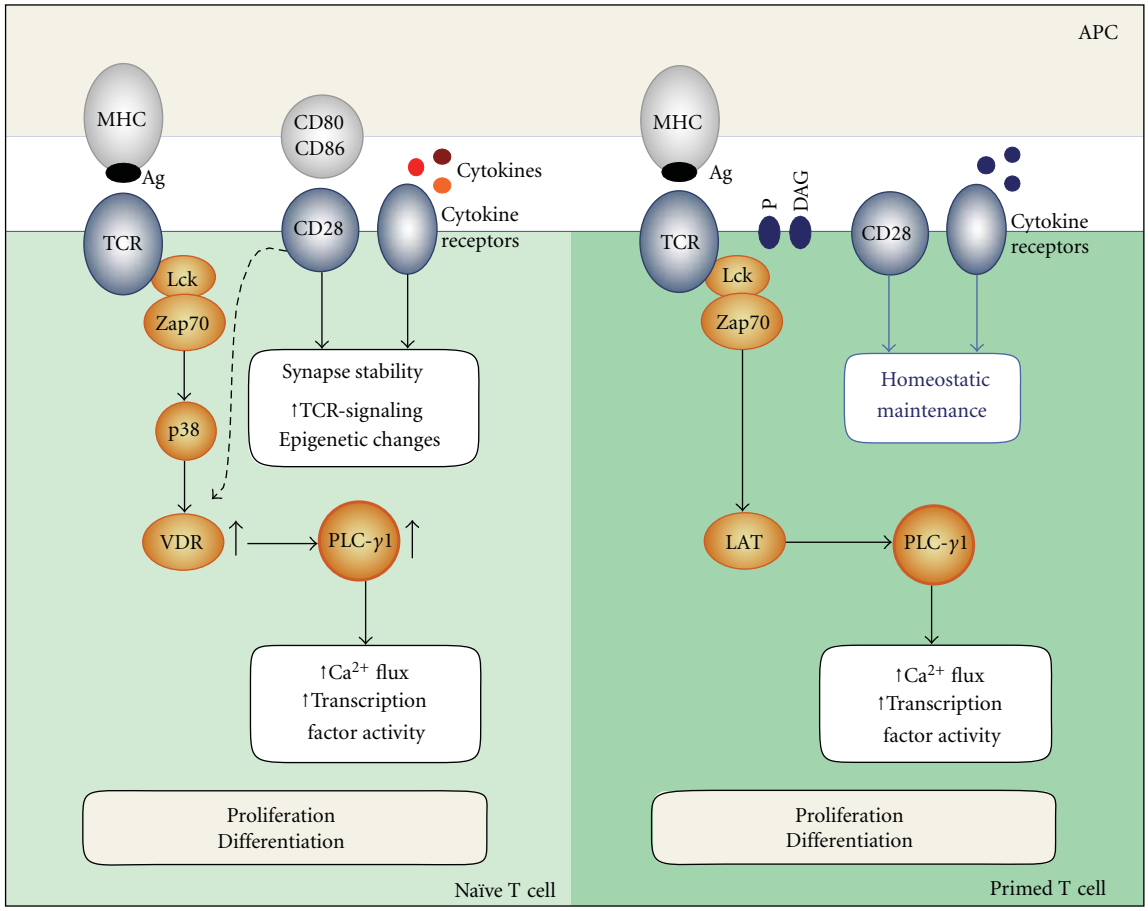

Figure 1: Simplified model illustrating the differences in T-cell signaling between naïve and primed T cells. In naïve human T cells, TCR engagement leads to activation of p38 through Zap70 resulting in upregulation of VDR and then PLC-γ1 mandatory for the naïve T cells to be activated. For activation, naïve T cells also require CD28 and cytokine receptor signals to induce and stabilize membrane structures and intracellular signaling molecules. In contrast, primed T cells already express PLC-γ1, have a higher DAG and phosphoprotein (P) basal level in specialized membrane structures with a high association of the CD45 molecule. In addition, signaling in primed T cells is rather independent of CD28 costimulatory signals as well as "third-signal" inflammatory cytokines, overall leading to a far more prompt antigenic response.

4. Molecular Mechanisms of Functional Avidity Maturation

As discussed in this paper and summarized in Figure 1, fundamental differences in activation of naïve and primed T cells exist. This includes both the requirement for the three antigenic-induced signals as well as intrinsic differences in the signaling machinery. CD28 and cytokine receptor signaling are central components of naïve T-cell activation as they help induce and stabilize both membrane structures and intracellular signaling molecules crucial for T-cell activation. In this way, the signaling machinery is already optimized for signal transduction in primed T cells prior to antigen reencounter. As a result, primed T cells respond much faster and stronger when an antigen is eventually engaged. It therefore seems as the T cells retain a permanent imprint of a prior response to antigen. But how is such an imprint formed? Accumulating evidence suggest that epigenetic changes are likely to be a contributing factor. For example, Northrop et al. demonstrated that stable demethylation of the regulatory region of the IL-2 gene takes place during priming of naïve T cells resulting in a gain of IL-2 expression in the primed T cells [60], a discovery validated by Murayama and co-workers [61]. In addition, Thomas et al. published the observation that CD28 costimulation during T-cell priming induces a stable histone acetylation and demethylation at the IL-2 promoter, suggesting that CD28 in part function through epigenetic mechanisms [62]. A personal observation of ours shows that CD28 signaling greatly increases the TCR induced upregulation of VDR in naïve T cells. In parallel with this, Kim et al. recently published that transcription of the gene CYP27B1 is controlled by methylation of its promoter [63]. The CYP27B1 gene product controls synthesis of active vitamin D, which is a prerequisite for VDR activity and hence for upregulation of PLC-γ1 in naïve T cells. Moreover, it has been speculated that the "third-signal" cytokines IL-12 and IFN-α drive chromatin remodeling events during initial priming of naïve

T cells [50]. It therefore seems likely that the more rapid and robust responses of primed T cells in comparison to naïve cells partly are a result from epigenetic changes in crucial genes, and furthermore that these changes may be driven by CD28 costimulation and "third-signal" cytokines during the initial priming phase. Despite the progress made in recent years, we still lack a clear understanding of some of the key aspects of functional avidity maturation. A better understanding of the molecular mechanisms involved in improving antigen-specific T-cell responses would be of great therapeutic value, for example, to advance vaccine efficiency.

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Review Article

HEB in the Spotlight: Transcriptional Regulation of T-Cell Specification, Commitment, and Developmental Plasticity

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The development of T cells from multipotent progenitors in the thymus occurs by cascades of interactions between signaling molecules and transcription factors, resulting in the loss of alternative lineage potential and the acquisition of the T-cell functional identity. These processes require Notch signaling and the activity of GATA3, TCF1, Bcl11b, and the E-proteins HEB and E2A. We have shown that HEB factors are required to inhibit the thymic NK cell fate and that HEBAlt allows the passage of T-cell precursors from the DN to DP stage but is insufficient for suppression of the NK cell lineage choice. HEB factors are also required to enforce the death of cells that have not rearranged their TCR genes. The synergistic interactions between Notch1, HEBAlt, HEBCan, GATA3, and TCF1 are presented in a gene network model, and the influence of thymic stromal architecture on lineage choice in the thymus is discussed.

1. T-Cell Progenitors and Lineage Plasticity

During hematopoiesis, pluripotent progenitors are sequentially restricted in lineage potential and progressively committed to a single lineage choice. Lineage commitment is, therefore, established in part by the inability to respond to environmental cues, migrate to inductive environments, and/or express key lineage regulatory factors that direct the acquisition of alternative fate choices [1]. However, the thymus, a site where T cells are generated, does not produce stem cells, and the generation of T cells depends solely on the intermittent input of progenitors from adult bone marrow [2]. Circulating progenitors such as lymphoid-primed multipotent progenitors (LMPPs) or common-lymphoid progenitors (CLPs) enter the thymus at the corticomedullary junction (CMJ). During development, T-cell progenitors transition through two functionally distinct zones of the thymus: immature cells migrate outward through the cortex, while the more mature cells migrate inward toward medulla [1]. The developmental status of thymocytes can be identified by their cell-surface marker expression. The most immature progenitors lack the expression of CD4 and CD8 (double negative, DN) and are further discriminated based on the expression of CD44 and CD25 into four sequential stages: DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$), and DN4 ($CD44^-CD25^-$) [3].

The DN1 population is quite heterogeneous and has the capacity to generate multiple lineages [4]. Since DN1a (c-kit^+CD24^-) and DN1b (c-kit^+CD24^+) cells generate T cells efficiently and exhibit a strong proliferative capacity, they are considered to be the canonical early T-cell progenitors (ETP). The remaining DN1 subsets, DN1c (c-$kit^{int}CD24^-$), DN1d (c-kit^-CD24^+), and DN1e (c-kit^-CD24^-), are noncanonical T-cell progenitors because they lack the proliferative potential and differ substantially in their capacity to generate T cells. The heterogeneity of the DN1 population reflects the variety of non-T-cell lineages that are generated in the thymus. While DN1c and DN1d cells give rise to B cells, DN1a, DN1b, and to a small degree DN1e cells can produce natural killer (NK) cells [4]. The DN1c, DN1d, and DN1e subsets have also been shown to have the potential to generate dendritic cells (DCs) in the thymus [5, 6]. In addition, ETPs can be further separated into two subsets based on the expression of Flt3; the $Flt3^+$ ETPs can give rise to B cells, while $Flt3^-$ ETPs no longer possess B-cell potential [7]. Lastly, ETPs have the potential to generate myeloid cells in

the thymus [8]. These studies indicate that B-cell potential is lost before myeloid potential in T-cell precursors prior to T-lineage commitment.

2. T-Cell Development: Gene Specification, Commitment, and Developmental Checkpoints

Specification into the T-cell lineage occurs during the transition from the DN1 to the DN2 stage, when lymphoid- and T-lineage-specific genes are turned on [9]. Some of the most important targets of T-lineage regulators include *Rag* genes, interleukin 7 receptor α (*IL7Rα*), *lck*, *Bcl11b*, *pTα*, and *CD3* genes. Based on the expression of lck and c-kit, DN2 cells can be further separated into DN2a (lck^-, $c\text{-}kit^{hi}CD25^+$) and DN2b (lck^+, $c\text{-}kit^{int}CD25^+$) subpopulations, which display differential lineage potential; while DN2a can give rise to myeloid, NK, and DC cells, DN2b are T-lineage restricted [10, 11]. However, the revised model of hematopoiesis, in which the lymphoid-myeloid segregation occurs after the T-B segregation [8], has been recently challenged by a study involving IL7R-reporter mice [12]. In this study, myeloid cells did not arise from the cells that had a history of IL7R expression as tracked by a fate-mapping reporter gene, even in the DN1a and DN1b fractions [12]. These results suggested that myeloid cells in the thymus may not share a common intrathymic precursor with T-cells. Additional studies are needed to resolve this issue.

T-lineage-restricted DN2b cells progress to the DN3 stage. At the DN3 stage, the *TCRβ* gene is rearranged and expressed. Successfully produced TCRβ chains pair up with invariant pTα chains, and with the CD3 components into a pre-TCR complex. Signaling through the pre-TCR grants survival and differentiation to the DN4 stage. In addition, the cells turn off the expression of *Rag* genes in order to prevent rearrangement of a second TCRβ allele, a process called allelic exclusion. Finally, the cells proliferate and differentiate into the DN4 stage. The overall process resulting in allelic exclusion as well as cellular survival, proliferation, and differentiation is referred to as β-selection and represents the first checkpoint in T-cell development [13]. This checkpoint ensures that cells lacking productive *TCRβ* genes do not proceed further in development. The cells that have not received a pre-TCR signal die by apoptosis, unless they were previously predisposed to differentiate into the $\gamma\delta$ T-cell lineage by the expression of TCRγ and TCRδ chains. Interestingly, pre-TCR signaling has also been linked to the inhibition of the tumour suppressor gene, p53, which functions in response to DNA damage [14]. An accumulation of p53 causes a cell-cycle arrest by activation of cell-cycle inhibitor genes such as p21, to support DNA repair. Alternatively, unrepaired DNA damage can also cause p53-induced death by activation of proapoptotic molecules. The mechanisms that link pre-TCR signaling to p53 induction have yet to be established.

Following β-selection, CD8 is upregulated slightly earlier than CD4 in mice, resulting in cells at the immature CD8 single positive (ISP) stage. ISP cells can be distinguished from the mature $CD8^+$ single positive (SP) cells by lack of cell surface TCRβ. As the cells progress into the $CD4^+CD8^+$ (double positive, DP) stage, the expression of *Rag* genes is reinstated and *TCRα* gene rearrangements take place. TCRα chains pair up with the TCRβ chains and the CD3 components to form the mature TCR$\alpha\beta$ complex, which interacts with peptide-MHC complexes expressed by the thymic stromal cells or thymus-resident APCs. TCR interactions with MHC and self-peptide result in positive and negative selection of DP thymocytes, which represent a second checkpoint in T-cell development and result in the generation of mature $CD4^+$ and $CD8^+$ SP cells.

3. Critical Regulators of Early T-Cell Development

Proper development of T cells depends on the timing and level of transcription of lineage-specific regulatory genes. During hematopoiesis, transcription factors coordinate complex developmental events by modulating an array of genes that reduce multilineage potential and steer development toward particular lineage fates [15]. The activity of the transcription factors depends on their dosage, availability of their partners, as well as their overall binding specificity and affinity for a consensus DNA sequence. Transcription factors that are important for T-cell specification and commitment include Notch/CSL, GATA-3, TCF1, Bcl11b, and E proteins.

3.1. Notch Signaling. Notch signaling is an evolutionarily conserved mechanism that influences cell fate through cell-cell interactions. Notch proteins are transmembrane receptors that signal in a ligand-dependent manner. Flies have one Notch receptor, and two ligands: Serrate and Delta. Mammals, however, possess four Notch receptors (Notch1 to 4) and five ligands: two Serrate-like ligands called Jagged-1 and Jagged-2, and three Delta-like (DL) ligands called DL1, DL3, and DL4. Upon receptor-ligand engagement, a series of proteolytic cleavages take place that liberate the intracellular segment of Notch (ICN). ICN is the active form of Notch, which binds to CSL (CBF-1/RBP-Jκ in mammals, Suppressor of Hairless in *Drosophila*, Lag-1 in *C. elegans*) displacing the Groucho corepressor and recruiting coactivators such as Mastermind to the complex. These events initiate transcription of Notch-target genes, such as *Hes1, Deltex1, CD25, pTα*, and *TCRβ* [16].

Among the four Notch receptors, Notch1 plays an indispensable role in T-cell development, particularly in the T/B lineage choice. Mice deficient for Notch1 in HSCs display an arrest at the DN1 stage of T-cell development and generate B cells intrathymically (Figure 1(a)) [17]. Furthermore, conditional inactivation of Notch1 at the DN stages has shown that Notch1 signaling is necessary for *TCRβ* rearrangement and for generation of $\alpha\beta$ but not $\gamma\delta$ T cells from DN3 progenitors [18–20]. Although thymocytes also express Notch2 and Notch3, mice deficient for either of these receptors do not have pronounced disturbances in T-cell development [21–23]. Likewise, Notch4-deficient mice do not have any detectable defects in T-cell development [21]. Interestingly,

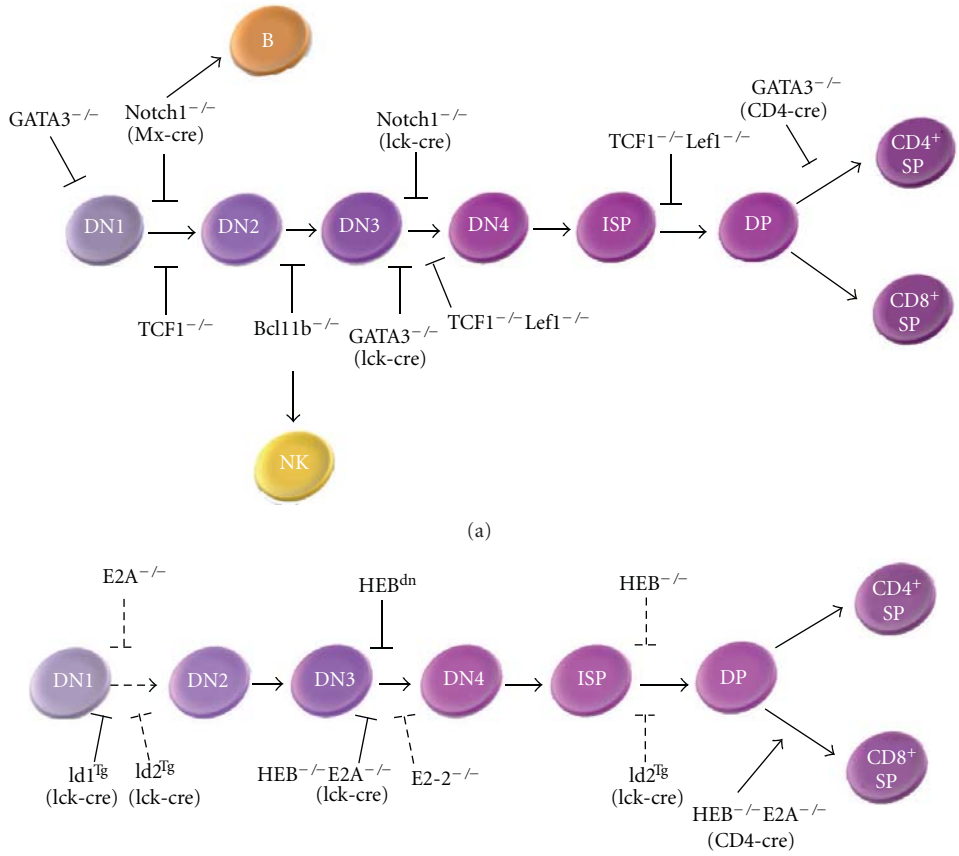

Figure 1: Key transcription factors in early T-cell development. Regulation of early T-cell development occurs through the coordinated action of transcription factors. (a) GATA3 is important for the generation of DN1 cells, and GATA3$^{-/-}$ mice fail to produce any T-cells. Inactivation of GATA3 during DN stages results in a block at the DN3 stage due to defects in TCRβ expression. When GATA is inactivated at the later stages of T-cell development, no CD4 SP cells can be generated. Notch1 signalling is indispensable for T-cell specification and commitment since mice deficient in Notch1 give rise to intrathymic B cells at the expense of T cells. Notch1 inactivation during DN stages arrests T-cell development at the DN3 stage due to the defects in V-(D)J rearrangements of the *TCRβ* locus. Inactivation of TCF1 and LEF1 simultaneously results in the partial block at the DN3 stage and a complete block at the ISP stage as cells fail to rearrange *TCRα* locus. Lastly, Bcl11b is essential for the specification into the T-cell lineage. Bcl11b$^{-/-}$ cells fail to progress past the DN2 stage of T-cell development, and instead, differentiate into NK cells. (b) T-cell blocks associated with mutations in HEB, E2A and/or E2-2 E-proteins and their antagonists, Id1 and Id2. Solid blunt lines indicate complete developmental arrest, while dotted blunt lines indicate a partial developmental arrest. DN: double negative, DP: double positive, SP: single positive, ISP: immature single positive, NK: natural killer, B: B lymphocytes.

progenitors constitutively expressing ICN develop into T cells in bone-marrow at the expense of B cells [24], indicating that the bone marrow environment is well equipped to support T cell development apart from the lack of DL Notch ligands [25]. Although Notch1 receptor has the capacity to interact with either DL1 or DL4 [26], DL4 represents the primary physiological partner for Notch1 receptor in T-cell development [27, 28]. Thymic stroma, therefore, provides essential Notch ligands that are not expressed by the bone-marrow stroma, which helps to explain the unique capacity of the thymus to support T-cell development.

3.2. GATA3. The GATA family includes three zinc-finger transcription factors, GATA1, GATA2, and GATA3, which bind to the consensus GATA motif in DNA. Within the hematopoietic system, all three GATA factors are expressed in the hematopoietic progenitors; however, GATA1 is also expressed in the cells of the myeloid origin, such as erythrocytes, mast cells, eosinophils, and megakaryocytes, while the expression of GATA2 is limited to mast cells and megakaryocytes [29]. GATA3, on the other hand, is most abundantly expressed in T cells and NK cells [30–32]. During T-cell development, the expression of GATA3 gradually increases from the DN1 to the DN3 stage then diminishes at the DN4 stage. GATA3 is repressed at the DP stage and becomes upregulated again in the CD4 SP cells, but it stays off in the CD8 SP cells [33].

GATA3$^{-/-}$ mice die at E11 due to defects in the development of the central nervous system [34]. The essential role of GATA3 in the generation of T cells was revealed in experiments involving antisense oligos against GATA3 [35], and by generation and examination of blastocyst chimeras

from GATA3$^{-/-}$ and Rag-2$^{-/-}$ embryonic stem cells (Figure 1(a)) [36]. GATA3 is also necessary for the generation of ETPs [32]. Conditional inactivation of GATA3 at the DN stage of T-cell development has revealed that GATA3 is also required for passage through β-selection and for the proper expression of TCRβ protein [37]. Inactivation of GATA3 at the later stages of T-cell development has shown that GATA3 is also essential for the generation of CD4$^+$ SP cells [37, 38]. GATA3 binds to the promoter regions and directly regulates the expression of other genes important for T-cell development, such as the *Rag* genes [39] and Th-POK, a CD4 cell specifying transcription factor [40]. Elevated levels of GATA3 in early T-cell development inhibit T-cell development by downregulating genes involved in T-cell specification [41].

3.3. TCF1. *Wnt* genes encode numerous Wnt factors, which are soluble glycoproteins secreted by thymic epithelial cells. Wnt factors provide intracellular signaling to different cell types, including developing thymocytes. Wnt-mediated signaling is initiated when Wnt binds to Frizzled receptors and the low-density lipoprotein receptor-related protein (LRP)-5 and LRP6 on the cell surface of developing thymocytes [42]. The signaling cascade stabilizes cytoplasmic β-catenin, which translocates into the nucleus and displaces a corepressor called Groucho from the T-cell factor 1 (TCF1) and the lymphoid enhancer factor 1 (LEF1) transcription factors. Stabilized β-catenin collaborates with pre-TCR signaling to ensure thymocyte survival [43, 44]. In the absence of Wnt, β-catenin is targeted for degradation by ubiquitination [45], thus leaving the TCF1/Groucho complex to function as a transcriptional repressor.

TCF1 and LEF1 share a homology domain with proteins of the high mobility group (HMG) family. The expression of TCF1 is restricted to T cells, with the highest expression occurring across the β-selection checkpoint and at the ISP stage of T-cell development [46]. T-cell development is impaired at multiple stages in TCF1-deficient mice (Figure 1(a)). First, there is a complete block at the DN1 stage when TCF1$^{-/-}$ stem cells are cultured on OP9-DL4 stroma, which support T-cell development *in vitro* [47]. Second, in TCF1$^{-/-}$ mice, there is a partial block at the DN1 to DN2 transition. Lastly, there is a marked accumulation of cells at the ISP stage and reduced overall numbers of thymocytes [46, 48, 49]. LEF1$^{-/-}$ mice have many abnormalities, but none that are associated with thymopoiesis [50]. The potential redundancy between the two factors was tested by generating mice deficient in both TCF1 and LEF1 [50]. Indeed, T-cell development was partially blocked at the DN3 stage and completely blocked at the ISP stage in TCF1/LEF1$^{-/-}$ mice due to the impaired expression of the *TCR*α gene.

3.4. Bcl11b. Bcl11b (B-cell lymphoma/leukemia 11b) is a tumour suppressor gene that encodes for three zinc-finger transcription factors: α, β, and γ. Bcl11bα and β are expressed at high levels in the thymus, while the expression of γ is low [51]. The gene was discovered while studying the thymic lymphomas in mice with mutations or deletions in the Bcl11b gene locus [52]. A Bcl11b homologue exists called Bcl11a, which functions as an oncogene in certain B-cell leukemias that involve Ig heavy-chain gene translocations [53].

An appreciation for the importance of Bcl11b in T-cell development stemmed from studies involving Bcl11b knockout mice. Bcl11b$^{-/-}$ thymocytes have severe defects in V-(D)J *TCR*β gene rearrangements resulting in apoptosis and arrest at the ISP stage [51]. The timing of developmental arrest suggested that Bcl11b has a regulatory connection with TCF1, and recent evidence suggests that Bcl11b is a direct target of TCF1 [47]. Furthermore, conditional inactivation of Bcl11b at earlier stages of T-cell development revealed a block at the DN2 stage and an increased production of NK cells [54, 55]. Bcl11b, like TCF1, is directly upregulated by DL-Notch signaling, implicating it as a mediator of the impact of Notch1 on alternative lineage choice. These studies have identified Bcl11b as a critical factor for early T-cell development (Figure 1(a)).

3.5. E Proteins. E proteins belong to the class I basic helix-loop-helix (bHLH) family of transcription factors. They control a variety of developmental processes in vertebrates such as myogenesis, neurogenesis, pancreatic development, and lymphopoiesis [56]. All E proteins possess a stretch of basic amino acids capable of binding DNA. Furthermore, E proteins function as homodimers as well as heterodimers with other E proteins or HLH factors. The crystal structure of the bHLH domain revealed that each subunit of the dimer contacts one half of the E-box site [57]. The contact with DNA is established via the basic region, while the HLH domain participates in dimerization. Binding to DNA, however, is not sufficient to activate transcription; rather, E proteins possess one or two activation domains (AD1 and AD2) [58–60], which mediate transcription by recruiting coactivators or corepressors to the complex. A repressive function may be conferred on E proteins upon binding to ETO factors, whereas activation may be enhanced by recruiting p300 to the transcription complex [61]. These factors competitively bind to the AD1 domain, enabling context-dependent regulation of gene expression.

The E protein family is comprised of three members, *E2A*, *E2-2*, and *HEB*; the timeline of their discovery is outlined in Figure 2. E proteins are indispensable for the generation of LMPPs and HSCs, and for normal B-cell, T-cell, and plasmacytoid DC (pDC) development. Each gene encodes two proteins, as illustrated in Figure 3(a). The genes have alternative names as follows: E2A (aka TCF3 or ALF2), E2-2 (aka ITF2 or TCF4), HEB (aka TCF12, ALF1, or ME1). The *E2A* gene locus gives rise to E47 and E12 by alternative splicing [72]. The *HEB* gene locus on the other hand, has two transcription start sites which are responsible for generating the long form of HEB, called HEBCan, and the short form of HEB, called HEBAlt [71]. The *E2-2* gene locus has the same type of genomic structure as the *HEB* gene locus, and also produces two forms, E2-2Can and E2-2Alt. As shown in Figure 3(b), the *HEB* gene locus is organized into 21 exons and spans a genomic area that is over 200 kb in size [71].

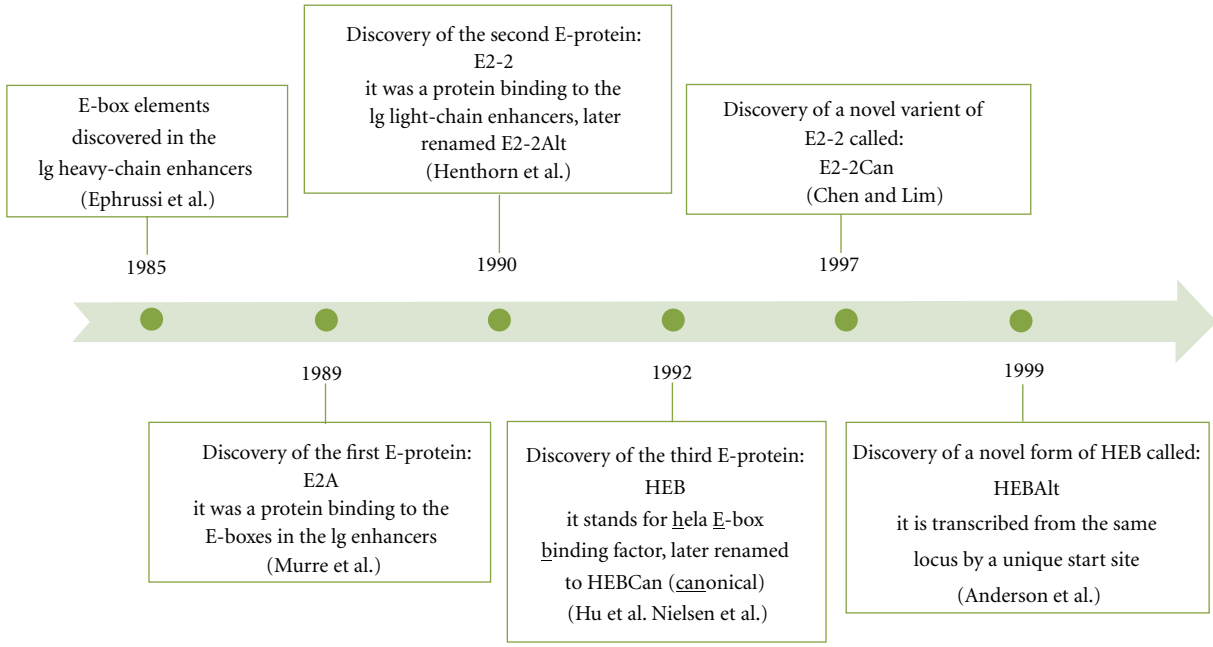

Figure 2: Timeline of E-protein discovery. In 1985, Ephrussi et al. identified regions in the immunoglobulin (Ig) heavy chain gene enhancer (μE1–μE5) that were occupied by unidentified DNA-binding proteins in the B-cell lines, but not in nonlymphoid cells [62]. The regions had a CANNTG consensus sequence, which was later named an E-box. In 1989, Murre et al. discovered that E-boxes in the Ig-heavy and light-chain enhancers were occupied by two novel proteins, which they named E47 and E12 [63]. These were the first E-proteins discovered, so called because they bind to the E-box sites. In 1990, the search for transcription factors that bind Ig light-chain enhancer sites (κE1–κE3) revealed a second E-protein, ITF-2A [64], later named E2-2Alt [65]. Concurrent studies by Hu et al. involved the use of the μE2 sequence to screen a cDNA library from HeLa cells, a human cell line, which led to the discovery of the third E-protein in 1992 [66]; the mouse counterpart was discovered later that year [67]. This protein was named HEB (HeLa E-box binding factor). In 1997, a splice variant of E2-2 was identified [68] and named ITF-2b (now called E2-2Can), which in contrast to E2-2Alt had an inhibitory effect on the promoter of a muscle-specific gene [69]. In 1999, Anderson et al. set out to identify transcription factors involved in T-cell specification by screening a SCID (severe combined immunodeficient)-thymocyte cDNA library. The search revealed a novel HEB clone [70], which was transcribed from the HEB locus from its own transcriptional start site located near a unique alternative (Alt) exon, homologous to E2-2Alt [71]. The presence of the Alt exon resulted in naming this E-protein HEBAlt, and referring to the canonical HEB as HEBCan.

HEBCan is encoded by exons 2–20, and excludes the Alt exon by alternative splicing. The transcription of HEBAlt initiates just upstream of a unique Alt exon, and the transcript shares exons 9–20 with HEBCan. An ankyrin-like exon can be included in HEBCan but does not appear to be present in transcripts cloned from thymocyte cDNA libraries [70, 71]. The Alt exon encodes for a 23 amino acid Alt domain, which is 80% identical to the Alt domain of E2-2Alt. Amino acid alignment of Alt domains from HEBAlt cDNA from fish, chicken, mouse, and human revealed a high degree of identity, indicating that the Alt domain plays an important and conserved function in vertebrates.

4. Negative Regulation of E Protein Function

E proteins are expressed widely in mouse tissues. Their functions are negatively regulated by three mechanisms: through direct competition for the E box DNA binding sites, by posttranslational modifications, or through protein-protein interactions. The transcription factor ZEB has been shown to compete for the E-box binding sites within the Ig heavy-chain gene enhancer, thus inhibiting E protein activity in a cell-specific manner [73]. Posttranslational modification, such as ubiquitination of E2A proteins upon signaling through Notch1 receptor [74] or calmodulin-mediated inactivation of E2A [75], represents another potential mechanism by which E protein function is regulated. In addition, HEB-Tal1 heterodimers suppress expression of some HEB target genes through competitive binding to the E box sites [76]. Lastly, Id factors, which lack DNA-binding capacity, antagonize E protein function by forming stable inactive Id/E protein heterodimers [77]. This form of negative regulation seems to be the most well-understood mechanism by which E-protein function is regulated during T-cell development.

There are four mammalian Id factors, Id1, Id2, Id3, and Id4 [78], which vary in tissue distribution. Id1 and Id3 factors are widespread in adult and embryonic mouse tissues [65]. In contrast, Id2 transcripts are only detected in bone marrow, testes, and brain of adult mice and in fetal livers after 13.5 days of gestation [79]. Id4 is not expressed in the fetal liver or any of the adult lymphoid tissues; its expression is limited to kidney, testes, and brain [80]. The importance

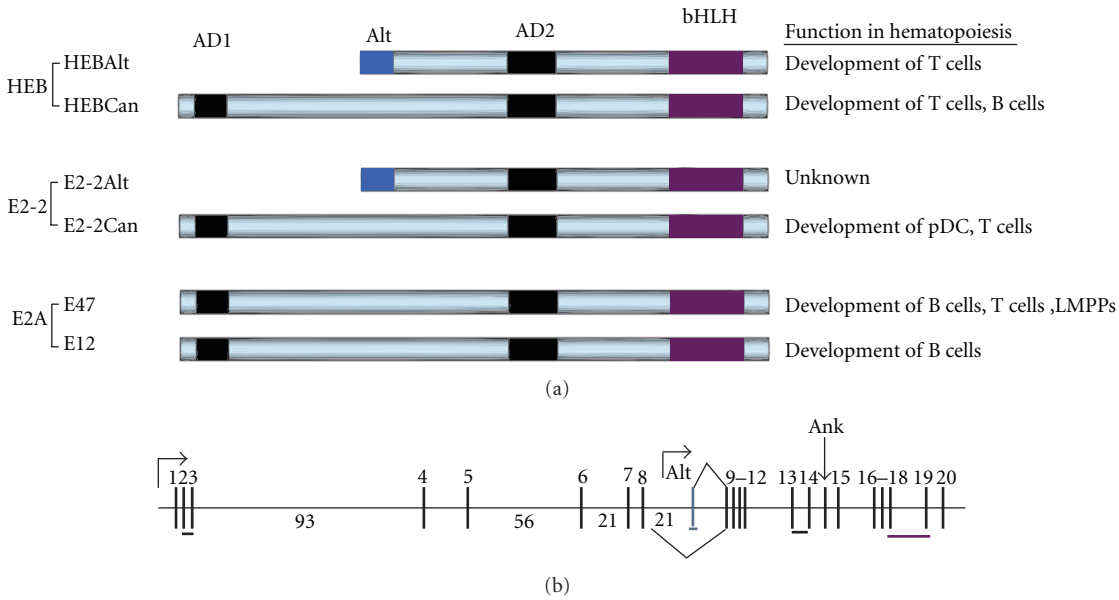

Figure 3: Structure of E-proteins. (a) E-proteins belong to the basic helix-loop-helix (bHLH) family of transcription factors. There are three genes, each encoding for two proteins: HEB (HEBCan and HEBAlt), E2-2 (E2-2Can and E2-2Alt), and E2A (E47 and E12). While E2A proteins are produced by alternative splicing, HEB and E2-2 factors are generated by independent transcription start sites and alternative splicing. All six transcription factors have a basic helix-loop-helix (bHLH) domain, which enables transcription factor dimerization and binding to the DNA. Activation domains 1 (AD1) and AD2 help recruit coactivators to the transcriptional complex. The Alt domain replaces AD1 found in the canonical forms of E-proteins and is conserved between mouse HEBAlt and E2-2Alt as well as through vertebrate evolution. (b) Organization of HEB gene. Vertical grey bars represent exons. Protein domains encoded by exons are shown as horizontal bars. Numbers above exons represent exon numbers, while numbers between exons indicate genomic distance in kb. pDC: plasmacytoid dendritic cells, LMPPs: lymphoid primed multipotent progenitors.

of Id factors in lymphoid development has been revealed by gene knockout and transgenic studies. Studies involving Id2$^{-/-}$ mice revealed that this factor is essential for the generation of NK cells [81] and DCs [82]. Transgenic expression of Id1 under the control of the *lck* promoter led to a severe block at the DN1 stage of T-cell development [83]. Lastly, Id3 overexpression promoted NK cell development at the expense of T cells [84]. Collectively, these studies have shown that Id factor interference with E-protein activity leads to severe perturbations during lymphoid development.

5. E2A and E2-2 in Hematopoiesis

The functions of E proteins have been most extensively studied in the context of B lymphopoiesis. In B cells, E2A proteins function as homodimers, stabilized by disulfide bonds in a B-cell specific manner [85]. E2A$^{-/-}$ mice lack B cells in fetal liver, bone marrow, and spleen and are prone to die shortly after birth [86]. In the absence of E2A, the early progenitors fail to activate early B-cell developmental genes, such as early B-cell factor (EBF) and the paired box protein 5 (Pax-5), as well as the B-cell specific expression of *Rag* genes [87–89]. As a result, E2A$^{-/-}$ cells fail to undergo *Ig* gene rearrangements and are arrested at the earliest stage of development [88]. In T-cell development, deletion of E2A results in an early partial arrest at the DN1 stage, inappropriate traversal through β-selection, and increased positive selection of DP thymocytes (Figure 1(b)) [90–92]. Since E proteins have been shown to compensate for each other [93, 94], studies involving a simultaneous deletion of E2A and HEB were done. These studies revealed defects that were not observed upon deletion of either gene alone. Deletion of E2A and HEB during DN stages revealed a role for E proteins in suppressing proliferation prior to pre-TCR signaling [95]. When both E proteins were deleted in later stages of T-cell development, DP cells developed to the CD8^{+} lineage in the absence of TCR, indicating inappropriate positive selection [96]. In addition, E2A also regulates the expression of *Rag* genes in CLPs [89] and LMPPs [97]. In contrast to E2A, the function of E2-2 is not as well characterized. The most prominent known function of E2-2 is in the regulation of pDC development [98, 99]. In T-cell development, E2-2 has been suggested to play a role at β-selection since E2-2$^{-/-}$ mice display an accumulation of DN3 cells (Figure 1(b)) [100].

6. HEB in Hematopoiesis

The importance of HEB factors in lymphopoiesis was revealed by studies involving HEB mutant mice. First, HEB$^{-/-}$ mice were generated by deleting a segment of the bHLH domain, thereby targeting both HEBCan and HEBAlt [93]. In contrast to E2A knockout mice, HEB$^{-/-}$ mice produce B cells, although in reduced numbers [93]. When compared to

other E proteins, loss of HEB has the most profound effect on T-cell development (Figure 1(b)). HEB$^{-/-}$ mice have reduced thymic cellularity and display an accumulation of CD8^{+} ISP cells [101], reminiscent of the arrest seen in TCF1$^{-/-}$ and Bcl11b$^{-/-}$ mice. Since the defects observed in HEB$^{-/-}$ thymocytes could not be repaired with anti-CD3 treatment or upon transgenic TCR expression, the functions of HEB were proposed to be either parallel with or downstream of pre-TCR signaling [101]. Moreover, mutant mice expressing HEB without the basic region of the DNA-binding domain render HEBCan and HEBAlt capable of dimerizing but incapable of binding DNA. This dominant negative mutation (HEB^{dn}) resulted in a severe block at the DN3 stage of T-cell development [102]. Since T-cell precursors failed to produce V-(D)J rearrangements, HEB was implicated in the regulation of *TCRβ* gene rearrangement. HEB is also involved in the regulation of *pTα* [103] and *CD4* [104] gene expression, as well as the rearrangement of *TCRα* gene [105]. However, the relative contributions of HEBAlt and HEBCan to these processes are not well understood.

The arrest at the ISP stage of development in TCF1$^{-/-}$, Bcl11b$^{-/-}$, and HEB$^{-/-}$ mice brings up the question of how these genes are connected, and how they might impact the expression of CD4. We have shown that IL7R signaling is sustained in HEB$^{-/-}$ DN cells [106]. It is, therefore, possible that HEB aids in the downregulation of IL7R signaling after β-selection, which is necessary to prevent interference with the upregulation of TCF1, LEF1, and RORγ genes and transition past the ISP stage of development [107]. HEBCan plays an important role in initiation of CD4 gene expression [104], raising the question of whether the CD8^{+} ISP cells in the HEB$^{-/-}$ mice represented DP cells in disguise. However, the cycling profile and intracellular TCRβ chain expression of these cells suggested otherwise [101]. GATA3 is essential for CD4 gene expression, whereas Runx3 is a direct repressor of CD4 [108, 109]. We found that although HEB deficiency at the DN3 stage did not affect the expression of GATA3, transgenic reconstitution of HEB$^{-/-}$ cells with HEBAlt resulted in the upregulation of CD4 to generate DPs [110]. Therefore, another possibility is that HEB factors, and HEBAlt in particular, function in repressing Runx3 protein expression or activity. This remains to be tested.

Our studies involving the retroviral overexpression of either HEBCan or HEBAlt have shed light on the functions of individual HEB factors in lineage specification and developmental fate decisions. For instance, ectopic overexpression of HEBAlt in LSK cells led to enhanced specification into the T-cell lineage [71] and a reduced capacity to generate myeloid cells [111] in presence of DL1-Notch1 signaling. During B-cell development, HEBAlt overexpression suppressed B-cell potential, even in the absence of DL-Notch1 signals [111]. Lastly, HEBAlt was also shown to play a role in lympho-myeloid specification since precursors with a strong myeloid potential adopted the T-cell fate upon overexpression of HEBAlt [112]. However, the precise mechanisms by which HEBAlt guides T-cell development and fate choice remain to be determined.

In our recent studies, we have shown that HEB$^{-/-}$ mice have an early block in T-cell development, which was alleviated in part upon the addition of an HEBAlt transgene driven by the *lck* promoter. Furthermore, we identified pTα and CD3 signaling components as specific targets of HEBAlt during β-selection [110]. In addition, HEB$^{-/-}$ mice also had a defect in T-cell commitment, with compromised Notch1 function and a tendency to become DN1-like cells [106]. The DN1-like cells could be induced to differentiate into thymic NK cells, revealing a role for HEB in the T/NK cell lineage decision. Importantly, a new set of interactions were revealed among HEB, Notch1, and GATA3, which regulate the T-cell fate choice in developing thymocytes. Conditional inactivation of either HEBCan or HEBAlt alone will allow for dissociation of their individual functions during T-cell development.

7. HEB in the Gene Regulatory Network Controlling the Early T-Cell Development

The gene networks that operate during early T-cell development integrate developmental regulatory states with the appropriate environmental signals to generate T cells. Although many individual factors have been identified, the connections that exist among them have not yet been well established. Bcl11b, HEBAlt, and TCF1 are positively regulated by Notch signaling in thymic precursors, and both Bcl11b and HEBAlt are sharply upregulated at the DN2a stage of T-cell development, just prior to commitment [47, 54, 71]. Moreover, precursors from both Bcl11b$^{-/-}$ and HEB$^{-/-}$ mice generate NK cells, suggesting that both of these factors are needed to suppress the NK cell fate. Since Bcl11b$^{-/-}$ thymocytes are arrested at the DN2 stage, whereas HEB$^{-/-}$ cells are arrested later in development, it could be proposed that HEBAlt expression is downstream of Bcl11b. However, HEBAlt expression is not considerably reduced in Bcl11b$^{-/-}$ precursors at early stages of development [54]. Likewise, Bcl11b is not reduced in Rag1$^{-/-}$HEB$^{-/-}$ DN3 cells as compared with Rag1$^{-/-}$ DN3 cells (M. Braunstein and M. K. Anderson, unpublished results). Moreover, constitutive Notch signaling did not rescue T-cell development in the absence of HEB [106], indicating that Notch target genes are not sufficient to drive T-cell development in the absence of HEB factors. We, therefore, propose that HEBAlt and Bcl11b function in parallel downstream of Notch signaling to specify the T-cell fate, as illustrated in our gene regulatory network model (Figure 4).

In early thymocytes, E2A is necessary for the initiation of Notch1 expression, which in turn activates HEBAlt gene expression. TCF1 is also required for the acquisition of the T-cell identity [47]. HEBAlt, therefore, must collaborate with TCF1 to enhance specification to the T-cell lineage, whereas Bcl11b promotes T-cell development indirectly by inhibiting NK-cell development. Indeed, HEBAlt and TCF1 regulate the expression of components of the pre-TCR signaling pathway [47, 110], whereas none of the pre-TCR genes were shown to be affected by the loss of Bcl11b [54]. Together these studies indicate that HEB factors are required for the integration of pre-TCR and Notch signals at β-selection and suggest that HEBAlt in particular plays a crucial role in this process.

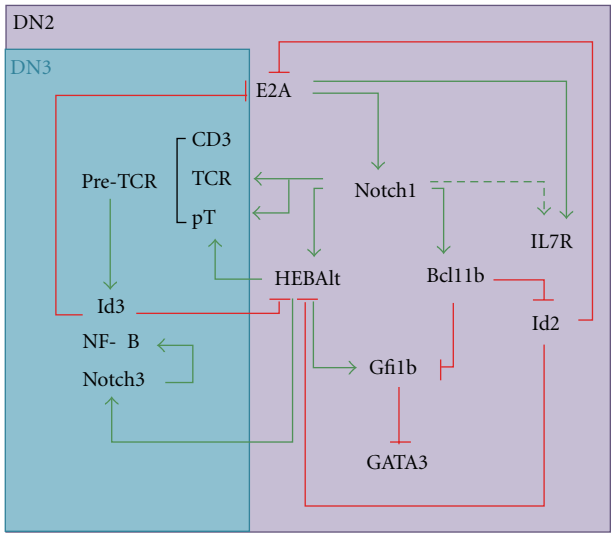

Figure 4: Gene regulatory network model operating in early T-cell development. E2A positively regulates Notch1 expression, which induces the expression of HEBAlt, Bcl11b, and IL7R. HEBAlt positively regulates T-cell genes, such as pTα and Notch3, which in turn upregulates NF-κB signaling. Bcl11b negatively regulates Id2 and Gfi1b to balance the expression of GATA3, thus limiting the NK-cell potential. HEBAlt may also regulate GATA3 indirectly through Gfi1b. HEBAlt and Notch1 upregulate pTα and TCRβ, the components of pre-TCR, thus promoting transition from the DN2 to the DN3 stage of T-cell development. Pre-TCR signaling upregulates Id3, which inhibits the activity of E2A and HEBAlt at the β-selection checkpoint. The inhibition of HEBAlt activity past the DN3 stage is important as it disrupts the positive feedback loop between Notch3 and NF-κB, which may, otherwise, lead to leukemogenesis. Green arrows show positive inputs, red blunt arrows show negative inputs. Established connections are shown by solid arrows, and indirect or proposed connections are shown by dashed arrows.

Although enforced Notch1 signaling was insufficient to support the DN to DP transition in HEB$^{-/-}$ precursors, it was able to effectively restore T-cell potential and suppress NK cell potential in these precursors [106]. It is tempting to speculate that under these conditions it was the induction of Bcl11b by Notch1 signaling that inhibited NK cell development. Bcl11b inhibits the expression of Id2 [54], allowing E2A and HEB factors to maintain the expression of Notch1 and pre-TCR complex genes. GATA3 is negatively regulated by Gfi1b [113]. Therefore, the induction of Gfi1b by E2A and HEB [114] coupled with the repression of Gfi1b by Bcl11b [54] allows fine tuning of the GATA3 levels needed for T-cell development. Our recent results indicate that the transgenic expression of HEBAlt is insufficient to prevent transition into the DN1-like state, consistent with an inability of HEBAlt to upregulate Bcl11b and diversion to the NK cell lineage (Figure 5). Taken together, these studies indicate that HEBAlt and Bcl11b function in parallel during early T-cell development and suggest that whereas Bcl11b inhibits NK and stem-cell gene expression, HEBAlt collaborates with TCF1 to induce T-cell gene expression.

8. Life and Death at the β-Selection Checkpoint

A lack of HEB gives cells a survival advantage in the absence of DNA rearrangement [106]. Initiation of TCRβ rearrangements is a key event orchestrating the normal outcomes of β-selection. During rearrangement, double-stranded DNA breaks are introduced which, if not repaired, result in death. This removes cells that may otherwise have oncogenic potential. For example, cells that are deficient for the enzyme DNA-dependent protein kinase (DNA-PK) are unable to resolve D-J breaks, which leads to a developmental arrest at the DN3 stage. As a consequence, the cells die via a p53-dependent pathway. On the other hand, T-cell progenitors that do not express *Rag* genes are unable to initiate DNA breaks during D-J rearrangement. Indeed, these cells have very low amounts of p53 compared to cells that are DNA-PK deficient, indicating that they escape death initiated by the p53-dependent pathway. However, Rag-deficient T-cell precursors still die. Although the mechanism of death has yet to be determined, it is likely to involve a combination of events that include upregulation of proapoptotic molecules, such as Bim, by the FOXO factors and the absence of pro-survival signals that emanate from the pre-TCR, Notch1 and IL7 signaling pathways [115]. Both pre-TCR and IL7R signal via PI3K, which inhibits the activity of FOXO factors [116]. Bim is also upregulated directly by E2A [115] and could be a direct target of HEB as well. In one scenario, accumulation of E proteins in DN3 cells that lack TCRβ rearrangements would result in upregulation of Bim and elimination by apoptosis. Interestingly, Notch1 signaling also mediates survival via Akt, not only in normal DN3 cells but also in Rag-deficient DN3 cells [117].

HEBCan and E2A factors suppress proliferation by upregulating cell-cycle inhibitors [118], which normally keep DN3 cells without rearrangements in check. Interestingly, an alternative outcome was available to certain HEB$^{-/-}$ DN3 cells at the time of β-selection: development into the thymic NK cell lineage. Although HEB$^{-/-}$ T-cell precursors with rearranged TCRβ genes and intact Notch1 signaling had the ability to turn into DN1-like cells, the majority of the cells that became DN1-like lacked TCRβ rearrangements and had downregulated Notch signaling. Even though restoring full Notch signaling did not restore the ability to pass through β-selection in the absence of the pre-TCR, it did restore the natural outcome of DN3 cells without rearrangements: death. The mechanism by which Notch signaling could overcome HEB deficiency to induce death is unknown. The tumour-suppressive function of E2A [119] and likely HEBCan is in contrast with the activity of HEBAlt, as we have observed that HEBAlt transgenic mice develop lymphoma, possibly through sustained Notch1 signaling (M. Braunstein and M. K. Anderson, unpublished results). Under normal circumstances, both HEBAlt and Notch1 are downregulated at β-selection. In the transgenic mice, however, Id3 was likely insufficient to block the activity of HEBAlt, which might have led to lymphomagenesis by maintaining Notch1 signaling across the β-selection checkpoint.

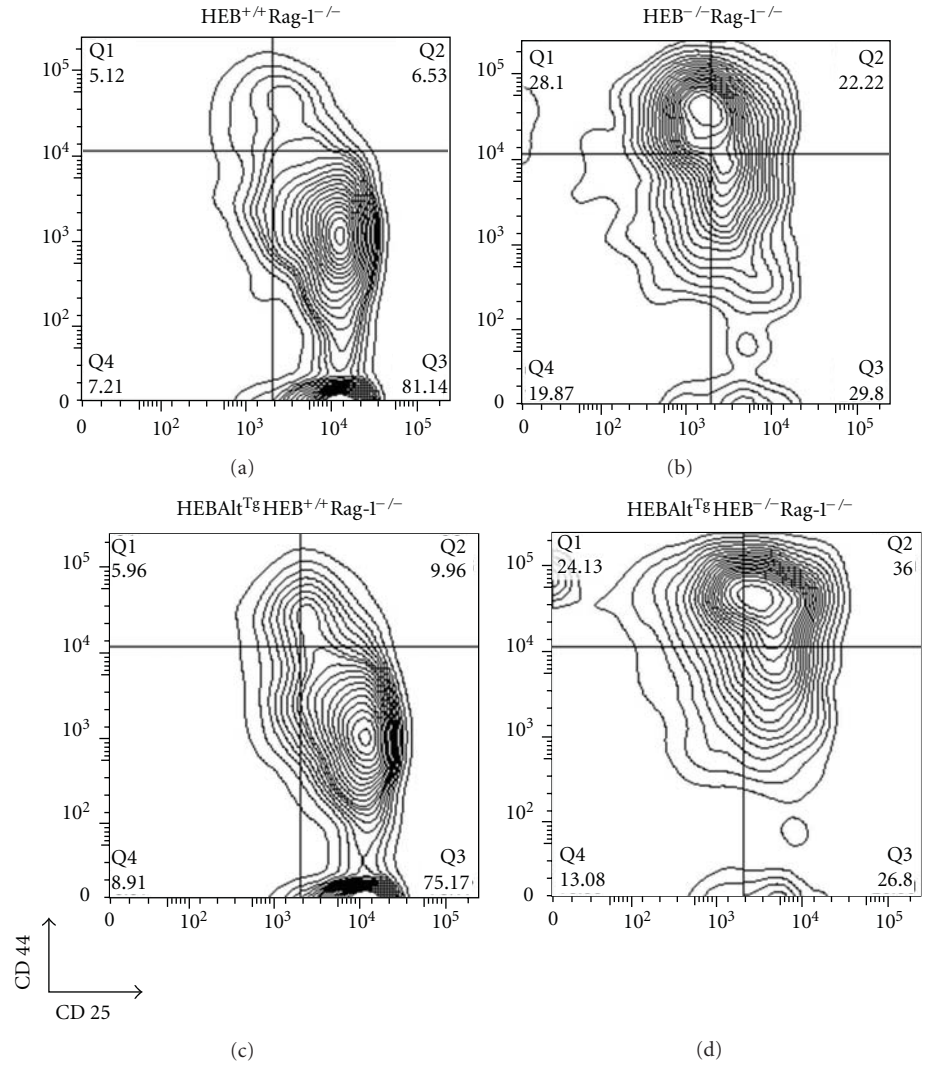

Figure 5: Developmental phenotype of $HEB^{-/-}$Rag-$1^{-/-}$ T-cell progenitors expressing transgenic HEBAlt. $HEB^{+/-}$ mice were bred with Rag-$1^{-/-}$ mice to generate $HEB^{+/-}$Rag-$1^{-/-}$ mice, which were timed mated to generate (a) $HEB^{+/+}$Rag-$1^{-/-}$ and (b) $HEB^{-/-}$Rag-$1^{-/-}$ embryos. Similarly, $HEB^{+/-}$Rag-$1^{-/-}$ mice were bred with $HEBAlt^{Tg}$ mice to generate $HEBAlt^{Tg}HEB^{+/-}$Rag-$1^{-/-}$ mice, which were timed mated to generate (c) $HEBAlt^{Tg}HEB^{+/+}$Rag-$1^{-/-}$ and (d) $HEBAlt^{Tg}HEB^{-/-}$Rag-$1^{-/-}$ embryos. Fetal livers were genotyped, lineage depleted (lineage positive fraction: B cells, myeloid cells, red blood cells). Fetal liver LSK (lineage negative, $Sca1^+$, $ckit^+$) cells were sorted and cultured on OP9-DL1 for 7 days to allow developmental progression to the DN3 stage. At day 7, lymphocytes were gated on the $CD45^+CD4^-CD8^-$ fraction and sorted for the DN3 cells ($CD44^-CD25^+$), which were cultured on fresh OP9-DL1 stroma with 5 ng/mL IL7 and Flt3L. Four days later, whole cell cultures were analysed by flow cytometry. All plots were gated on the $CD45^+CD4^-CD8^-$ fraction.

9. Development of T versus tNK Cells in the Thymus

From an evolutionary standpoint, Notch signaling is an ancient pathway, whereas pre-TCR signaling is a relatively new acquisition. The NK cell gene program, therefore, may represent a default route for early progenitors in the ancient thymus, which later in evolution became circumvented to generate cells with rearranged receptors. Indeed, NK cells are generated first in the fetal thymus prior to any $\alpha\beta$ T cells [120]. Moreover, the requirements for GATA3 and IL7R are common between T cells and thymic NK cells, and while the development of thymic NK cells may not depend on Notch1 signaling [121], evidence for a role of Notch in thymic NK cell development does exist [122]. Therefore, the evolutionary divergence of the thymic NK and T-cell lineages may be mirrored by the developmental steps that give rise to each lineage. HEB may in part be responsible for the separation of these lineages, by modulating Notch signaling and selective survival of β-selected T-cell progenitors, and by regulating the levels of GATA3.

In our studies, thymic NK cells were derived from $HEB^{-/-}$ DN3 cells that would not have survived or developed in the absence of Notch signaling, suggesting an initial role for Notch in specifying a common T/NK progenitor. It is also possible that tNK cells normally arise from noncanonical

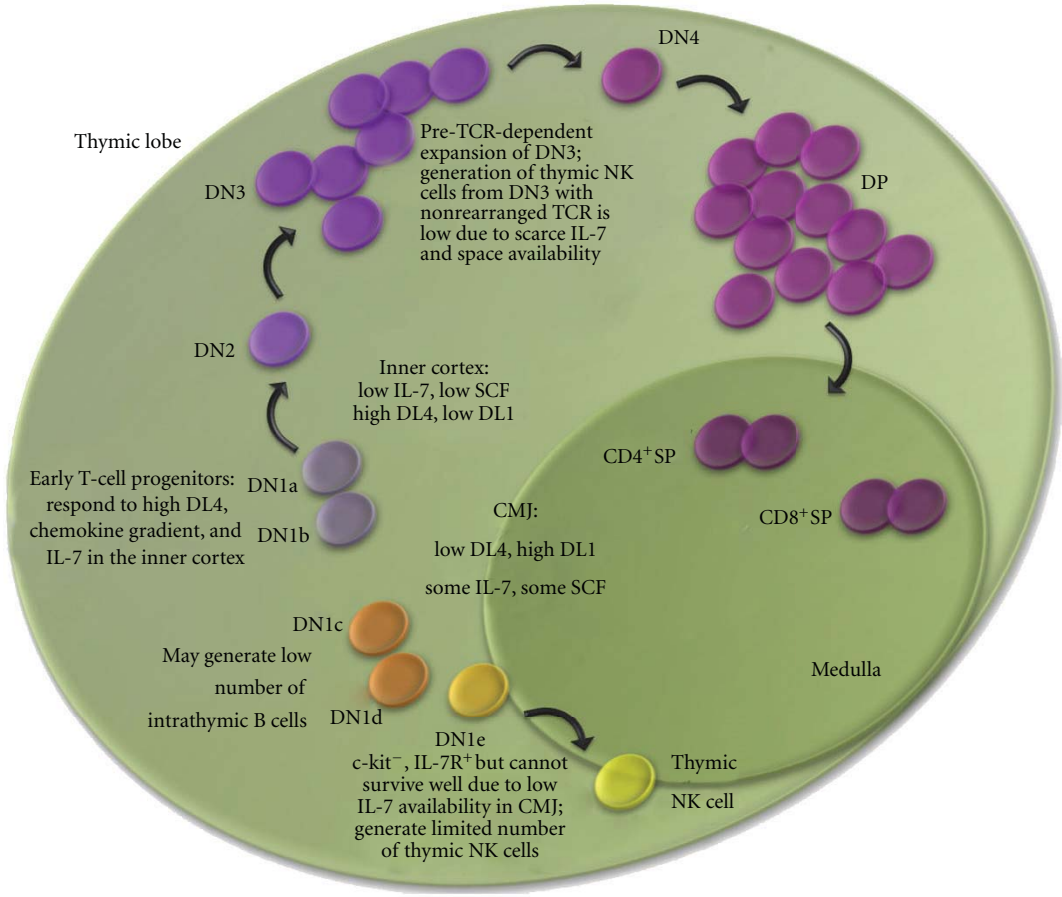

Figure 6: Development of T versus tNK cells in the thymus. DN1 cells enter thymus at the corticomedullary junction (CMJ). This area is low in DL4 but high in DL1 ligand; in addition, there is a low amount of IL-7 and SCF dispersed throughout a thymus. DN1c and DN1d cells may be progenitors to a small number of B cells generated intrathymically. DN1a and DN1b cells represent canonical early T-cell progenitors (ETPs), which migrate to the inner cortex, the area of high DL4 ligand concentration. In response to Notch signalling, ETPs turn on many of the T-lineage specific genes and develop into DN2 cells. At the DN3 stage, the cells rearrange TCRβ genes and undergo β-selection, thus expanding and taking up most of the space in the outer cortex. This is disadvantageous for those DN3 cells that have not rearranged TCRβ, which may give rise to the thymic NK cells. Thus, a small percentage of NK cells may be generated in a thymus, mostly from the DN1e progenitors, which are likely to remain near the CMJ region where the DL4 ligands are low and the IL-7 concentration is sufficient for the thymic NK cell development.

precursors such as DN1c, DN1d, or DN1e cells. DN1e cells are of particular interest because they already express high levels of IL7R and Id2 [4, 6]. Although they do not have strong proliferative potential, they do generate both T and NK cells [4]. Interestingly, culturing DN1e cells on OP9 stroma that lacks DL expression yielded only 3% NK cells, whereas culturing ETPs on OP9 stroma generated approximately 40% NK cells. This raises the intriguing possibility that DN1e cells are primed to become thymic NK cells but need intermittent and/or low DL-Notch signals to give rise to thymic NK cells (Figure 6). Consistent and/or high DL-Notch1 signaling, on the other hand, would be expected to promote noncanonical T-cell development from DN1e cells.

The DN1 and DN2 stages of T-cell development express many progenitor-like genes [123, 124] that allows for their experimental reprogramming into mast cells and NK cells. Under normal conditions, however, the DN3 stage marks the point of no return; at this stage, the cells either commit to the T-lineage or die. The question then arises: what defines the DN3 stage and T-cell commitment? Development to the DN3 stage does not require the rearrangement of TCRβ genes or the expression of *Rag* genes, as indicated by the ability Rag-1$^{-/-}$ thymocytes to acquire the T-lineage phenotype up to this stage. Instead, the upregulation of many other T-cell specification genes must be used as the criteria to determine the developmental status of an early T-cell progenitor. Commitment, on the other hand, is defined as the inability to adopt alternative lineage choices. HEB$^{-/-}$ DN3 cells display an interesting and aberrant gene expression pattern that speaks to these criteria: they have a partially activated T-cell program, and they maintain a limited ability to differentiate into an alternative fate. Therefore, it is unlikely that the HEB-deficient DN3 cells, which can give rise to thymic NK cells, reflect DN2 cells in disguise. Rather, the transition from the DN3 to DN1-like state involves a true loss of T-cell identity in the absence of cell death.

The thymus provides a highly structured and ordered environment, where Notch ligands and cytokines become available in varying doses and in specific niches, tightly controlling cellular development [125]. These restrictions promote early T-cell development and limit the selection of both DN3 cells that lack TCRβ rearrangements and thymic NK cell development. A T-cell progenitor entering the thymus through the CMJ is exposed to the DL1 ligand and SCF, but low IL-7 availability (Figure 6). At this point, progenitors such as DN1e cells, which are c-kit$^-$ and IL7R$^+$, could potentially respond to DL1 but would be limited in their survival and thus fail to generate abundant thymic NK cells. The distribution and levels of IL15 within the thymus still need to be determined; however, it is likely that IL15 is only scarcely available throughout the thymus given the small number of thymic NK cells that are generated even in a Rag-deficient thymus. Lastly, the expression of chemokine receptors on DN1e cells suggests that these cells may migrate towards the medulla rather than the cortex, which could provide an alternative set of signals that would promote NK cell development [6]. ETPs, on the other hand, lack IL7R but express c-kit and chemokine receptors that would help with transition from the CMJ to the cortical region. In the cortex, the rapid expansion of β-selected cells allows the T-cell precursors to outcompete and thus limit the survival and developmental capacity of DN3 cells lacking TCRβ rearrangements. Thymic epithelial cells express abundant levels of IL7 throughout the entire fetal thymus from day E12.5 to E13.5 [126]. Therefore, the availability of IL7 within an E12.5–E13.5 fetal thymus would be expected to encourage thymic NK cell development. Indeed, thymic NK cells develop in the fetal thymus before any DP cells are generated. After E15.5, however, the thymus size increases due to proliferating thymocytes and the proportion of epithelial cells producing IL7 is correspondingly reduced. Approximately 15% of the cells in fetal thymic organ culture are thymic NK cells, whereas adult Rag-deficient thymus contains approximately 4% thymic NK cells. By contrast, thymic NK cells represent only 0.013% of the adult thymocyte population. Our results showed that, although HEB$^{-/-}$ precursors had downregulated Notch signaling and indeed gained DL independence, they were nonetheless still dependent on IL7 to survive [106].

10. Summary

In summary, HEB factors are essential mediators of T-cell lineage specification and commitment. HEBAlt and HEBCan play distinct roles in these processes, with HEBAlt inducing T-lineage genes and suppressing myelopoiesis within the thymus, whereas HEBCan appears to be more involved in repressing the NK cell fate. These factors interface with Notch1, TCF1, GATA3, Bcl11b, and Gfi1b to form a network of interactions that not only initiates the T-cell program but also incorporates positive feedback loops that sustain it. Further study will be needed to address the question of how HEBAlt and HEBCan function as homodimers, heterodimers with each other, or heterodimers with E2A, but our work has clearly shown that both HEBCan and HEBAlt are central factors in the early stages of T-cell development.

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Research Article

The Modulation of PPARγ1 and PPARγ2 mRNA Expression by Ciglitazone in CD3/CD28-Activated Naïve and Memory CD4+ T Cells

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Given their roles in immune regulation, the expression of the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) 1 and 2 isoforms was investigated in human naïve (CD45RA+) and memory (CD45RO+) CD4+ T cells. Stimulation of both types of cells via the CD3/CD28 pathway resulted in high expression of both PPARγ receptors as measured by real-time PCR. Treatment with the PPARγ agonist, ciglitazone, increased PPARγ1 expression but decreased PPARγ2 expression in stimulated naïve and memory cells. Furthermore, when present, the magnitude of both PPARγ receptors expression was lower in naïve cells, perhaps suggesting a lower regulatory control of these cells. Similar profiles of selected proinflammatory cytokines were expressed by the two cell types following stimulation. The induction of PPARγ1 and suppression of PPARγ2 expressions in naïve and memory CD4+ T cells in the presence of ciglitazone suggest that the PPARγ subtypes may have different roles in the regulation of T-cell function.

1. Introduction

Peripheral CD4+ T cells can be divided into two broad functional groups based on their expression of distinct isoforms of the CD45 surface molecule, CD45RA representing naïve CD4+ T cells and CD45RO representing memory CD4+ T cells [1]. Memory CD4+ T cells require a shorter lag time to proliferate when they are stimulated by antigens and are less dependent on costimulation than are naïve CD4+ T cells [2]. On the other hand, naïve CD4+ T cells have been reported to be the source of autoreactive lymphocytes in multiple sclerosis [3, 4], suggesting a differential regulatory mechanism for these cells.

The peroxisome proliferator-activated receptors (PPARs) are ligand-activated receptors that belong to the nuclear receptor superfamily [5]. Three isoforms of PPARs have been identified and are encoded by separate genes, namely, PPARα, γ, and β/δ [6, 7]. PPARγ is predominantly expressed in adipose tissue, colon, spleen, adrenal gland, and monocytes/macrophage [6, 7]. This isoform is further divided into four subtypes: PPARγ1, γ2, γ3, and γ4 due to alternative promoter use and RNA splicing [8]. PPARγ1, PPARγ3, and PPARγ4 encode for the same protein product, while the PPARγ2 protein contains an additional 28 amino acids at its N-terminus. PPARγ ligands include the naturally occurring arachidonic acid metabolite, 15-deoxy-D12,14-prostaglandin J2 (15d-PGJ2), as well as the thiazolidinedione (TZD) group of drugs such as ciglitazone and certain novel non-TZD insulin-sensitizing agents [9, 10].

PPARγ expressed in murine T-cells plays a regulatory role in T-cell activation [11]. Previous experiments showed that murine helper-T-cell clones and freshly isolated splenocytes express PPARγ1 but not PPARγ2 mRNA and that 15d-PGJ2 and ciglitazone inhibited the proliferative responses and IL-2 production of these cells when stimulated with the specific antigen and anti-CD3 antibodies, respectively [11].

Similarly, it was reported that 15d-PGJ2 and troglitazone suppressed IL-2 production of PHA-stimulated peripheral blood T cells [12]. PPARγ has been shown to physically bind to the transcription factors AP-1 and NFAT [12, 13], which regulate the IL-2 promoter thus blocking their binding to the promoter and hence inhibiting the transcription of the IL-2 gene.

These studies indicate an important immunoregulatory role for PPARγ in T-cell function. It will, therefore, be interesting to investigate whether naïve and memory CD4+ T cells behave in the same manner with regard to the expression of PPARγ and whether their activation modulate the expression of the PPARγ receptor differently. It would also be important to explore the impact on cytokine expression in these T-cell subsets upon activation of PPARγ, in particular selected proinflammatory cytokines, which are important in autoreactivity such as autoimmune diabetes [14].

Most studies on the role of PPARγ have used semi-quantitative measurements to assess the mRNA level of the receptor. Since subtle changes in PPARγ levels may result in significant changes to various downstream events as postulated by other types of receptor-signaling molecules [15], an accurate quantification of PPARγ isoform levels following cellular activation would need to be carried out.

We propose to study the expression of PPARγ1 and PPARγ2 in unstimulated and stimulated naïve and memory CD4+ T-cell subsets using quantitative real-time PCR. To further dissect the role of PPARγ1 and PPARγ2 in immune activation, the PPARγ agonist, ciglitazone, was used to modulate the activation status of these cell types and assess the modulation of their expression levels as well as those of selected proinflammatory cytokines in these cells.

2. Materials and Methods

2.1. Isolation of Naïve and Memory CD4+ T Cells from Peripheral Blood. Peripheral blood collection has prior approval from the Universiti Sains Malaysia Ethics Committee and collected after informed consent was obtained. Human naïve and memory CD4+ T cells were isolated from the peripheral blood by immunomagnetic separation. Briefly, blood was obtained from normal donors, and the peripheral blood mononuclear cells (PBMCs) were isolated by the Ficoll gradient centrifugation and incubated with a panel of biotin-conjugated monoclonal antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR$\gamma\delta$, and glycophorin A (Miltenyi Biotec, Germany). CD45RA and CD45RO microbeads were added reciprocally for the negative isolation of memory and naïve CD4+ T cells. The purity of the isolated naïve and memory CD4+ T cells were generally 90–95% as determined by flow cytometric analysis.

2.2. In Vitro Stimulation of Naïve and Memory CD4+ T Cells. Naïve and memory CD4+ T cells were suspended at 2×10^5 cells/mL in complete RPMI 1640 medium (10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin) containing CD3/CD28 beads at a 1 : 1 cell/bead ratio in 25 cm^2 tissue culture flasks. Twenty μM of ciglitazone solution was added when required at day 0 of culture. This concentration of ciglitazone was determined based on the minimum concentration required to cause a reduction in cell proliferation as reported in the literature [11, 13, 16]. The flasks were incubated for 5 days in a humidified incubator at 37°C in 5% CO_2.

2.3. Proliferation Assay. Naïve and memory CD4+ T cells were suspended in 200 μL of complete RPMI 1640 medium at a concentration of 1×10^3/well in triplicate wells of a 96-well flat-bottom plate and stimulated with CD3/CD28 beads for 5 days as previously described [17]. When required, ciglitazone (20 μM) was added at day 0 of culture. Ten μL of diluted [^{3}H] thymidine (1 μCi) was added to each well at 0, 24, 48, 72, and 96 h after stimulation. After incubation for another 20–22 h, the cells were harvested to represent day 1, 2, 3, 4, and 5, respectively, using the Innotech cell harvester system (Innotech AG, Switzerland). The incorporation of [^{3}H] thymidine into DNA was quantified using a liquid scintillation counter by Hidex data analysis software (Hidex, USA).

2.4. Total RNA Extraction and cDNA Synthesis. Total RNA was extracted from unstimulated and stimulated naïve and memory CD4+ T cells with or without ciglitazone treatment using the RNeasy Mini kit (Qiagen, USA) and QIAshredder (Qiagen, USA) according to the manufacturer's instructions. Briefly, the cells were lysed in RLT buffer and the beads were depleted using Dynal MPC. The lysed cells were applied onto the QIAshredder column followed by the RNeasy Mini spin column after addition of 70% ethanol. The sample column was then centrifuged, and the flow-through discarded before 700 μL of RW1 buffer was added into the column. Following centrifugation, the mixture was washed twice in 500 μL RPE buffer before 50 μL of RNase free water was added into the column to dissolve the total RNA. The RNA was eluted by centrifugation, and its integrity was assessed by gel electrophoresis while RNA purity and concentration were measured by spectrophotometry (Biophotometer, Eppendorf, Germany).

Total RNA (between 0.5 to 5 μg) was reverse transcribed into cDNA using the RevertAid H Minus first strand cDNA synthesis kit (MBI Fermentas, USA) in the presence of 0.5 μg oligo(dT)$_{18}$ primer in nuclease-free deionized water. The mixture was firstly incubated at 70°C for 5 minutes. The reaction mixture was then mixed with 4 μL of 5x reaction buffer, 20 unit ribonuclease inhibitor, and 2 μL of 10 mM dNTP mix, followed by incubation at 37°C for 5 minutes. The process of reverse transcription was performed at 42°C for 1 hour using 200 unit of RevertAid H Minus M-MuLV. Finally the process was terminated by heating at 70°C for 10 minutes. The success of cDNA synthesis was confirmed by running a PCR using human β-actin primer (Maxim Biotech, USA).

2.5. Competitive Real-Time PCR. The PPARγ1 gene was amplified and quantified using the following primers/probe: forward primer 5′-CTT TAT GGA GCC CAA GTT TGA

GTT-3′; reverse primer 5′-GGC TTC ACA TTC AGC AAA CCT-3′ and TaqMan probe 5′-TGC CAA GTC GCT GTC ATC TAA TTC CAG TG-3′. The PPARγ2 gene was amplified and quantified using the following primers/probe: forward primer 5′-GGG TGA AAC TCT GGG AGA TTC TC-3′; reverse primer 5′-GAT GCC ATT CTG GCC CAC-3′ and TaqMan probe 5′-TGA CCC AGA AAG CGA TTC CTT CAC TGA-3′. A total volume of 22.5 μL master mix, which included the TaqMan Universal Master Mix (ABI, USA), TaqMan probe, forward and reverse primers, and sterile distilled water was added in each well of the PCR plate prior to the addition of 50 ng of target cDNA. The master mix contains a dye (ROX) for normalization. Five dilutions of internal standards (plasmids containing the PPARγ1 and PPARγ2 genes) were chosen from the range of 10^{-4} pmol to 10^{-8} pmol. For nontemplate control (NTC) wells, only water was added. The reaction plate was sealed with an optical adhesive cover, centrifuged briefly to avoid any bubbles, and placed in the real-time PCR apparatus to begin the reaction. All samples were run in triplicates.

The reaction was initiated at 50°C for 2 min. This step was required for optimal AmpErase uracil-N-glyco-syl-ase (UNG) enzyme activity to decontaminate any DNA carryover. The temperature was increased to 95°C for 10 min to activate the AmpliTaq Gold enzyme. This was followed by 45 cycles of denaturation at 95°C for 15 sec and primer annealing and extension stages at 60°C for 1 min each.

2.6. Multiplex PCR (MPCR). The expression levels of TGFβ, IL-1β, IL-8, TNFα, GM-CSF, and IL-6 were measured in unstimulated and stimulated naïve and memory CD4+ T cells with or without treatment with ciglitazone using the MPCR kit for Human Inflammatory Cytokines Genes Set-1 (Maxim Biotech, USA). The expression of the house-keeping gene, GAPDH, was used for normalization. The MPCR was carried out according to the manufacturer's instructions. Briefly, 1x MPCR buffer, 1x MPCR primer mix, 2.5 units of Taq polymerase, and 0.1 μg cDNA template were mixed in a 50 μL reaction; the optimum annealing temperature for the MPCR analysis was 66°C and subjected to 35 cycles of PCR, with denaturing, annealing, and extension temperatures at 94, 58, and 70°C for 1 min each, respectively. Following MPCR, the products were fractionated electrophoretically in a 2% agarose gel containing 0.5 μg/mL ethidium bromide and analysed by the Image Master Total Lab v1.00 (Amersham Pharmacia, USA).

2.7. Statistical Analysis. The profiles of [^{3}H] thymidine incorporation of naïve and memory CD4+ T cells after *in vitro* stimulation with or without ciglitazone treatment were compared and analysed using the Kruskal-Wallis test. The PPARγ1 and PPARγ2 expression and cytokine profiles of unstimulated and stimulated naïve and memory CD4+ T cells with or without ciglitazone treatment were compared and analysed using the Mann-Whitney U test by statistical program for social science (SPSS) version 11.0 computer program (SPSS Inc., USA).

3. Results

3.1. Proliferative Response of CD3/CD28-Stimulated Naïve and Memory CD4+ T Cells. The proliferative response of purified naïve and memory CD4+ T cells following *in vitro* stimulation with CD3/CD28 was assessed. Anti-CD3/CD28 enhanced proliferation in both naïve and memory CD4+ T cells as depicted by the incorporation of [^{3}H] thymidine (Figure 1). From day 1 to 5 after stimulation, the cell proliferation rate increased by more than 20-fold. There was no significant difference in the proliferation rate between the naïve and memory CD4+ T cells. The addition of ciglitazone decreased the degree of proliferation in naïve and memory CD4+ T cells by about 10-fold. Ciglitazone significantly decreased the proliferation rate of activated naïve CD4+ T cells on days 3, 4, and 5 ($P < 0.05$) and that of activated memory CD4+ T cells on days 4 and 5 ($P < 0.05$).

3.2. Quantification of PPARγ1 and PPARγ2. Unstimulated naïve and memory CD4+ T cells expressed low constitutive levels of PPARγ1 mRNA, whereas stimulated naïve and memory CD4+ T cells expressed significantly higher levels of the receptor in both cell types (average of 7×10^4 and 1.2×10^5 mRNA transcripts/μg of total RNA, for naïve and memory CD4+ T cells; resp., $P > 0.05$; Figure 2(a)). Stimulated memory CD4+ T cells displayed higher PPARγ1 expression than naïve CD4+ T cells ($P < 0.05$). Ciglitazone treatment significantly increased the expression of PPARγ1 by about 70-fold and 160-fold in naïve and memory CD4+ T cells ($P < 0.01$), respectively. PPARγ1 expression remained significantly higher in stimulated memory compared to stimulated naïve CD4+ T cells in the presence of ciglitazone ($P < 0.01$).

Unstimulated naïve and memory CD4+ T cells expressed 10-fold lower constitutive levels of PPARγ2 mRNA compared to PPARγ1 (Figure 2(b)). Stimulated naïve and memory CD4+ T cells express very high levels of PPARγ2 mRNA in both cell types (average of 3.9×10^6 and 5.5×10^6 mRNA transcripts/μg of total RNA, in naïve and memory CD4+ T cells, resp.). PPARγ2 expression in stimulated memory CD4+ T cells expressed higher levels of the receptor compared to naïve CD4+ T cells. In contrast to PPARγ1, the addition of ciglitazone significantly decreased the expression of PPARγ2 by about 470-fold and 150-fold in naïve and memory CD4+ T cells, respectively ($P < 0.01$). However, after treatment with ciglitazone, PPARγ2 expression was significantly higher in stimulated memory compared to stimulated naïve CD4+ T cells ($P < 0.01$).

Figure 3 shows an example of a gel electrophoresis of the MPCR products of selected inflammatory cytokines in unstimulated and stimulated naïve and memory CD4+ T cells with or without ciglitazone treatment. The expression of various cytokines was compared by densitometric analyses and expressed as a ratio of GAPDH. The results were then plotted as histograms as depicted in Figure 4.

As shown in Figure 4(a), the expression levels of TGFβ gene were higher in unstimulated naïve and memory CD4+ T cells but decreased significantly in their stimulated state ($P < 0.01$). The addition of ciglitazone did not significantly

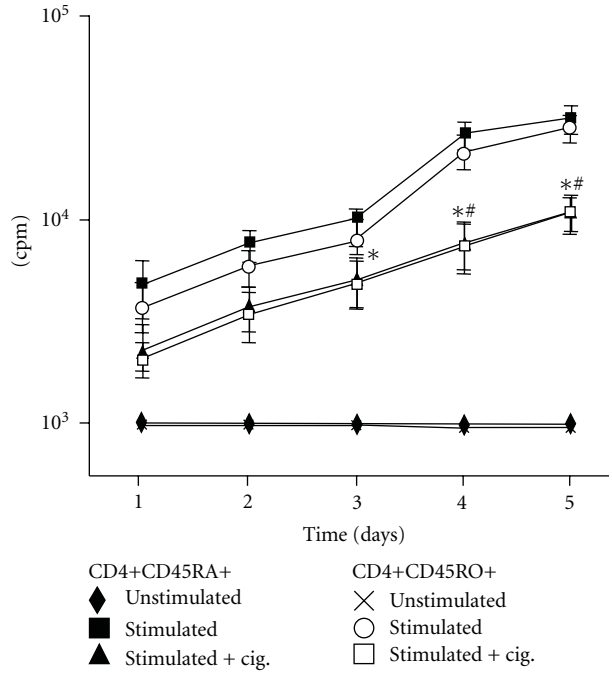

Figure 1: Proliferation assay. [^{3}H] thymidine incorporation of naïve (CD45RA+) and memory (CD45RO+) CD4+ T cells following *in vitro* stimulation with CD3/CD28 beads, in the presence or absence of ciglitazone. Data are expressed as the mean cpm of triplicate cultures ± SEM. The experiments were repeated three times. Statistical analyses were performed using the Kruskal-Wallis test.$^*P < 0.05$ (for naïve CD4+ T cells) or $^\#P < 0.05$ (for memory CD4+ T cells) of ciglitazone-treated, compared to untreated stimulated cells.

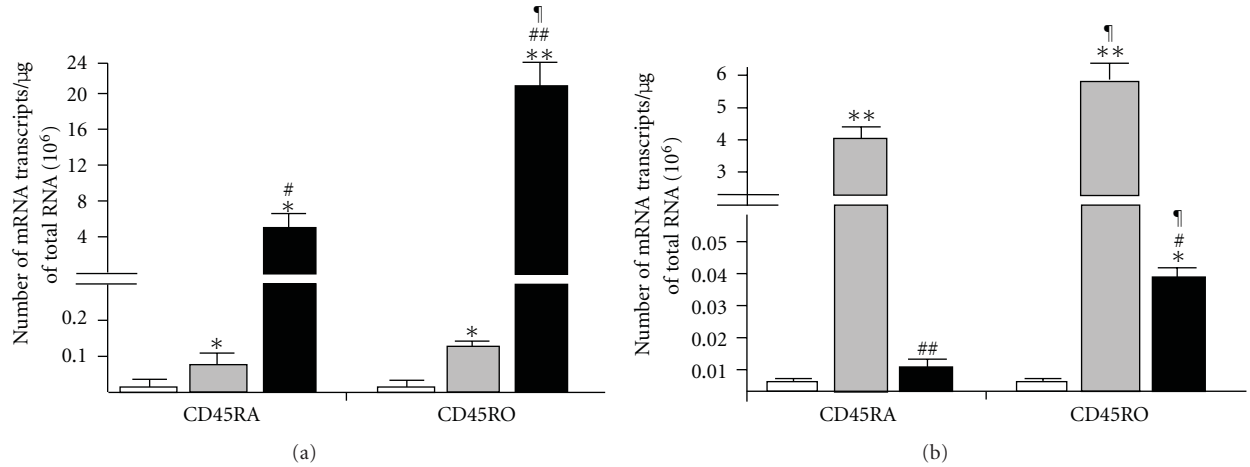

Figure 2: PPARγ1 and PPARγ2 mRNA expression. (a) PPARγ1 and (b) PPARγ2 gene expression levels in unstimulated (open bar, $n = 13$) and stimulated (grey bar, $n = 13$) naïve and memory CD4+ T cells or those treated with ciglitazone (solid bar, $n = 8$). The PPARγ1 and PPARγ2 gene expression levels were calculated as the number of mRNA transcripts per μg total RNA. The data plotted is the mean mRNA transcripts ± SEM. Statistical analyses were performed using the Mann-Whitney U test. $^*P < 0.05$; $^{**}P < 0.01$—significantly different from unstimulated cells. $^\#P < 0.05$; $^{\#\#}P < 0.01$—significantly different from CD3/CD28–stimulated cells. $^\P P < 0.05$—significantly different from correspondingly treated CD45RA+ cells.

alter the expression of TGFβ in both stimulated cells. IL-1β gene expression was also higher in unstimulated naïve and memory CD4+ T cells but decreased significantly in their stimulated state ($P < 0.01$). Ciglitazone further decreased the expression of IL-1β in stimulated naïve ($P < 0.01$) but not in stimulated memory CD4+ T cells (Figure 4(b)). IL-8 gene was expressed at low levels in unstimulated naïve and memory CD4+ T cells but significantly increased in both cell types upon activation ($P < 0.01$). IL-8 expression decreased in memory and naïve CD4+ T cells to its unstimulated states upon addition of ciglitazone ($P < 0.01$) (Figure 4(c)).

Figure 4(d) shows the *de novo* TNFα expression in stimulated naïve and memory CD4+ T cells. There was no significant difference in the expression of TNFα in both cell

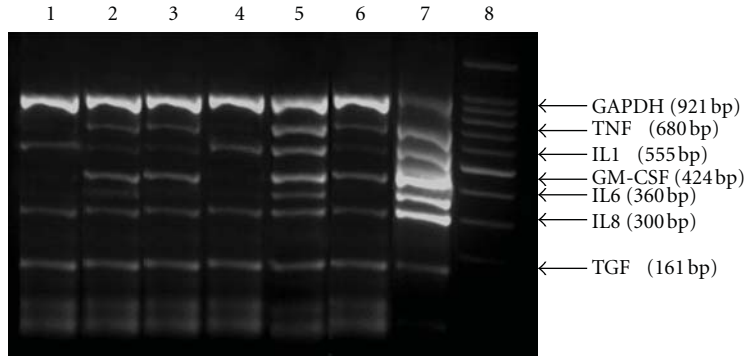

Figure 3: Example of multiplex PCR readout of inflammatory cytokine gene expression. Lane 1: unstimulated naïve CD4+ T cells. Lane 2: stimulated naïve CD4+ T cells. Lane 3: stimulated naïve CD4+ T cells + ciglitazone. Lane 4: unstimulated memory CD4+ T cells. Lane 5: stimulated memory CD4+ T cells. Lane 6: stimulated memory CD4+T cells + ciglitazone. Lane 7: positive control. Lane 8 : 100 bp marker.

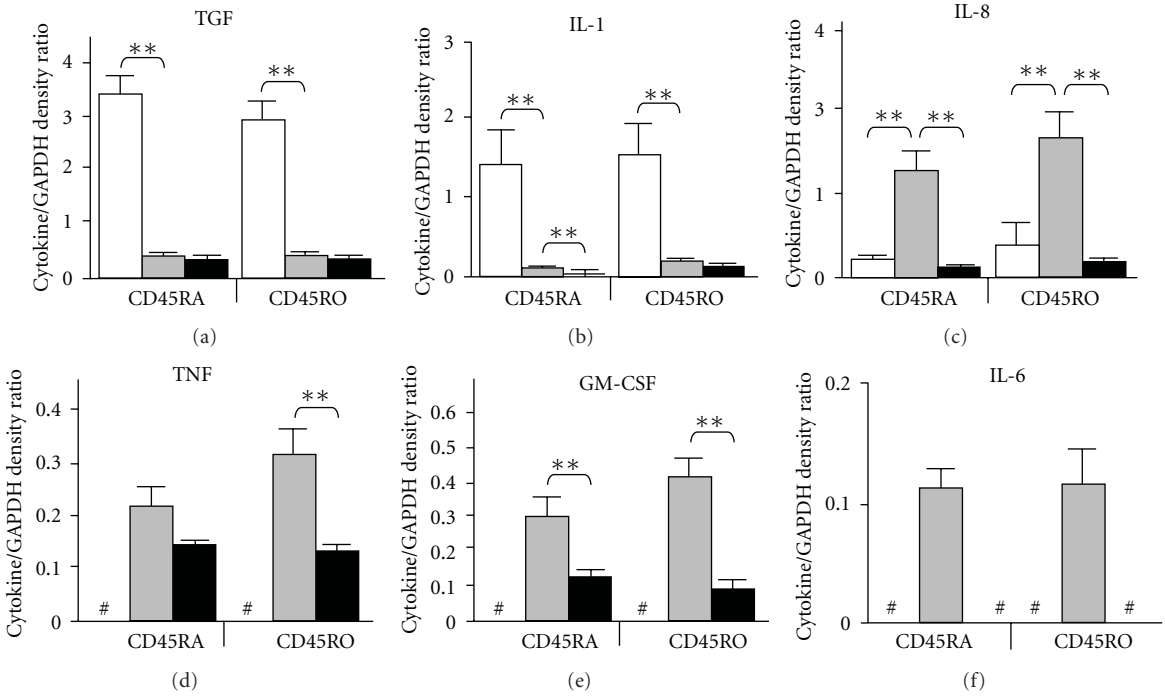

Figure 4: Inflammatory cytokine expression**.** Relative mRNA expression levels of selected cytokine genes in naïve and memory CD4+ T cells following CD3/CD28 stimulation in the presence or absence of ciglitazone ($n = 5$). Untreated cells (open bar), stimulated cells (grey bar), and ciglitazone-treated cells (solid bar) were assessed for their relative expression of (a) TGFβ, (b) IL-1β, (c) IL-8, (d) TNFα, (e) GM-CSF, and (f) IL-6, as a ratio of GAPDH. Statistical analyses were performed using the Mann-Whitney U test. $^{*}P < 0.05$, $^{**}P < 0.01$. $^{\#}$Significance levels cannot be analyzed because the gene expression was not detectable.

types after activation. Ciglitazone significantly decreased the expression of TNFα in stimulated memory ($P < 0.01$) but not in naïve CD4+ T cells. GM-CSF was also expressed in stimulated naïve and memory CD4+ T cells but not in their unstimulated state. There was no significant difference in the expression of GM-CSF in both cell types after activation. GM-CSF expression was significantly reduced in stimulated naïve and memory CD4+ T cells in the presence of ciglitazone ($P < 0.01$; Figure 4(e)). Figure 4(e) shows that only stimulated naïve and memory CD4+ T cells expressed IL-6. The addition of ciglitazone completely abolished the expression of IL-6 in both stimulated cells. The results clearly show *de novo* expression of TNF-α, GM-CSF, and IL-6 upon activation of naïve and memory CD4+ T cells.

4. Discussion

It is now established that PPARγ is involved in the regulation of T-cell function, as well as macrophage and dendritic cell activities [18–20]. In view of the fact that human naïve and memory CD4+ T cells differ in the requirements for activation and magnitude of their cellular responses [21]

and autoreactivity [3, 4], we investigated the effect of the PPARγ agonist, ciglitazone, on the mRNA expression of PPARγ1 and PPARγ2 and on a number of inflammatory cytokines produced by these cells. No previous studies on the expression of PPARγ1 and PPARγ2 in human naïve and memory CD4+ T cells have been reported.

Consistent with previous reports [11, 13, 16, 20], ciglitazone treatment resulted in a tenfold reduction in the proliferative response of both CD3/CD28-stimulated naïve and memory CD4+ T-cell subsets. Inhibition of proliferation in activated naïve T cells by PPARγ agonists, such as ciglitazone, has been previously attributed to apoptosis [16], although whether this occurs via a PPARγ-dependent or independent pathway remains to be elucidated.

Using RT-PCR, PPARγ1 and PPARγ2 were found to be highly expressed in both naïve and memory CD4+ T cells upon activation through the TCR and costimulatory CD28 pathway. Consistent with previous findings [21], only low expression levels of both transcripts in unstimulated CD4+ T cells were recorded. Interestingly, previous studies reported that PPARγ is constitutively expressed in human peripheral blood mononuclear cells [6, 22]. However, this may be due to its expression by other cell subsets in the mononuclear cell population such as monocytes [18], B cells [23], and NK cells [24].

It is interesting to note the low level expression of PPARγ1 and PPARγ2 in resting human naïve and memory CD4+ T cells. This may suggest that their roles are primarily in the regulation of responding T cells. It is also noteworthy that higher levels of both transcripts are found in activated memory CD4+ T cells as opposed to their low level expression in activated naïve T cells, suggesting that regulation of memory CD4+ T cells may require higher-level expression of PPARγ compared to naïve CD4+ cells.

Treatment with ciglitazone enhanced the expression of PPARγ1 but greatly diminished that of PPARγ2 in both the naïve and memory CD4+ T cells. Previous studies have reported that PPARγ agonists such as troglitazone [12] and pioglitazone [22] attenuated the expression of the receptor. Here, we report that ciglitazone enhances the expression of PPARγ1 but greatly diminishes the expression of PPARγ2 in both naïve and memory CD4+ T cells. This apparent discrepancy can be attributed to the fact that the above studies did not distinguish between the two PPARγ isoforms. PPARγ1 can be regarded as a "subset" of PPARγ2 which contains additional 28 amino acids at its N-terminus. Thus, measuring PPARγ expression without distinguishing the two isoforms may not provide an accurate reflection of the receptor's role in immune regulation. The lack of specific antibodies against PPARγ1 has however impeded our attempt to differentiate the protein expression of these receptors in the current study. The decrease in PPARγ2 expression cannot be attributed to cell death via apoptosis [16] since the expression of PPARγ1 was enhanced and that the cell recovery after 5 days was above 90% (results not shown).

The different roles played by the two PPARγ isoforms in CD4+ T-cell regulation can be inferred from their expression levels displayed at pre- and posttreatment with ciglitazone. Thus, although the fold increase in PPARγ2 expression was higher than that observed for PPARγ1, it was almost completely abrogated upon addition of ciglitazone. A previous report [12] showed that troglitazone and 15d-PGJ2 inhibited IL-2 production in the PPARγ2-expressing but not in PPARγ2-nonexpressing transfected Jurkat T cells, suggesting that PPARγ2 is involved in regulating T cell function. The almost complete abrogation of PPARγ2 expression following treatment with ciglitazone is interesting and requires further investigations, such as inhibition studies. The present lack of specific chemical inhibitors for PPARγ2, however, would complicate such studies for the time being.

As mentioned above, activation of PPARγ by its ligands has been shown to induce apoptosis in T cells [16, 25]. Hence the question arises whether cells that express higher levels of PPARγ2 are more prone to apoptosis, resulting in the preferential "elimination" of PPARγ2-expressing cells. Single-cell analyses, including the measurement of PPARγ1 and PPARγ2 protein levels, should be carried out to address these questions. However, as anti-PPARγ1 antibodies are not available, such experiments may prove currently challenging. It will also be important to investigate the molecular regulation of PPARγ1 and PPARγ2 promoters in order to understand the possible differential control of their expression.

Since differential expression of PPAR has been shown to correlate with selected cytokine production [26, 27] and that naïve and memory CD4+ T cells may play a differential role in autoimmunity [3, 4], the level of various proinflammatory cytokines that were expressed in the resting and activated naïve and memory CD4+ T cells with or without treatment with ciglitazone was subsequently determined. While TGFβ, IL-8, and IL-1β expression in resting naïve and memory CD4+ T cells has previously been reported [28], their expression in activated naïve and memory CD4+ T cells has not been previously studied.

Activated naïve and memory CD4+ T cells displayed low expression levels of both TGF-β and IL-1β, further reduced upon stimulation with ciglitazone (in the case of IL-1β, further reduction was only observed in activated naïve CD4+ T cells). These findings are in agreement with those previously reported [13, 19]. Unstimulated naïve and memory CD4+ T cells displayed low levels of IL-8 which significantly increased upon activation. However, the addition of ciglitazone dramatically reduced IL-8 expression. This observation is in contrast to a previous finding that 15d-PGJ2, another PPARγ ligand, induced the expression of IL-8 in human T cells via a PPARγ-independent manner [29]. Thus there may be distinct response against different ligands with regard to the function of these receptors. Future studies will, therefore, need to include the use of several PPARγ ligands to determine the detailed mechanistic roles of the receptors in immune response.

Activation of both naïve and memory CD4+ T cells induced *de novo* expression of TNFα, GM-CSF, and IL-6, whereas treatment of these activated cells with ciglitazone diminished TNFα and GM-CSF expression, and totally abrogated IL-6 expression. Previous studies showed significant reduction in the release of LPS-stimulated TNFα upon activation of placental, amnion, and choriodecidual, tissues with

both 15d-PGJ2 and troglitazone [30]. Ciglitazone, troglitazone, and 15d-PGJ2 also inhibited RSV-induced release of TNFα in A549 epithelial cells [31]. As previously reported [19], the expression of GM-CSF in activated naïve and memory CD4+ T cells may play a role in inducing the expression of PPARγ1 and PPARγ2 in both activated cells. Reduction of GM-CSF expression after ciglitazone treatment has also been reported in mast cells where a PPARγ agonist decreased the antigen-induced GM-CSF production [32].

The present observation that IL-6 is produced in similar levels by both naïve and memory CD4+ T cells has previously been reported [33]. IL-6 plays an essential role in activating naïve and memory CD4+ T cells through the CD2 molecule [34]. Unlike naïve T cells, CD4 memory T cells can undergo proliferation when stimulated with anti-CD2 in the absence of APCs since they are able to use self-produced IL-6 [35]. However, the current study shows that activation of naïve CD4+ T cells via the CD3 and CD28 pathways also induced the production of IL-6. This may have occurred through the engagement of the CD28 molecule which may act by amplifying the activation signals in an autocrine fashion.

A previous report [36] supports our observation that ciglitazone completely abolished the expression of IL-6 in activated naïve and memory CD4+ T cells. There is also evidence that chronic IL-6 treatment suppressed the expression of PPARγ [26], and the suppression of PPARγ functions resulted in excessive production of the cytokine [37]. The mechanism through which ciglitazone affects cytokine production remains to be elucidated. There is evidence [11, 12, 19] to suggest that this may occur through activation of transcription factors such as AP-1, STAT-1, and NF-κB. Since there are no reports to suggest that the *cis*-element of inflammatory cytokine genes contains PPARγ binding site, inhibition may occur indirectly via transrepression as described above [13]. It was also reported that 15d-PGJ2 treatment rendered IκB resistant to degradation upon cellular activation [38], hence, preventing NF-κB activation. However, since ciglitazone is structurally different from 15d-PGJ2, the mechanism of inhibition of NF-κB and AP-1 activity by ciglitazone may differ from its inhibition by 15d-PGJ2.

5. Conclusions

PPARγ1 and PPARγ2 have differential regulatory roles in responding naïve and memory CD4+ T cells. Overall, naïve CD4+ T cells seem to be more sensitive to PPARγ activation, although further studies need to be carried out to confirm this observation. The availability of specific antibodies and specific antagonists against these two isoforms is needed to enable a more precise elucidation of their purported differential functions in T-cell regulation. In addition, the precise mechanism of how PPARγ1 and PPARγ2 regulate the response of naïve and memory cells or the immune response in general will require further investigations utilizing single-cell analytical tools.

Acknowledgments

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Review Article

Myeloid-Derived Suppressor Cells Participate in Preventing Graft Rejection

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Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of cells and have a tremendous potential to suppress immune responses. MDSCs accumulate during tumor progression, autoimmunity, chronic infection, transplantation, and other pathological conditions and can potently suppress T-cell function. Here, we discuss recent findings that describe the molecular mechanisms of MDSCs suppressing T-cell immune responses as well as recent observations that MDSCs may have roles in transplant tolerance.

1. Introduction

Immature myeloid cells (IMCs) are part of the normal process of myelopoiesis, which takes place in the bone marrow and is controlled by a complex network of soluble factors. Haematopoietic stem cells differentiate into common myeloid progenitor cells and then into IMCs [1]. In normal individuals, IMCs migrate into different peripheral organs, where they quickly differentiate into macrophages, dendritic cells, or granulocytes. However, factors that are produced during acute or chronic infections, trauma, or sepsis and in the tumor microenvironment promote the accumulation of IMCs at these sites, prevent their differentiation, and induce their activation. These cells exhibit immunosuppressive functions and are therefore known as myeloid-derived suppressor cells (MDSCs) [2]. MDSCs are not a defined subset of myeloid cells but rather a heterogeneous population of activated IMCs that have been prevented from fully differentiating into mature cells. MDSCs lack the expression of cell-surface markers that are specifically expressed by monocytes, macrophages, or dendritic cells and comprise a mixture of myeloid cells that have the morphology of granulocytes or monocytes. Early studies showed that 1–5% of MDSCs can form myeloid cell colonies and that about one-third of this population can differentiate into mature macrophages and dendritic cells in the presence of the appropriate cytokines in vitro and in vivo [3].

MDSCs are a heterogeneous population of cells that consist of myeloid progenitors and immature macrophages, immature granulocytes, and immature dendritic cells [4]. MDSCs were first characterized more than 20 years ago in tumor-bearing mice and in patients with cancer [5]. There are many tumor-derived factors that can promote the expansion of MDSCs through the stimulation of myelopoiesis and inhibit the differentiation of mature myeloid cells, such as vascular endothelial growth factor (VEGF), prostaglandin E2 (PGE2), granulocyte-macrophage colony-stimulating factor (GM-CSF), transforming growth factor-β (TGF-β), interleukin- (IL-) 1β, IL-10, IL-6, and macrophage colony-stimulating factor (M-CSF) [6]. Glucocorticoids are also believed to have inhibitory effects on the maturation of IMCs. Most tumor-derived factors exert the inhibiting effects on differentiation and maturation of myeloid cells through signal transducer and activator of transcription 3 (STAT3) signaling pathway [7]. In animal tumor models and cancer patients, MDSCs, induced by tumor-derived factors, accumulate in large numbers in the blood, bone marrow, spleen, and tumor masses, mediating the downregulation of T-cell immunity, thus leading to tumor escape, progression, and metastasis [8].

Although initial observations and most of the current information on the role of MDSCs in immune responses have come from studies in the field of cancer research, accumulating evidence has shown that MDSCs also regulate

Table 1: Summary of phenotype of MDSCs in tumors and transplantation models.

Tumour type	Reported phenotype	References
Renal cell carcinoma	$CD11b^+/CD14^-/CD33^+/CD15^+$	
	$CD66b^+/VEGF1^+$	Rodriguez et al. [16]
Non small cell lung cancer	$CD11b^+/CD14^-/CD15^+/CD33^+$	Liu et al. [17]
Colon carcinoma	$Lin^-/HLA\text{-}DR^-/CD33^+/CD11b^+$	Diaz-Montero et al. [18]
Breast carcinoma	$Lin^-/HLA\text{-}DR^-/CD33^+/CD11b^+$	Diaz-Montero et al. [18]
Prostate cancer	$CD14^+/HLA\text{-}DR^{low/neg}$	Vuk-Pavlović et al. [19]
Malignant melanoma	$CD14^+/CD11b^+/HLA\text{-}DR^{low/neg}$	Filipazzi et al. [20]
	$CD80^+/CD86^+$	Poschke et al. [21]
Hepato-cellular carcinoma	$CD14^+/HLA\text{-}DR^{low/neg}$	Hoechst et al. [22, 23]
Hodgkin lymphoma (HL)	$CD11b^+$, $CD13^+$, $CD34^+$, CD14-, CD45+	Parrinello et al. [24]
Non hodgkin lymphoma	$CD14^+/HLA\ DR^{low/neg}/CD120^{low}$	Lin et al. [25]
Myelodysplastic syndrome (MDS)	$LIN^-/HLA\text{-}DR^-/CD33^+$	Wei et al. [26]
Transplantation model	Reported phenotype	References
Rat kidney allograft	$CD11b^+CD6^-CD80/86^+NKRP\text{-}1^+$	Dugast et al. [15]
Mouse skin allograft	$CD40^+/CD80^+/F480^+/IL\text{-}4R\alpha^+$	Adeegbe et al. [27]
	$CD11b^+/Gr1^+$	Zhang et al. [28], De Wilde et al. [29]
Mouse hepatic islet allograft	$CD11b^+/CD45^+$	Chou et al. [30]
Mouse cardiac allograft	$CD11b^+/Gr\text{-}1^{int}/F4/80^+$	Turnquist et al. [31]

immune responses during infectious diseases, autoimmune disorders, and transplantation [9–14]. Evidence for a role of MDSCs in transplantation is emerging from various animal models. An expansion of MDSCs was first described in a rat model of kidney allograft tolerance induced by anti-CD28 antibodies [15]. Recently, MDSCs have been considered a key role in several transplantation models, and study on the mechanism of MDSC-induced immune suppression may generate new insights into our understanding of allograft tolerance and improve therapeutic efficiency in transplantation. In this paper, we discuss the phenotype and subsets, the mechanisms of suppressive function of MDSCs, and the possible role of these cells in organ transplantation.

2. Phenotype and Subsets of MDSCs

MDSCs represent a heterogeneous population of myeloid cells at different stages of differentiation that comprises myeloid progenitor cells and immature myeloid cells (macrophages, granulocytes, and dendritic cells). There is no strict cell-surface-marker-guided classification of MDSC available at present (Table 1).

In mice, MDSCs are commonly identified as the cell membrane that simultaneously expresses two markers: one is CD11b, an adhesion molecule also known as Mac-1, the other is Gr1 antigen, a 21–25 kDa glycosylphosphatidylinositol- (GPI-) anchored protein. Normal mouse bone marrow contains 20–30% of cells with this phenotype, but these cells make up only a small proportion (2–4%) of spleen cells and are absent from the lymph nodes [32]. More recently, according to MDSCs morphological and functional features, as well as their expression of the two molecules lymphocyte antigen 6 complex, locus C(Ly6C) and lymphocyte antigen 6 complex, locus G(Ly6G), MDSCs were subdivided into two different subsets of granulocytic MDSCs (CD11b+ Ly6G+ Ly6Clow) and monocytic MDSCs (CD11b+ Ly6G− Ly6Chigh) [33]. In addition to CD11b and Gr1, MDSCs express additional markers of early myeloid differentiation, such as CD31, ER-MP54, and ER-MP58, and low levels of costimulatory molecules [34]. Some researchers also identified a more specific population of MDSCs that express Gr1 and CD115, which has much stronger suppressive activity compared with the classic Gr1+ CD11b+ MDSCs [35].

In humans, MDSCs are even less well defined owing to the lack of specific markers. Human cells do not express a marker homologous to mouse Gr1. MDSCs are most commonly defined as CD14-CD11b+ cells or, more narrowly, as cells that express the common myeloid marker CD33 but lack expression of markers of mature myeloid and lymphoid cells and of the MHC class II molecule HLA-DR [36]. MDSCs have also been identified within a CD15+ population in human peripheral blood. In healthy individuals, IMCs constitute ~0.5% of peripheral blood mononuclear cells. MDSCs in human were also subdivided into two subsets: granulocytic MDSCs express CD15+ CD33+ CD11b+ with minimal or no HLA-DR expression, while monocytic MDSCs express CD14 with minimal or no HLA-DR expression, CD49d (also known as integrin α4) and low levels of CD15 [37].

The terminally differentiated granulocytic MDSCs represent 70–80% of MDSCs. Monocytic MDSCs, accounting for 20–30% of MDSCs, retain the ability to differentiate into mature dendritic cells and macrophages (Table 2). Although these subsets can have various functions and distributions depending on their environment, their capacity to induce T-cell hyporesponsiveness is generally considered equal [38].

Table 2: Phenotype of monocytic and granulocytic MDSCs subsets in murine and human.

	Granulocytic MDSCs	Monocytic MDSCs
Murine	$CD11b^+$ $Ly6G^+$ $Ly6C^{low}Gr\text{-}1^{high}CD49d^-$	$CD11b^+$ $Ly6G^-Ly6C^{high}Gr\text{-}1^{int}CD49d^+$
Human	MHC class $II^{low}CD33^+CD11b^+$ $CD14^-CD15^+$	MHC class $II^{low}CD33^+CD11b^+$ $CD14^+CD66b^+$

3. Suppressive Function of MDSCs

A growing body of evidence suggests that MDSCs have a remarkable suppressive effect on T-cell proliferation. Most studies have shown that the immunosuppressive functions of MDSCs require direct cell-cell contact, which suggests that they act either through cell-surface receptors or through the release of short-lived soluble mediators [4]. Here, we will elaborate the mechanismsby which MDSCs suppress T-cell responses and the effects of MDSCs in organ transplantation.

3.1. Arginase-1 (Arg-1) and Inducible Nitric Oxide Synthase (iNOS). Historically, the suppressive activity of MDSCs has been associated with the metabolism of L-arginine. L-arginine serves as a substrate for two enzymes, iNOS (which generates NO) and Arg-1 (which converts L-arginine to urea and L-ornithine). MDSCs express high levels of both Arg-1 and iNOS, and a direct role for both of these enzymes in the inhibition of T-cell function is well established [39].

Although the first experiments underlying the importance of L-arginine metabolism in cancer were performed more than 50 years ago, only recently has the role of Arg-1 in tumor growth and escape from the immune surveillance been clarified [40]. Arg-1 can be released or expressed by either cancer cells or tumor-associated myeloid cells, including putative MDSCs. Recent data suggest that there is a close correlation between the availability of L-arginine and the regulation of T-cell proliferation. The increased activity of Arg-1 in MDSCs leads to enhanced L-arginine catabolism, which depletes this nonessential amino acid from the microenvironment. The shortage of L-arginine inhibits T-cell proliferation through several different mechanisms, including decreasing their expression of CD3 ζ-chain and preventing their upregulation of the expression of the cell cycle regulators cyclin D3 and cyclin-dependent kinase 4 [41]. An expansion of MDSCs was detected in immunoglobulin-like transcript 2 (ILT2) transgenic mice [28]. In this model, adoptive transfer of MDSCs from ILT2 mice significantly delayed the rejection of major MHC-II-mismatched skin allografts. This effect was associated with a unique MDSCs transcriptional profile including upregulation of Arg-1, but not iNOS. Highfill et al. [42] found that exogenous IL-13 produced an MDSCs subset that was more potently suppressive and resulted in Arg-1 upregulation. These MDSCs were more effective to inhibit graft-versus-host disease (GVHD). GVHD inhibition was reduced when Arg-1 deficient MDSCs were used.

iNOS can be induced in myeloid cells by different tumor-secreted factors such as VEGF, GM-CSF, and IL-6. MDSCs expressing iNOS can inhibit mitogenic and peptide-specific responses through NO production. MDSC-mediated T-cell inhibition is associated with the impairment of the main signaling pathways coupled to the IL-2 receptor as demonstrated by the lack of JAK3, STAT5, extracellular signal-regulated kinase, and Akt phosphorylation in response to IL-2 [43]. NO is able to induce a reversible type of T-cell anergy by reducing phosphorylation of tyrosine residues on JAK3 and STAT5. NO also can reduce MHC-II expression, either by downregulating IFN-γ-induced expression of class II transactivator or by inhibiting DNA binding of transcription factor NF-Y at the class II promoter Y box [44]. MHC-II expression is critical for antigen-specific immunity. In a model of MHC-mismatched rat kidney allograft, treatment with anti-CD28 antibodies induced long-term survival and was associated with the presence, in tolerated allografts, of MDSCs that operated through iNOS activity [15]. The action of NO production was critical to the immunosuppression mediated by MDSCs and in maintaining the tolerant state in vivo. In this kidney transplantation model, the injection in tolerant animals of amino guanidine, which inhibits iNOS, broke the established tolerance and led to graft rejection. These results suggest that MDSCs, accumulated in the blood of tolerant kidney recipients, release high levels of NO after contact with activated effector T cells and specifically control their proliferative response.

3.2. Heme Oxygenase-1 (HO-1). HO-1 catabolizes pro-oxidant heme groups into carbon monoxide, biliverdin and ferritin, three metabolites involved in immunoregulatory processes [45, 46]. Recently, De Wilde et al. [29] reported the observation of HO-1-dependent MDSCs-mediated alloreactive T-cell suppression, which was cell-to-cell contact dependent and requires IL-10 activity. They found that transfer of MDSCs from LPS-treated mice in untreated recipients significantly prolonged skin allograft survival. To specifically address the role of HO-1 in this MDSCs-mediated delay of allograft rejection was tested by incubating purified MDSCs with the HO-1-specific inhibitor SnPP pretreatment before an adoptive transfer in female mice. SnPP treatment of MDSCs abrogated the inhibition of allograft rejection. This demonstrates that HO-1 activity is a dominant effector of in vivo immune suppression mediated by MDSCs.

3.3. Radical Oxygen Species (ROS). The production of ROS also contributes to the suppressive activity of MDSCs, as increased ROS levels in MDSCs induce the upregulation of several subunits of the NADPH oxidase [47]. ROS can induce DNA damage in immune cells resident in the tumor microenvironment, inhibit the differentiation of MDSCs into functional dendritic cells, and recruit MDSCs to the tumor site. Moreover, extracellular ROS catalyzes the nitration of the TCR, which consequently inhibits the T-cell-peptide-MHC interaction resulting in T-cell suppression [48]. The

involvement of ROS in the suppressive activity of MDSCs is not restricted to neoplastic conditions. Indeed, inflammation and microbial products are also known to induce the development of an MDSC population that produces ROS following its interaction with activated T cells.

3.4. Regulatory T Cells (Treg). Recently, MDSCs have been shown to enhance the development of Treg, possibly through interactions between CD80 expressed by MDSCs and CTLA-4 expressed by Treg, production of IL-10, and/or preferential inhibition of activated T cells through NO [35, 49]. In a mouse model of lymphoma, MDSCs were shown to induce Treg expansion through a mechanism that involved Arg-1 and the capture, processing, and presentation of tumor-associated antigens by MDSCs but was independent of TGF-β [50]. In a mice model of skin transplantation, recipents were injected with recombinant G-CSF, or IL-2 complex(IL-2C), Gr1+ CD11b+ MDSC or CD4+ Foxp3+ Treg were induced in circulation of recipients [27]. They found that although treatment with either IL-2C or G-CSF led to a significant delay of MHC-II disparate allogeneic donor skin rejection, the combinatorial treatment was superior to either alone, confirming that MDSCs and Treg prolonged skin allograft survival in mice. Karp and Mannon [51] summarized identified an HLA-Dqα-class-II-derived peptide that was a potent inducer of CD11b+CD115+Gr1+ MDSCs. Moreover, this peptide prolonged the survival of fully mismatched mouse cardiac allografts associated with the induction of Foxp3+ Treg. Depletion or inhibition of function of MDSCs reversed the prolonged survival and decreased Treg in the recipient.

3.5. CD8+ T Cells. MDSCs can take up soluble antigens, including tumor-associated antigens, and process and present them to T cells. Blocked MDSCs-T cells interactions with a MHC-I specific antibody abrogate MDSC-mediated inhibition of T-cell responses in vitro. The MHC-I restricted nature of MDSC-mediated CD8+ T cell suppression has also been demonstrated in vivo in tumor models [52, 53]. MDSCs can abrogate the expression of L-selectin on CD8+ T-cell, suppressing the homing of these cells to the tumor site, where they would be activated. MDSCs cleave L-selectin from T cells because they constitutively express ADAM17 at their cell surface and, as a result, T cells cannot traffic to tumor draining lymph nodes, where they normally would have access to tumor antigens and consequently can not be activated [54]. One study showed a potential mechanism for IL-10- and IFN-γ-dependent MDSCs regulation of CD8+ T cell function mediated through programmed cell death-1 (PD-1) and PD-1 ligand interaction [55]. They testified the PD-1 signaling pathway inducing the apoptosis of CD8+ T cells and phagocytosed CD8+ T cells, contributing to CD8+ T cell exhaustion. However, whether PD-1 signaling pathway plays a role in MDSCs-mediated T cell suppression remains controversial, and further investigations are needed.

3.6. T-Helper 2 (Th2) Cells. A recent study found that MDSCs can impair tumor immunity not only by suppressing T-cell activation but also by interacting with macrophages to increase IL-10 and decrease IL-12 production, thereby promoting a tumor-promoting type 2 response [56]. Many literatures have reported that MDSCs inhibit antigen-specific and nonspecific T-cell functions via several different mechanisms, including Arg-1, NO, ROS, IL-10, and TGF-β [35]. Furthermore, using a depleting antibody, Delano et al. [57] demonstrated that expansion of MDSCs in vivo contributed to the induced Th2 polarization of antibody responses after sepsis. Challenging mice with T-cell-dependent antigens, such as NP-KLH, offers the opportunity to explore in vivo the shift in antibody class switching to IgG_{2a} or IgG_1 production, which is dependent on cytokines, including IFN-γ and IL-4, and reflects this predilection toward a Th2 versus a Th1 CD4+ T-cell response. Turnquist et al. [31] found that IL-33 administration greatly increased splenic MDSCs in normal and transplanted mice. It has been suggested that IL-33 prolongs cardiac allograft survival by promoting Th2 responses. Administration of IL-33 concurrent with cardiac allotransplantation increased systemic levels of IL-5 and IL-13, increased IL-5+CD4+ cells, and decreased CD8+INF-γ+ T cells. Notably, IL-13 is implicated in tolerance, particularly by targeting myeloid cells and activating the suppressive function of MDSCs.

4. Summary

MDSCs aid tumor development by exerting a profound inhibitory activity on T cells. The mechanism of MDSCs possessing a direct role in the inhibition of T-cell function is well established in tumors. Their potential role in organ transplantation requires far more investigation. Recently, Qian's group have found that cotransplantation with in vitro generated MDSCs can effectively protect islet allografts from host immune attack [30]. Our study also demonstrated that MDSCs can be propagated in vitro from bone-marrow-derived myeloid precursor cells under the influence of hepatic stellate cells. Adoptive transfer of these in vitro generated cells can prolong cardiac allograft survival. However, the mechanism of MDSCs causing immunosuppression in this model has not yet been explored. A detailed understanding of MDSCs regulation of T-cell immune function in transplantation will undoubtedly lead to the design of more effective strategies to achieve transplant tolerance in the clinic.

Authors' Contribution

X. Gu and Y. Wang have equally contributed to this work. They are joint first authors.

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Review Article

CD4$^+$T Cells: Differentiation and Functions

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CD4$^+$T cells are crucial in achieving a regulated effective immune response to pathogens. Naive CD4$^+$T cells are activated after interaction with antigen-MHC complex and differentiate into specific subtypes depending mainly on the cytokine milieu of the microenvironment. Besides the classical T-helper 1 and T-helper 2, other subsets have been identified, including T-helper 17, regulatory T cell, follicular helper T cell, and T-helper 9, each with a characteristic cytokine profile. For a particular phenotype to be differentiated, a set of cytokine signaling pathways coupled with activation of lineage-specific transcription factors and epigenetic modifications at appropriate genes are required. The effector functions of these cells are mediated by the cytokines secreted by the differentiated cells. This paper will focus on the cytokine-signaling and the network of transcription factors responsible for the differentiation of naive CD4$^+$T cells.

1. Introduction

The human immune system consists of the ancient innate immune system passed on along the evolution from invertebrates and the recently acquired adaptive immune system uniquely present in vertebrates. The principal functions of the immune system are the recognition with subsequent elimination of foreign antigens, formation of immunologic memory, and development of tolerance to self-antigens. The lymphocyte population is mainly made up of the thymus-derived lymphocytes (T-lymphocytes), bone-marrow-derived (B-lymphocytes), and the natural-killer cells (NK cells). T-lymphocytes mediating the cellular immunity, along with B lymphocytes mediating humoral immunity, provide adaptive immunity, which work in close collaboration with the innate immune system. B-lymphocytes mature in the bone marrow itself, while the T-lymphocytes require the thymus to mature, before being deployed to the peripheral lymphoid organs for further antigen-mediated differentiation. A small subset of the CD4$^+$cells, including natural regulatory cells and natural killer T cells (NKT cells), are already distinct differentiated cells on release from the thymus.

CD4$^+$T cells along with CD8$^+$T cells make up the majority of T-lymphocytes. CD4$^+$T cells after being activated and differentiated into distinct effector subtypes play a major role in mediating immune response through the secretion of specific cytokines. The CD4$^+$T cells carry out multiple functions, ranging from activation of the cells of the innate immune system, B-lymphocytes, cytotoxic T cells, as well as nonimmune cells, and also play critical role in the suppression of immune reaction. Continuing studies identified new subsets of CD4$^+$ cells besides the classical T-helper 1 (Th1) and T-helper 2 (Th2) cells. These include T-helper 17 (Th17), follicular helper T cell (Tfh), induced T-regulatory cells (iTreg), and the regulatory type 1 cells (Tr1) as well as the potentially distinct T-helper 9 (Th9). The differentiation of the different lineages depends on the complex network of specific cytokine signaling and transcription factors followed by epigenetic modifications. This paper will be focusing on the cytokine milieu and lineage specific transcription factors

required for the differential development of the antigen-activated $CD4^+$T cells, and also will cover a brief overview of the development pathway of mature naïve $CD4^+$T cells, and finally the effector functions of each subtype will be summarized.

2. Lymphopoiesis

T cells precursors originating from a common lymphoid hematopoietic stem cell leave the bone marrow to reach the thymus for maturation. Initially thought to be an evolutionary remnant with negligible function, the thymus is in fact a primary lymphoid organ indispensable for T-lymphocyte development. The thymus provides a suitable microenvironment with specific combination of stromal cells, cytokines and chemokines to generate functional T cells from T-cell precursors (thymocytes). T-cell receptor (TCR) gene rearrangement and thymocyte selection are the critical steps in the development of mature T-lymphocytes capable of recognizing an infinite range of antigens. During the differentiation process, the migration of thymocytes through discrete thymic microenvironments and contact with peptide-MHC complex (pMHC) on distinct thymic antigen-presenting cells (APCs), including the cortical thymic epithelial cells (cTECs), medullary thymic epithelial cells (mTECs), and dendritic cells (DCs), play a pivotal role in the shaping of the T cell repertoire for antigen recognition, the selection process, and the expression of surface molecules such as CD4 and CD8 [1–3]. The selection process can be depicted by the affinity model, whereby the thymocytes expressing TCR with negligible affinity to pMHC die and those with very high affinity are destroyed (negative selection). Only thymocytes with TCR of intermediate affinity to pMHC undergo positive selection and further differentiation into mainly $CD4^+$ and $CD8^+$ mature T-lymphocytes [1, 4]. TCR consists of $\alpha\beta$ or $\gamma\delta$ chains bonded with five CD3 subunits (γ, δ, μ, π, and Σ). TCR interacts with antigen-MHC complex, while CD3 mediates T-cell activation signals [5]. TCR α chain is encoded on chromosome 14 and consists of V (variable) and J (joining) genes. The β chain genes are located on the 7 chromosome with V, J, and D (diversity) gene segments. The γ chain is on chromosome 7, and the δ chain on chromosome 14. A vast repertoire of TCR $\alpha\beta$ is generated by gene rearrangement between exons of the variable domains of the V-J segments of α chain and V-D-J segments of β chain [6]. Moreover junctional diversity V-N-J, V-N-D, and D-N-J are produced by random insertions/deletions at these regions [7, 8]. The diversity is expressed in the complementary determining regions (CDRs), that make up the antigen-recognition site of the TCR. The T-cell precursor, that is the double positive $CD4^+CD8^+$ thymocyte, differentiates into several mature T cell lineage. Based on the interaction of $CD4^+CD8^+$ cell TCR with pMHC I or II, some nonconventional lineages are also produced along with the classical naïve $CD4^+$CD8-T cells and $CD4^-CD8^+$ T cells. The $CD4^+$ expressing non-conventional T cells include the $FOXP3^+CD4^+CD25^+$ natural T-regulatory cells (nTreg cells), and the CD1d-reactive natural killer T (NKT) cells, whereas the $CD8^+$ ones are the MHC1b $CD8^+$T cells, and the major histocompatibility molecule-related 1(MR1)-restricted mucosa-associated invariant T cells [9]. The NKT cells can be $CD4^+$ or $CD4^-CD8^-$. Mature naïve $CD4^+$ T cells are then deployed to secondary lymphoid organs, including the spleen, lymph nodes, and the mucosa-associated lymphoid tissue, where they constantly survey for pMHC II molecules, for antigen recognition [10].

3. $CD4^+$T Cells Activation and Differentiation

The initial step of differentiation of the naïve cells is the antigenic stimulation as a result of interaction of TCR and CD4 as co-receptor with antigen-MHC II complex, presented by professional antigen presenting cells (APCs). TCR coupled with CD3 activation consequently induces a network of downstream signaling pathways, that eventually lead to naïve cell proliferation and differentiation into specific effector cells. Lineage-specific differentiation depends on the cytokine milieu of the microenvironment, as well as on the concentration of antigens, type of APCs, and costimulatory molecules [11, 12]. Among the APCs, the dendritic cells (DCs) are considered to be most important due to their enhanced ability to stimulate naïve T cells [13]. Dendritic cells are activated through the recognition of pathogenic antigens by cell surface pattern recognition receptors, such as toll-like receptor and intracellular pathogen sensing receptors such as the nucleotide oligomerization domain (NOD)-like receptors [14, 15]. DCs consist of different subsets which interfere with the differentiation lineage. In mice, $CD8\alpha^+$ DC were involved with Th1 lineage, while the $CD8\alpha^-$ subsets were linked to Th2 differentiation, through the secretion of IL-12 and IL-6, respectively [16]. Costimulatory signals augment TCR signals, thereby promoting proliferation and differentiation. The main co-stimulatory receptor is CD28, which is expressed in all naïve T cells. The ligands of CD28 on the DC are the CD80 (B7-1) and CD86 (B7-2), which are upregulated upon activation of DC. Other less potent co-stimulatory molecules include CD28 homolog inducible co-stimulator (ICOS), members of TNF receptor family (CD27, 4-1BB, and OX-40). These receptors have their ligands expressed on DC [17, 18]. The initial source of cytokines are from the APCs as well as other members of the innate immune cells. Subsequently, some of the cytokines produced by the differentiating cells can create a positive feedback loop, whereby the differentiation and response are marginally enhanced.

3.1. Th1 Differentiation. Interleukin 12 (IL12) and interferon γ (IFNγ) are the critical cytokines initiating the downstream signaling cascade to develop Th1 cells [19]. IL12 is secreted in large amounts by APCs after their activation through the pattern recognition receptors [14, 15, 20]. The IL12, in turn, induces natural killer cells(NK) to produce IFNγ.

Several transcription factors in coordination induce full differentiation of the Th1 cells (Table 1). The master regulator for Th1 differentiation, the T-box transcription factor (T-bet), is defined not only by its ability to activate the set of genes to promote differentiation of a particular

Table 1: Cytokines and transcription factors (the master regulators are underlined).

$CD4^+$ Subset	Cytokines	Transcription factors	Inhibitory transcription factors
Th1	IL12, **IFNγ**	T bet, STAT1, STAT4, Runx 3, Eomes, Hlx	GATA3
Th2	**IL4**, IL2	GATA3, STAT6, STAT5, STAT3, Gfi-1, c-Maf, IRF4	T-bet, Runx3
Th17	IL6, **IL 21**, IL 23, TGF-β	RORγt, STAT3, RORα, Runx1, Batf, IRF4, AHR	T-bet$^+$ Runx1, Smad3 Runx1$^+$FOXP3
Tfh	IL6, IL21	Bcl6, STAT3	
iTreg	TGF-β, IL2	FOXP3, Smad2, Smad3, STAT5, NFAT	
Th9	TGF-β, IL4	IRF4	
Tr1	IL27, IL10	c-Maf, AhR	

phenotype, but also by that of being able to suppress the development of opposing cell lineages [21, 22]. T-bet is the principal transcription factor, as it significantly enhances the production of IFNγ, and plays important role in suppressing the development of Th2 and Th17 [22, 23]. T-bet expression was found to be strongly dependent on signal transducer and activator of transcription 1 (STAT1), rather than on IL12–dependent STAT4 [21, 24]. STAT1, is in turn activated by IFNγ. T-bet further induces IFNγ production by the differentiating cells, thereby amplifying T-bet expression and upregulating the expression of IL12Rβ2. The latter cells can then be selected by the abundant IL12 from the APCs, thus ensuring selective expansion of the differentiating Th1 cells [21]. T-bet suppresses development of Th2 cell by inhibiting the crucial IL4 gene and impairing the function of the Th2 master regulator GATA3 [25, 26]. Th17 lineage is inhibited by the interaction of T-bet with Rorc promoter, which encodes RORγt, the principal transcription factor of Th17 [23].

IL12-induced STAT4 is another important transcription factor involved in the Th1 cell differentiation [27]. STAT4 induces IFNγ production, thereby creating a positive feedback loop for further T-bet and IL12Rβ2 expression. STAT4 and T-bet are involved directly in the transcription of IFNγ locus through the creation of activating marks at the locus, while STAT6 and GATA3 in Th2 differentiation establish repressive histone marks at the said locus, thereby indicating that the activation of IFNγ locus dictates Th1 differentiation [28]. However, STAT4 and T-bet do not function in a linear way in the differentiation of Th1 cell, with each having their unique signaling pathway. But for complete Th1 cell differentiation, these-lineage specific transcription factors need to operate in coordination with one another [29]. In later stages of differentiation, IL12/STAT4 pathway upregulates IL-18Rα. IL12 along with IL18 induces IFNγ production independent of TCR activation, thus creating a pathway for enhancing Th1 response.

Runt-related transcription factors also participate in the differentiation process. Runx1 and Runx3 were found to promote Th1 cell differentiation [16, 25, 30]. Runx3, in coordination with T-bet, binds to the IFNγ promoter and silences the genes encoding IL4, leading to the Th1 lineage differentiation [25]. Moreover, Runx3, through interaction with GATA3, leads to the inhibition of Th2 differentiation [16]. Runx1 together with T-bet inhibits Th17 development by interfering with the RORγt master regulator [23].

Recent studies identified a novel role of T-bet as a transcriptional repressor. T-bet through the induction of transcriptional repressor, Bcl-6, represses the activity of IFNγ locus in later stages of Th1 differentiation, with the consequence of reducing the overproduction of IFNγ and hence acts as a protective mechanism to avoid immunopathology [31].

Eomesodermin (Eomes), also a member of the T-box gene family, is important in regulating $CD8^+$ cells development and functions, and also plays a role in the Th1 lineage commitment. IL 21 represses Eomes expression. Exposure of naïve cell to IL21 led to the reduction of IFNγ production by the developing Th1 cells [32].

Hlx, another transcription factor induced downstream to T-bet activation, has been found to enhance IFNγ production by Th1 cells [33].

3.2. Th2 Differentiation. IL4 and IL2 are critical for Th2 differentiation. The major transcription factor involved in Th2 lineage differentiation includes the IL4-induced STAT6, which upregulates the expression of the master regulator GATA3 (GATA-binding protein) [34–36]. 3 distinct mechanisms of GATA3 involvement in Th2 differentiation have been postulated, including enhanced Th2 cytokine production, selective proliferation of Th2 cells through recruitment of Gfi-1, and inhibition of Th1 differentiation presumably by interacting with T-bet [37]. Moreover, GATA3 was found to suppress Th1 differentiation by downregulating STAT4 [38]. In vivo, GATA3 is indispensable for Th2 response. In GATA3 deficient mice, differentiation of naïve cells was diverted towards the Th1 lineage [39]. Absence of GATA3 leads to the interruption of Th2 differentiation [37, 39, 40]. Recent studies showed that GATA3 by itself cannot regulate all the Th2-specific genes, but instead needed the collaboration of STAT6 [41]. Although IL4 and IL2 are required for Th2 cells development in vitro, there is evidence of IL4-independent

Th2 differentiation in vivo. But since GATA3 is indispensable for Th2 cells differentiation in vivo, it can be suggested that there exist an IL4-independent GATA3 activation pathway [42, 43]. Continuing researches showed that Th2 cell differentiation involves several other transcriptional factors activated downstream to several cytokines, including IL2, IL6, and IL21.

STAT5 has an important role in the Th2 lineage commitment. It is readily activated by IL2 [44, 45]. STAT5 activation is independent of IL4 signaling and does not induce GATA3 expression [46]. For full differentiation of Th2 cells, the coordinated activity of STAT5 and GATA3 is required, since GATA3 alone cannot induce the production of IL4. This is due to the fact that GATA3 and STAT5 bind to different sites of the IL4 locus. GATA-3 binds to DNaseI hypersensitive site Va and CNS-1 sites of the IL4/IL13 loci, while STAT5 binds to the DNase I hypersensitive sites (HSII and HSIII) in the second intron of the IL4 locus [37, 45].

Recent studies identified the role of STAT3 in Th2 differentiation. STAT3 is required by STAT6 for interaction with relevant gene loci in the developing T cells. It was found that in the absence of STAT3, STAT6 was normally activated, but its interaction with loci was impaired, suggesting the role of STAT3 as a mediator to access to the loci [47, 48]. In STAT3 deficient mice, allergic inflammation was aborted, thereby proving the importance of its presence for the proper development of Th2 cells [47].

IL6, abundantly produced by APCs as well as by nonimmune cells, plays a dual role in Th2 lineage differentiation. It promotes Th2 differentiation, while simultaneously inhibiting the Th1 lineage [49, 50]. The downstream signaling pathway of IL6, in favor of Th2 differentiation, is IL4-dependent. IL6 enhances IL4 production by naïve $CD4^+$ cells, through the upregulation of nuclear factor of activated T cells (NFAT). Then IL4 signaling pathway ensures the differentiation as described above. The inhibition of the Th1 development occurs through the IL6-induced upregulation of suppressor of cytokine signaling-1 (SOCS-1) expression, which interferes with STAT1 activation downstream to IFNγ signaling [49, 50].

Growth factor independent-1 (Gfi-1) is a transcription repressor, induced by the IL4/STAT6 pathway, as well as by TCR signaling alone. It promotes Th2 cell expansion by selectively enhancing proliferation of GATA3-high cells. In Gfi-1 deficient mice, Th2 cell expansion was significantly reduced [51, 52]. c-Maf selectively upregulates IL4 gene transcription and consequently promotes Th2 cell differentiation by IL4-dependent mechanism [53]. However, c-Maf is not involved in the production of other Th2 cytokines, except for IL4 [46]. Interferon regulatory factor 4 (IRF4) is another transcription factor useful in the lineage specific differentiation of Th2. It coordinates with nuclear factor of activated T cells 2 (NFATc2) to activate IL4 promoter [54]. It has been shown that in the absence of IRF4, IL4 could not induce Th2 differentiation, and GATA3 could not be upregulated despite IL4 treatment. However, the fact that over expression of GATA3 restored Th2 differentiation pathway, one may conclude that IRF4 upregulates GATA3 [55].

3.3. Th9 Cells. Initially characterized as a subset of Th2 cells, ongoing researches tend to classify IL9 secreting-Th9 cells as a distinct subset of $CD4^+$ T cells. TGF-β was found to divert the differentiation of Th2 towards the development of Th9 cells. Moreover, TGF-β in combination with IL 4 directly induces the differentiation of Th9 cells [56]. IRF4 also plays an important role. IRF4 was found to directly bind to the IL9 promoter [57]. However, more research need to be conducted to get more insights about the Th9 cells, before being classified as a distinct lineage of $CD4^+$ cells.

3.4. Th17 Cells Differentiation. IL6, IL21, IL23, and TGF-β are the major signaling cytokines involved in Th17 cells differentiation, and retinoic acid receptor-related orphan receptor gamma-T (RORγt) is the master regulator. The differentiation process can be split into 3 stages, including the differentiation stage mediated by TGF-β and IL6, the self-amplification stage by IL21, and the stabilization stage by IL23.

TGF-β is the critical signaling cytokine in Th17 differentiation [58–62]. However, TGF-β signaling pathways also play significant role in the development of iTreg. Th17 and iTreg are antagonistically related. TGF-β alone, at high concentration, can divert lineage differentiation towards iTreg development, through the induction of FOXP3 [63, 64]. However, at low concentration and in the presence of IL6, TGF-β induces Th17 differentiation, production of IL21 and upregulates expression of IL23R [58–60, 64]. Since TGF-β signaling,unlike IL6, IL21, and IL23, does not activate STAT3, its role appears to involve in the enhancement of STAT3 activation. TGF-β inhibits IL6/IL21-induced expression of suppressor of cytokine signaling 3 (SOCS3), which negatively regulates STAT3 signaling pathways [65]. Downstream TGF-β signaling pathway in the presence of IL6 leads to the activation of RORγt [66, 67]. Forced expression of RORγt induces the production of IL-17A and IL-17F. Besides the master regulator RORγt, several other transcription factors need to collaborate for full differentiation of Th17 cells. As such, deficiency of RORγt does not lead to complete interruption of Th17 cytokine expression [67].

STAT3, activated downstream to IL6, IL21, IL23 signaling plays an important role in the differentiation process. It induces RORγt expression. STAT3 deficiency was found to cause enhanced expression of T-bet and FOXP3, which are involved in the development of opposing cell lineages [68]. STAT3 binds to IL-17A and IL-17F promoters [69].

RORα, another member of the ROR family, also participates in the lineage commitment pathway. Together RORα and RORγt synergistically enhance Th17 differentiation, and their absence completely aborted the development of Th17 cells [67].

Runx1 also influences Th17 differentiation. Runx1 through the induction of RORγt, promotes differentiation. However, Runx1/FOXP3 interaction negatively regulates Th17 development [70]. Moreover, T-bet in collaboration with Runx1 leads to the interruption of Runx1-mediated transactivation of Rorc, thereby suppressing Th17 development [71].

Aryl hydrocarbon receptor (AHR), a ligand-dependent transcription factor, was found to promote Th17 differentiation, presumably through the inhibition of STAT1 and STAT5, which negatively regulate Th17 development. However, its absence did not cause complete abortion of Th17 differentiation, but was associated with inability to produce IL22 [72, 73]. Recently identified, activator protein (AP-1) transcription factor, Batf, also plays an important role in the differentiation process. Batf$^{(-/-)}$ mice had defective Th17 response, but Th1 and Th2 development was unaffected [74]. IRF4 was found to be important not only in the differentiation of Th2, but also in that of Th17. Irf4$^{(-/-)}$mice failed to enhance expression of RORγt and subsequently did not develop experimental autoimmune encephalitis as a result of impaired Th17 response [75]. IRF4 activity is negatively regulated by IRF4 binding protein (IBP), leading to a control of IL17 and IL21 production. Overproduction of the latter cytokines is associated with the development of multiple autoimmune diseases. Mice with deficiency of IBP, rapidly developed rheumatoid arthritis-like joint disease and vasculitis [76].

The self-amplification phase is a crucial step in the differentiation process. It is required in order to mount a robust immune response. Unlike Th1 and Th2 differentiation mechanisms, where their respective major cytokine IFNγ and IL4 act as amplifying cytokines, the main cytokine IL17 of Th17 cell does not amplify its differentiation. Instead it is IL21, produced in significant amount by Th17, that in collaboration with TGF-β amplify Th17 differentiation. This phase does not require IL6, thereby creating a TCR-independent mechanism of differentiation [77, 78].

The third phase is conducted by IL23, mainly produced by APCs. IL23 is principally required for expansion and maintenance of the Th17 population [58, 79]. IL6 and IL21 downstream signaling induces the expression of IL23R on Th17 cell surface [80]. Moreover IL23 has been shown to induce its own receptor independently [79]. Although thought to be unable to induce Th17 differentiation, recently IL23 in association with IL-1β was shown to induce the development of T-bet$^+$RORγt$^+$Th17 cells independent of TGF-β [81].

3.5. Regulatory Cells Differentiation. iTreg cells are FOXP3$^+$ CD4$^+$CD25$^+$ cells, which are developed in the peripheral lymphoid organs after antigen priming, in contrast to the natural Treg (nTreg) which are released from the thymus as a distinct lineage with FOXP3 already expressed [82]. TGF-β is the critical cytokine responsible for the initiation of the iTreg cell lineage commitment [82–85]. Forkhead transcription factor FOXP3 is specifically expressed in CD4$^+$CD25$^+$Treg cells and is the major lineage-specific transcription factor involved in iTreg differentiation [85–87]. FOXP3 is induced downstream to TGF-β signaling, after interaction with TCR [82, 85]. Fatal immunopathology followed as a result of FOXP3 deletion/mutation, which resulted in defective and decreased iTreg cells [86, 87]. As with the differentiation of the other subsets of CD4$^+$ cells, FOXP3 along with other transcription factors is needed for full differentiation of the iTreg cells.

Smad2 and Smad3, which are also activated through TGF-β signaling pathways, are involved in the iTreg differentiation process by inducing FOXP3 [85, 88, 89]. Moreover, Smad 2 and Smad 3 were also found to induce differentiation via FOXP3-independent pathway. Smad3 can differentially enhance iTreg development by upregulating FOXP3 expression and inhibit Th17 differentiation by blocking RORγt [90].

STAT5-induced downstream to IL2 signaling is required for the differentiation of iTreg [91–94]. STAT5 was found to enhance FOXP3 expression and subsequently downstream to FOXP3 signaling and promote iTreg development. STAT5 and STAT3, which bind to multiple common sites across the IL17 locus, function closely and antagonize each other. Activation of STAT5 by IL2 signaling impair STAT3 binding to the locus sites and consequently enhance iTreg differentiation. Conversely, defective IL2-STAT5 signaling suppresses iTreg, and thus Th17 pathway is favored [91, 95].

NFAT through interaction with FOXP3 promoted Th17 differentiation [89, 96]. Impaired interaction of mutated FOXP3 gene and NFAT led to decreased expression of Treg markers-CTLA4 and CD25 [96].

Among the regulatory cells,Tr1 is being extensively studied. These IL10-producing cells play important role in suppressing inflammation and autoimmune processes. IL27 and IL10 are the principal cytokines involved in driving the Tr1 cells differentiation [97, 98]. IL10 signaling pathways in the induction of the differentiation remains to be elucidated. IL27 signaling leads to the activation of three key factors required for the differentiation. They include the transcription factor c-Maf, IL21, and the costimulatory receptor ICOS. c-Maf is the main factor, whose activation leads to enhanced production of IL21. IL21 acts as an autocrine growth factor driving the expansion of Tr1 cells [99]. ICOS promotes the IL27-induced differentiation of Tr1. Recently, Aryl hydrocarbon receptor (AhR), also induced by IL27, was found to be important in the differentiation of Tr1 cells. AhR and c-Maf act synergistically to mediate the differentiation [100].

3.6. Follicular Helper (Tfh) T Cells. Tfh are C-X-C motif receptor-5 (CXCR 5$^+$) expressing cells and are located in follicular areas of lymphoid tissue, where they participate in the development of antigen-specific B-cell immunity [101, 102]. IL6 and IL21 are the main cytokines involved in the differentiation process [103, 104]. STAT3, activated downstream to cytokine signaling, is an important transcription factor of Tfh. However, unlike in Th17 development, TGFβ does not participate, and RORγt is not induced. In vitro, IL21 in the absence of TGFβ resulted in Tfh differentiation [105]. Inducible costimulator (ICOS), member of CD28 family, is also required for Tfh development [106, 107]. In mice with ICOSL deficiency, Tfh differentiation was downregulated. More recently Bcl6, a transcription factor selectively expressed in Tfh, was found to play important role in the differentiation. It is activated downstream to IL6 and IL21 signaling, and its overexpression induced Tfh differentiation, while inhibiting opposing cell lineages [108].

4. Plasticity of $CD4^+$ Cells

Unlike Th1 and Th2 cells, which are considered to be terminally differentiated, Th17 and Treg have shown plasticity, thereby suggesting that they are not terminally differentiated (Figure 1). However, recent studies found that even Th2 cells exhibit plasticity. TGF-β caused Th2 cells to switch their characteristic cytokine profile into a IL9 predominating one, suggesting the conversion into Th9 cells [56]. Th17 in the presence of IL12 switched to Th1 phenotype, and interaction with IL4 led to the differentiation into Th2 cells [109, 110]. Treg showed tendency to convert to Th17 and Tfh. In the presence of IL 6, $CD4^+CD25^+FoxP3^+$ cells upon activation reprogrammed into Th17 [111]. $FoxP3^+$Treg in Peyer's patches differentiated into Tfh, with subsequent interaction with B cells and production of Ig A [112]. IRF4 inactivation in $Foxp3^+$ cells resulted in Th2 development and increased germinal centre formation [113].

5. Effector Functions

5.1. Th1 Cells. Th1 cells are involved with the elimination of intracellular pathogens and are associated with organ-specific autoimmunity [114]. They mainly secrete IFNγ, lymphotoxin α (Lfα), and IL2. IFNγ is essential for the activation of mononuclear phagocytes, including macrophages, microglial cells, thereby resulting in enhanced phagocytic activity [115]. IFNγ is believed to exert its effect through the activation of IFNγ-responsive genes, which account for more than 200 [116]. One of the well studied is the gene encoding IFNγ-inducible GTP-binding protein (IGTP) [105, 117]. IGTP is a member of p47 GTPase family also known as IRG family, is strongly induced by IFNγ, and induces the elimination of intracellular pathogens [117, 118]. Lfα is a member of the TNF super family. Lfα is associated with autoimmune diseases. The depletion of Lfα has shown to inhibit the development of experimental autoimmune encephalitis [119, 120]. IL2 promotes proliferation of $CD8^+$T cells with acquisition of cytolytic phenotype [121, 122]. Besides its role as T cell growth factor, IL2 was also found to promote the development of $CD8^+$ memory cells after antigen priming, and thus participating in ensuring a robust secondary immune response [123]. Natural Treg (thymus derived) need IL2 for survival and activation. Downstream IL2 signaling leads to the activation of STAT5 and eventually to enhanced expression of FOXP3 in naïve cells, thereby acquiring potent suppressive ability [124].

5.2. Th2 Cells. Th2 cells mount immune response to extracellular parasites, including helminthes, and play major role in induction and persistence of asthma as well as other allergic diseases [114, 125]. The key effector-cytokines include IL4, IL5, IL9, IL13, IL10, IL25, and amphiregulin. IL4 is a major cytokine involved in allergic inflammation. It is involved in IgE switching and secretion by B cells. IL4 also upregulates low-affinity IgE receptor (FcεRI) on B-lymphocytes and mononuclear phagocytes, and also high-affinity IgE receptor (FcεRII) on mast cells and basophils, with subsequent degranulation of the cells and release of several active metabolites, including histamine and serotonin [126]. IL4 also induces the increase of several other proinflammatory mediators, including IL6, GM-CSF (granulocyte-macrophage colony-stimulating factor), VCAM-I adhesion molecule [127]. IL5 mainly targets eosinophils and its precursors, since these cells have relatively higher amounts of IL5R expressed on their surface, and subsequently leads to their activation with upregulation of CD11b and inhibition of apoptosis [128]. IL9 participates actively in the immunopathogenesis of asthma. It activates the function of several cells, including mast cells, B cells, eosinophils, neutrophils as well as airway epithelial cells. Along with hypersecretion of mucus, IL9 was found to release chemoattractant factors, leading to allergic airway inflammation [129]. One of IL13 main roles is to combat gastrointestinal helminthes. IL13, through the activation of cell-mediated immunity, helps in the elimination of intracellular pathogens, such as Leishmania. It also plays a major role in the induction of allergic asthma, through activation of eosinophils, enhanced mucus secretion, and airway hyperresponsivity. Potent stimulation of tissue fibrosis at sites of inflammation was also associated with IL13 [130]. IL10 is an anti-inflammatory cytokine. After pathogen clearance in the course of an immune response, IL10 helps achieve homeostasis through the inhibition of Th1 cells as well as other immune cells of the innate system [131]. IL25, previously known as IL17E, is a member of the IL17 family of cytokines. It is structurally similar to IL17, but functionally different. It promotes Th2 responses [132–134]. It induces increased mucus production, eosinophilia, IgE switching, and enhanced Ig secretion, as a result of upregulation of IL4, IL5, and IL13, thereby amplifying aTh2 response. It was found to induce pathologies of lungs and digestive tract, due to enhanced expression of IL13 [132]. Novel role of IL25 was identified to be the suppression of Th17 response, and consequently the regulation of the development of autoimmune disease. In $IL25^{(-/-)}$ mice, the susceptibility to acquire experimental autoimmune encephalitis was found to be significantly raised, and disease course was accelerated [133]. IL25 suppressed Th17 response by increasing the expression of IL13, which directly inhibit production of cytokines required for development Th17, including IL23, IL1β, and IL6 by activated dendritic cells. Moreover, $IL25^{(-/-)}$ mice failed to expel helminthes *Nippostrongylus brasiliensis*, thereby indicating a poor Th2 response [134]. Amphiregulin is a member of the epidermal growth factor (EGF) family. It directly induces epithelial cell proliferation. Its deficiency was associated with delayed expulsion of nematode *Trichuris muris* [135]. The Th9 cell secretes large quantities of IL9, with effects as stated above. At present, Th9 cells are viewed as major culprits in the the development of allergic pathologies, especially asthma [136].

5.3. Th17 Cells. Th17 is responsible to mount immune response against extracellular bacteria and fungi. They are also involved in the generation of autoimmune diseases [137–139]. The key effector cytokines include IL17A, IL17F, IL21, and IL22. IL17A and IL17F signaling occurs through a common receptor, IL17RA, thereby suggesting similar

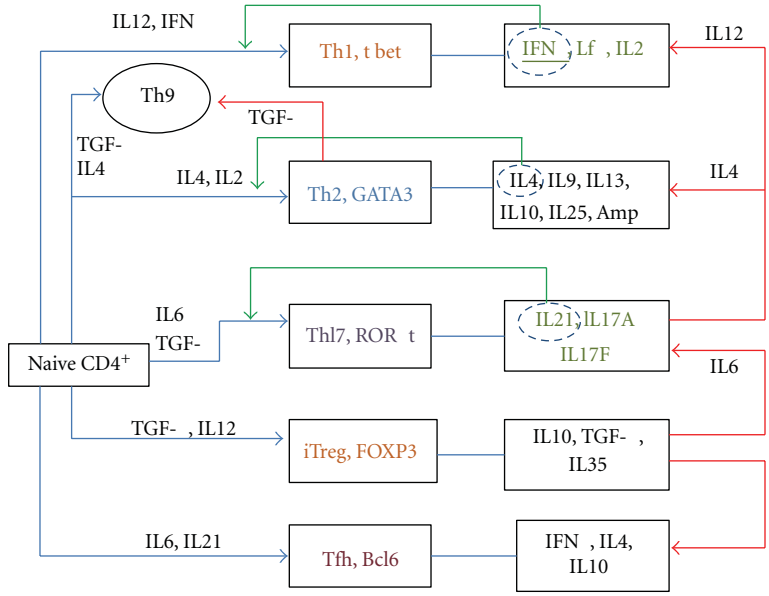

Figure 1: Influence of distinct cytokine milieu in the differentiation of CD4$^+$T cells. Blue arrows show the differentiation of naïve cells in the presence of particular cytokines. The green arrows represent the self-amplification phase by the encircled cytokines. Plasticity of T cell subset under the influence of specific cytokine is represented by the red arrows. Along with Th subset, the master regulator is shown. However, Bcl6 has not yet been identified as the master regulator, but it plays major role in the differentiation of Tfh.

functions [140]. Since the receptor IL17RA is expressed in multiple tissues, such as hematopoietic tissue, skin, lung, intestine, and joints, the effect of IL17 extends beyond T cell-mediated inflammatory response. IL17 leads to the induction of proinflammatory cytokines, including IL6, IL1, TNFα, and also proinflammatory chemokines ensuring the chemotaxis of inflammatory cells to sites of inflammation [139, 141]. IL21, along being an amplifying cytokine for TH17 development, has pleiotropic functions, including activating T cells, inducing B cells to differentiate into plasmocytes and memory cells, and activating NK cells [142, 143]. IL22 is known to mediate both inflammatory response and exhibits tissue protective properties. IL22 participates actively in mucosal host defense against bacterial pathogens, by inducing antimicrobial peptides and increasing cell proliferation [144]. In acute liver disease, IL22 was shown to be involved in limiting liver tissue damage [145].

5.4. Regulatory CD4$^+$T Cells. Treg exists as natural thymus-derived subset with expressed FOXP3, and as peripheral-induced Treg cells, which arise from naïve CD4$^+$CD25-cells after antigen priming in a relevant cytokine milieu [82]. Treg and Tr1 play important role in the maintenance of immunologic tolerance to self and foreign antigen. After clearance of pathogens, they negatively regulate the immune response, thereby protecting against immunopathology [32, 146]. Their main effector cytokines include IL10, TGF-β, and IL35. IL10 is a potent inhibitory cytokine, with the ability to suppress proinflammatory response and thus limits tissue damage by the inflammatory process [131, 147, 148]. IL10 and TGF-β potently suppress IgE production, thereby showing their important role in attenuating allergic inflammation [149]. Mice with T-cell-specific deletion of Tgfb1 gene, developed fulminant immunopathology as a result of uncontrolled differentiation of proinflammatory T cells, and hence showing the relevance of TGF-β in regulating immune response [150].

5.5. Follicular Helper (Tfh) T Cells. After TCR interaction and subsequent differentiation from the CXCR5$^-$ CCR7$^+$CD4$^+$ naïve cells, these CXCR5+CD4+T (Tfh) cells play significant role in mediating humoral immu-nity through interaction with B-lymphocytes. After having lost CCR7, the differentiated CXCR5$^+$CCR7-pMHCII-specific Tfh cells enter the pregerminal centre for initial interaction with antigen-primed B cell, with subsequent differentiation of the B cells into Ig-producing plasma cells. In the germinal area, they are involved in the development of long-live B memory cells. According to the predominant cytokine secreted, Tfh cells have been classified into Tfh1, Tfh2, and Tfh10. Tfh1 by secreting IFNγ promotes IgG2a production. Tfh2 secretes IL4, which favors the production of IgG1 and IgE. Tfh10 through the secretion of IL10, promotes IgA secretion [151].

6. Conclusion

Clearly the CD4$^+$T cells represent a unique branch of the adaptive immune system that is crucial in achieving a regulated effective immune response to pathogens, and their proper functioning is vital for survival. Through their distinct phenotypes with their respective cytokine profile, they modulate the functions of the innate immune cells as well as the members of the adaptive immune system.

During the recent years, subsets with more specialized and more defined properties have been identified, such as the Tfh and Th9, thereby reinforcing their control over the immune system. Thanks to new technologies, more will be learned about the epigenetic modifications that occur during the differentiation process, and hence we will gain more insights in their development, which will prove useful for later clinical use. Once considered terminally differentiated after antigen-mediated activation, recent studies have been showing the plasticity of the different subsets, particularly the Treg and Th17 cells. This plasticity makes the potential use of Treg risky in autoimmune diseases and organ transplant, since the Treg cells can reprogram into proinflammatory phenotypes in the presence of relevant cytokine milieu and cause more harm. Moreover, aberrantly functioning $CD4^+$ cells are associated with the development of multiple autoimmune and allergic pathologies. More research will bring new insights about the epigenetic program of the current and probably novel subsets of $CD4^+$T cells and their mechanism and means of functioning, thus subsequently becoming a valuable asset, which clinicians can use against immune-mediated diseases.

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Research Article

Improved Activation toward Primary Colorectal Cancer Cells by Antigen-Specific Targeting Autologous Cytokine-Induced Killer Cells

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Adoptive therapy of malignant diseases with cytokine-induced killer (CIK) cells showed promise in a number of trials; the activation of CIK cells from cancer patients towards their autologous cancer cells still needs to be improved. Here, we generated CIK cells *ex vivo* from blood lymphocytes of colorectal cancer patients and engineered those cells with a chimeric antigen receptor (CAR) with an antibody-defined specificity for carcinoembryonic antigen (CEA). CIK cells thereby gained a new specificity as defined by the CAR and showed increase in activation towards CEA^+ colon carcinoma cells, but less in presence of CEA^- cells, indicated by increased secretion of proinflammatory cytokines. Redirected CIK activation was superior by CAR-mediated CD28-CD3ζ than CD3ζ signaling only. CAR-engineered CIK cells from colon carcinoma patients showed improved activation against their autologous, primary carcinoma cells from biopsies resulting in more efficient tumour cell lysis. We assume that adoptive therapy with CAR-modified CIK cells shows improved selectivity in targeting autologous tumour lesions.

1. Introduction

Although a variety of therapeutic options for metastatic colon cancer were evaluated during the last decade, most patients in advanced stages of the disease have no hope for cure by standard therapies. Alternative therapeutic approaches including immunotherapy are currently explored [1]. One of the major pitfalls in the adoptive immunotherapy of cancer is the strikingly low activation of T cells from cancer patients compared to healthy donors due to reduced expression of TCR/CD3 components [2]. The need for alternative effector cells in targeting colorectal carcinoma becomes obvious by the fact that T cells infiltrating colon cancer metastases have reduced CD3ζ chain expression and lack tumour-specific activation [3]. Compared to firstly activated effector T cells, *ex vivo* generated cytokine-induced killer (CIK) cells have a number of advantages since they exhibit properties different from effector or central memory T cells, that is, CIK cells are activated in an MHC-independent fashion [4, 5], produce proinflammatory cytokines, mainly IFN-γ and IL-4 [6, 7], and exhibit antigen-independent cytolytic activities against a variety of tumour cells. CIK cells are generated *ex vivo* by extensive stimulation of $CD3^+$ $CD56^-$ $CD8^+$ T cells with IFN-γ and CD3 and prolonged propagation in presence of high-dose IL-2 [4]. After 2-3 weeks in culture, the majority of cells exhibit a large granular lymphocyte morphology and express both NK and T-cell markers including CD8, CD11a, CD49d, CD56, and NKG2D, while lacking most NK-cell-associated activating and inhibitory receptors [8]. The $CD45RA^+$ $CCR7^-$ $CD62L^{(+)}$, $CD27^+$, $CD28^-$, $MIF\text{-}1a^+$ CIK phenotype coincides with that for terminally differentiated memory T cells [9]. CIK cells display extraordinary cytolytic

capacities toward a broad array of malignant cells [10] and traffic efficiently to the tumour side after systemic delivery [11]. Upon activation, CIK cells upregulate perforin and FasL as well as DAP10 which couples NKG2D signaling to perforin-based cytotoxicity [12], thereby recognizing a class of stress-associated ligands, NKG2D ligands, expressed on the tumour cell surface. Consequently, CIK cells exhibit MHC-unrestricted cytotoxicity and do not rely on a particular antigen. Based on these and other properties, CIK cells attracted interest for adoptive immunotherapy particularly in advanced stages of the disease where repression of MHC expression or defects in the antigen-processing machinery frequently occur. For application in adoptive therapy, CIK cells display the advantage that they do not require priming but can rapidly be expanded in culture [13] and are less associated with graft-versus-host disease than conventional effector T cells [14]. CIK cells have been adoptively transferred in phase I trials to treat leukemia/lymphoma and various solid tumours including hepatocellular carcinoma, colon carcinoma, astrocytoma, melanoma, and renal cell carcinoma [15–17]. CIK therapy showed low toxicity [18], however, limited therapeutic efficacy; CIK therapy is consequently assumed to require large numbers of CIK cells to be transferred to achieve efficient tumour clearance.

In this situation, we asked to improve CIK cell activation against autologous tumour cells. We therefore made use of the concept to redirect T cells towards defined target cells by a recombinant chimeric antigen receptor (CAR) which is expressed on the surface of T cells and provides both antigen-targeting specificity and T-cell activation [19]. The CAR in the extracellular moiety is composed of a single-chain fragment of variable region (scFv) antibody for target binding and in the intracellular moiety of the CD3ζ signaling chain to initiate T-cell activation upon binding. To furthermore increase T-cell activation, the costimulatory CD28 endodomain was linked to CD3ζ in a combined signaling moiety [20]. We here demonstrate that *ex vivo* generated CIK cells from colon carcinoma patients can be engineered with a tumour-specific CAR; such "designer" CIK cells increase cytokine secretion and cytolysis when engaging autologous, primary colon carcinoma cells. Data suggest such CAR-engineered CIK cells to improve the antitumour response in the adoptive immunotherapy of colon carcinoma.

2. Materials and Methods

2.1. Patient Characteristics and Evaluation. Patients with colorectal carcinoma were treated by surgery of the primary tumour lesion. Approval of the local ethics committee was obtained. Diagnosis of CEA$^+$ colorectal carcinoma was confirmed by immunohistology in a pathology reference centre.

2.2. Cells, Cell Lines, and Reagents. T cells were isolated from heparinized peripheral blood by Ficoll density centrifugation. CIK cells were generated as previously described [21]. In brief, nonadherent peripheral blood mononuclear cells were stimulated in RPMI 1640 medium, 10% (v/v) FCS, and 25 mM HEPES with human recombinant IFN-γ (1,000 U/mL; Roche Biochemicals, Mannheim, Germany). After 24 h, 50 ng/mL OKT3 monoclonal antibody (mAb) (Orthoclone; Cilag, Sulzbach, Germany), 100 U/mL IL-1β, and 300 U/mL IL-2 (Roche, Mannheim, Germany) were added. Cells were propagated in a density of 3×10^6 cells/mL in presence of IL-2. Primary colon carcinoma cell cultures were established from patients' carcinoma specimens obtained during surgery as described [22]. In brief, tissue specimens were incubated in HBSS buffer containing 100 U/mL DNase I (Roche Biochemicals), 50 U/mL collagenase III (Biochrom, Berlin, Germany), 150 U/mL hyaluronidase (Sigma, Deisenhofen, Germany), and 0,08 U/mL insulin (Hoechst, Bad Soden a. Ts., Germany) at 37°C for 15 min. Cells in the supernatant were centrifuged for 5 min at $400 \times$ g and erythrocytes eliminated by incubation in 10 mL "erythrocyte-lysis buffer" (8,29 g/L NH_4Cl, 1 g/L $KHCO_3$, 0,0371 g/L EDTA) and for 15 min. Cells were washed and resuspended in Leibovitz medium, 10% (v/v) FCS, 1 mM L-glutamine, 1x MEM vitamins, 2.5 mg/mL transferrin, 1 g/L sodium bicarbonate, 1 g/L glucose, 80 U/mL insulin, and 10 mg/mL gentamycin (all from Gibco Invitrogen, Karlsruhe, Germany). Cultures contaminated with fibroblasts were removed. Carcinoma cells grown in culture were monitored for CEA expression by immunohistochemical analysis using an anti-CEA mAb 1C3 (Abcam, Cambridge, MA) and a peroxidase-conjugated Fab anti-mouse Ab (1 : 50) (Roche Diagnostics) and visualized by 3-Amino-9-ethylcarbazole (AEC; Sigma). 293T cells are human embryonic kidney cells that express the SV40 large T antigen. LS174T is a CEA$^+$ colorectal carcinoma line (ATCC, CL-188), and Colo201 is a CEA$^-$ adenocarcinoma line (ATCC CCL 224). OKT3 (ATCC CRL 8001) is a hybridoma cell line that produces the anti-CD3 mAb OKT3. 293T cells were propagated in DMEM medium supplemented with 10% (v/v) FCS, and all other cell lines were cultured in RPMI 1640 medium, 10% (v/v) FCS (all Life Technologies, Paisly, UK). OKT3 monoclonal antibody (mAb) was affinity purified from hybridoma supernatants utilizing goat anti-mouse IgG2a antibodies (Southern Biotechnology, Birmingham, AL, USA) that were immobilized on N-hydroxy-succinimid-ester-(NHS)-activated sepharose (Amersham Biosciences, Freiburg, Germany). Human IgG1 antibodies and the phycoerythrin-(PE-)conjugated anti-CD3 mAb UCHT1 were purchased from Dako, Hamburg, Germany, and the goat antihuman IgG antibody and its FITC- and PE-conjugated F(ab′)$_2$ derivatives from Southern Biotechnology. The antihuman IFN-γ mAb NIB42 and the biotinylated anti-human IFN-γ mAb 4S.B3 were purchased from BD Bioscience, San Diego, CA, USA.

2.3. Engineering of CIK Cells and Receptor-Mediated Activation. The generation of the expression cassettes for the CEA-specific CARs BW431/26-scFv-Fc-ζ (no. 439) and BW431/26-scFv-Fc-CD28-ζ (no. 607) was previously described [20]. CIK cells were engineered with the CAR by retroviral gene transfer, and CAR expression was identified by flow cytometry utilizing a PE- or FITC-conjugated F(ab′)2 anti-human IgG1 antibody (1 μg/mL), which recognizes the CAR extracellular IgG1 Fc spacer, and the anti-CD3 mAb (UCHT-1,

1 : 20). Flow cytometry was performed using an FACScan cytofluorometer equipped with the CellQuest research software (Becton Dickinson, Mountain View, CA, USA). Engineered CIK cells were cocultivated in round bottom 96-well microtiter plates ($1.25-10 \times 10^4$ engineered cells/well) with CEA^+ and CEA^- tumour cells (5×10^4 cells/well), respectively. After 48 hrs, culture supernatants were analyzed by ELISA for IFN-γ. Briefly, IFN-γ was bound to the solid phase anti-human IFN-γ mAb NIB42 (1 μg/mL) and detected by the biotinylated anti-human IFN-γ mAb 4S.B3 (0.5 μg/mL) (both from Pharmingen). The reaction product was visualized by a peroxidase-streptavidin conjugate (1 : 10,000) and ABTS (both from Roche Diagnostics).

2.4. Cytotoxicity Assay. Specific cytotoxicity of receptor-grafted T cells against target cells was monitored by an XTT-based colorimetric assay. Briefly, XTT (2,3-bis(2-methoxy-4-nitro-5sulphonyl)-5[(phenyl-amino)carbonyl]-2H-tetrazolium hydroxide) reagent (1 mg/mL) ("Cell Proliferation Kit II," Roche Diagnostics) was added to the cells and incubated for 30–90 min at 37°C. Reduction of XTT to formazan by viable tumour cells was monitored colorimetrically at an adsorbance wavelength of 450 nm and a reference wavelength of 650 nm. Maximal reduction of XTT was determined as the mean of wells containing tumour cells only, and the background as the mean of wells containing culture medium only. The nonspecific formation offormazan due to the presence of effector cells was determined from triplicate wells containing effector cells in the same number as in the corresponding experimental wells. The cytotoxicity towards tumour cells was calculated as follows: cytotoxicity [%] = 100 −{[OD (exp. wells-corresponding number of effector cells)/OD (tumour cells without effectors − medium)] × 100}.

2.5. ELISpot Assay. IFN-γ-producing cells were determined using the human "IFN-γ ELISpot kit" (Hölzel, Cologne, Germany). Peripheral blood lymphocytes (4×10^4 cells) were plated on nitrocellulose 96-well plates (Millipore, Bedford, MA) coated with anti-IFN-γ antibody. Cells were stimulated either with phytohemagglutinin (10 μg/mL, Sigma) or with 100 Gy irradiated tumour cells (1×10^3 tumour cells per 4×10^4 CIK cells) for 48 hrs. Bound IFN-γ was detected by a biotinylated antibody and visualized by streptavidin alkaline phosphatase and BCIP/NBT (BioRad, Munich, Germany) as substrate. Spots were recorded using the Bioreader 2000 (Bio Sys, Karben, Germany).

2.6. Statistical Analyses. Statistical analyses were performed using the two-tailed Student's *t*-test if not otherwise described.

3. Results

$CD3^+$ $CD56^+$ CIK cells were generated *in vitro* from peripheral blood lymphocytes of a healthy donor by incubation with IFN-γ, IL-1beta, and the agonistic anti-CD3 mAb OKT3 and propagated in the presence of IL-2 as previously described [21]. After 2-3 weeks, CIK cells showed their characteristic repertoire of surface molecules (Table 1). CIK cells are activated upon coincubation with CEA^+ Colo205 and CEA^- Colo201 colon carcinoma cells, respectively, indicated by the increase in the number of IFN-γ-secreting CIK cells (Figure 1). Activation of CIK cells occurred equally upon binding to CEA^+ and CEA^- tumour cells confirming the known property of CIK cells of antigen-independent antitumour activation.

We asked to furthermore improve CIK cell activation selectively towards CEA^+ colorectal carcinoma cells by engineering with a CEA-specific CAR. Therefore, CIK cells were retrovirally transduced to express either the anti-CEA CAR BW431/26scFv-Fc-CD3ζ with the CD3ζ or alternatively the anti-CEA CAR BW431/26scFv-Fc-CD28-CD3ζ with the combined CD28-CD3ζ signaling domain, both harboring the same CEA binding domain (Figure 2(a)). Both CARs

Table 1: Characterization of CIK cells.

Marker	Positive cells (%)
CD3	98.6
CD4	22.1
CD8	67.8
CD14	0.0
CD33	9.3
CD56	31.7
HLA-DR	67.4

CIK cells were generated from peripheral blood lymphocytes from a healthy donor as described in Section 2. After 2-3 weeks of propagation, cells express the phenotype of $CD3^+CD56^+$ CIK cells. Data of a representative CIK cell induction are shown.

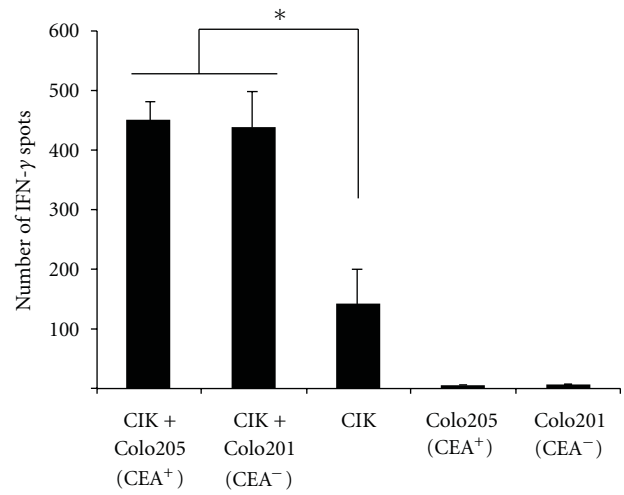

Figure 1: CIK cells are equally activated upon binding to CEA^+ and CEA^- tumour cells. CIK cells (4×10^4 cells) generated from blood lymphocytes of healthy donors were coincubated with CEA^+ Colo205 or CEA^- Colo201 tumour cells (10^3 tumour cells) for 48 hrs. IFN-γ production was monitored by ELIspot analysis. Data represent the mean ± standard error of the mean; a representative experiment out of three is shown. $^*P < 0.05$.

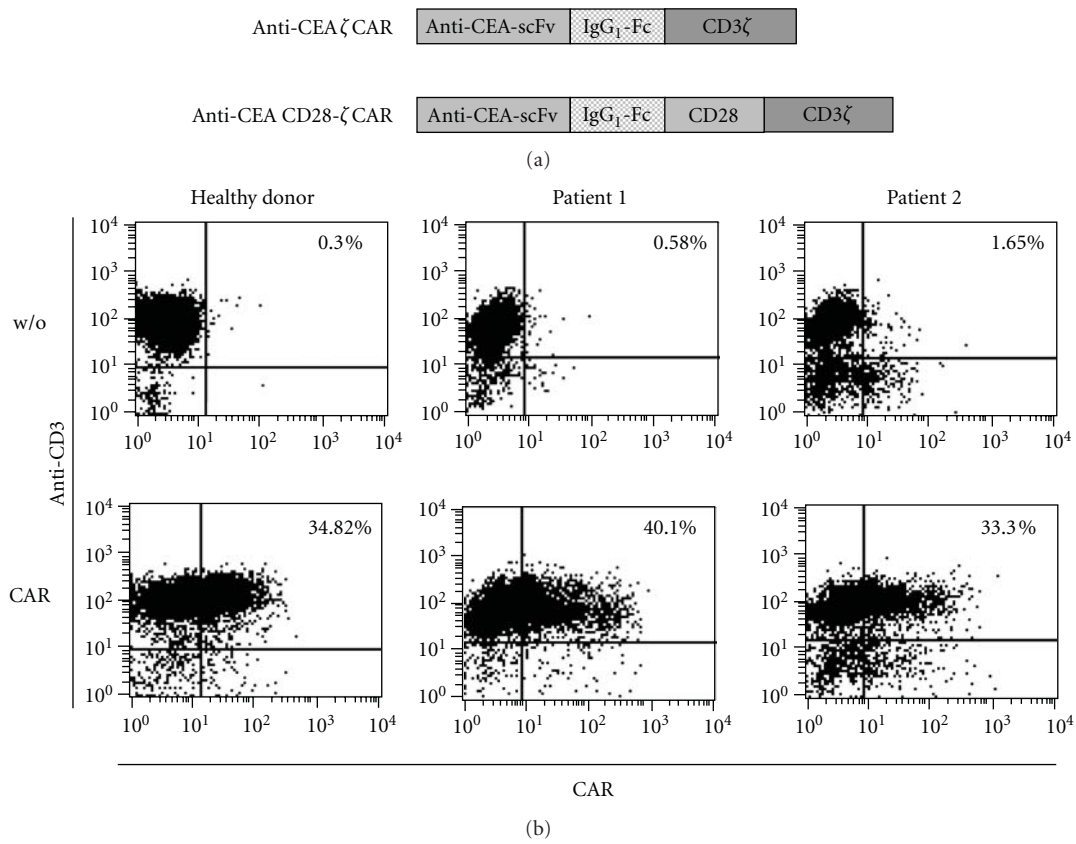

Figure 2: Genetic engineering of CIK cells with CARs. (a) Schematic diagram of the expression cassettes coding for the CEA-specific CAR BW431/26scFv-Fc-CD3ζ and BW431/26scFv-Fc-CD28-CD3ζ used in this study. (b) CIK cells were generated from mononuclear cells and subsequently transduced by retroviral infection to express the anti-CEA CAR. Mock-transduced cells (w/o) served as controls. CAR expression was recorded by flow cytometry using the anti-CD3 antibody OKT3 and the anti-human IgG-Fc antibody which detects the CAR extracellular IgG1 Fc spacer domain.

were efficiently expressed on the CIK cell surface as recorded by flow cytometry using an antibody directed towards the CAR extracellular IgG1 Fc spacer domain (Figure 2(b)). CAR expression on engineered cells was similar on CIK cells from healthy donors and from colorectal cancer patients.

In order to record antigen-redirected activation, CIK cells with engineered anti-CEA CAR were coincubated with CEA^+ colon carcinoma cells and with CEA^- carcinoma cells as controls. CAR CIK cells increased IFN-γ secretion upon coincubation with CEA^+, but not upon co-incubation with CEA^- tumour cells, whereas CIK cells without CAR did not increase IFN-γ secretion (Figure 3(a)). IFN-γ secretion was more increased when CIK cells were activated by the CD28-CD3ζ than the CD3ζ CAR. Moreover, redirected cytolytic activity of CAR-engineered CIK cells towards CEA^+ tumour cells is increased compared to CIK cells without CAR. The cytolytic activity towards CEA^- tumour cells was not substantially altered by engineering with a CEA-specific CAR as control. We conclude that CIK cells can specifically be improved in activation by CAR engagement.

To confirm antigen specificity in CAR-mediated CIK activation, we blocked the CAR by incubation with the anti-idiotypic antibody BW2064, which is directed toward the BW431/26-scFv domain for antigen binding. As summarized in Figure 3(b), both IFN-γ secretion and cytolysis of CAR CIK cells were repressed in presence of the anti-idiotypic mAb, whereas an isotype-matched IgG1 antibody of irrelevant specificity had no effect. Data demonstrate that the anti-tumour activation of CIK cells is mediated by the engineered CAR in a CEA-dependent fashion.

We now explored whether activation of CIK cells from colorectal cancer patients toward their autologous tumour cells can be improved by CAR-mediated engagement of target cells. Colorectal carcinoma cells were isolated from surgical specimens and confirmed by immunostainings to express CEA (data not shown). Colorectal carcinoma cells were coincubated with engineered autologous CIK cells with the CD3ζ or the combined CD28-CD3ζ CAR. As summarized in Figure 4, activation of CAR CIK cells is substantially increased against autologous tumour cells compared to nonmodified CIK cells of the same patient. Increase in IFN-γ secretion was higher upon stimulation by the CD28-CD3ζ than by the CD3ζ signaling CAR. Increased IFN-γ secretion is due to increased numbers of activated CIK cells as indicated by the increased numbers of IFN-γ-ELISpots. Activation is antigen specific since the same CAR-engineered CIK cells did not increase

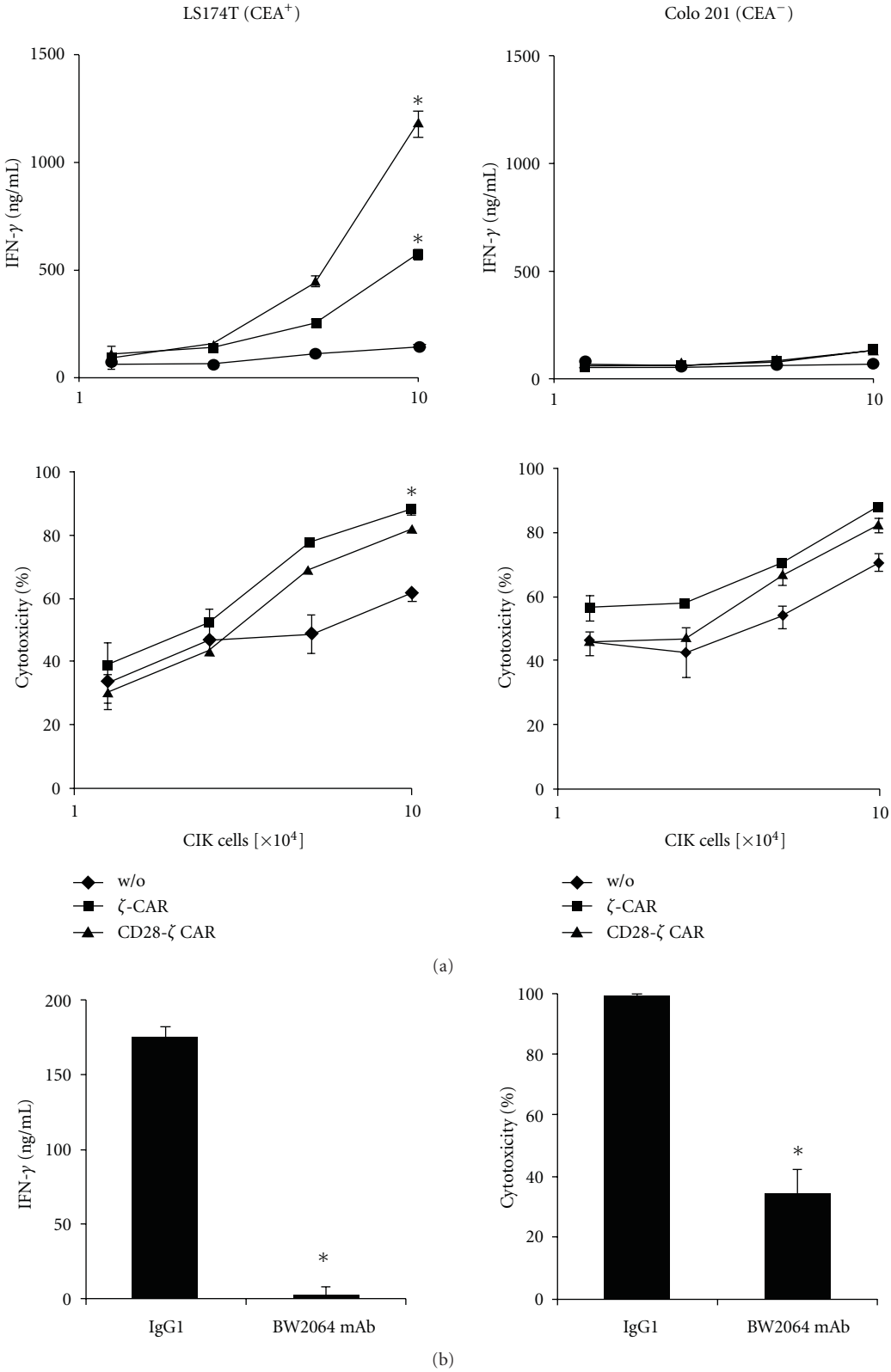

Figure 3: CAR engagement produces higher activation of engineered CIK cells toward colorectal carcinoma cells. (a) CIK cells were engineered with the CEA-specific ζ or CD28-ζ CAR ($1–10 \times 10^4$ CAR^+ cells/well) and incubated for 48 hrs with CEA^+ LS174T and CEA^- Colo201 cells (5×10^4 cells/well). Mock-modified CIK cells without CAR (w/o) served as control. IFN-γ secreted by activated CIK cells into the culture supernatants was recorded by ELISA (upper), and cytolysis of tumour cells was monitored using the XTT-based viability assay (lower). $^*P < 0.05$ compared to nonmodified CIK cells (w/o). (b) To block the CEA-specific CAR binding domain, CIK cells with ζ CAR were incubated in presence of the anti-idiotypic antibody BW2064, which binds to the CAR BW431/26-scFv domain, or with an isotype-matched IgG1 control antibody of irrelevant specificity together with CEA^+ LS174T tumour cells for 48 hrs. IFN-γ secreted into the culture supernatant and specific cytotoxicity toward LS174T cells were monitored. Data show a representative experiment out of three. $^*P < 0.05$.

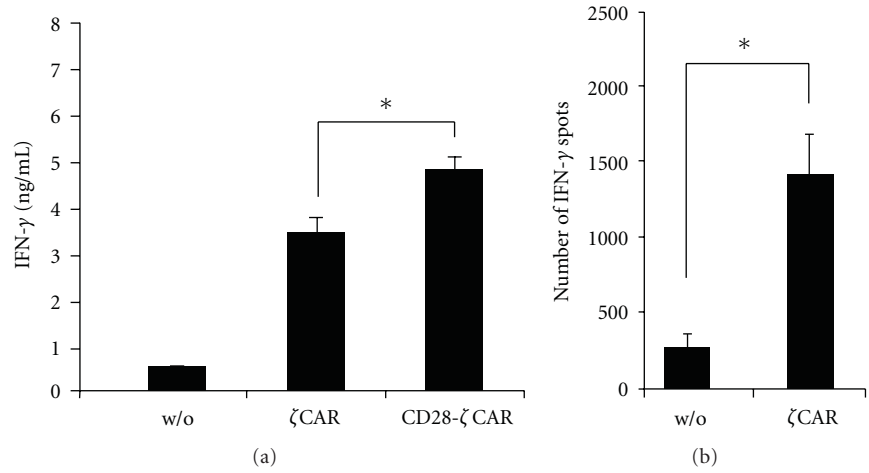

Figure 4: Activation of CIK cells from cancer patients against autologous colon carcinoma cells. Primary colon carcinoma cells were isolated from a colon carcinoma biopsy as described in Section 2 and cultured *in vitro* for short term. CIK cells from the same patient were generated *in vitro* and engineered with the ζ and the CD28-ζ CAR. CIK cells were coincubated for 48 hrs with the autologous CEA$^+$ colon carcinoma cells (4×10^4 CIK cells; 10^3 tumour cells). Secreted IFN-γ in the culture supernatant was monitored by ELISA. Data represent the means of triplicates $\pm$ standard error of the mean. One representative experiment out of three is shown. $^*P < 0.05$.

IFN-γ secretion in presence of CEA$^-$ Colo201 cells (data not shown). Taken together activation of CIK cells from colorectal cancer patients toward autologous tumour cells can be improved by CAR engagement of target cells and is furthermore increased by combined CD28-CD3ζ CAR signaling.

4. Discussion

To improve CIK cell activation towards autologous tumour cells, we here revealed that (i) CIK cells generated *ex vivo* from peripheral blood lymphocytes can be engineered with a CAR as a targeting and activating receptor, (ii) engineered CIK cells increase activation upon CAR engagement which is superior upon CD28-CD3ζ signaling, and (iii) redirected by the CAR, CIK cells from tumour patients exhibit improved activation towards autologous tumour cells. While CIK cells recognize tumour cells in an antigen-independent fashion, CAR-engineered CIK cells gain antigen specificity as defined by the CAR binding domain. CAR-engineered CIK cells show improved tumour specificity indicated by increased IFN-γ secretion upon contact to CEA$^+$ compared to CEA$^-$ tumour cells, while nonmodified CIK cells do not increase IFN-γ in presence of CEA$^+$ compared to CEA$^-$ tumour cells. Improved CIK cell activation requires antigen engagement since activation is blocked by an anti-idiotypic antibody directed against the CAR binding domain. While CIK cells are susceptible to CD3ζ signaling, furthermore increase in IFN-γ secretion by designer CIK cells upon CD28-ζ CAR stimulation likely contributes to improve antitumour activity *in vivo* through activation of bystander cells. Cytolytic activity is predominantly mediated by perforin triggered by NKG2D in CIK cells [12]; other mechanisms may additionally contribute since blocking of NKG2D did not completely eliminate the cytotoxic activity of CIK cells [23].

To evaluate the specific situation in cancer patients, we confronted CIK cells from a colon cancer patient with the autologous colon cancer cells from a biopsy *in vitro*. Similarly as CIK cells from a healthy donor, CAR-engineered CIK cells from cancer patients showed improved activation against autologous tumour cells indicated by increase in IFN-γ secretion compared to CIK cells without CAR. Previous reports by Sheen et al. [24] and our group [25] demonstrated efficient targeting of CD3$^+$ effector T cells from cancer patients toward autologous colon carcinoma cells; CIK cells herewith expand the panel of effector cells suitable to target autologous tumour cells.

Soluble CEA in the serum of cancer patients, particularly in advanced stages of the disease, may prevent CAR-mediated activation of engineered CIK cells by blocking the binding domain. In the case of the BW431/26 scFv CAR domain, we previously demonstrated that CEA in concentrations up to 20 μg/mL does not block CAR-mediated T-cell activation and does not inhibit induction of cytolytic activities [26]. We therefore do not expect that serum CEA interferes with the activity of anti-CEA CAR-modified CIK cells in colorectal cancer patients.

Given the insufficiencies in activating CIK cells and the difficulties in generating sufficient quantities for clinical applications, improved CIK activation upon CAR signaling is assumed to decrease the numbers of CIK cells required to elicit a therapeutic response. Previous strategies to overcome limitations in specific T-cell activation used bispecific antibodies which target CD3 on effector cells and the tumour-associated antigen CA125, Her2/neu, or other tumour-associated antigens on tumour cells [27, 28]. Redirecting CIK cells from patients with ovarian cancer with bispecific antibodies increased lysis of primary ovarian cancer cells [27]. Most recently, CIK cells were modified with a CD33-specific CAR for targeting acute myeloid leukemia cells [29] and with

a CD19-specific CAR with 4-1BB costimulatory signal for targeting B-lineage acute lymphoblastic leukemia [29, 30].

5. Conclusion

CAR-mediated redirection of CIK cells from colon carcinoma patients improves their activation towards autologous tumour cells in an antigen-dependent fashion.

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Review Article

A Key Role for NF-κB Transcription Factor c-Rel in T-Lymphocyte-Differentiation and Effector Functions

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The transcription factors of the Rel/NF-κB family function as key regulators of innate and adoptive immunity. Tightly and temporally controlled activation of NF-κB-signalling pathways ensures prevention of harmful immune cell dysregulation, whereas a loss of control leads to pathological conditions such as severe inflammation, autoimmune disease, and inflammation-associated oncogenesis. Five family members have been identified in mammals: RelA (p65), c-Rel, RelB, and the precursor proteins NF-κB1 (p105) and NF-κB2 (p100), that are processed into p50 and p52, respectively. While RelA-containing dimers are present in most cell types, c-Rel complexes are predominately found in cells of hematopoietic origin. In T-cell lymphocytes, certain genes essential for immune function such as *Il2* and *Foxp3* are directly regulated by c-Rel. Additionally, c-Rel-dependent IL-12 and IL-23 transcription by macrophages and dendritic cells is crucial for T-cell differentiation and effector functions. Accordingly, c-Rel expression in T cells and antigen-presenting cells (APCs) controls a delicate balance between tolerance and immunity. This review gives a selective overview on recent progress in understanding of diverse roles of c-Rel in regulating adaptive immunity.

1. Introduction

c-Rel is a member of the Rel/NF-κB family of eukaryotic transcription factors, which also includes the proteins RelA (p65), RelB, NF-κB1 (p105/p50), and NF-κB2 (p100/p52). NF-κB transcription factors can form various homo- and heterodimers possessing unique specificities in regulating target gene expression [1]. Despite some redundancy, functional studies on mice lacking one or more NF-κB proteins revealed that distinct NF-κB subunits play specific role in regulating T-cell development and effector functions [2–5]. NF-κB complexes are held in the cytoplasm by interacting with a family of inhibitory proteins known as the IκB proteins. In general, binding of IκB proteins to NF-κB dimers masks the nuclear localization signals of NF-κB proteins and inhibits both, nuclear import of NF-κB complexes as well as binding to their specific DNA binding site (κB site) [6]. Activation of NF-κB complexes in T lymphocytes requires T-cell receptor (TCR) stimulation, which provides a signal for phosphorylation and degradation of IκB proteins via the ubiquitin-proteasome system in order to initiate nuclear translocation and DNA binding of active NF-κB dimers [7]. In the past decade, several studies have been conducted to identify genes that are directly regulated by the transcription factor c-Rel [8–12]. Despite extensive research, not all c-Rel-controlled genes have been identified yet. Most information about the role of c-Rel in T lymphocytes has come from *in vivo* analyses of c-Rel deficient mice suggesting an important function for this protein in regulating T cell development, differentiation, and effector function in thymus and peripheral lymphoid tissues. This review attempts to highlight various nonredundant physiological functions of c-Rel, particularly with regard to regulation of T-cell-mediated immunity.

2. c-Rel-Signalling Pathway in T Lymphocytes

Three main NF-κB activating pathways exist in mammalian cells [13]. The so-called canonical NF-κB pathway by which cytokines and other various signals initiate activation of

RelA/p50 and c-Rel/p50 heterodimers has been investigated in greatest detail. A central component in NF-κB regulation is a serine-specific IκB kinase (IKK), a complex composed of three subunits: IKKα (IKK1), IKKβ (IKK2), and IKKγ (NEMO). In T lymphocytes, the canonical pathway is triggered by TCR and CD28 engagement resulting in activation of IKKα/IKKβ/IKKγ complex. Following stimulation, activation of IKK results in phosphorylation of IκBs on specific serine residues, recruitment of the $SCF^{\beta\text{-TrCP}}$ ubiquitin ligase complex, rapid polyubiquitination, and subsequent degradation of IκB inhibitory proteins by the 26S proteasome [14]. IκBα is phosphorylated by IKKβ on two N-terminal serine residues, Ser32 and Ser36, which creates a binding site for the receptor subunit (β-TrCP) of specific ubiquitin E3 ligase SCF. Once liberated from IκB molecules, p65/50 and c-Rel/p50 dimers participate in the transcriptional regulation of distinct genes involved in adaptive immunity functions. In contrast, the alternative NF-κB activation pathway is induced by a subset of TNFR family members (e.g., LTβR and BAFFR) involving NIK and IKKα-mediated p100 processing and generation of transcriptionally active p52/RelB heterodimers [15, 16] (Figure 1). The major function of this pathway is related to the development and organization of secondary lymphoid organs (downstream of LTβR) and homeostasis of B cells (downstream of BAFFR) [6, 17]. Although T cells express a number of costimulatory TNFR family members such as OX40, CD30, and GITR that are assumed to induce processing of p100 via activation of NIK and IKKα homodimers [18, 19], it remains largely unclear how the alternative NF-κB pathway exactly regulates T-cell differentiation, effector functions, and memory responses. The third NF-κB pathway, also called p105 pathway, is initiated by IKKβ through phosphorylation of p105 precursor protein at Ser927 and Ser932. It uses the same IKK complex as the canonical pathway and its activation lead to complete degradation of the p105 molecule and release of docked molecules [14, 20, 21].

Since the discovery of NF-κB proteins 25 years ago, there have been many questions with respect to the selectivity and diversity of NF-κB functions. Novel studies have begun to reveal how the complex networks of positive and negative regulatory signals and crosstalk between activating pathways shape the NF-κB response in a cell-type-dependent and stimulus-specific way [17, 22–24]. In naïve T cells, TCR stimulation and subsequent IKKβ-dependent phosphorylation of IκBα lead to the nuclear translocation of active NF-κB dimers. The protein kinase C isozyme PKC-θ is a central molecule for recruiting additional factors required for IKK-mediated NF-κB activation in T cells. TCR-mediated activation of p65/p50 and c-Rel/p50 dimers in T cells includes activation of kinases of the Src and the Syk families. Furthermore, CD28 and TCR costimulation facilitates phosphorylation of CARMA1 and its recruitment into signalling complex with Bcl10 and MALT1 (CBM complex, Figure 1). Although not completely elucidated, mechanisms such as linear ubiquitination of NEMO and phosphorylation of IKKβ (probably by TAK1) lead to the activation of the IKK complex and phosphorylation of IκBs [16]. Cellular localization of NF-κB proteins is controlled by three IκB isoforms: IκBα, IκBβ and IκBε. Interestingly, the rate of degradation and resynthesis of each IκB isoform may vary in cell-specific way [25]. Whereas IκBα mediates rapid NF-κB activation and strong negative feedback loop regulation, IκBβ and IκBε allow a relatively stable NF-κB response by responding more slowly and acting to dampen oscillatory NF-κB activation profile [25–27]. An important question is how the closely related RelA and c-Rel proteins can operate distinctly in T lymphocytes and whether the inhibitory IκBs play a central role in these processes. Recent studies suggest that triggering the TCR/CD3 complex results in rapid translocation of active p65-containing dimers into the nucleus and slower activation of c-Rel complexes. As consequence, c-Rel-dependent gene transcription in T cells is slower as compared to p65-mediated responses. In unstimulated T cells, c-Rel is primarily associated with IκBβ, and the proportion of c-Rel bound to IκBα can be substantially increased after activation of cells with TNF-α and IL-1β [28, 29]. In particular, IκBα is degraded more rapidly than IκBβ and IκBε. Taken together, in naïve T cells, two members of the classical NF-κB activating pathway, p65 and c-Rel, seem to be differentially regulated by forming distinct complexes with IκBs. c-Rel dimers cannot be easily activated as c-Rel is mainly complexed to IκBβ. Costimulatory signals transmitted by CD80/86/CD28 and the presence of proinflammatory cytokines secreted by APCs increase IκBβ degradation and c-Rel is consequently shifted to IκBα-associated complexes [29].

Remarkably, turnover of c-Rel itself seems to be regulated by the ubiquitin-proteasome pathway adding another level of the complexity to its regulation [30]. A novel study has described that the E3 ubiquitin ligase Peli1 mediates polyubiquitination of c-Rel and subsequent degradation of this protein by the 26S proteasome. This prevents aberrant accumulation of c-Rel during T-cell activation. Interestingly, Peli1 deficiency in mice results in nuclear accumulation of c-Rel, T-cell hyperactivation, and spontaneous development of autoimmunity associated with multiorgan inflammation and production of autoantibodies [31]. This finding emphasizes that regulation of c-Rel expression in T cells might play an important role in the maintenance of peripheral T-cell tolerance.

3. Cell-Autonomous Role of c-Rel in T-Lymphocyte Differentiation

The differentiation of the $CD4^+$ T-cell lineage into T effector cells is a crucial prerequisite for a successful host immune defense against pathogens. Functional specialization is coordinated by a complex genetic network, initiated and terminated in a time-dependent manner. Several studies have attempted to identify transcriptional signatures and master transcription factors driving the differentiation of individual T-cell subsets. Recently, a discovery of huge range of the functional plasticity and heterogeneity of T-cells has drawn much attention [32, 33]. As several subpopulations have only been examined *in vitro*, it is still unclear if they

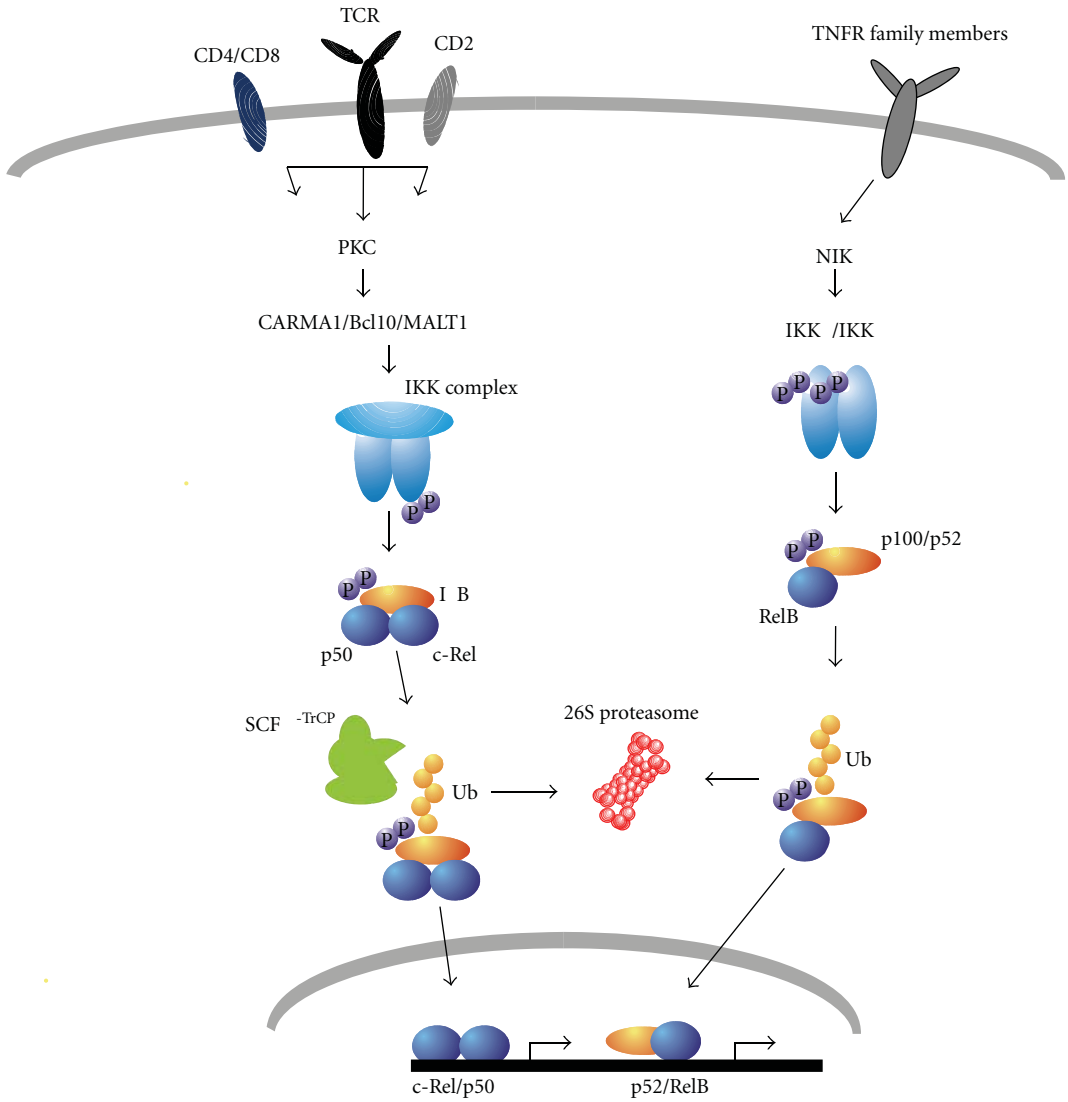

Figure 1: Canonical and alternative NF-κB activation pathways in T cells. Several signals are required for the activation of the canonical NF-κB-signalling pathway. The inhibitory IκB proteins typically bind to dimers of the NF-κB family such as p65/p50 (not shown in the figure) and c-Rel/p50 to generate inactive complexes that are sequestered in the cytosol. PKCθ is a central molecule for TCR-mediated NF-κB activation. Phosphorylation of CARMA by PKCθ results in formation of stable CARMA/Bcl10/MALT1 complex and activation of IKK. Activated IKKβ kinase mediates phosphorylation of IκB molecules and recruitment of SCF$^{\beta\text{-TrCP}}$ ubiquitin ligase. Activation of alternative NF-κB pathway is triggered by a subset of TNFR family members and is mediated by NIK and IKKα that phosphorylates p100. The ubiquitin-proteasome pathway is involved in activation of NF-κB via specific degradation of IκBs and processing of p100 to produce p52.

should be considered as distinct T cell subsets or whether expression of characteristic molecules is just an adaptation of already known and well-described Th1, Th2, and Th17 cells to certain microenvironment. This review aims to discuss the data that will allow us to understand how c-Rel influences the development and effector functions of most important T-cell subsets. Th1 and Th2 cells have distinct immunological functions by producing their key cytokines IFN-γ and IL-4, respectively. Recently, IL-17-producing cells that express transcription factors IRF-4 and RORγt, named Th17 cells, have been described to develop via a unique lineage, independently of the Th1 and Th2 master transcription factors T-bet and GATA-3 [34–37]. Another population of CD4$^+$ T cells, so-called follicular helper T cells (T_{FH}), preferentially reside in germinal centres where they help B cells to generate high-affine antibodies [38, 39]. Finally, regulatory T (Treg) cells are characterized by their expression of transcription factor Foxp3 and are essential for tolerance and prevention of autoimmunity [40, 41].

The transcription factor c-Rel has emerged to be an important molecule that can mediate proliferation, differentiation, and cytokine production of T cells. However, the extent and impact of the described defects in c-Rel-deficient T-cells vary considerably. Experiments with c-Rel-deficient mice have revealed that this protein is crucial for optimal IL-2 production and expression of IL-2Rα

(CD25) in T cells [42, 43]. Normally, immature T cells are unable to produce IL-2. However, once dendritic cells (DCs) encounter danger signals at the site of infection and get fully maturated, differentiation, of naïve $CD4^+$ T cells is driven effectively through antigen recognition, cytokine milieu, and costimulation by CD80 and CD86. In response to antigens, T cells start producing IL-2 and IL-2/IL2-R-signalling becomes crucial for their activation and expansion. In light of the finding that c-Rel complexes are mainly bound to IκBβ and that stimulation via CD28 leads to degradation of IκBβ and activation of c-Rel signalling pathway, it is evident why c-Rel-deficient T cells cannot respond appropriately to T-cell activation signals. With regard to activated naïve T cells, c-Rel signalling (acting downstream of TCR and CD28) may also be essential for secretion of other IL-2-dependent cytokines. IL-2 is known to be required for optimal IL-4 and IFN-γ expression by T-helper cells and for expression of granzyme and perforin by cytotoxic T lymphocytes (CTL) [44, 45]. Since c-Rel, AP-1, and NFAT act in concert to regulate IL-2 expression and T-cell proliferation, IL-2 secretion is reduced but not completely abrogated in c-Rel-deficient T cells. Thus, some defects in Th differentiation observed under *in vitro* polarizing conditions in the absence of c-Rel may indirectly result from decreased T-cell proliferation. Interestingly, in mature effector T cells that differ from naïve ones by producing cytokines more rapidly after TCR stimulation, IL-2 and IFN-γ gene expression seems to occur independently of c-Rel-mediated signal transduction [29].

It is likely that regulatory functions of c-Rel on target gene promotors are accomplished by heterodimerization with p50 or by forming c-Rel/c-Rel homodimers. There is also evidence that c-Rel/p50 dimers cooperate with other NF-κB family members. For example, c-Rel and p65 complexes bind together to IL-2Rα promoter and even cooperate with other transcription factors such as SRF to increase expression of IL-2Rα gene [46]. Recently, a c-Rel binding site was identified in proximal promoter of *Il21* gene implicating an important role for c-Rel in development of IL-21-dependent T and B subsets [47]. IL-21 has been reported to be essential for both T_{FH} development and regulation of B-cell function [38, 48–51]. Accordingly, the frequencies of T_{FH} cells and germinal centre (GC) B cells were significantly reduced in c-Rel-deficient mice immunized with $MOG_{35–55}$ [47]. We have also found reduced IL-21 production and GC formation in Peyer's patches of c-Rel-deficient mice (A. Visekruna, unpublished data). However, our recent unpublished results show that, at least in response to IL-6 stimulation, there was no significant difference between WT- and c-Rel-deficient $CD4^+$ T cells with respect to IL-21 production. This suggests that c-Rel might be involved in IL-6-independent signal transduction pathways leading to induction of IL-21 expression. Although c-Rel binds to the promoter of the *Il21* gene, many other transcriptional activators such as STAT-3, IRF-4, and NFATc2 seem to be more important for optimal *Il21* gene expression [52, 53].

More recently, c-Rel has been shown to control the differentiation of Treg cells in the thymus by promoting formation of so-called *Foxp3*-specific "enhanceosome [*sic*]" containing p65, Smad3, NFATc2, and CREB [54–58]. It has also become evident that c-Rel protein and RNA expression are specifically upregulated in $CD4^+CD25^+$ thymocytes as compared to other T-cell populations in the thymus indicating the importance of this factor for development and maintenance of emerging Treg population. Intriguingly, although c-Rel-deficient mice exhibit diminished Treg cell numbers, c-Rel appears to be dispensable for immune suppressive activity of Treg cells, as c-Rel-deficient Treg cells are able to inhibit T-cell proliferation *in vitro* and suppress development of T-cell-induced colitis [58]. Three highly conserved noncoding DNA sequences (CNSs) in the *Foxp3* locus have been identified and named CNS1-3. *In silico* analysis has revealed that c-Rel complexes but not p65 complexes bind to CNS3 region of *Foxp3* locus resembling the CD28 response element (CD28RE) in the *Il2* locus, also known to be occupied by c-Rel homodimers [59]. Given the importance of Foxp3 expression in Treg differentiation and effector functions, an interesting consideration point is to better understand how intracellular signalling molecules and adapters are involved in NF-κB activation in Treg population. Although engagement of TCR and IL-2 signalling is crucial for both thymic and peripheral development of Treg cells, the overall "quality" of peripheral signals may not mimic all facets of Treg development in thymus. While TCR signalling via c-Rel provides an instructive signal to open the *Foxp3* locus during thymic development, additional factors are probably involved in the generation of peripheral Treg (iTreg) cells. Very recently, we have demonstrated that, in the presence of TGF-β, the addition of exogenous IL-2 is sufficient to drive iTreg differentiation and to upregulate Foxp3 expression in c-Rel-deficient naïve $CD4^+$ T cells [60]. Further, our unpublished data suggest that *in vivo* treatment with immune complexes consisting of IL-2 and anti-IL-2 mAb (JES6-1) leads to a widespread increase in Treg cell frequencies not only in WT but also in c-Rel deficient mice. The paradoxical observation that frequencies of Treg cells increase substantially in c-Rel deficient mice implies that, at least in the periphery, control of the *Foxp3* locus by c-Rel is not required for maintaining the homeostasis and expansion of Treg cells. Interestingly, thymic and peripheral $CD4^+Foxp3^+$ Treg cell frequencies are also significantly reduced in mice deficient in upstream components of c-Rel-activating pathway such as PKC-θ, CARMA1, Bcl10, and MALT1 [56, 61–63]. It will be of interest to determine if iTreg cells generated from these mice induce Foxp3 after *in vitro* exposure to IL-2 and TGF-β similarly to c-Rel-deficient T cells. These findings collectively suggest that c-Rel has an important nonredundant function for Treg cells by inducing Foxp3 expression during thymic Treg cell development.

Additionally to its role in several $CD4^+$ T subsets, c-Rel might play an important role for $CD8^+$ T-cell function. One mechanism in particular is regulation of IL-2 production as consumption of this cytokine has a crucial influence on various aspects of $CD8^+$ T-mediated immunity. Current experimental data indicate that the PKC-θ/c-Rel-signalling axis is a crucial survival pathway in activated $CD8^+$ T lymphocytes. Interestingly, exogenous IL-2 can bypass survival and proliferative defects in PKC-θ- and c-Rel-deficient $CD8^+$ T cells [64]. Additionally, in the presence of

exogenous IL2, c-Rel-deficient CTL have normal cytotoxicity *in vitro*. *In vivo* studies have shown normal capacity of c-Rel-deficient $CD8^+$ T cells to clear influenza infection [65]. Major contribution of c-Rel to functional CTL responses might comprise regulation of the inflammatory environment (e.g., regulation of cytokines produced by APC and $CD4^+$ T cells) rather than playing substantial intrinsic role in cytotoxic T cells.

4. Crucial Role of c-Rel in Regulating Inflammation and Immune Defense against Microbial Pathogens

In vitro analyses of c-Rel-deficient cells have revealed selective requirement for c-Rel during IL-12 p40 induction in macrophages [66]. Similarly, p50/c-Rel dimers have been described to bind to the proximal promoter of IL-12 p35 and IL-23 p19 subunits in murine macrophages and DC [67–70]. Both proteins, IL-12 and IL-23, play a crucial role for the differentiation of T lymphocytes and immunity against pathogens. Importantly, maturation of DC is not affected in the absence of c-Rel, whereas the loss of this protein in APC compromises DC-mediated $CD4^+$ T-cell activation [71]. Thus, c-Rel appears to be a crucial link between innate immune signals and primary T-cell responses by substantially influencing a delicate balance between Th1, Th17, and Treg cells.

Complex *in vivo* functions of different NF-κB family members following exposure to pathogens remain partially controversial. Infected mice devoid of specific NF-κB proteins display distinct phenotypes probably reflecting the ability of individual members to regulate expression of different sets of target genes associated with innate and adoptive immunity. One of the fundamental immunological challenges is to understand how the immune system can decide what type of immune responses to launch against different classes of pathogens. The capacity of Th1 and Tc1 responses to protect against intracellular pathogens is well known. For example, the control of infection with protozoan parasite *Leishmania major* has been attributed to IL-12-mediated differentiation and expansion of $CD4^+$ Th1 cells with subsequent IFN-γ secretion, activation of infected macrophages, and NO-mediated killing of parasite. Two studies have shown that mice lacking c-Rel display a high susceptibility to *L. major* infection. The reduced levels of IL-12 p70 in DC as well as defective IFN-γ secretion by T cells and NO production by macrophages in both *L. major*-infected MyD88 and c-Rel-deficient mice suggest that the high susceptibility of such animals is dependent on TLR-induced activation of c-Rel-signalling pathway with subsequent development of IL-12-mediated protective Th1 response against *Leishmania* parasites [72–74]. One might assume that this mechanism displays a general dependency of protective Th1 immunity on c-Rel, particularly involving regulation of IL-12 production by this transcription factor in APC. Although the failure of c-Rel deficient mice to control infection with another intracellular parasite *Toxoplasma gondii* was also associated with defective Th1 responses, in contrary to infection with *L. major*, this effect appears to be rather dependent on T-cell-intrinsic expression of c-Rel [75]. Thus, the evidence that c-Rel is essential for the production of IL-12 in response to LPS and *Leishmania*, but dispensable for IL-12 production in response to *Toxoplasma,* suggests that this transcription factor is associated with various complex aspects of regulation of innate and adaptive responses required to control infections [76].

There are emerging insights that c-Rel might play a key role in inflammatory diseases. Recent studies from several groups have shown that c-Rel is essential for the development of both colitis as well as experimental autoimmune encephalomyelitis (EAE). Impaired Th1 and Th17 development seems to occur in parallel with protection from EAE in c-Rel-deficient mice [47, 77, 78]. While potentially multiple roles of c-Rel in the inductive and effector stages of EAE are still partially elusive, its innate function in the control of proinflammatory responses during an intestinal inflammation is well known [79, 80]. A defect in the intestinal epithelial barrier function is an important etiologic factor leading to development of inflammatory bowel disease (IBD) in humans. After encountering microbial agents, activation of c-Rel in DC leads to induction of IL-23 and IL-12 expression. IL-23 strongly enhances production of IL-17 by previously primed $CD4^+$ T cells and probably by recently described innate lymphoid cells (ILCs) [81]. The regulation of IL-23 by c-Rel within APC has a critical role in mediating chronic intestinal inflammation. A recent genetic study in humans and several studies in mice have uncovered IL-23 as a key factor in the pathogenesis of Crohn's disease [82–85]. The role of c-Rel and other NF-κB family members can be regulated at many different levels. Two very recent studies have provided important clues to the underlying mechanisms of Th17-cell mediated diseases, showing that c-Rel is required for RORγt expression in T cells [78, 86]. Therefore, both c-Rel expressed by $CD4^+$ T cells regulating directly the expression of a Th17 lineage-specific transcription factor RORγt as well as c-Rel expression by myeloid cells contribute to differentiation and maintenance of Th17 cells. Results obtained from mouse models and human specimens show that, besides c-Rel-mediated Th17 cell differentiation, IFN-γ-mediated induction of immunoproteasomes has an important role for activation of NF-κB and enhancement of chronic inflammation in the gut [80, 87, 88]. Collectively, induction of inflammation in the gut caused by imbalanced activation of DC expressing high level of c-Rel and immunoproteasomes contributes to IBD by augmenting proinflammatory Th1 and Th17 responses (Figure 2). Novel data have also indicated that, in addition to T cells, ILC might be important factors driving intestinal inflammation in mice and humans [89, 90]. However, the role of NF-κB transcription factor c-Rel in regulating various effector functions of these cells has not been characterized yet.

5. Conclusions and Future Directions

In last 25 years, major steps forward have been made in understanding how NF-κB regulates different aspects of the

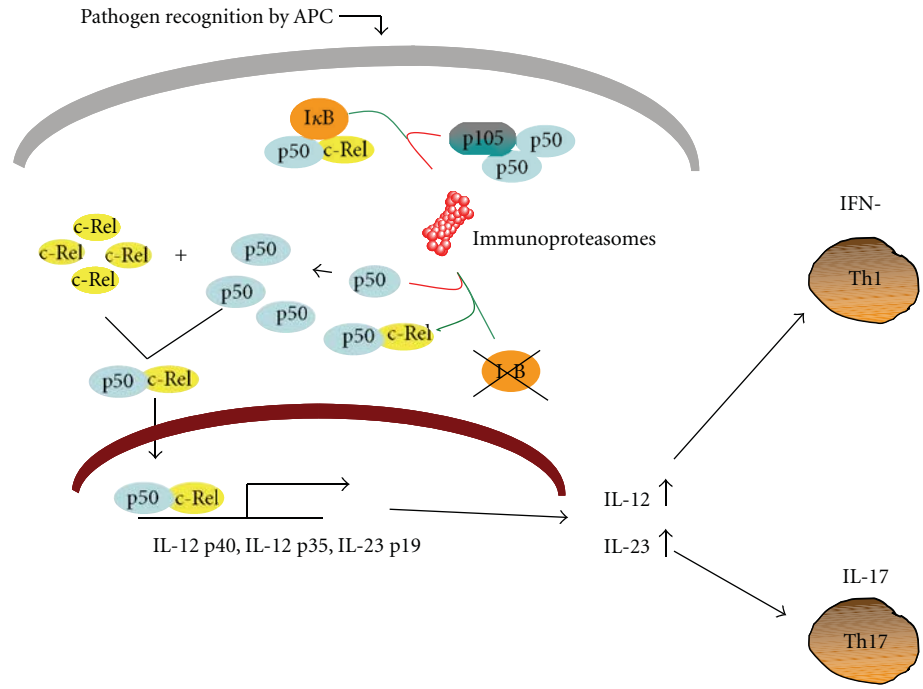

Figure 2: Induction of immunoproteasomes and c-Rel signalling in APC during intestinal inflammation. After disruption of intestinal barrier, the activation of DC by innate immunity triggers such as TLR ligands results in signalling cascade that induces expression of immunoproteasomes. The immunoproteasome is highly active form of proteasome that enhances activation of NF-κB signalling. Additionally, stimulation of TLR releases c-Rel/p50 dimers from IκB to bind to the p40, p35, and p19 promoter. The synergy between immunoproteasomes and c-Rel leads to an increase of IL-12 and IL-23 secretion by APC contributing directly to T-cell-mediated immune responses and exacerbation of intestinal inflammation.

immune system. Several studies have begun to examine the role of specific NF-κB family members in regulating infections and chronic inflammatory disorders. c-Rel has emerged to play a critical role in inducing inflammatory and immune responses against pathogens by regulating a crucial set of T-cell stimulatory genes. In addition to dominant effects of c-Rel on promoting Th1- and Th17-mediated immune responses, this transcription factor also plays an important role by providing an initial signal for opening of the *Foxp3* locus in thymic Treg cells. Although the impact of c-Rel on DC activities to induce Th2 responses has not been examined extensively, c-Rel-deficient mice seem to be capable of mounting sufficient Th2 responses. Previous studies suggest that this protein is not essential for control of Th2-mediated intestinal inflammation following *Trichuris muris* infection. In contrast, NF-κB1- and NF-κB2-deficient mice fail to clear helminth infections [91]. Such data reflect nonoverlapping functions of individual NF-κB family members suggesting that targeting specific NF-κB proteins might be a promising therapeutic approach in inflammation and infectious diseases. Especially, molecules that specifically regulate c-Rel-signalling pathway such as E3 ubiquitin ligase Peli1 might be of particular interests as c-Rel exhibits a unique dual capacity to regulate both tolerogenic and inflammatory responses.

Abbreviations

APC: antigen presenting cell(s)
BAFF: B-cell-activating factor
Bcl10: B-cell lymphoma 10
CREB: cAMP response element-binding protein
DC: dendritic cell(s)
GC: germinal centre
ICAM: intracellular adhesion molecule
IFN: interferon
IκB: inhibitory-κB protein
IKK: IκB kinase
ILC: innate lymphoid cell(s)
IRF: interferon regulatory factor
LT: lymphotoxin
mAb: monoclonal antibody
MALT: mucosa-associated lymphoid tissue lymphoma translocation protein 1
MAPK: mitogen-activated protein kinase
NEMO: NF-κB essential modifier
NFAT: nuclear factor of activated T cells
NF-κB: nuclear factor-κB
NIK: NF-κB inducing kinase
NO: nitric oxide
PKC: protein kinase C
RORγt: retinoic acid-related orphan receptor γt
Ser: serine

SRF: serum response factor
STAT: signal transducer and activator of transcription
TCR: T-cell receptor
TGF: transforming growth factor
TLR: toll-like receptor.

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Review Article

From Murine to Human Nude/SCID: The Thymus, T-Cell Development and the Missing Link

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Primary immunodeficiencies (PIDs) are disorders of the immune system, which lead to increased susceptibility to infections. T-cell defects, which may affect T-cell development/function, are approximately 11% of reported PIDs. The pathogenic mechanisms are related to molecular alterations not only of genes selectively expressed in hematopoietic cells but also of the stromal component of the thymus that represents the primary lymphoid organ for T-cell differentiation. With this regard, the prototype of athymic disorders due to abnormal stroma is the Nude/SCID syndrome, first described in mice in 1966. In man, the DiGeorge Syndrome (DGS) has long been considered the human prototype of a severe T-cell differentiation defect. More recently, the human equivalent of the murine Nude/SCID has been described, contributing to unravel important issues of the T-cell ontogeny in humans. Both mice and human diseases are due to alterations of the FOXN1, a developmentally regulated transcription factor selectively expressed in skin and thymic epithelia.

1. Introduction

Primary immunodeficiencies (PIDs) are severe disorders of the immune system in which patients cannot produce a proper protective immune response, leading to an increased susceptibility to infections. Nowadays, more than 200 well-characterized genetic immune deficiencies have been identified thanks to the advances in molecular genetics and immunology. PIDs are classified according to the component of the immune system that is primarily involved including T, B, natural killer (NK) lymphocytes, phagocytic cells, and complement proteins [1].

Primary T-cell defects are rare disorders, accounting for approximately 11% of reported PIDs [2]. These diseases may be considered true experiments of the nature in that the recognition of the molecular mechanisms underlying their pathogenesis led to clarify the phases of the T-cell differentiation process and the physiological mechanisms of the T-cell responses. Studies in this field led to unravel the checkpoints, which play a pivotal role in these processes, which mostly rely on a proper intercellular interaction between thymocytes and the thymic microenvironment.

2. T-Cell Development and Thymus

The thymus is the primary lymphoid organ that supports T-cell differentiation and repertoire selection [3, 4]. The intrathymic development of T cells consists of several phases that require a dynamic relocation of developing lymphocytes within multiple architectural structures of this organ. As shown in Figure 1, these steps are (1) the entry of lymphoid progenitor cells into the thymus, (2) the generation of $CD4^+$ $CD8^+$ double positive (DP) thymocytes in the cortex, (3) the positive selection of DP thymocytes in the cortex, and (4) the interaction of positively selected thymocytes with medullary thymic epithelial cells (mTECs) to complete the thymocyte maturation and, eventually, the export of mature T cells from the thymus [5].

Thymus anlagen arises as bilateral structures from the third pharyngeal pouch in the embryonic foregut [6, 7]. The interaction of the epithelial component with the lymphoid progenitor takes place as early as embryonic day 11.5 in mice and at the eighth week of gestation in humans [8, 9].

At an early stage, these precursors have both lymphoid and myeloid potential [10, 11] and are characterized by

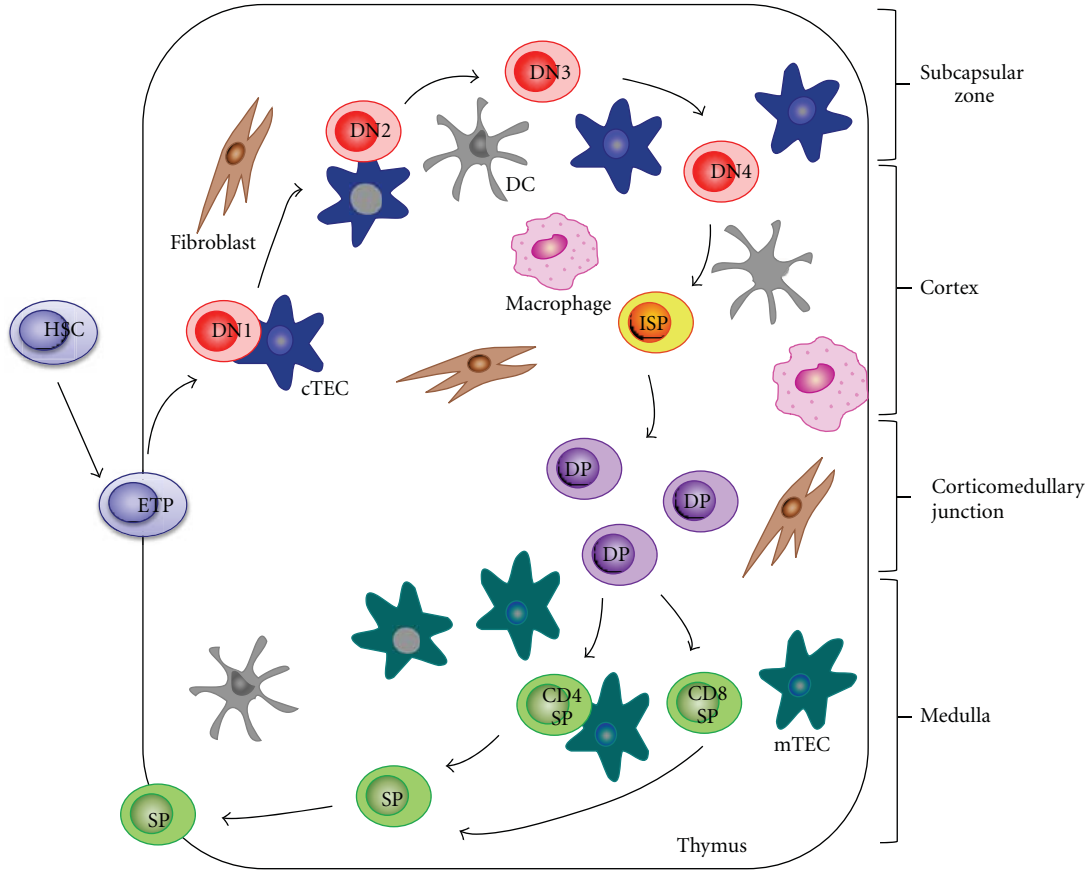


Figure 1: Steps of T-cell development. The lymphoid progenitor cell goes into the thymus through the cortico-medullary junction. DN thymocytes ($CD4^-CD8^-$) migrate across the subcapsular region and then the outer cortex. Interaction between DN cells and cTECs generates DP thymocytes ($CD3^+CD4^+CD8^+$). Positively selected thymocytes interact with mTECs to complete the maturation process. In the medulla, self-reactive thymocytes are deleted, SP ($CD3^+CD4^+$or $CD3^+CD8^+$) thymocytes are generated, and, eventually, the export of mature T cells from the thymus takes place.

the expression of the CC-chemokine receptor 9 (CCR9), that, along with the CCR7, plays a central role in this precocious stage of thymus colonization. At this stage of differentiation, lymphoid cells also express the stem- and progenitor-cell markers KIT (also known as CD117), the stem-cell antigen-1 (SCA-1), and the growth-factor-receptor tyrosine kinase type 3 (FLT3) [12–14].

Following the entry into the thymus through the corticomedullary junction, lymphoid progenitor cells begin their commitment toward the T-cell lineage. The developmental pathway is traditionally divided into three subsequent steps, as defined by peculiar immunophenotypic patterns: the $CD4^-CD8^-$ double negative (DN) stage, the $CD4^+CD8^+$ double positive (DP) stage, and the $CD4^-CD8^+$ or $CD4^+CD8^-$ single positive (SP) stage. In mice, an immature single positive (ISP) $CD8^+CD4^-$ cell may be detected between the DN and DP stages. This population can be easily distinguished from the mature SP cell by the high levels of expression of T-cell receptor (TCR) β and CD3 and the low level of CD24 (heat stable antigen, HSA). DN cells in mice can be further subdivided based on the expression of CD44 and CD25 in the following populations: $CD44^+$ $CD25^-$(DN1), $CD44^+CD25^+$ (DN2), $CD44^-CD25^+$(DN3), and $CD44^-CD25^-$(DN4) [15].

From the early T-cell lineage progenitor (ETP) stage to the double-negative 3 (DN3) stage, T-cell differentiation is independent from the TCR and is dependent on the migration through the distinct thymic structures [16]. These phases are regulated by the expression levels of specific transcription factors and by a fine tuned interplay between them (Figure 1).

At the beginning, ETPs and DN2 cells exhibit a high proliferative capability. Differently, at the DN3 stage, when a fully rearranged TCR occurs, the proliferation stops. In the initial thymocyte development till the DN3 stage, Notch-mediated signals play a pivotal role [17, 18] also supported by signals delivered through the interleukin-7 receptor (IL-7R) [19, 20].

The immature thymocytes journey through the thymus has also the additional effect of promoting the differentiation of thymic stromal precursors into mature thymic epithelial cells, thus playing an important role in the formation of

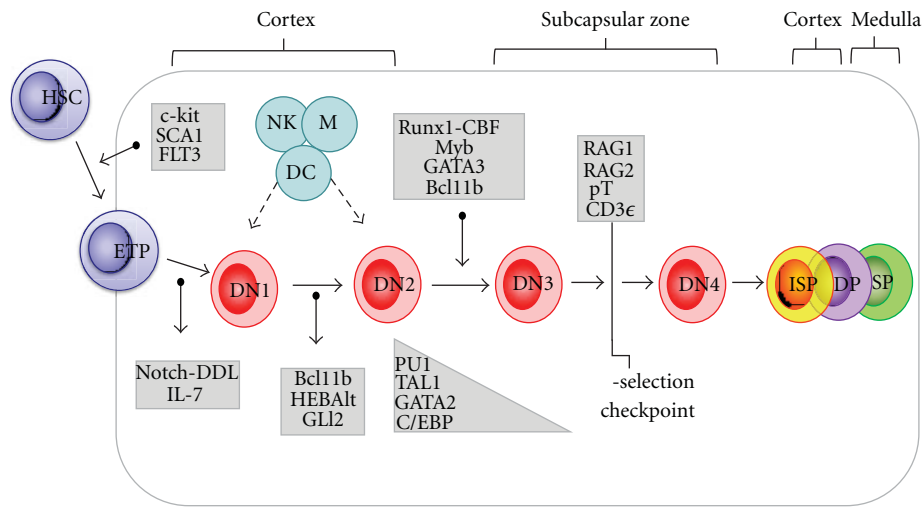


Figure 2: Differential gene expression profile, which modulates the discrete stages of the T-cell development. The lymphoid progenitors, entering into thymus and expressing the markers of HSCs, are primed to Notch and IL-7 signaling until DN1 stage. During the transition DN1/DN2, immature thymocytes lose multilineage potential through the downregulation of genes involved in the differentiation towards other cellular lineages, as PU.1, TAL1, GATA-2, and C/EBPα. At the DN2 stage, Myb, GATA-3, HEBalt, GLI-2, and Bcl-11b are upregulated. At the DN3 stage, the genes required for a proper TCR assembly as Rag-1, Rag-2, and pTα are expressed, thus leading to the β-selection. Following β-selection check-point, DN4 cells are fully committed to the TCR$\alpha\beta^{+}$ T-cell lineage.

the thymic microenvironment [21–24]. In particular, thymocytes during the DN1-DN3 stages participate to the differentiation process of TEC precursor cells into cortical TECs (cTECs).

The DN1 cell thymocytes keep the potential to differentiate into B, T, myeloid, NK, and dendritic cells (DCs) [25–27]. The transition to DN2 is characterized by the upregulation of a number of genes involved in the process, including genes needed for rearrangement and/or expression of the pre-TCR signaling complex components (Figure 2) [28]. At this stage, the thymocytes lose the multilineage potential due to silencing of genes involved in the differentiation towards other cellular lineages. Nevertheless, this potential is not completely lost, since cells with the DN2 phenotype can still differentiate into NK cells, DCs, or macrophages under certain circumstances [29, 30].

DN2 stage T cells are fully responsive to IL-7 and SCF due to the high expression of IL-7Rα and c-kit. The DN2 stage is characterized by the upregulation of CD25 molecule (interleukin-2 receptor α, IL-2Rα) and CD90 (Thy-1) [28]. Moreover, the genes which favor the myeloid, NK, and dendritic fate, so-called T-cell antagonists, as PU.1, stem-cell leukemia (SCL also known as TAL1), GATA binding protein-2 (GATA-2), and CCAAT-enhancer binding protein α (C/EBPα) are silenced before that β or $\gamma\delta$ selection takes place (Figure 2) [31]. During this phase only a few transcription factors, including the zinc-finger transcription factor, the tumor suppressor factor B-cell lymphoma/leukemia 11b (BCL-11b) [32], basic helix-loop-helix (bHLH) transcription factors alternative (HEBalt) [33], and, more transiently, glioma-associated oncogene 2 (GLI-2), a transcription factor involved in the sonic hedgehog signaling [34], are expressed (Figure 2).

The following DN2 to DN3 stage transition requires the expression of different arrays of genes, as Runt-related transcription factor 1-Core binding factor β (Runx1-CBFβ) complexes, the transcription factor Myb, GATA-3, and Bcl-11b, which allow full TCRβ gene rearrangement in thymocytes, that become competent to undergo β-selection [35–37]. Several important events occur during the DN2/3 transition, as the induction of recombinase activating gene-1 (Rag-1) and Rag-2, the upregulation of pre-Tα (pTα), and the rearrangement of TCRδ and γ. CD3ε and IL-7Rα (CD127) are also upregulated at this phase [38] along with the turn-on of the *lck* tyrosine kinase implicated in the pre-TCR and TCR signaling [39]. At this point, T-cell precursors lose their capability to follow a non-T-cell fate choice [28].

The cells overcoming β-selection express the pre-TCR complex on their surface and reach the DN3 stage [40]. Thereafter, the E-proteins E2A and HEB play a crucial role in several processes and are required for the progression of the T-cell development. In fact, these proteins are involved in the TCR gene rearrangement [41], in conferring the competence to undergo β-selection, and in the arrest of thymocyte proliferation at the DN3 stage [42].

At the DN3 stage, pre-TCR signaling results in the downregulation of CD25, pTα, Rag-1, and Rag-2, which leads to the appearance of DN4 cells. These cells are fully committed to the $\alpha\beta$ T-cell lineage [43, 44]. After β-selection, the thymocytes, which have properly rearranged TCRβ chains, show a burst of proliferation and a subsequent upregulation of CD8 and then CD4. At this point, the cells become double positive (DP). Eventually, DP cells rearrange TCRα gene, leading to TCRα assembly into a TCR complex.

The newly generated DP thymocytes are localized in the cortex and express low levels of the TCR$\alpha\beta$ complex. This

DP population consists of T cells with an unselected repertoire [45, 46]. Following that, positive and negative selections take place. In the cortex, the DP thymocytes interact through their TCR with peptide-MHC complexes expressed by stromal cells, as cTECs and dendritic cells [47]. When TCR interacts with low-avidity with the peptide-MHC ligands, DP thymocytes receive survival signals. This process, referred to as positive selection, allows "productive" T cells to potentially react to foreign antigens, but not to self-antigens [5]. Lately, positively selected DP thymocytes are ready to differentiate into SP cells, that is, $CD4^+CD8^-$ or $CD4^-CD8^+$ and relocate into the medulla. At this site, newly generated SP thymocytes are further selected by the medullary stromal cells, including autoimmune regulator- (AIRE-) expressing mTECs. The cells which are reactive to tissue-specific self antigens are deleted, thus avoiding autoimmunity [5]. SP thymocytes egress from the thymus as recent thymic emigrants (RTEs), naïve cells expressing the CD62 ligand (CD62L), also known as lymphocyte- (L-) selectin, CD69, and the CD45RA isoform. These RTE cells are fully mature T cells that exert proper functional capabilities of cell-mediated immunity [48–50].

3. Pathogenetic Mechanisms of T-Cell Defects

Most of the pathogenic mechanisms underlying primary T-cell disorders are related to molecular alterations of genes selectively expressed in hematopoietic cells. However, since the differentiation process requires a crosstalk among thymocytes and thymic microenvironment, a severe T-cell defect may also be due to alteration of the stromal component of the thymus.

T-cell disorders include a wide spectrum of disorders that affect T-cell development and/or function. The severity of the T-cell defect varies a lot ranging from the syndrome of severe combined immunodeficiency (SCID), characterized by a complete absence of T-cell functions to combined immunodeficiency disorders, in which there are a low number of T cells whose function is not adequate [51].

SCIDs comprise a heterogeneous group of monogenic disorders characterized by a virtual lack of functional peripheral T cells. To date, more than 20 different genetic defects involved in the pathogenesis of SCID in humans have been identified [52, 53]. Typically, patients with SCID show a severe defect in T-cell differentiation and a direct or indirect impairment of B-cell development and function. On the basis of the involvement of different cell lines in the pathogenesis of the disease and of the subsequent different clinical phenotypes, SCIDs have been till now classified according to the presence or absence of T, B, and NK cells (Table 1). Impaired survival of lymphocyte precursors is observed in reticular dysgenesis (RD) and in adenosine deaminase (ADA) deficiency. In RD the mutations of the adenylate kinase 2 gene (AK2) result in increased apoptosis of myeloid and lymphoid precursors. As a consequence, patients with RD show marked lymphopenia and neutropenia [54, 55]. ADA deficiency is characterized by the accumulation of high intracellular levels of toxic phosphorylated metabolites of adenosine and deoxyadenosine that cause apoptosis of lymphoid precursors in the bone marrow and thymus [56, 57].

Table 1: SCIDs classification. SCIDs have been so far classified according to the presence or absence of T, B, and NK cells, as a consequence of different molecular defects.

Lymphocyte phenotype	Gene defect	Form of SCID
$T^-B^-NK^-$	Adenylate kinase	Reticular dysgenesis
	Adenosine deaminase	ADA deficiency
$T^-B^+NK^-$	IL-2Rγ	SCID-X1
	Jak3	SCID-AR
$T^-B^+NK^+$	IL-7Rα	IL-7Rα deficiency
$T^-B^-NK^+$	Rag-1 or Rag-2 artemis	Omenn syndrome Artemis deficiency

The majority of SCIDs in human subjects derive from alterations of the cytokine-mediated signaling apparatus. SCID-X1 represents the most common form of SCID and is caused by mutations of the IL-2 receptor γ gene (IL-2Rγ), which encodes for the common γ-chain (γ-c) shared by cytokine receptors, including those for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. Patients usually have few or no T and NK cells but a normal or elevated number of B cells which fail to produce immunoglobulins normally [58]. γ-c also plays effects on cell cycle control and participates to the growth of tumoral cells, as well [59, 60]. Defects of JAK3, an intracellular tyrosine kinase physically and functionally coupled to γ-c, result in a syndrome whose immunologic phenotype is undistinguishable from that of SCID-X1 [61]. Mutations in the gene encoding for the α-chain of the IL-7R abrogate T lymphocyte development but leave B and NK cell development intact [62]. Mutations in critical genes needed for the expression of pre-T-cell receptor, as Rag-1 and Rag-2, result in a functional inability to form antigen receptors through genetic recombination, compromising the production of functional T cells. These proteins recognize recombination signal sequences and introduce a DNA double-stranded break, permitting V, D, and J gene rearrangements [63, 64]. Lymphocyte phenotype differs from those of patients with SCID caused by γ-c, Janus kinase-3 (Jak-3), IL-7Rα, or ADA deficiencies in that they lack both B and T lymphocytes since pre-TCR and pre-B-cell receptor (BCR) share similar molecular mechanisms requiring Rag-1 and 2 expression [65]. Defects of pre-TCR and pre-BCR expression might also reflect mutations in genes that encode proteins involved in nonhomologous end-joining (NHEJ) and DNA repair and, in particular, Artemis, DNA protein-kinase catalytic subunit (DNA-PKcs), Cernunnos/XLF, and DNA ligase IV [65–69]. In all these diseases, the generation of both T and B lymphocytes is severely compromised. However, it should be noted that a functional T-cell defect may also be due to infections [70, 71] or during the reconstitution phase following stem cell transplantation [72].

It is noteworthy that all the genes whose alterations lead to the above mentioned forms of SCID selectively impair

the lymphocyte functionality and the ability of these cells to proceed in the developmental pathway. In some cases, as in the case of TrkA mutation [73], the gene has pleiotropic effects resulting in complex multisystemic disorders associated to immunodeficiency.

4. The Murine Model of Athymia: nu/nu Mice

The first example of SCID not primarily related to a hematopoietic cell abnormality but rather to an intrinsic thymic epithelial cell defect is the Nude/SCID phenotype, whose identification contributed to unravel important issues of T-cell ontogeny.

The "nude" phenotype, identified for the first time in mice, results from inactivating mutations in a single gene, originally named winged-helix-nude (whn) and recently known as forkhead box n1 (foxn1) [74]. This murine model was described by Flanagan in 1966, when spontaneously appeared in the Virus Laboratory of Ruchill Hospital in Glasgow (UK) [75–77]. Mice homozygous for the mutation "nude" are hairless, have retarded growth, decreased fertility, and die by 5 months of life for infections. The hairlessness is due to the coiling of the incomplete hair shafts in the dermis caused by the absence of free sulfhydryl groups in the midfollicle region [78]. The "nude" *foxn1* gene does not affect the growth of hair follicles, but the epidermal differentiation process, regulating the balance between proliferation and differentiation of keratinocytes in the hair follicle [79, 80]. The "nude" mice are affected by severe infertility and show small ovaries with low egg counts in the females and no motile sperm in the males [78]. This condition may be the result of changes in hormonal status, as demonstrated by altered serum levels of estradiol, progesterone, and thyroxine [81]. The thymus is absent at birth [82] and there are very few lymphocytes in the thymus dependent areas of the spleen and lymph nodes [83].

Since the abnormal, or even absent, thymus is the hallmark of the "nude" phenotype, these animals develop a profound T-cell deficiency and a severely impaired immune response of either cell-mediated and, indirectly, humoral immunity. In "nude" mice, when the thymus is present in the first days of life**,** it reveals no normal structure, consisting of a thymic rudiment composed of vesicles or canaliculi delimited by epithelial-like cells, with no trace of lymphoid cells. By the day 14, the "nude" thymus is much smaller compared to the normal [84].

Nu/nu mice show lymphopenia and also low immunoglobulin levels. In the absence of normal T cells originated from the thymus, the development of the antibody forming cells is delayed, although "nude" mice do not lack precursors of antibody forming cells. This indicated that antibody forming cells may mature in the absence of the thymus, albeit at a slower rate [85]. In "nude" mice lymph nodes, the outer cortex with primary nodules and the medullary cords are normal. In the spleen sections from the nu/nu mice, the proportion of red to white pulp is greater than normal and, in some cases, an unusually high number of megakaryocytes are seen in the red pulp. In some spleens, Malpighian follicles, although present, are fewer and smaller than in controls and a depletion of lymphocytes is constant in the close proximity of the central arteriole in the thymus-dependent area. The depletion in the splenic thymus-dependent areas is not as prominent as in the lymph nodes [83]. In man, the prototype of an athymic disorder has long been considered the DiGeorge's Syndrome (DGS), even though main features of athymic murine model and human disease, including immunological signs, are not completely overlapping.

5. The Athymic DiGeorge Syndrome

The DGS, along with velocardiofacial syndrome and conotruncal anomaly face syndrome, is frequently associated to a common heterozygous intrachromosomal deletion in 22q11.2. However, a DGS-like phenotype can have alternative etiologies, including maternal diabetes, fetal alcohol syndrome, and teratogenesis, even though the molecular mechanisms underlying these forms are still unknown [86]. DGS has an estimated incidence of 1 in 4000 live births [87, 88] and, thus, it is the most common microdeletion syndrome in humans and the second most common chromosomal disorder after Down's syndrome. The deletion is due to a meiotic nonallelic homologous recombination between flanking 250 kilobases (kb), mapping in 22q11.2 chromosomal region and consisting in low-copy repeats/segmental duplications in the termed LCR22 [89, 90]. Although most cases of DGS occur as *de novo* deletions, approximately 5% of cases are inherited as an autosomal dominant trait [91–93]. In the 90% of patients, a hemizygous 3 Mb deletion, containing about 30 genes [89, 90, 94, 95], is found, whereas approximately 8% of patients carry a smaller deletion of 1.5 Mb, encompassing 24 genes [96], even though no difference in the clinical presentation is appreciable in the smaller deletion [86].

The main features of this syndrome are mild facial dysmorphism, submucous cleft palate, velopharyngeal insufficiency, speech delay, recurrent infections, variable immunodeficiency secondary to thymic aplasia or hypoplasia, and cardiac anomalies [97, 98]. Most of the patients have learning disabilities and behavioral disorders, including schizophrenia in some cases [99–102]. Children with the DGS, according to the aplasia or hypoplasia of the thymus**,** are classified as complete or partial DGS. The "complete" form represents a small percentage of patients, accounting to the 0.5% of all patients. These patients show a severe combined immunodeficiency phenotype with near absent T lymphocytes. The majority of patients have a "partial" phenotype and an immune defect usually manifesting as mild to moderate T lymphocytopenia. The T-cell proliferation is usually normal or in very few cases low normal. These patients have been reported to have a moderate increase of the number of infections than predicted on the basis of the immunological impairment, suggesting that anatomical defects, gastroesophageal reflux, allergies, cardiac disease, and poor nutrition may also contribute to recurrent infections [103]. It should be underlined that never "partial" DGS patients have severe infections as reported in SCID and, moreover, T-cell proliferation is usually normal. A moderate CD4 lymphocytopenia with low to normal CD8 T lymphocytes is usually found. An age-related decrease of T lymphocytes is also seen in DGS patients. TCR

repertoire analysis in 22q11.2 deletion patients has shown significant oligoclonal peaks and Vβ family dropouts when compared to controls. In a study of nine patients with a negative infectious history, a decreased diversity in $CD4^+$ and $CD8^+$ TCR repertoire, using both flow cytometric and third complementarity determining region (CDR3 spectratyping) fragment analysis, has been documented [104]. In another study, the spectratyping showed alterations in the repertoire, which, however, improved over the time [105].

Immune deficiency in these patients seems to be associated to an increased incidence of autoimmune diseases [106–108], in particular cytopenias [109, 110], arthritis [111], and endocrinopathies [112].

The chromosomal region usually deleted contains several genes, which may be candidate of the DGS phenotype. TBX1, which belongs to the family of T-box transcription factors, which share a common DNA binding domain is called "T-box" [113]. A specific role for Tbx1 in DGS and thymus development came out from the peculiar expression pattern in both the third pharyngeal pouch endoderm and the adjacent mesenchyme and not in the neural crest cells [114]. Furthermore, the homozygous loss of *Tbx1* causes thymic hypoplasia, as well [96, 115–117]. Of note, mice heterozygous for a null allele of *Tbx1* demonstrate only a mild phenotype without thymus anomalies [118]. Thus, evidence would suggest, at least in mice, that gene dosage of *Tbx1* is crucial in the pathogenesis of DGS. However, in the same region there are other genes potentially implicated in the pathogenesis of DGS, such as *Crkl*, which encodes an adaptor protein implicated in growth factor and adhesion molecule signaling. Homozygous *Crkl* gene deletion results in multiple defects in neural crest derivatives including aortic arch arteries, thymus, and craniofacial structures [96] and in prenatal death. However, the deletion at the heterozygous state does not cause any clinical sign, thus indicating that a combination of gene alterations is needed for the full expressivity of the phenotype [119].

6. The Human Nude/SCID Phenotype

The human equivalent of the "nude" murine phenotype was first described in two sisters in 1996, after more than 30 years from the initial mouse description and, subsequently, associated to *FOXN1* gene alterations.

The human Nude/SCID is an autosomal recessive disorder [120], whose hallmark is the T-cell immunodeficiency due to the complete absence of the thymus. This immunodeficiency presents in a quite similar fashion to the classical SCID phenotype, thus being more severe than DGS. Along with the severe infections, other features of the syndrome are ectodermal abnormalities, as alopecia and nail dystrophy [121]. Of note, the nail dystrophy can be observed also in subjects carrying the genetic alteration in heterozygosity. The most frequent nail alteration is the koilonychia (spoon nail), characterized by a concave surface and raised edges of the nail plate, associated with significant thinning of the plate itself; a canaliform dystrophy associated to a transverse groove of the nail plate (Beau line) may also be found (Figure 3). However, the most specific phenotypic alteration is leukonychia, characterized by a typical arciform pattern resembled to a half-moon and involving the proximal part of the nail plate. These alterations of digits and nails have also been reported in a few strains of "nude" mice. FOXN1 is known to be selectively expressed in the nail matrix where the nail plate originates, thus confirming that this transcription factor is involved in the maturation process of nails and suggesting nail dystrophy as an indicative sign of heterozygosity for this molecular alteration [121].

Interestingly, additional studies have also reported on anomalies of brain structures, suggesting a potential role of this transcription factor in brain embryogenesis, as also suggested by its expression in epithelial cells of the developing choroids plexus, a structure filling the lateral, third, and fourth ventricles. However, the severe neural tube defects, including anencephaly and spina bifida, have been only inconstantly reported, thus probably indicating that the genetic alteration represents a cofactor and is not sufficient *per se* to alter brain embryogenesis. The anomalies of brain structure have been considered potentially responsible for the high rate of mortality *in utero* observed in the geographic area with the high frequency of *FOXN1* alteration [122].

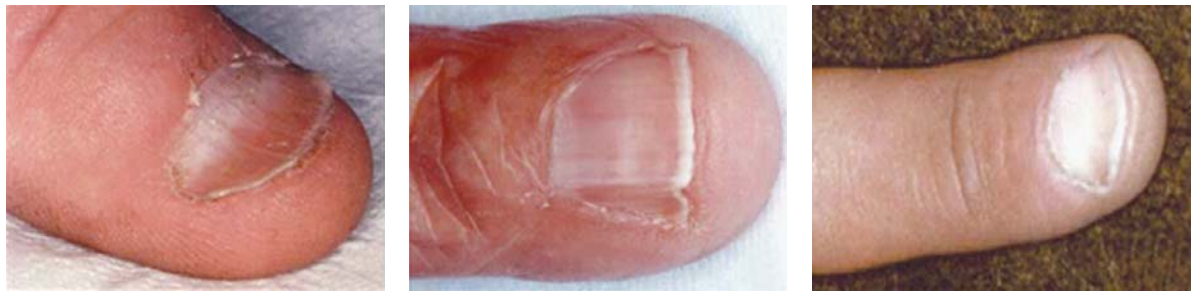
Prenatal alteration of the *FOXN1* gene in humans prevents the development of the T-cell compartment as early as at 16 weeks of gestation [123]. By contrast, stem cells, B, and NK lymphocytes are normal. $CD4^+$ cells are more affected than $CD8^+$ cells, even though the latter are also profoundly reduced. No $CD4^+CD45RA^+$ naive cells can be usually found [123]. CD8 cells coexpressing CD3 are very scarce and a few $CD3^+CD8^+CD45RA^+$ naïve cells can be detected [123]. Overall, a substantial reduction of T cells bearing TCR$\alpha\beta$, but not of lymphocytes expressing TCR$\gamma\delta$, is observed [123]. TCR gene rearrangement, although altered, occurs to some extent, suggesting the possibility of an extrathymic and FOXN1-independent site of differentiation. However, it should be emphasized that these few T cells, which escape the blockage, are unable to sustain a productive immune response into the periphery.

Taken together, the data so far available underline the crucial role of FOXN1 in the early prenatal stages of T-cell ontogeny in humans [123].

7. Role of FOXN1 in Immune System

FOXN1 belongs to the forkhead-box gene family that comprises a diverse group of "winged helix" transcription factors implicated in a variety of cellular processes: development, metabolism, cancer, and aging [124]. These transcription factors share the common property of being developmentally regulated and of directing tissue specific transcription and cell fate decisions. While during embryogenesis *FOXN1* is expressed in several mesenchymal and epithelial cells, including those of the liver, lung, intestine, kidney, and urinary tract, later, its expression is confined to skin and thymus epithelia, where FOXN1 is absolutely required for the normal differentiation of hair follicles and TECs.

FOXN1 gene, spanning about 30 kb [125, 126], is an epithelial cell-autonomous gene and is highly conserved in sequence and function in rodents and humans. Interestingly,



(a) (b) (c)

Figure 3: Nail dystrophy patterns in subjects carrying heterozygous mutations in FOXN1 gene: (a) koilonychias, (b) canaliform dystrophy, and (c) leukonychia.

an extensive screening of cDNA clones obtained from skin cells revealed the presence of two different noncoding first exons [126], the exons 1a and 1b, that undergo to alternative splicing to either of two splice acceptor sites of the exon 2, located upstream of the initiation codon. This suggests the presence of two distinct promoters of exons 1a and 1b [125]. The alternative usage of the exon 1a or 1b seems to direct the tissue specificity [126], in that promoter 1a is active in thymus and skin, while promoter 1b is active only in skin.

The molecular mechanisms by which *FOXN1* expression and activity are regulated are only incompletely understood. It is suggested that FOXN1 might, subsequently, upregulate the expression of fibroblast growth factor (FGF) receptors, which in turn modulate the thymic stroma differentiation and thymopoiesis [127]. *In vitro* exposure of thymic epithelial cells to some Wnt proteins is sufficient to upregulate FOXN1 protein expression in both an endocrine and paracrine fashion [128]. Wnts belong to a large family of secreted glycoproteins that have important roles in cell-fate specification [127].

The prenatal thymus development, the maintenance of a proper thymic microenvironment, and the efficient T-cell production require an appropriate crass-talk between thymocytes and thymic stromal cells [12]. Postnatally, the thymic involution results in dramatically reduced T-cell generation in an age-dependent fashion [129].

Indeed, recent evidence has implicated both TEC- and hematopoietic stem cell- (HSC-) intrinsic defects in involution of the organ [130–133]. *Foxn1* is expressed in all TECs during initial thymus organogenesis and is required for the initial phase of their differentiation [75, 134, 135]. *Foxn1* exerts an important role [136] in inducing both cortical and medullary differentiation [137, 138]. Although foxn1 has long been studied, most of the studies thus far available are restricted to fetal differentiation process, while its postnatal role in the mature thymus still remains to be fully elucidated.

However, it is largely unknown whether the role of foxn1 in the thymus and skin is identical. One important difference is that foxn1 is involved in morphogenesis of the three-dimensional thymic microstructure, which is important for the functionality of the thymus [139]. Moreover, the differentiation of the immature epithelial cells into functional cTECs and mTECs is foxn1-dependent. In particular, foxn1 mainly regulates TEC patterning in the fetal stage [140] and TEC homeostasis in the postnatal thymus [141]. TECs are implicated in either thymus organogenesis or in most stages of maturation of thymocytes [142, 143]. The inborn null mutation in *foxn1* [76] causes a differentiation failure in TECs thereby halting thymic development at a rudimentary stage. The thymic lobar architecture is still present but the epithelial cells lack the ability to induce the entrance of hematopoietic precursor cells (HPCs) into the epithelial cluster and thus preclude the generation of thymocytes [144]. These results argue strongly for a failure in thymocytes-epithelial crosstalk, thus, explaining the blockage of thymic lymphopoiesis [75, 136]. The organ is, therefore, an alymphoid two-dimensional (2D) rudiment with a cystic structure [72, 82, 120, 123].

Because of the significant expression levels of FOXN1 in skin elements, keratinocytes have been successfully used to support a full process of human T-cell development in vitro, resulting in the generation of mature T cells from HPCs. This finding would imply a role for skin as a primary lymphoid organ [145].

8. Conclusion and Future Research

Primary T-cell defects are rare disorders, accounting for approximately 11% of reported PIDs. These disorders include a wide spectrum of diseases that affect T-cell development and/or function. The pathogenic mechanisms are mostly related to molecular alterations of genes selectively expressed in hematopoietic cells. However, they can also be due to alterations of the stromal component of the thymus, which is the primary lymphoid organ that supports T-cell differentiation and repertoire selection. In this organ, the dynamic relocation in multiple architectural structures requires the crosstalk between thymocytes and thymic microenvironment. The Nude/SCID syndrome results from inactivating mutations in the gene encoding the *FOXN1* transcriptional factor selectively expressed in skin and thymic epithelia. In mice and humans its alteration leads to thymic agenesia and severe T-cell deficiency. The Nude/SCID immunodeficiency is much more severe than DGS, indicating that the *FOXN1* expression is absolutely required for an efficient production of mature T cells. The studies on the human Nude/SCID

phenotype greatly contributed to unravel important issues of the T-cell ontogeny and, in the near future, may help define potential extrathymic and thymus-independent sites of differentiation in man.

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Research Article

Adaptive Immune Response to Model Antigens Is Impaired in Murine Leukocyte-Adhesion Deficiency-1 Revealing Elevated Activation Thresholds *In Vivo*

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Absence of β_2 integrins (CD11/CD18) leads to leukocyte-adhesion deficiency-1 (LAD1), a rare primary immunodeficiency syndrome. Although extensive *in vitro* work has established an essential function of β_2 integrins in adhesive and signaling properties for cells of the innate and adaptive immune system, their respective participation in an altered adaptive immunity in LAD1 patients are complex and only partly understood *in vivo*. Therefore, we investigated adaptive immune responses towards different T-dependent antigens in a murine LAD1 model of β_2 integrin-deficiency ($CD18^{-/-}$). $CD18^{-/-}$ mice generated only weak IgG responses after immunization with tetanus toxoid (TT). In contrast, robust hapten- and protein-specific immune responses were observed after immunization with highly haptenated antigens such as $(4\text{-hydroxy-3-nitrophenyl})_{21}$ acetyl chicken γ globulin (NP_{21}-CG), even though regularly structured germinal centers with specificity for the defined antigens/haptens in $CD18^{-/-}$ mice remained absent. However, a decrease in the hapten/protein ratio lowered the efficacy of immune responses in $CD18^{-/-}$ mice, whereas a mere reduction of the antigen dose was less crucial. Importantly, haptenation of TT with NP (NP-TT) efficiently restored a robust IgG response also to TT. Our findings may stimulate further studies on a modification of vaccination strategies using highly haptenated antigens in individuals suffering from LAD1.

1. Introduction

Adaptive immune responses require the integration of complex antigen receptor and costimulatory signals on T cells as well as B cells to override activation thresholds that lead to lymphocyte differentiation and eventually, to antibody production. The β_2 integrin LFA-1 (CD11a/CD18) is particularly prominent in promoting the interaction between APC and naïve T cells [1] as well as between T cells and B cells [2, 3]. In this, LFA-1 signaling is an important determinant of phenotypic outcome in naïve T-cell and B-cell maturation and effector function [4–6].

A prerequisite for T-cell activation is a stable T-cell stimulation which is essential to be sustained up to several hours [4] to achieve commitment to cytokine synthesis (IL-2, IFN-γ) [7–9]. It has been reported that the duration of stimulation directly contributes to the commitment of $CD4^+$ T cells to division thus correlating with the development of effector functions and the extent of memory generation [10]. Furthermore, it has been assumed that the adhesive interaction mediated by LFA-1 increases the avidity of T cell : APC interaction allowing T cells to sample large numbers of specific peptides presented on MHC class II on the surface of APC which then leads to a more efficient TCR engagement. Increased adhesion can thus lower the effective dose of antigen required to reach a minimal threshold number of activated TCR complexes [1, 5, 11, 12]. Furthermore, LFA-1 facilitates the clustering of surface molecules such as the TCR, CD28, and CD40 in the immunological synapse between T cells and APC (quantitative signal enhancement) [13, 14]. In addition to its above-described adhesive functions, LFA-1 may also provide unique signals that are primarily independent of TCR signaling (qualitative signal modulation) [5, 8]. Model systems used to study T-cell activation have shown that even a mere increase in antigen "quantity" at a large scale could not initiate naïve $CD4^+$ T-cell proliferation or cytokine synthesis in the absence of ICAM/LFA-1 interaction [15]. Collectively, it is well established that LFA-1 stimulation increases the number of activated T cells favoring Th1 differentiation, distinctly enhances activation velocity in particular by supporting early IL-2 production, leading to entry of naïve cells into the cell cycle and reduces activation thresholds of T cells. Hence, LFA-1/ICAM signaling significantly supports T-cell activation and polarization towards the Th1 phenotype [5, 7]. In the absence of functional LFA-1, Th1 differentiation is severely impaired, whereas Th2 response is strongly enhanced, both *in vitro* and *in vivo* [9, 16, 17].

Interestingly, the relevance of LFA-1/ICAM for T : B immunological synapses has also been documented for membrane-mediated B-cell activation by Th cells [2]. Signaling from antigen-specific T cells to B cells to induce activation required LFA-1/ICAM-1 ligation and is based on tight physical contact of T : B in an immune synapse [18]. In this context, Carrasco and coworkers showed that inclusion of ICAM-1 in the immunological synapse decreases the B-cell avidity threshold by at least 10-fold [2]. At low antigen densities, LFA-1 can help B-cells adhering, forming a synapse, and becoming activated. Thus, in analogy to the T cell : APC interaction, synergy of BCR crosslinking and ICAM-1-mediated signals can reduce threshold barriers for B-cell activation. Vice versa, effective B : T cell synapses are of even greater importance for T-cell activation by antigen-presenting B cells (B-APC). Engagement of the BCR by polyvalent antigen can rapidly elicit expression of B7-2 (CD86) on B cells resulting in a robust costimulatory signal that is sufficient even to drive naïve Th cell responses [19, 20].

Although detailed studies on the adhesive and differentiation-inducing functions of LFA-1-mediated binding for APC : T cell and T : B cell contacts are available, it still remains incompletely understood how the observed *in vitro* functions combine and contribute to the clinical picture of immunodeficiency in individuals lacking β_2 integrins *in vivo*. Absence of β_2 integrins (CD11/CD18) in humans leads to leukocyte-adhesion deficiency-1 (LAD1), a severe primary immunodeficiency syndrome. Expression of less than 1% of CD18 causes a severe form of the disease with recurrent life-threatening bacterial or fungal infections resulting in death of patients early in childhood [21, 22]. Impaired adaptive immune responses to common vaccination protocols have been observed as one important feature of the disease [23, 24]. We have previously reported on a murine model for LAD1 carrying a CD18 null mutation that shares all major features with the human syndrome [25–29]. Using this murine model, we herein dissect the role of β_2 integrins in functional and structural components of the adaptive immune response *in vivo*.

Our results demonstrate that in absence of β_2 integrins, mice display a severely impaired adaptive immune response *in vivo*. A marked elevation of activation thresholds excluded the commonly potent antigen TT as an immunogen, whereas haptenation of carrier proteins could override the activation threshold and elicited robust adaptive immune responses. These findings indicate that modifying vaccination approaches towards the use of highly haptenated antigens may be more successful in LAD1 patients.

2. Materials and Methods

2.1. Mice. All mice were maintained on a mixed 129Sv × C57BL/6 background. $CD18^{-/-}$ homozygotes [25] and $CD18^{+/+}$ WT controls were derived from heterozygote crosses. Immunization trials were performed under specific pathogen-free (SPF) conditions using mice at an age of 8–12 weeks. All experiments were done in compliance with the German Law for Welfare of Laboratory Animals.

2.2. Immunization of $CD18^{-/-}$ Mice. For immunization of mice, protein antigens conjugated with a hapten at different haptenation ratios were used. These hapten-coupled proteins were prepared according to a previously established protocol [30, 31]. In brief, per animal, a solution of 200 μL PBS containing 10 or 100 μg of (4-hydroxy-3-nitrophenyl) acetyl (NP; Bioresearch Technologies, Inc., Novato, CA) coupled to chicken γ globulin (CG; Calbiochem, Schwalbach, Germany) with a ratio of 21 or 4 NP molecules per molecule CG was precipitated by adding 200 μL 10% $KAl(SO_4)_2$ (alum; Merck

Chemicals, Darmstadt, Germany) and was then titrated using 5 N NaOH. Or else, uncoupled CG was precipitated. The alum precipitates were injected intraperitoneally into mice. All animals were reinjected with 10 or 100 μg of the soluble, unprecipitated NP-CG or CG at the indicated time points.

Immunization with tetanus toxoid (TT) was performed in a similar fashion. "Tetanus-Impfstoff Mérieux" vaccination suspension was purchased from Aventis Pasteur MSD (Lyon, France). Eight- to tewlve-week-old mice were injected with a dose of either 2.0 Lf (flocculation units) or 0.2 Lf of alum-precipitated TT. In a further trial, mice were immunized with 2.0 Lf of alum-precipitated TT haptenated with NP molecules at an unknown ratio, according to a previously published protocol for the hapten conjugation of protein carrier molecules [30, 31]. All mice were reinjected with the same dose of TT or NP-TT, respectively, at indicated time points of the trials.

2.3. Measurement of NP-Specific Ig. Immune responses were estimated by ELISA detection of NP hapten-specific IgM, IgG_1, κ, and λ light chain Ab in the sera of mice immunized with NP-CG as described elsewhere [30, 32]. Ninety-six-well plates (Greiner Bio-One, Frickenhausen, Germany) were coated with 10 μg/mL NP_{14}-BSA in PBS at 4°C overnight and were then blocked with 0.5% BSA in PBS. Serially diluted sera obtained at the indicated time points after immunization were added and incubated at 4°C overnight. On each plate, equally diluted anti-NP mAb standards with corresponding isotypes (prepared at the Institute of Genetics, University of Cologne according to previously published protocols [30, 31]) were included to obtain appropriate standard curves. After intermittent washing steps with H_2O, biotinylated detection antibodies (goat anti-mouse IgM, IgG, κ and λ; Southern Biotechnology Associates Inc., Birmingham, AL) at a dilution of 1 : 1000 and alkaline-phosphatase-(ALP-) conjugated streptavidin (1 : 3000; Roche, Mannheim, Germany) were added. ALP activity was visualized using ALP substrate solution (0.4 mg/mL; Roche) and subsequently, OD was measured at 405 nm versus 570 nm. The concentrations were determined by comparing to standard curves created from above mentioned anti-NP standards.

2.4. Affinity Maturation of NP-Specific Antibodies. Assessment of affinity maturation of NP-specific antibodies was carried out by ELISA using two different coupling ratios of NP-BSA as described previously [32, 33]. Briefly, 96-well plates (Greiner) were coated with 10 μg/mL NP_4-BSA or NP_{14}-BSA in PBS at 4°C overnight. After blocking with 0.5% BSA in PBS, sera obtained at the indicated time points after immunizations were serially diluted and plated out. On each plate, anti-NP-specific mAb standards of the same isotype but with different affinity constants (*Ka*) (prepared at the Institute of Genetics, University of Cologne according to previously published protocols [30, 31]) were added to obtain standard curves for affinity assessment. The final steps of the ELISA were then performed as described above using biotinylated goat anti-mouse λ light chain and IgG_1 (data not shown) detection Ab. To estimate the affinity of NP-binding antibody in the sera, ratios of NP_4-binding antibody to NP_{14}-binding antibody were calculated.

2.5. Measurement of Protein-Carrier-Specific IgG. For assessment of anti-TT- or anti-CG-specific IgG Ab, sera obtained by bleeding from tail veins were analyzed by ELISA. Briefly, for anti-TT detection, human Tetanus IgG ELISA kits were purchased from IBL (Hamburg, Germany) and ELISA performed according to a slightly modified protocol, as distributed by the manufacturer. Sera were initially diluted 1 : 10 in assay diluent and subsequently plated out in 1 : 5 or 1 : 6 dilution steps using assay diluent. For detection of murine anti-TT IgG Ab, a horseradish peroxidase-conjugated rat anti-mouse IgG mAb (X56; Pharmingen, BD, Heidelberg, Germany) was used at a dilution of 1 : 1000. Tetramethylbenzidine (TMB, IBL) served as a substrate for the color reaction. Plates were read at 450 nm within 60 minutes after addition of 1 M H_2SO_4. Anti-TT IgG titers were calculated from the last dilution step where the OD was still above the background level. Assays for measurement of anti-CG IgG were performed accordingly, except with the modification that, initially, 96-well plates (Greiner) were coated with 10 μg/mL soluble CG in PBS and were blocked with 0.5% BSA. Subsequently, all further procedures were carried out as described above.

2.6. Antibodies and Fluorochrome-Coupled Proteins. GL7-FITC (Ly77), CD18-PE (C71/16) mAbs were purchased from Pharmingen, CD19-PE (6D5) was from SBA, and IgD (HB250) and IgM (HB88) were obtained as described earlier [34]. Peanut agglutinin (PNA)-FITC was purchased from Sigma (Taufkirchen, Germany) and DAPI from Roche (Grenzach-Wyhlen, Germany).

For detection of antigen-specific cells, NP_4-CG and CG were labeled with Cychrome 5 (Cy5), and conjugates purified using NAP columns, as recommended by the manufacturer (Amersham/Pharmacia, Freiburg, Germany). NP_4-CG and CG were used instead of NP_{21}-CG for fluorochrome coupling to allow sufficient binding of Cy5 to free sites of the CG.

2.7. Histology. Spleens were removed at the indicated time points after immunization and were embedded in Tissue-Tek O.C.T. compound (Fisher Scientific, Bridgewater, NJ) for cryosections. Immunohistologic analysis of adult lymphoid tissues was done as described earlier [34] using a motorized Axiovert M200 microscope (Carl Zeiss, Germany). Frozen sections of 6–10 μm thickness were mounted on slides and fixed in cold acetone. Cryosections were blocked with rat serum and stained with mAb and lectins against the indicated markers. Overviews of spleen sections shown in Figure 3(a) were achieved using automated image assembly applying the KS300 MosaiX software (Carl Zeiss, Oberkochen, Germany).

2.8. FACS Analysis. Cells were obtained from spleens, or from BM flushed out of femurs of mice. Red blood cells were removed using an osmotic lysis buffer (0.15 M NH_4Cl, 1.0 M $KHCO_3$, 0.1 M Na_2EDTA, pH 7.2). The remaining

leukocyte fraction was adjusted to 1×10^6 cells per 50 μL and unspecific binding was blocked with 2% rat serum. Subsequently, 50 μL of the cell suspension were stained with ≤ 1 μL of the fluorochrome-conjugated mAbs (at a stock concentration of 0.5–1 mg/mL, dependent on the respective mAb-fluorochrome conjugate) for 30 min at 4°C. Stained cells were analyzed using a FACSCalibur (BD, Heidelberg, Germany).

2.9. Statistics. For statistical evaluation of the differences in serum Ig levels, Mann-Whitney U test was used. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Impaired Humoral Immune Response in CD18$^{-/-}$ Mice upon Immunization with Tetanus Toxoid. LAD1 patients suffer from a severe immunodeficiency due to an absence of functional CD18 heterodimers. Patients [23, 24] as well as cattle [35] deficient in CD18 have been described to respond poorly to T-dependent antigens or vaccines such as bacteriophage ϕX174 or tetanus toxoid (TT). Since TT is a well-characterized and commonly potent immunogen that has been frequently employed to detect T-dependent immunodeficiency by others before, we determined anti-TT IgG titers in a TT vaccination trial in the murine LAD1 model. CD18$^{-/-}$ and WT mice were immunized with either 2.0 or 0.2 flocculation units (Lf) of TT/alum. For assessment of memory B-cell function and amplification of specific Ig production during secondary immune response, animals were boost-immunized with the same doses at day 34. Serum levels of anti-TT IgG were detected by ELISA at different time points throughout the trial. At all time points analyzed, anti-TT IgG titers were significantly lower in CD18$^{-/-}$ mice than in WT controls independent of the TT dose injected ($P < 0.05$) (Figure 1). After secondary immunization, anti-TT IgG titers of CD18$^{-/-}$ mice were about three logs below WT control titers. Whereas in WT mice a strong amplification of the immune response occurred, CD18$^{-/-}$ mice were not able to amplify their anti-TT IgG production any further after reimmunization with the antigen. However, TT-specific IgG titers were measurable also in CD18$^{-/-}$ mice, confirming that class switch was not impaired.

3.2. Robust T-Dependent Humoral Immune Response in CD18$^{-/-}$ Mice upon Immunization with NP-CG. To address the question, whether defective adaptive immunity in CD18$^{-/-}$ mice relied on particular TT-specific properties, CD18$^{-/-}$ and WT mice were immunized with the alum-precipitated antigen NP_{21}-CG at a dose as high as 100 μg per mouse, in an analogous immunization trial. For measurement of memory B-cell function and amplification of specific Ig production during secondary immune response, animals were boost-immunized with 100 μg of soluble NP_{21}-CG at day 34. Serum levels of anti-NP-specific Ig were detected and further differentiated into subclasses by ELISA. Surprisingly, a slightly lower production of anti-NP IgG_1 was detectable only during the primary immune response

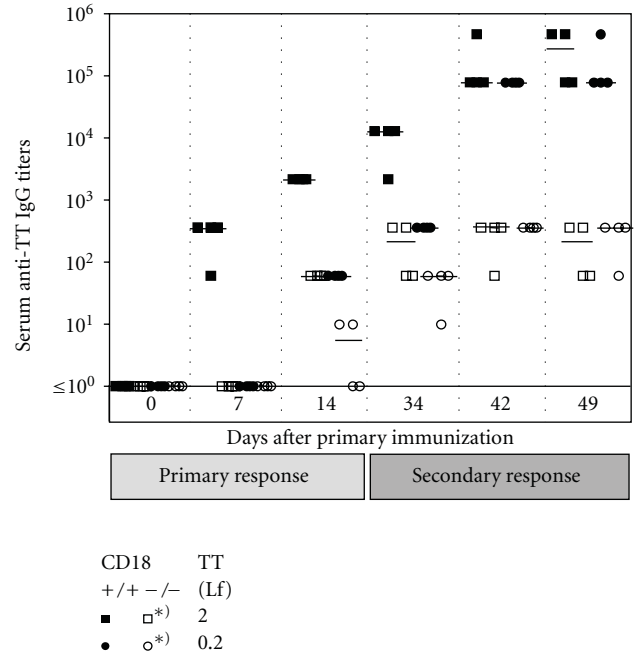

Figure 1: Defective humoral immune response upon TT in CD18$^{-/-}$ mice. Eight- to twelve-week-old CD18$^{-/-}$ (open symbols) and WT (filled symbols) mice were immunized intraperitoneally with 2.0 (squares) or 0.2 Lf (circles) of tetanus toxoid (TT)/alum. Animals were reimmunized with the same dose of the antigen at day 34. For assessment of the primary immune response, sera were collected at days 0, 7, and 14, for secondary immune response at days 34, 42, and 49. Subsequently, sera were diluted 1 : 10, and plated out on TT-coated plates in 1 : 6 dilution steps. Serum titers of anti-TT specific IgG_1 were determined from the last dilution step where the optical density was still above the background level of the assay. Bars represent the median of each group. *Indicates a $P < 0.05$ for the marked cohorts at all times points shown, from day 14 on.

in CD18$^{-/-}$ mice (Figure 2(a)). At day 7, titers of CD18$^{-/-}$ mice were about 4.5-fold reduced when compared to WT controls ($P < 0.005$), whereas at day 14, this difference had decreased to 2.5-fold ($P < 0.005$). No significant differences in NP-specific Ig titers of CD18$^{-/-}$ and control mice occurred after rechallenge with the soluble antigen, from day 42 onwards ($P > 0.05$). These results demonstrate a slight shift in the kinetics of the primary immune response in CD18$^{-/-}$ mutants, with an initial decrease in hapten-specific IgG_1 production, whereas primary NP-specific IgM were not reduced in CD18$^{-/-}$ mice (data not shown). However, overall hapten-specific IgG peak titers mounted by CD18$^{-/-}$ mice after immunization were in the same range as in WT controls showing that class switch as such was not impaired. After boosting, amplification of the immune response was as high in CD18$^{-/-}$ as in WT mice reflecting a normal memory B-cell generation and function. Besides, antibody composition of either κ or λ light chains was comparable to those of WT controls and revealed a marked prevalence of λ light chains during the primary IgG response to NP, a typical feature of the C57BL/6 mouse strain (data not shown) [30].

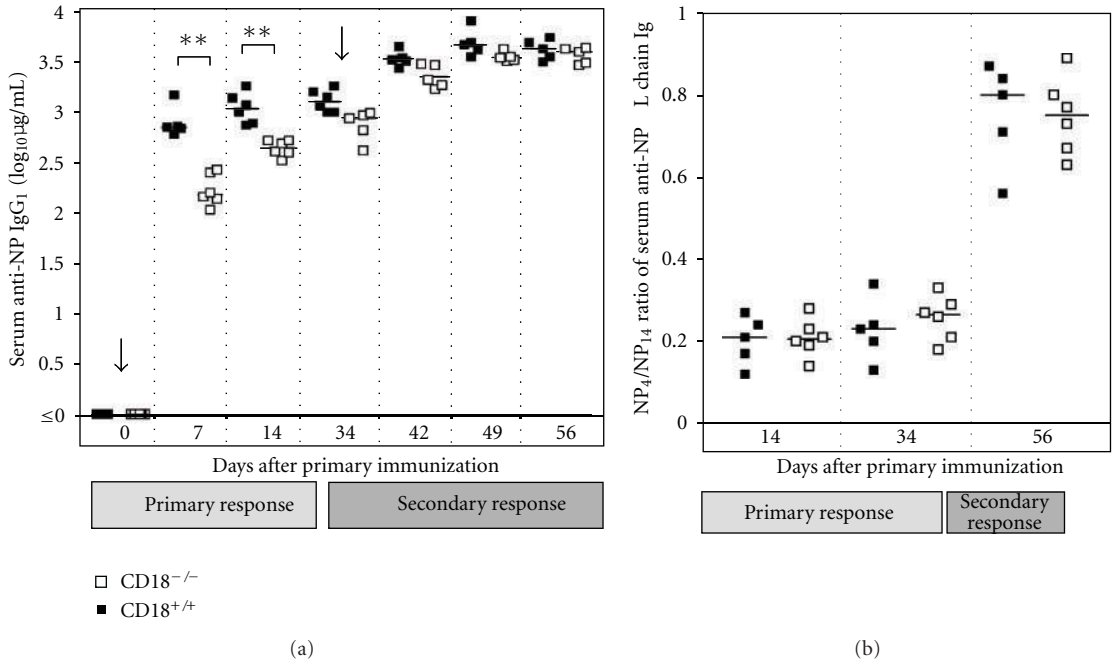

Figure 2: Functional humoral immune response upon NP-CG in $CD18^{-/-}$ mice. Eight- to twelve-week-old $CD18^{-/-}$ and WT mice were immunized intraperitoneally with 100 μg of alum-precipitated NP-CG. Animals were reinjected with 100 μg of soluble NP-CG at day 34. Sera were collected at days 0, 7, and 14 during primary immune response, and at days 34, 42, 49, and 56 during secondary immune response. (a) Serum levels of anti-NP-specific IgG_1 were subsequently detected by ELISA on NP_4-coated ELISA plates and calculated by comparison to an IgG_1 standard. (b) Anti-NP-specific Ig carrying λ or κ L chains were differentially detected on high-density (NP_{14}-BSA) and low-density (NP_4-BSA) hapten-coated ELISA plates in sera obtained at days 14, 34, and 56. Affinity maturation of NP-specific antibodies was estimated as ratio of NP_4- to NP_{14}-binding antibodies for each of the three time-points. Bars represent the median of each group. $^{**}P < 0.005$.

To determine affinity maturation of NP-specific antibodies, sera collected during the immunization trial were analyzed for their contents of low and high affinity antibodies for the hapten NP. Our data clearly demonstrate that affinity maturation occurred to the same extent in $CD18^{-/-}$ as in WT mice, both showing a sharp increase in anti-NP affinity after repeated antigen challenge ($P < 0.05$) (Figure 2(b)).

3.3. No Formation of Antigen-Specific GC after Immunization with NP_{21}-CG. $CD18^{-/-}$ mice have a severely disturbed architecture of secondary lymphoid organs such as the spleen and the lymph nodes [25, 28, 36]. Since adaptive immunity in $CD18^{-/-}$ mice was nevertheless functional upon immunization with NP_{21}-CG, we set out to detect germinal centers with specificity for the injected antigen NP_{21}-CG. To exclude artefacts skewing histological analysis of $CD18^{-/-}$ mice, mice were used for histology at an age of 8–12 weeks when lymphoid architecture had not yet succumbed to secondary lymphoid and myeloid hyperplasia. As described above, $CD18^{-/-}$ and control mice were injected with 100 μg NP_{21}-CG/alum. Secondary lymphoid tissues were removed at day 14, when GC formation in mice is at its maximum (Figure 3). Cryosections of WT spleens showed typical germinal centers ($GL\text{-}7^+PNA^+$, IgD^-IgM^-) with numerous GC that stained positive for the antigen NP-CG coupled with Cy5 (NP-CG-Cy5) (Figures 3(a) and 3(b)). In contrast to WT, $CD18^{-/-}$ mice had considerably fewer GC, of a smaller size and altered structure. In addition, none of the GC-like structures but only some disseminated cells stained for the antigen NP_{21}-CG in $CD18^{-/-}$ mutants. Importantly, these cells were situated extrafollicularly and were not organized in clusters as are GC.

Since no classical NP-CG-$Cy5^+$ GC structures could be detected in immunized $CD18^{-/-}$ mice by histology, whereas IgG with high affinity for NP_{21}-CG was abundant in the sera, we analyzed lymphoid tissues for NP-CG-$Cy5^+$ cells with a GC-like phenotype ($CD19^+GL7^+$ or $CD19^+PNA^+$) [32, 37] by flow cytometry (Figures 3(c) and 3(d)). As observed by immunofluorescent microscopy, NP_{21}-CG immunized WT revealed a prominent $CD19^+$ B cell population that stained for the GC marker GL-7, or PNA (data not shown), and NP-CG-Cy5. In contrast, in $CD18^{-/-}$ mice, $CD19^+$ B-cells staining double positive for GL-7 and NP-CG-Cy5 were 5 times less frequent compared to WT mice (Figure 3(c)). Hence, GC-like cells specific for NP-CG were present, but without a distinct structural correlative in histology.

In case of altered lymphocyte trafficking and disrupted secondary lymphoid tissue, BM can function as site of primary immune response [38]. B cells, which have acquired a typical PNA^+ GC phenotype, have been described to seed to the BM, where they further differentiate into antibody-forming cells (AFCs) [39]. Thus, BM may, to some extent, serve as a refuge for late B-cell development and maturation. To trace B cells showing a GC phenotype in BM [33] of

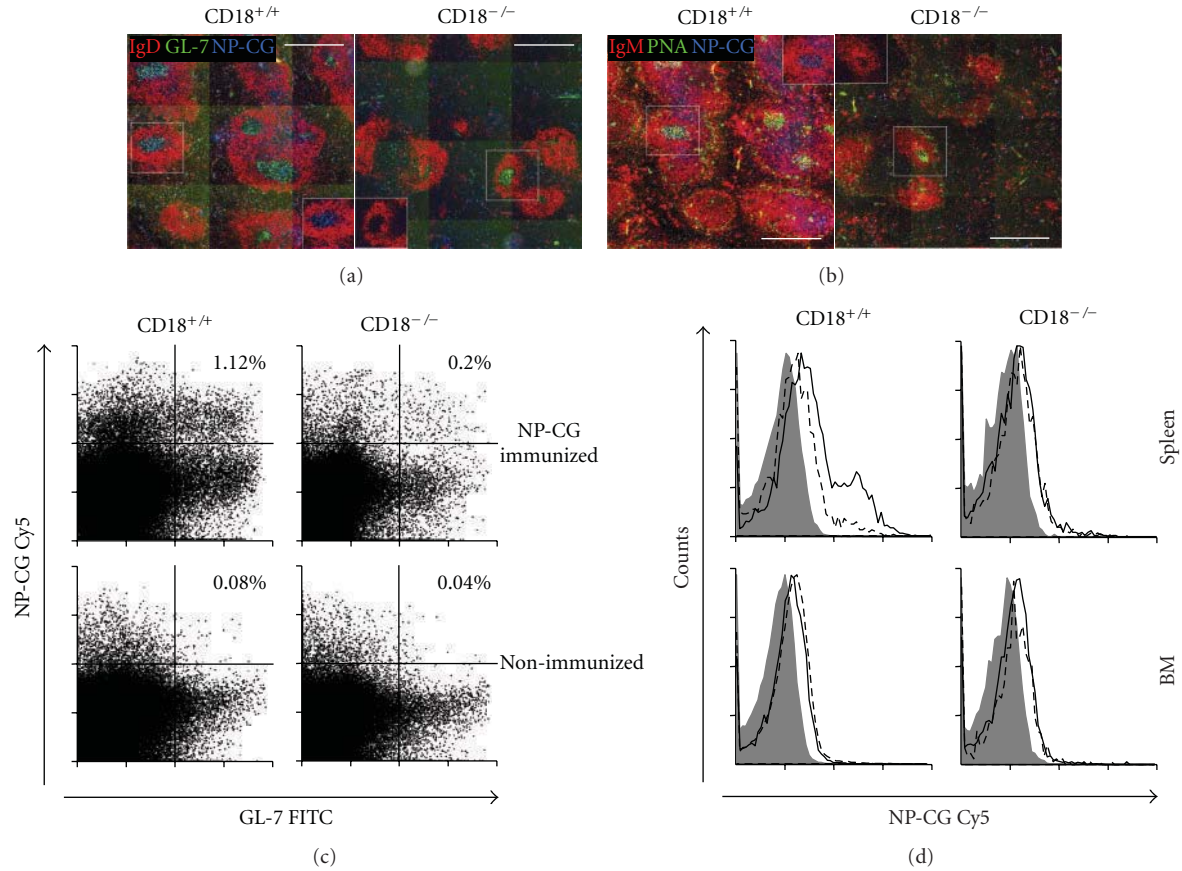

Figure 3: No induction of GC with NP_{21}-CG specificity after immunization in $CD18^{-/-}$ mice. Two weeks after immunization, spleens and BM of WT and $CD18^{-/-}$ mice were subjected to immunofluorescence microscopy (spleens; (a), (b)) or flow cytometry (BM, spleens; (c), (d)). B-cell follicles identify as IgD^+ (b) or IgM^+ (a) (both in red), whereas GC locate within the follicles and stain IgD^- $GL\text{-}7^+$ (a) or IgM^- PNA^+ (b) (GL-7 and PNA both in green). Specificity for NP-CG was assessed by staining with NP-CG-Cy5 (blue). (a) and (b) show representative sections of spleens solely from immunized mice. Scale bars, 500 μm. Spleen ((c); (d), upper histograms) or BM ((d), lower histograms) cells were analyzed by FACS. (c) displays representative dot plots of splenic cells gated for $CD19^+$ IgM^{low}. B cells with a GC phenotype additionally stain positive for GL-7 and are situated in the right quadrants of each plot. GC B cells with specificity for NP-CG are located in the upper right quadrants. Percentages for size-gated cells are indicated for immunized (upper dot plots) and mock-immunized (lower dot plots) mice. (d) shows representative histogram plots of cells gated for $CD19^+$ IgM^{low} $GL\text{-}7^+$, representing GC B cells. The continuous lines indicate samples obtained from mice immunized with NP_{21}-CG; dashed lines: mock-immunized mice; grey areas: irrelevant control conjugates.

$CD18^{-/-}$ mice, mononuclear cells were isolated from BM and stained for GC markers. Only very few $CD19^+IgM^{low}GL\text{-}7^+$ cells could be detected using flow cytometry. These cells did not differ in fluorescence intensities for NP-CG-Cy5 in NP_{21}-CG immunized and nonimmunized cohorts, neither in $CD18^{-/-}$ nor in WT mice (Figure 3(d)). Thus, in $CD18^{-/-}$ mice no evidence for a compensatory function of the BM in hosting GC-like cells was detected. Our data reveal a ubiquitous deficiency for specific GC formation upon immunization with NP_{21}-CG in all lymphoid tissues of $CD18^{-/-}$ mice subjected to analysis, postulating a salvage mechanism or alternative pathway in generating high-affinity AFC that yet remains unclear.

3.4. Efficacy of the Immune Response Directly Correlates to the Hapten/Protein Ratio of Antigens in $CD18^{-/-}$ Mice. To better understand potential reasons for the in part contradictory results obtained by immunization with NP_{21}-CG and TT, we modified our immunization protocols with regard to antigen quantity and quality. Given the high dose of 100 μg NP_{21}-CG administered in the initial trial, we repeated the experiment with a low dose injecting 10 μg NP_{21}-CG/alum for the induction of primary immune responses, and 10 μg of soluble NP_{21}-CG/PBS for boosting. Furthermore, to test whether the degree of haptenation may be crucial for the induction of a full immune response in $CD18^{-/-}$ mice, we have used a reduced NP/CG ratio of 4/1, or nonhaptenated CG without NP. This time, besides anti-NP specific IgG, also anti-CG-specific IgG were determined from the sera to rule out that in $CD18^{-/-}$ mice, hapten-specific responses may be functional but carrier/protein directed IgG production, as, for example anti-TT IgG, may be not. For this reason, sera obtained during the initial immunization trial with 100 μg NP_{21}-CG were additionally subjected to anti-CG IgG ELISA.

As shown by Figure 4, $CD18^{-/-}$ mice were well able to mount anticarrier/protein-specific IgG titers to a similar extent as they mounted antihapten-specific IgG titers if provided with a suitable antigenic stimulus. Our studies demonstrate that reduced anti-NP IgG levels (Figure 4(a)) were paralleled by a decrease in anti-CG IgG levels (Figure 4(b)), both in WT and in $CD18^{-/-}$ mice and preclude a general deficiency of producing protein-specific antibody in our murine LAD1 model.

However, in contrast to WT, $CD18^{-/-}$ mice exhibited a definite impairment in their primary and secondary IgG responses upon decreased antigen quantity or hapten coupling. Whereas upon doses as low as 10 μg NP_{21}-CG, $CD18^{-/-}$ mice revealed a significant reduction of NP- and CG-specific IgG production only during the secondary immune response ($P < 0.05$), a reduced NP/CG ratio of 4/1 ($P < 0.05$), and more distinctly, uncoupled CG ($P < 0.005$) significantly lowered antigen-specific IgG throughout all time points analyzed. When the hapten/protein ratio was decreased to 4/1, the gap between the $CD18^{-/-}$ and WT cohort was more pronounced than after a mere reduction of the antigen dose to 10 μg NP_{21}-CG. Furthermore, even after injection of a dose as high as 100 μg nonhaptenated CG, $CD18^{-/-}$ mice mounted only very poor anti-CG IgG titers that remained more than 4 logs below the WT cohort, also after boosting (Figure 4(b)). In the $CD18^{-/-}$ cohort that had been administered 100 μg NP_4-CG, anti-CG IgG levels still were 3 logs below those of the WT cohort. Using 100 μg NP_{21}-CG, $CD18^{-/-}$ mice produced anti-CG IgG at WT levels. Altogether these data demonstrate a gradual dependence of adaptive humoral immunity in $CD18^{-/-}$ mice on CG haptenation. These data furthermore indicate that the deficiency in adaptive humoral immunity of $CD18^{-/-}$ mice can be compensated by the amount of antigen injected for immunization. However, haptenation of proteins is pivotal and can effectively rescue antigen-specific IgG production in $CD18^{-/-}$ mice turning a weak protein antigen into a strong immunogen in this system.

3.5. $CD18^{-/-}$ Mice Secrete Normal Levels of Anti-TT IgG after Immunization with NP-TT. To test the validity of our conclusions drawn from immunization with the haptenated carrier NP-CG also for other T-dependent antigens, TT vaccine was conjugated with NP, and subsequently used for immunization as described above. Figure 5 shows a slight initial delay of anti-TT IgG production at day 7 after first injection of 2 Lf NP-TT, reflecting results obtained during the primary immune response to 100 μg NP_{21}-CG. At all later time points assessed, production of anti-TT IgG in $CD18^{-/-}$ mice occurred at equal levels as in WT mice, compared to immunization with 2 Lf nonconjugated TT, which had failed to induce sufficiently high anti-TT IgG titers. Collectively, these findings demonstrate, for the first time, that the elicitation of a full adaptive immune response can be obtained upon immunization with a highly haptenated TT analogue in complete absence of CD18. This argues towards the use of highly haptenated antigens as vaccines in LAD1.

4. Discussion

Effective induction of an adaptive immune response relies on a fine-tuned orchestration of cell-cell interactions. This requires integrity of functional as well as structural components of the immune response. Despite the involvement of CD18 in cognate interactions between APCs, T and B lymphocytes, and a marked impairment of the adaptive immune response in patients deficient in CD18 (LAD1) [23, 24, 35], we here report that a suitable, multivalent antigenic stimulus can distinctly overcome the necessity of CD18 for adhesion and intracellular signaling leading to a robust antigen-specific humoral immune response *in vivo*. Using the protein antigens CG or TT gradually haptenated with NP as immunizing agents, we could induce a full adaptive immune response in a murine model of LAD1. This shows that CD18 is dispensable for the elicitation of a complete adaptive immune response *in vivo* under the here further defined vaccination conditions. We here demonstrate that CD18 deficiency with specific defects in intercellular adhesion required for cellular communication and activation at several stages of adaptive immunity can nevertheless be overcome in $CD18^{-/-}$ mice *in vivo* by increasing antigen concentrations or by modification of antigen quality towards carrier haptenation. These results are potentially valuable for patients suffering from LAD1 or similar immunodeficiency.

A critical event in the initiation of adaptive immune responses is the activation of T lymphocytes. LFA-1 (CD11a/CD18) is known to participate critically in the biochemical and structural organization of immunological synapses during T-cell activation [14]. It is a prerequisite for a sustained TCR/MHC-peptide engagement [1, 3, 40] to achieve commitment to cytokine synthesis (IL-2, IFNγ) [9] and T-cell proliferation [7, 8]. Accordingly, our previous *in vitro* data showed that $CD18^{-/-}$ mice exhibited a severely impaired activation of T-cells in MLR [25]. However, full T cell activation was possible, when priming was done using a sufficient amount of antigen, antigenic restimulation [36], or IL-2 substitution [36, 41].

We here show that the requirement for LFA-1 also in T : B cell contacts is overall not essential for generating an adaptive immune response *in vivo*. Our major finding is that highly haptenated antigens do not depend on CD18 to elicit complete adaptive immune responses. This may be due to the fact that crosslinking of BCR does not require CD18. Indeed, increased numbers of epitopic binding sites obtained by multivalent haptenation cause a profound reduction in both the minimal concentration and affinity requisites for B-cell activation [20, 42]. One key mechanism likely to contribute to this phenomenon may be the marked increase in IL-2 release due to an efficient crosslinking of BCRs by multivalently haptenated antigen [43, 44]. This may have contributed to the rescue of the previously demonstrated deficiency in IL-2 release in absence of LFA-1 or CD18 [9, 36, 41]. Accordingly, an enhanced release of IL-2 from B cells due to crosslinking by polyhaptenated antigen may have compensated for the intrinsic defect of $CD18^{-/-}$ T cells to secrete IL-2. In addition, B-APC may be superior to DC in antigen presentation and subsequent activation of $CD4^+$

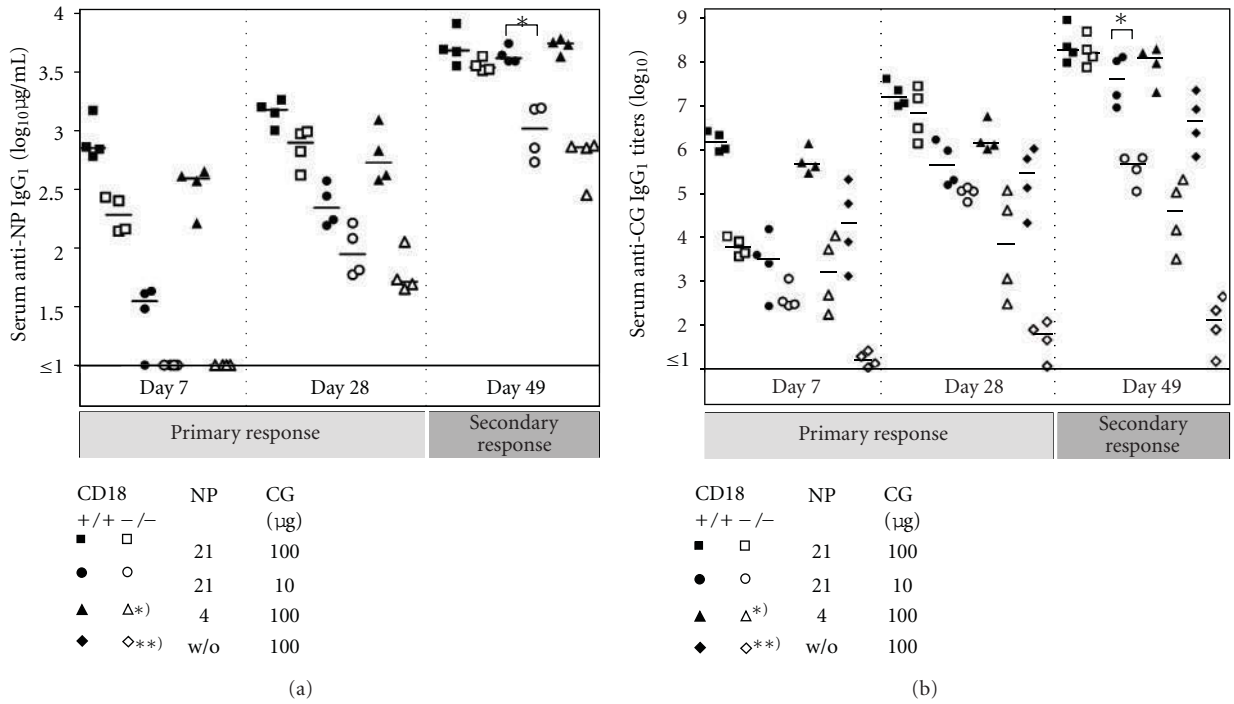

Figure 4: High NP/CG ratios are critical for elicitation of hapten- and protein-specific IgG titers in CD18$^{-/-}$ mice. Eight- to twelve-week-old CD18$^{-/-}$ (open symbols) and WT (filled symbols) mice were immunized intraperitoneally with 100 μg of NP_{21}-CG/aluM (squares), 10 μg NP_{21}-CG/alum (circles), 100 μg NP_4-CG/alum (triangles), or 100 μg CG/alum (diamonds). (a) Serum levels of anti-NP specific IgG_1 were subsequently detected by ELISA on NP_4-BSA coated ELISA plates and calculated in μg/mL by comparison to IgG_1 standards, as described above. (b) For detection of CG-specific IgG_1, sera obtained by bleeding from tail veins were diluted 1 : 10, and then plated out on CG-coated plates in 1 : 5 dilution steps. Serum titers of anti-CG-specific IgG_1 were determined from the last dilution step where the optical density was still above the background level of the assay. For assessment of the primary immune response, results from sera collected at days 7 and 28 are displayed. Besides, measurements for day 49 are shown, and depict IgG_1 titers representative also for further time points assessed during secondary immune responses. Bars represent the median of each group. $^*P < 0.05$; $^{**}P < 0.005$. Asterisks used in the key box indicate significant differences for the marked cohorts at all times points shown.

T cells upon encounter with protein antigen as compared to peptide antigen [45]. Haptenated carriers even multiply this effect. Altogether, these mechanisms may contribute to the herein observed effect that polyhaptenated proteins help to mount an effective immune response even under conditions of an impaired synapse formation in the absence of CD18.

Our results furthermore provide circumstantial evidence that several redundant pathways may exist *in vivo* substituting for each other to obtain a sufficient immune response. However, impairment of a distinct accessory pathway may limit or suppress immune responses that were previously robust by critically elevating activation thresholds, as has been shown upon injection of TT in CD18$^{-/-}$ mice. Such thresholds have earlier been described in detail for the different types of cellular interactions *in vitro* [1, 2, 5, 14, 36]. *In vivo*, also spatial availability of both lymphocytes and APCs in a timely highly regulated fashion is pivotal for the elicitation of adaptive immunity [46]. But with regard to interstitial tissue locomotion of leukocytes (i.e., in three-dimensional environments), the role of CD18 (and also of other integrins) seems to be rather limited [6, 47]. Nevertheless, in CD18$^{-/-}$ mice, structural integrity of lymphoid organs is severely affected [25]. This is most likely due to impairments in cell trafficking in the context of systemic leukocyte recirculation [27, 48, 49] and the overall proinflammatory situation [28] in these mice.

Our data of an intact class switch and memory function support former reports. These reports revealed that a structural integrity of GC, which have been commonly called to account for class switch, affinity maturation, and memory B-cell generation [50, 51], is not compellingly required to mount a full adaptive immune response [51–54]. Also, mice deficient in Lyn kinase (LynK) exhibit absence of GC combined with a widely functional humoral immune response showing functional antibody production, class switch, or even affinity maturation [55]. Interestingly, both CD18 and LynK, apart from costimulatory signaling, are involved in cell-cell adhesion stabilizing membrane contacts required for cognate synapses in GC [56].

Several reports reveal that antigen-driven clonal selection of antibody-forming cells (AFCs) leading to an effective affinity maturation of secreted Ig strictly requires Th : B cell cooperation but can take place independently of classical GC structures [32, 37, 52], even in compartments such as the BM [33, 57, 58]. However, B-cells with GC phenotypic markers seem to be inevitable as an intermediate step

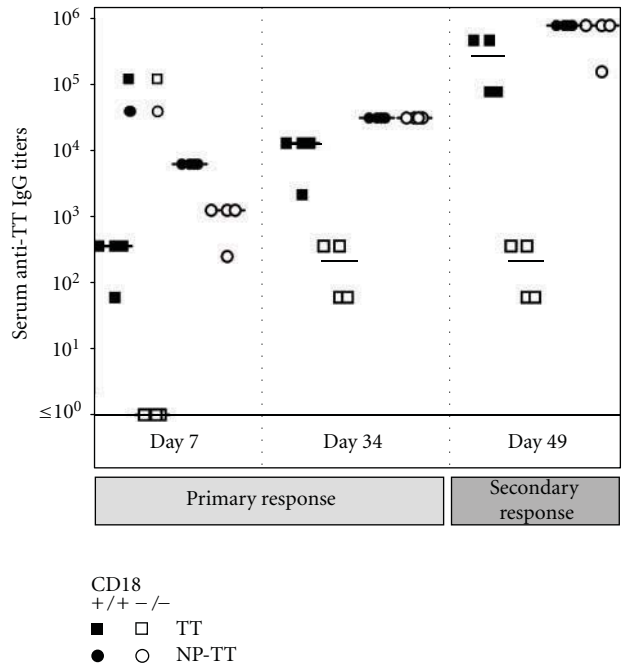

Figure 5: Adaptive immunity is functional upon immunization with NP-TT in CD18$^{-/-}$ mice. Eight- to twelve-week-old CD18$^{-/-}$ (open symbols) and WT (filled symbols) mice were immunized intraperitoneally with 2.0 Lf TT/alum (squares) or 2.0 Lf NP-TT/alum. For measurements of anti-TT IgG_1, blood was obtained by bleeding from tail veins. Prior to analysis, sera were diluted 1 : 10, and then plated out on TT-coated plates in 1 : 5 dilution steps. Serum titers of anti-TT-specific IgG_1 were determined from the last dilution step where the optical density was still above the background level of the assay. For assessment of the primary immune response, results from sera collected at days 7 and 34 are displayed. Besides, measurements for day 49 are shown, and depict IgG_1 titers representative also for further time points assessed during secondary immune responses. Bars represent the median of each group.

for AFC generation, independent of the respective type of lymphoid tissue where the AFC emerge [32, 58]. B cell maturation to AFC can be even achieved *in vitro* without forming the typical complex GC structures but still traversing intermediate stages with expression of GC markers [59]. GC phenotypic cells with specificity for the injected antigen were also detected in peripheral lymphoid tissues of CD18$^{-/-}$ mice at low numbers, although classical GC with an analogous specificity remained absent. Besides, careful examination revealed no hints for a compensatory hosting of GC structures or cells in the BM of CD18$^{-/-}$ mice. We therefore conclude that disseminated GC-phenotypic cells were functional in mediating an adaptive immune response including a normal affinity maturation of antibody in CD18$^{-/-}$ mice.

In summary, our data suggest that functional adaptive immunity in murine LAD1 depends on specific properties of the employed immunogen. Demonstrating that absence of CD18 largely reduces the spectrum of suitable immunogens, our data provide further insight into the role of β_2 integrins in adaptive immunity yielding novel results with regard to the complex *in vivo* situation. Based on our data, we suggest that distinct features of an immunization with highly haptenated NP conjugates only could lead to a rescue of function, though not to a rescue of structure with immunogen-specific GC remaining absent. Our data may stimulate further investigations in LAD1 patients.

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Research Article

B-Cell-Activating Factor Affects the Occurrence of Thyroid Autoimmunity in Chronic Hepatitis C Patients Treated with Interferon Alpha

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Chronic hepatitis C (CHC) patients frequently suffer from thyroid disorders during interferon therapy. However, the mechanism remains unclear. In this study, we investigated the association between serum B-cell-activating factor belonging to the TNF family (BAFF) levels and the presence of antithyroid peroxidase antibody (anti-TPO) in CHC patients treated with pegylated interferon alpha and ribavirin combination therapy. Six months after the therapy, anti-TPO antibody was detected in 10 (males, 1; females, 9) of 50 patients. The mean age of these patients was higher than that of the anti-TPO-negative patients (61 yr versus 55 yr). Before treatment, the serum BAFF levels of the anti-TPO-positive patients were higher than those of the anti-TPO-negative patients. After starting therapy, the serum BAFF levels of both the anti-TPO-positive and -negative patient groups were elevated. Our findings suggest that the serum BAFF concentration before therapy can predict the risk of thyroid autoimmunity in elderly female patients with CHC.

1. Introduction

Interferon alpha (IFNα) is a type I interferon that has been widely used as a therapeutic agent, mostly for infectious diseases, including chronic hepatitis C virus (HCV) infection [1]. IFNα therapy is associated with many side effects such as flu-like symptoms, hematologic disorders, and neuropsychiatric disorders [2]. One of the commonest side effects of IFNα therapy is autoimmune thyroid disorders manifesting as Hashimoto's thyroiditis, Graves disease, or the production of thyroid autoantibodies without any thyroid dysfunction [3–5]. However, the detailed mechanism of these effects is unknown.

B-cell-activating factor belonging to the TNF family (BAFF), which is also known as BLyS, TALL-1, zTNF4, or THANK, is part of the TNF family and is known to play an important role in the differentiation of B cells and the maintenance of mature B-cell shape [6–10]. BAFF is expressed on the surfaces of monocytes, dendritic cells, neutrophils, activated T cells, malignant B cells, and epithelial cells [6–10]. BAFF plays an important role in humoral immunity.

The N-terminal sequence of human BAFF contains a furin cleavage site that is responsible for the release of soluble BAFF [8]. After the development of ELISA using monoclonal antibody, high concentrations of BAFF were clinically measured in patients with autoimmune diseases such as rheumatoid arthritis, autoimmune diabetes, Sjögren's syndrome, and multiple sclerosis [11–15]. It was further found that BAFF affects the regulation of the interaction between antigen-presenting cells and T cells, resulting in the emergence of several autoantibodies [16].

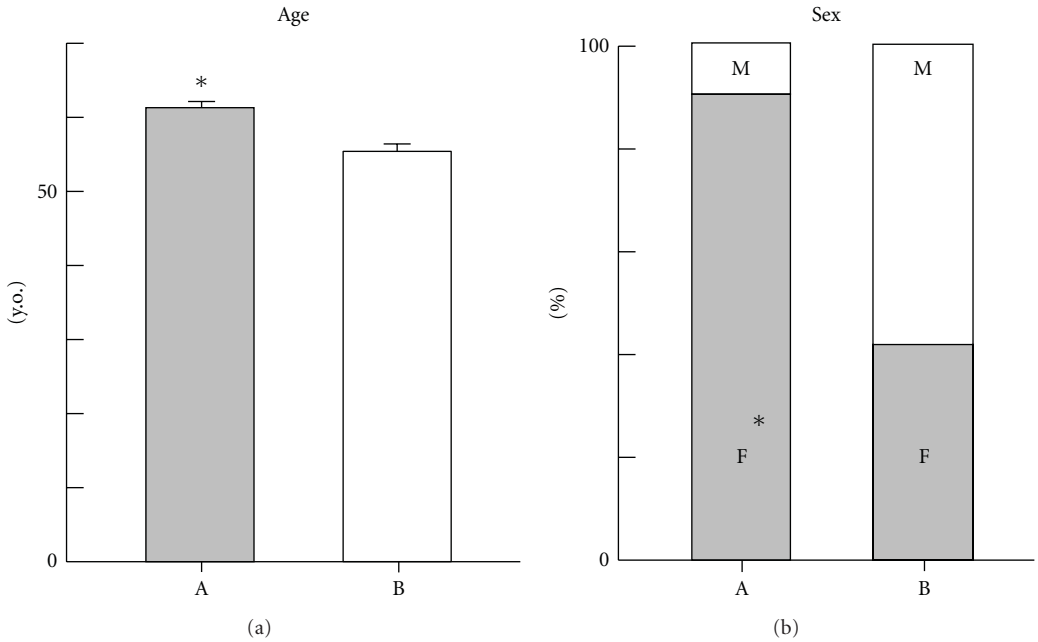

Figure 1: Clinical features (age, sex) of CHC patients. (a) patients who developed anti-TPO antibodies ($n = 10$) at six months after the start of peg-interferon and ribavirin therapy. (b) Patients who not developed anti-TPO antibodies ($n = 40$) at six months after the start of peg-interferon and ribavirin therapy. $^*P < 0.05$ was statistically significant.

So, in the present study, to address the onset mechanism of IFN-induced thyroid autoimmunity, we investigated anti-TPO antibodies and serum BAFF levels in chronic hepatitis C (CHC) patients treated with IFNα.

2. Materials and Methods

2.1. Patients. Fifty CHC patients (males, 24; females, 26) who received pegylated interferon alpha (PEG-IFNα 2b) and ribavirin therapy were enrolled in this study. Their mean age was 57.0 ± 7.1 years old. All patients were diagnosed with chronic hepatitis based on liver pathological findings and were positive for serum HCV RNA before therapy. After obtaining written informed consent, venous blood was collected by venipuncture and was permitted to clot. Serum samples were collected and stored at −70°C.

Serum anti-TPO antibody, TSH, and free T4 levels were examined prior to therapy and six months after the start of therapy using commercially available ECLIA kits (MBL, Nagoya, Japan and Eiken, Tokyo, Japan). The standard values of anti-TPO, TSH, and free T4 are less than 16 IU/mL, 0.5–5.0 μIU/mL, and 0.9–1.7 ng/dL, respectively. Prior to therapy, all patients were confirmed to be negative for anti-TPO and to be within normal limits for TSH and free T4.

2.2. Serum BAFF Concentration. The serum BAFF concentration was examined by a commercially available sandwich ELISA, the Quantikine Human BAFF/BLyS/TNFSF13B Immunoassay (R&D Systems, MI, USA), using monoclonal antibody specific to BAFF [11]. All of the subjects' serum samples were assayed on the same day. The standard serum BAFF value was set from sera of 72 healthy control subjects (males, 35; females, 37. mean age, 56.3 ± 6.2 y.o.).

2.3. Statistical Analysis. Data are expressed as the mean ± standard deviation (SD), and all analyses were performed using the nonparametric Mann-Whitney test and chi-square test. We considered P values of <0.05 to be significant.

3. Results

3.1. Anti-TPO Antibody, TSH, and Free T4 Levels. Before therapy, all study patients were negative for anti-TPO antibody. Six months after the start of treatment, anti-TPO antibodies were newly detected in 10 (20%) of 50 patients. Hereafter, the 10 patients in whom anti-TPO antibody was detected six months after the start of IFN therapy are referred to as group A and the other 40 patients are referred to as group B. As shown in Figure 1, the mean age of the group A patients (61.2 ± 3.8 y.o.) was significantly higher than that of group B (55.6 ± 7.9 y.o.) ($P = 0.03$). The female-to-male ratio of group A was 90% (males, 1; females, 9), and that of group B was 42.5% (males, 23; females, 17). The difference between the two groups was significant ($P = 0.001$). In group A, the mean TSH level before therapy was 1.7 ± 0.6 μIU/mL, and that at six months after the start of IFN therapy was 2.1 ± 1.4 μIU/mL. The mean free T4 level before therapy was 1.1 ± 0.1 ng/mL, and that at six months after the start of IFN therapy was 1.3 ± 0.3 ng/mL. There was no significant difference between the two groups. In addition, the sustained virological response rate in group A was 60% (6/10), and that in group B was 50% (20/40), which were not significantly different.

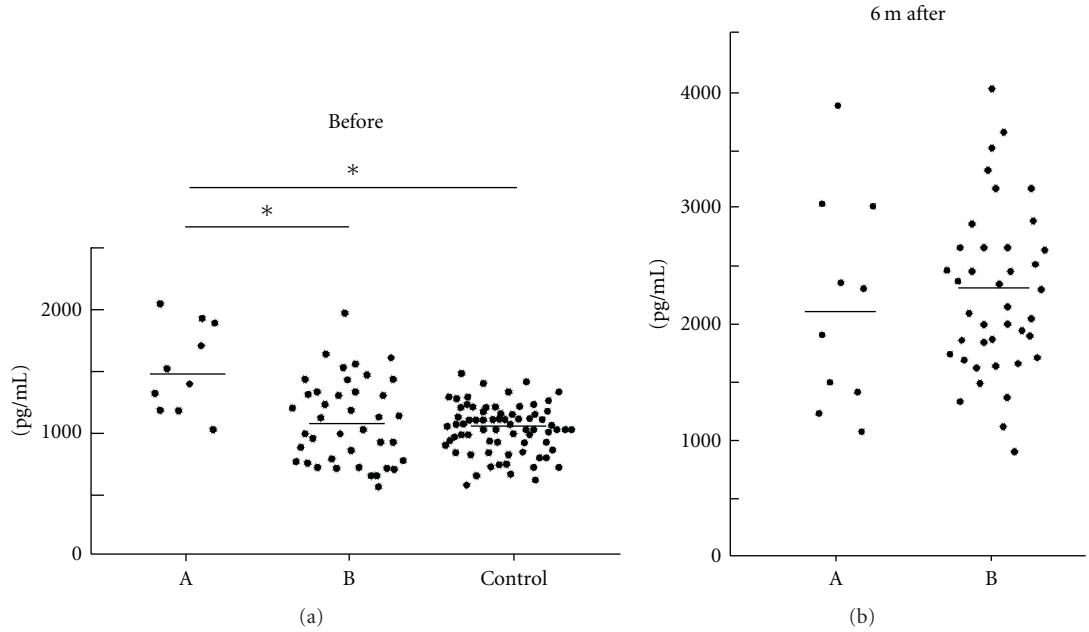

Figure 2: Serum BAFF concentrations of (a, b) (before treatment, at 6 months after the start of therapy) and healthy control subjects. $^*P < 0.05$ was statistically significant.

3.2. Serum BAFF Levels. As shown in Figure 2, the mean serum BAFF level prior to IFN therapy in group A (1497.4 ± 319.4 pg/mL) was significantly higher than that in group B (1139.5 ± 359.1 pg/mL) and healthy control subjects (1105.0 ± 215.2 pg/mL) ($P < 0.05$). The mean serum BAFF levels of both A and B groups were higher at six months after the start of IFN therapy than before therapy; however, they were not significantly different (group A: 2177.8 ± 753.3 pg/mL, group B: 2302.3 ± 660.6 pg/mL).

4. Discussion

Recent well-controlled studies demonstrated that both hypothyroidism and thyroid autoimmunity were significantly more common in patients with CHC than in the control population [17, 18]. Moreover, in CHC patients treated with IFNα, these thyroid disorders were well recognized as serious side effects. Previously, it was found that among CHC patients that received IFN therapy, elderly women were shown to have a 4.4 times higher risk of developing thyroid dysfunction than men [5]. Our findings were concordant with these previous findings. However, the detailed mechanism of IFN-induced thyroid autoimmunity remains unknown [19, 20].

Recently, BAFF was identified to be a 285-amino-acid protein that belongs to the TNF ligand superfamily [6–10]. After a serum BAFF assay was developed using a monoclonal antibody, clinical studies of several autoimmune diseases were conducted [11–15]. In particular, BAFF was found to be strongly associated with the emergence of several autoantibodies [16]. So, in the present study, we investigated the relationship between IFN-induced thyroid autoimmunity and serum BAFF.

First, the serum BAFF baseline levels before IFN therapy were significantly higher in group A than in group B. No difference was observed between group B and healthy control subjects. This result indicates that a high serum BAFF level before IFN therapy is a risk factor affecting the development of thyroid autoimmunity during IFN therapy. IFNα can cause a significant increase in anti-TPO levels in individuals who are positive for anti-TPO before IFN therapy [5]. Even in individuals in whom autoantibody tests were negative before IFN therapy, it was suggested that autoimmunity including thyroid disorders was amplified by IFNα therapy in patients showing high serum concentrations of BAFF before therapy.

Second, the mean serum BAFF levels detected at six months after the start of IFN therapy were significantly higher than those observed before therapy in both groups; that is, we found that serum BAFF levels were increased by IFN therapy.

Interestingly, there was a case undergoing type I IFN therapy developed RA, and this was also associated with increased levels of BAFF [21]. One potential consequence of high BAFF levels is the emergence of autoimmunity during IFNα therapy.

Finally, based on the hypothesis that BAFF might promote autoimmune diseases [22], clinical trials using BAFF inhibitors have been performed in RA and SLE patients [23]. These results could lead to the development of new strategies for treating IFN-induced thyroid autoimmunity.

5. Conclusion

Our findings suggest that the high values of serum BAFF concentration before IFN therapy can predict the risk of thyroid autoimmunity in elderly female patients with CHC.

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Research Article

Interplay of Polarity Proteins and GTPases in T-Lymphocyte Function

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Polarity refers to the asymmetric distribution of different cellular components within a cell and is central to many cell functions. In T-cells, polarity regulates the activation, migration, and effector function of cytotoxic T-cells (CTLs) during an immune response. The regulation of asymmetric cell division by polarity proteins may also dictate CTL effector and memory differentiation following antigen presentation. Small GTPases, along with their associated polarity and adaptor proteins, are critical for mediating the polarity changes necessary for T-cell activation and function, and in turn, are regulated by guanine exchange factors (GEFS) and GTPase activating proteins (GAPS). For example, a novel GEF, dedicator of cytokinesis 8 (DOCK8) was recently identified as a regulator of immune cell function and mutations in DOCK8 have been detected in patients with severe combined immunodeficiency. Both B and T-cells from DOCK8 mutant mice form defective immunological synapses and have abnormal functions, in addition to impaired immune memory development. This paper will discuss the interplay between polarity proteins and GTPases, and their role in T-cell function.

1. Overview of Polarity

Polarity refers to the asymmetric distribution of surface receptors, cytoskeletal components, vesicle trafficking, and signaling proteins within a cell [1]. Many polarity components are conserved between different cell types and organisms (reviewed in [2]). Polarity is an important factor in T-cell functions, such as immunological synapse (IS) formation, migration, target cell killing, asymmetric cell division (ACD), and differentiation [3–8]. In order to establish and maintain polarity in response to dynamic cell-cell interactions and extracellular cues, a T-cell must be able to orchestrate different signals to regulate the different recruitment of many cellular components. This process is highly regulated and involves both GTPases (reviewed in [9]) and a network of polarity proteins [1].

GTPases act as molecular switches to control cellular processes. The family of Rho GTPases includes Cdc42, RhoA, and Rac1 (reviewed in [10]). GTPases have two conformational states, which are dependent on the type of guanine nucleotide bound. The active state is induced by the binding of Guanosine-5′-triphosphate (GTP), and the inactive state is induced when Guanosine diphosphate (GDP) is bound. The loading of GTP and dissociation of GDP are regulated by different proteins: guanine exchange factors (GEFs) promote the exchange of GDP for GTP, GTPase activating proteins (GAPs) catalyze the activity of GTPase activity to their downstream effectors, and the guanine nucleotide dissociation inhibitors (GDIs) block regulation (reviewed in [11]). Activated Rho GTPases regulate cytoskeleton remodeling, which in turn influences morphology, migration, and protein trafficking (reviewed in [12]). Like other members of the Rho GTPase family, Cdc42 influences a large array of cellular activities. Its downstream effectors include a large number of kinases which activate many signaling pathways [13, 14] as well as nonkinase proteins, such as neuronal Wiskott-Aldrich Syndrome protein (N-WASP) [15] which promotes actin nucleation.

The evolutionarily conserved polarity proteins are localized into different regions of a cell to act as scaffolds for the

recruitment of other protein complexes (reviewed in [16]). The Scribble, Par and Crumbs polarity protein complexes are the most extensively studied. The Scribble complex consists of Scribble (Scrib), Discs large (Dlg), and Lethal giant larve (Lgl) proteins (reviewed in [17]). The Scribble and Par complexes regulate asymmetric cell division (ACD) of neuroblasts in *Drosophila* (reviewed in [18]). The Par complex, which consists of Par3, Par6, and atypical protein kinase C (aPKC), was first discovered in *C. elegans* embryos that have defective anterior-posterior partitioning [19]. The Crumbs complex consists of Crumbs, PatJ, and Pals1 (reviewed in [2]) and is important in mammalian epithelial cell polarity [20]. All of these proteins, with the exception of aPKC, consist of a variable number of binding motifs termed PSD-95/Dlg/ZO-1 (PDZ) domains [2]. The PDZ domain can interact with a number of signaling proteins; for example, Dlg1 can interact with protein tyrosine phosphatase and tensin homologue (PTEN) [21] as well as with other PDZ-containing proteins and the Par6-aPKC complex can interact with Lgl, Par3, and Pals1 (reviewed in ([1, 2]). Polarity proteins establish a network to orchestrate signals throughout the cell in response to extracellular cues. The polarity proteins can work cooperatively or antagonistically [17] to regulate cell polarity. Polarity proteins also work in conjunction with GTPases to establish and maintain cell polarity (reviewed in [22]).

2. Polarity in T-Cells: The Immunological Synapse

Two main classes of T-cells are produced after maturation and selection in the thymus: $CD8^+$ T-cells and $CD4^+$ T-cells, distinguished by their expression of either the cell surface marker, cluster of differentiation 8 (CD8) or 4 (CD4). $CD8^+$ T-cells function as cytotoxic T-lymphocytes (CTL) and have the ability to kill target cells, such as virus-infected cells, by releasing pore-forming perforin and serine protease granzymes via exocytosis [23]. To carry out their immune functions, CTLs must first be activated. CTL activation involves the interaction of the T-cell receptor (TCR) with pathogen-derived peptide antigen presented by antigen presenting cells (APCs) via their major histocompatibility complex (MHC) class I molecule. An immunological synapse (IS) is formed when a TCR interacts with peptide MHC (as reviewed in [24, 25]). T-cell activation also involves an important second signal, which is provided by the interaction between the costimulatory molecules on the T-cell and APC. The importance of the co-stimulatory signal in naïve T-cell activation has been demonstrated in many *in vitro* studies (reviewed in [26]).

During IS formation, many molecules and complexes are recruited towards, or away from, the cell-cell interface. Molecules such as the TCR and microtubule organizing centre (MTOC) are recruited to the interface, while CD43, a member of sialoglycoproteins, is polarized to the distal pole, away from the interface [27]. At the interface, compartmentalization of proteins was first described by Kupfer's group, where surface molecules are clustered to regions termed supramolecular activation clusters (SMACs) [28]. In a mature IS, the central region, or cSMAC, contains the TCR, CD28, and their associate signaling molecules. The cSMAC is surrounded by an outer ring of adhesion molecules including lymphocyte function-associated antigen 1 (LFA-1), and talin, a cytoskeleton protein that links integrins to the actin cytoskeleton [29, 30]. The formation of the IS is a dynamic process. Initial antigen-independent contacts between the T-cell and the target cell involve the interactions of adhesion molecules such as CD2 with LFA-3 [31] and LFA-1 with ICAM-1 [28]. LFA-1 and ICAM-1 localize to the cSMAC and TCR-MHC complexes to the pSMAC at the initial phase of IS formation. However, in a mature IS the situation inverts and the TCR-MHC complex resides in the cSMACs, while the antigen independent interactions are at the periphery [24]. An important implication of a polarized and compartmentalized IS is the regulation of T-cell activation, by controlling TCR signaling and TCR degradation [32]. Studies have shown that the cSMAC plays a role in TCR degradation in the event of strong agonist interactions [33], and it has been proposed that signals from weaker interactions are enhanced [34]. The exact role of the synapse is still controversial ([35, 36]), however, these studies highlight the importance of the polarized and compartmentalized nature of the immunological synapse.

While the nature and function of the TCR-MHC complex has been intensively studied, the role of LFA-1 and its interaction with its ligand, ICAM-1, in the pSMAC has only recently been elucidated. LFA-1 is part of the large family of leukocyte integrins and is expressed on T and B lymphocytes. It is involved in a wide range of T-cell functions including activation upon antigen presentation, CTL-mediated killing, cell adhesion, and migration. The importance of LFA-1 in the immune system is highlighted by patients with leukocyte adhesion deficiency (LAD) who have impaired pathogen clearance and suffer repeated infections [37]. Integrin $\beta 2$ chain (CD18)–deficient mice displayed defects in leukocyte adhesion and proliferation [38]. LFA-1 is critically involved in the initial contact of a T-cell with the APC [39]. This contact is essential for T-cell activation as it provides the stop signal for a migrating T-cell to scan the surface of the APC for peptide-MHC. The TCR-peptide-MHC interaction activates LFA-1 and increases its affinity and avidity, resulting in a stringent interaction with its ligands, such as ICAM-1 (reviewed in [40]). This stronger interaction is believed to be a stabilizer in T-cell dendritic cell (DC) interactions [41] therefore allowing sustained TCR signaling. LFA-1 is also needed for Erk1/2 signaling during antigen presentation [42]. The Erk1/2 signaling pathway promotes T-cell activation and proliferation. LFA-1 is one of the many proteins that regulate IS formation and, as discussed above, is critical for normal T-cell activation and proliferation.

To carry out its highly specialized functions, the IS and its associated signaling and adhesion proteins are tightly regulated. The change in morphology that occurs when a T-cell contacts a target cell is mediated by actin cytoskeleton rearrangement. TCR signaling induces phosphorylation of myosin II [43], which causes loss of myosin filaments [44]. This allows for the depolymerization of the actin

cytoskeleton in the midbody, and in the uropod, facilitating change in morphology. The Scribble complex is also believed to be involved in myosin II regulation [45]. Scribble and Dlg are transiently recruited to the cell-cell interface upon IS formation [6, 46]. TCR signaling induces dephosphorylation of pERM, which leads to relaxation of the cytoskeleton, allowing Scribble and Dlg to be recruited to the synapse. This process is mediated by cytoskeleton rearrangements that are regulated by Rho and Rac GTPases [47]. TCR signaling also leads to Vav-(a GEF for Cdc42) mediated cytoskeleton remodeling. After activation by TCR signaling, Vav activates Cdc42 and Rac1 [48, 49], which in turn activates WASP and PAK. WASP promotes actin nucleation, which generates a contracted actin network that serves as a scaffold for signaling molecule recruitment. Scribble may recruit Rac1 and Cdc42 to the IS through the p21-activated kinase [PAK]-interacting exchange factor (β-PIX) [50] and may bring the GTPases into close proximity to their downstream effectors and many signaling molecules (Figure 1(a)). β-PIX and Scribble have been shown to interact in other cell types, so this interaction may also provide a mechanism for recruitment of β-PIX to the IS following TCR stimulation. TCR signaling also leads to the activation of downstream transcription factors, which play a major role in regulating asymmetric cell division and differentiation, and polarity proteins may serve as an integrating platform for various signals.

3. Polarity in T-Cells: Asymmetric Cell Division

As discussed above, a naïve T-cell is activated after interacting with the peptide-MHC molecule on APCs, during which an IS is formed. Differentiated cells are well-characterized by the expression levels of specific cell surface markers and immune functions, as well as transcriptional events in the developmental pathway. However, there are competing hypotheses on the mechanism that gives rise to the large variety of functionally diverse subsets of T-cells [51–56]. In the "one cell, one fate" model, naïve cells are activated after receiving unique signals and give rise to a homogeneous populations of progeny cells. The generation of different subsets of cells is therefore determined by factors such as antigen availability over time and degree of maturation of the DCs [57]. The "one cell, multiple fates" model proposes that naïve cells undergo asymmetric cell division after activation and give rise to two daughter cells that are committed to different cell fates, thus generating a heterogeneous population of progenies [58]. Asymmetric cell division involves the establishment of an axis of polarity, which may be influenced by different external cues such as the microenvironment and the orientation of the mitotic spindle to the axis. Fate determinants are recruited into the two daughter cells and after division, each daughter cell inherits a different set of determinants, which set them on different paths of cell fate (reviewed in [59]). Asymmetric cell division has been observed in different cell types in mammalian cells [60] and is evolutionarily conserved across many organisms. One example is when a *Drosophila* sensory organ precursor (SOP) undergoes asymmetric cell division to produce a pIIa cell and pIIb cell. Following another round of asymmetric cell division, the pIIa daughter cell gives rise to a socket and a shaft granddaughter cell. One of the daughter cell of pIIb is programmed to die, while the other gives rise to a neuron and a sheath cell [61].

The first evidence to show that asymmetric cell division occurs in T-lymphocytes was reported by Chang et al. [5]. This study, and others since, has shown that polarity proteins, cell fate determinants such as Numb, and transcription factors, are asymmetrically distributed in T-cells during cell division [4, 5, 8]. Most interestingly, the putative "proximal" daughter cells (isolated by high expression of CD8) provided acute, but poor long-term, protection against *Listeria* infection after adoptive transfer of the daughter cells into recipient mice. In contrast, the low CD8 expressing daughter cells (putative "distal" daughters) gave long-term protection [5]. Scribble, aPKC and Par3 are all asymmetrically distributed during cell division in T-lymphocytes. However, the mechanisms of ACD, as well as how extracellular cues, such as the degree of DC maturation and the cytokine environment, can influence asymmetric cell division and ultimately cell fate, are poorly understood.

4. Polarity in T-Cells: Migration

Migration is particularly important in the context of T-cell activation and effector functions, as T-cells undergo a number of scanning steps before antigen recognition. When a T-cell migrates, it establishes a front-rear polarity with a leading edge and a trailing end (reviewed in [3, 62]). The leading edge of the cell, or lamella, has a high concentration of free actin filaments to generate contractile force [3], and chemokine receptors such as CCR2 and CCR5 [63] to facilitate effective homing of the lymphocyte. The posterior of the cell contains a protrusion, termed the uropod, which adheres to the substratum, allowing the lymphocyte to move forward (reviewed in [27, 45]). The MTOC, the TCR, ezrin, and adhesion molecules, such as CD43, and intercellular adhesion molecule-1 (ICAM) [64] are polarized to the uropod. GTPases and polarity proteins regulate the spatial organization of these cellular components.

The shape of a migrating T-cell is dynamic and requires continual rearrangement of the actin cytoskeleton in the lamellipodia [65]. Therefore, a T-cell must be able to remodel its cytoskeleton efficiently. GTPases are central to this process and the spatial regulation of their activity enables cell movement and controls directionality (reviewed in [66]). Rac1 and Cdc42 promote actin nucleation at the leading edge of a T-cell via WASP and Scar proteins, which induce Arp2 and Arp3 proteins to bind to actin monomers and promote nucleation. Nucleation of actin monomers catalyzes actin polymerization [1, 67]. Rac1 promotes protrusion and Cdc42 induces filopodia. Cdc42 is also essential for directing a migrating cell to extracellular cues [68]. Another important GTPase is RhoA. Activation of RhoA is required for uropod formation. ROCK protein kinase is one of the downstream effectors of RhoA [69]. ROCK signaling results in cell body contraction and rear end retraction (reviewed [1]).

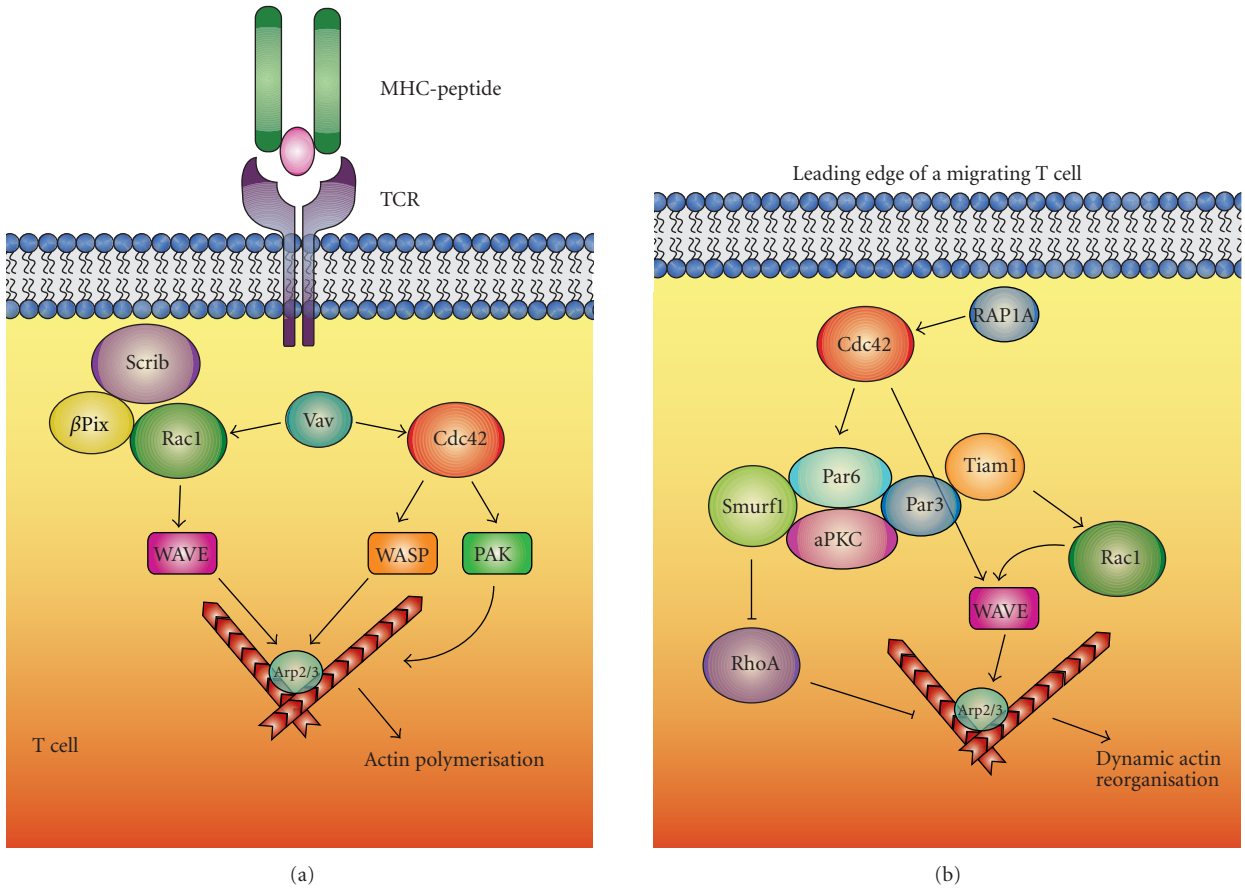

Figure 1: GTPases are important mechanical switches in T-lymphocyte function. (a) During antigen presentation, a T-cell undergoes dramatic changes in protein localization and morphology. The polarity protein, Scribble, is believed to be recruited to the synapse after TCR signaling and, through its potential association with βPIX, may recruit Rac1 and Cdc42 to close proximity to GEFs such as Vav. Activated Rac1 and Cdc42 in turn, activate downstream effectors such as WAVE, WASP, and PAK, enabling actin polymerization and thus, changes in morphology. (b) In a migrating T-cell, GTPases regulate actin polymerization to allow for cell moment. At the leading edge of the cell, Cdc42 is activated by the Ras-related protein RAP1a, which in turns activates members of the Par complex. Par3 recruits a RAC GEF, Tiam1, which in turn activates Rac1. Rac1 promotes actin reorganization, thus lamellipodium formation through proteins such as WAVE and Arp2/3. The Par complex also binds and activates the E3 ligase Smurf1. Smurf1 promotes degradation of another GTPase, RhoA, which, in its active form enables actin contractility in cells.

Polarity proteins have been shown to interact extensively with GTPases in T-cells and other cell systems. Following chemokine stimulation, Cdc42 at the leading edge is activated by RAP1A, a Ras-related protein, which activates the Par complex [22]. Tiam1, a Rac GEF, is recruited to the leading edge by Par3 [70, 71] and then activates Rac1, which in turn induces actin nucleation and therefore lamellipodium formation. The Par6-aPKC heterodimer also binds to E3 ligase Smurf1 and activates it. Smurf1 degrades RhoA [72] and therefore reduces actin contractility, resulting in the characteristic dynamic actin polymerization and depolymerization at the leading edge (Figure 1(b)). Scribble and Dlg are found to be asymmetrically distributed in the uropod of migrating T-cells and reduced expression of Scribble and Dlg by shRNA knockdown results in the loss of the uropod and the recruitment of the uropod markers, CD44, and Ezrin. The loss of Scribble also causes abrogation of T-lymphocyte migration [6].

5. DOCK8: A New Player in T-Cell Polarity

Apart from the more extensively studied polarity proteins, the protein Dedicator of Cytokinesis 8 (DOCK8), was recently identified as a potential regulator of polarity in immune cells. DOCK8 is a Rho-Rac guanine exchange factor [73] and was first discovered in a screen for binding partners of the Rho GTPase, Cdc42 using a yeast two-hybrid system [74]. The DOCK8 protein has extensive homology to the Ced-5/DOCK180/Myoblast city (CDM) family of proteins [75]. The members of this family of proteins share two conserved domains, DOCK homology regions (DHR) 1 and 2. The important GEF activity is situated in the DHR-2 domain. There are eleven members in the DOCK family [74] but only DOCK180 and DOCK2 have been extensively studied. DOCK2 is required for CD28-mediated Rac activation [76], translocation of the TCR after antigen presentation [77] and lymphocyte migration [78]. DOCK2

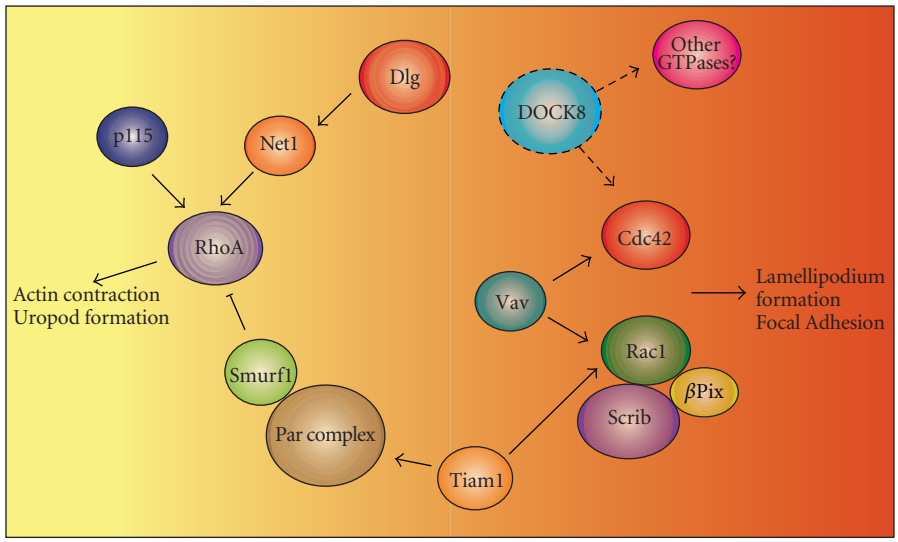

Figure 2: GEFs and polarity proteins are important GTPase regulators. GTPases function as switches in cells, controlling a large variety of pathways. They are tightly regulated by Guanine exchange factors (GEFS), GTPase activating proteins (GAPS) and polarity proteins. The recently discovered that GEF, DOCK8, may also be part of this large network. Evidence has shown that it interacts with Cdc42, an important GTPase in the regulation of cell morphology and motility. DOCK8 may also be a regulator of other GTPases that control different cellular functions important for T-cell function.

and DOCK180 are involved in cytoskeletal remodeling [78, 79] and in regulating the activation of Rac [77, 80]. Apart from binding to Cdc42 with high affinity in the yeast two-hybrid screen, Ruusala also demonstrated that DOCK8 is localized in the lamellipodia in porcine aortic endothelial cells [74] where extensive actin cytoskeleton remodeling occurs. Therefore, one can speculate that, similar to the other members in the family, DOCK8 is involved in some aspects of actin cytoskeleton regulation. This is reinforced by the fact that DOCK8 serves as a GEF for Cdc42, which is a regulator of cell morphology, migration, and proliferation.

Interestingly, loss-of-function mutations in *DOCK8* were recently identified in patients with severe combined immunodeficiency, characterized by repeated bacterial and viral infections [81, 82]. Analysis of patient lymphocytes revealed lower numbers of $CD4^+$ and $CD8^+$ T-cells, impairment of T-cell proliferation upon stimulation by anti-CD3 and anti-CD28 antibodies, and a moderate decrease in interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) [81, 82]. However, the $CD8^+$ T-cells had normal levels of cytotoxic activity as well as extravasation ability [82]. These studies demonstrated, for the first time, that DOCK8 is involved in the regulation of immune cells. DOCK8 has also been speculated to have tumour-suppressor functions, as a number of patients in the study had human papillomavirus infections and cutaneous T-cell lymphoma-leukemia [82].

Using a DOCK8 mutant mouse model, Primurus (*pri/pri*), where a point mutation changes a serine to a proline residue in the DHR-2 domain of the DOCK8 protein, Randall et al., [73] have characterized the role of DOCK8 in immune cell function. The *pri/pri* mutation is thought to break the contact between the DHR-2 domain with Cdc42, and therefore interfere with normal GTP exchange function. Analysis of the *pri/pri* mice revealed that there are defects in marginal zone B lymphocyte formation as well as in B-cell persistence in the germinal centers. The mutant B-cells are also unable to undergo affinity maturation, resulting in poor longevity in memory-mediated humoral response. The mutation also disrupts the accumulation of ICAM-1 to the pSMAC of the IS [73]. DOCK2 deficient mice also have impaired B-cell migration to lymph nodes but this phenotype is not observed in the *pri/pri* mice despite the high degree in homology between amino acid sequence between DOCK2 and DOCK8 [74]. This data suggests that DOCK8 may have a specialized role in immune cells.

The severe cutaneous viral infections typical of patients with DOCK8-deficiency in particular, suggest a role for DOCK8 in $CD8^+$ T-cell function. In two separate studies using the *pri/pri* mouse model, mutation of DOCK8 significantly decreased the number of peripheral naïve $CD8^+$ T-cells [83, 84]. Although phenotypically normal, the $CD8^+$ T-cells show delayed proliferation in response to dendritic cells presenting antigen *in vitro* [83]. Despite this phenotype, DOCK8 deficient mice mount a relatively normal primary immune response to viral infection *in vivo*, but show significantly impaired persistence and survival of memory $CD8^+$ T-cells [83, 84]. Interestingly, this defect correlated with abnormal polarization of LFA-1 and actin to the immunological synapse formed between naïve $CD8^+$ T-cells and antigen-presenting dendritic cells [73] suggesting a polarity defect that results in suboptimal synapse formation. These data, and others [85, 86], suggest that the quality of the IS and the downstream signals generated are critical for the development and persistence of memory T-cells.

6. Conclusions

It is now apparent that, similar to polarity of cells of solid tissues [16], polarity of immune cells may be controlled by a dynamic and two-way interaction between polarity proteins and Rho GTPases. The molecular links between the two groups of proteins seem to be predominantly built upon physical interactions between regulators of the Rho GTPases such as the GEFS, and different components of the polarity complexes (Figure 2). As we identify the specific role of each GEF in morphological changes of immune cells, we will begin to elucidate how the polarity proteins influence the localization of each GEF, but at this stage there are many gaps in our knowledge. For instance, new findings regarding DOCK8 clearly demonstrate important roles for this protein in immune cell polarization, but the molecular basis for its polarity is not yet known. In contrast, Tiam1 and βPIX have clear roles in T-cell polarity (particularly related to the immunological synapse) and are regulated by known interactions with members of the polarity network. Understanding how each of these players interact to dictate T-cell polarity will be the next big challenge.

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Research Article

Inhibition of Arterial Allograft Intimal Hyperplasia Using Recipient Dendritic Cells Pretreated with B7 Antisense Peptide

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Background. Low expression or absence of dendritic cell (DC) surface B7 molecules can induce immune tolerance or hyporesponse. Whether DCs could induce indirect allogeneic-specific cross-tolerance or hyporesponse to recipient T cells remains unclear. *Methods*. Generated from C3H/He mice bone marrow cells pulsed with donor antigen from C57BL/6 mice, recipient DCs were incubated with B7 antisense peptide (B7AP). Immune regulatory activities were examined *in vitro* by a series of mixed lymphocyte reactions. Murine allogeneic carotid artery orthotopic transplantation was performed from C57BL/6 to C3H/He. Recipients were given B7AP-treated DCs 7 days before transplantation. Allograft pathological analysis was done 2 months after transplantation. *Results*. B7AP-pretreated DCs markedly inhibited T-cell proliferation compared with untreated group. Pretreated T cells exhibited markedly reduced response to alloantigen versus third-party antigen. Pathological analysis of arterial allografts demonstrated significant reduction of intimal hyperplasia in B7-AP pretreated group versus control. *Conclusion*. Blockade of B7 molecules by B7AP could induce indirect allogeneic-specific hyporesponse and inhibit arterial allograft intimal hyperplasia, which may be involved in future strategies for human allograft chronic rejection.

1. Introduction

Graft loss from chronic rejection has become the major obstacle to the long-term success of whole organ transplantation [1]. It is accepted that direct and indirect recognitions, both mediated by donor and recipient dendritic cells (DCs), are the major causes for all types of organ transplant rejection [2, 3]. The direct activation of T lymphocytes by donor-derived antigen-presenting cells (APCs) is thought to be responsible for the vigor of acute rejection, while the indirect allorecognition has been implicated in the initiation of chronic allograft dysfunction [4–6].

The B7-CD28/CTLA4 costimulatory pathway plays a crucial role in the regulation of T-cell activation [7]. B7 molecules are expressed on the surface of APCs, providing a critical co-stimulatory signal to T cells by engaging CD28. Blockade of the B7-CD28 interaction *in vitro* can generate antigen-specific anergy [8–10]. Administration of monoclonal antibodies (MoAbs) against B7 or CTLA4-Ig fusion protein to block B7 has been shown to be promising as a treatment for allograft rejection [11–13]. It has been reported that the recipients immunized with donor resting B cells or immature DCs could induce specific immune tolerance and prolong allograft survival. This effect has been attributed to low-level expression or absence of B7 molecules on these cells [14, 15]. Although recent studies [16–18] have shown that modified or pretreated DCs can induce direct alloreactive T-cell hyporesponsiveness, it is not yet clear whether DCs can also induce indirect allogeneic-specific cross-hyporesponsiveness to recipient T cells. Recent years have witnessed an increasing interest in the development of nonimmunogenic peptide as an antagonist for protein-protein interaction in immunomodulatory therapeutics [19, 20]. Progress in antisense technology and molecular modeling over the past decade has made molecular recognition study possible [21–23]. Antisense peptides are short peptide sequences that specifically constitute one side of the binding sites of complementary protein pairs [24]. B7 antisense peptide (B7AP) is a peptide analogue of the CD28-binding region [12, 24]. It is characterized by higher affinity to B7

ligand, lower molecular weight, and lower immunogenicity and difficulty to be metabolized compared with CTLA4-Ig [12, 25] which would block the allorecognition in a longer period to take more effect. It has been reported [12] that blockade of B7 molecules on donor splenocytes pretreated with B7AP could induce specific immune hyporesponse and prolong allograft survival in the recipients. In this study, we tried to induce cross-hyporesponsiveness to recipient T cells in an indirect pathway by B7AP pretreated donor-pulsed recipient DCs. The results showed that the administration of this recipient DCs could induce indirect allogeneic-specific cross-hyporesponsiveness to recipient T cells and inhibit the intimal thickening of arterial allograft, which may lead to the suppression of allograft chronic rejection.

2. Materials and Methods

2.1. Animals. C57BL/6(H-2K^b), C3H/He(H-2K^k) and BALB/C(H-2K^d) male mice weighing 29–32 g, 8–12 weeks old, were purchased from Shanghai Laboratory Animal Center of Chinese Academy of Sciences (Shanghai, China), maintained in a specific pathogen-free facility at Fudan University (Shanghai, China). All animal surgical procedures were approved by the Institutional Animal Care and Use Committee of Fudan University.

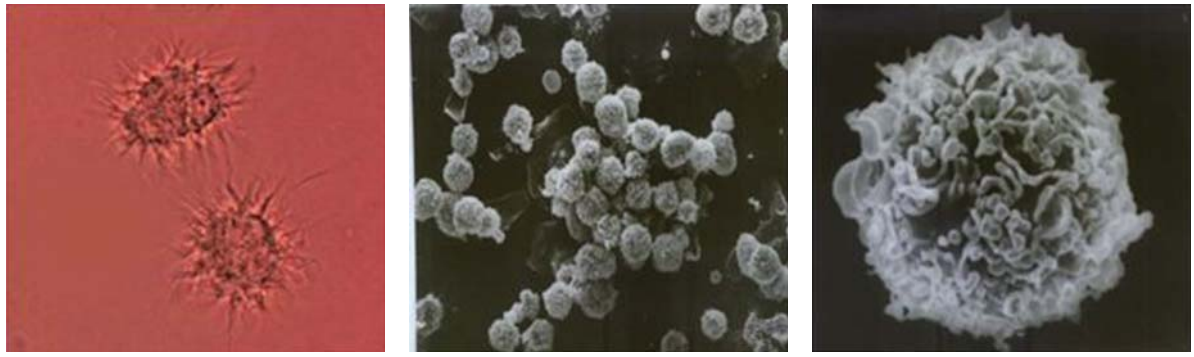
2.2. Synthesis and Purification of B7AP [26]. Antisense peptides were synthesized and purified by GL Biochem Ltd., (Shanghai). It has been reported that the MYPPPY motif is the core of CD28 binding sites to its ligand B7. Several different peptides containing the motif were screened by BIOPOLYMER and BINDING SITE ANALYSIS in the INSIGHT II molecular modeling software package, and B7AP was obtained with the sequence EFMYPPPYLD. The peptide was synthesized on a solid phase peptide synthesizer (Multiple Peptide Synthesizer; Genemed Synthesis, Inc., CA, USA). The crude peptide was purified by the Varian Prostar high performance liquid chromatography (HPLC) system using a C8 column (Varian Prostar HPLC system, CA, USA). Analytical HPLC was performed through a Varian C8 analytical column using a linear gradient of 0–100% acetonitrile in water containing 0.1% trifluoroacetic acid over a period of 20 min. The identity of the peptide was confirmed by mass spectrometry (Voyager Elite model, Perceptive Bio system, Applied Bio systems, WA, USA). The purity of the peptide (higher than 95%) was examined by HPLC analysis. Peptide was lyophilized and stored at −20°C. Serum-free RPMI 1640 was added to adjust the concentrations of B7AP before use.

2.3. Preparation of the Donor Antigen. Freshly harvested C57BL/6 mice spleens were first minced in 2 mL complete RMPI-1640 medium, filtered through a nylon mesh, and then transferred into a 15-mL centrifugal tube. After centrifugation at 1500 rpm for 5 min followed by discarding of the supernatant, complete RPMI-1640 medium was added to the splenocyte suspension. The suspension was then transferred to another centrifugal tube containing 3 mL lymphocyte separation medium and centrifuged at 2000 rpm for 20 min to get the lymphocyte layer. Then the lymphocyte layer was carefully transferred to another centrifugal tube, added with complete RPMI 1640 medium and centrifuged again at 1500 rpm for 5 min. After discarding the supernatant, complete RPMI 1640 medium was added and the cell concentration was adjusted to 1×10^6/mL to get the pure lymphocyte suspension. Lymphocyte suspension (1×10^6/mL) was frozen at −80°C (or liquid nitrogen) and thawed at 37°C for 6 times, then centrifuged at 7500 rpm for 10 min. The supernatant was harvested, filtered through 0.22 μm membrane, and stored at 4°C.

2.4. Propagation of Bone-Marrow-Derived DCs Loaded with Donor Antigen. Bone-marrow-derived dendritic cells (BM-DCs) were generated as previously described [27, 28] with some modification. Bone marrow cells harvested from the femurs and tibias of C3H/He mice were cultured in 24-well plates (1×10^6 cells per well). Culture medium contains 160 U/mL gentamycin, 2 mmol/L L-glutamine, 0.05 mmol/L 2-mercaptoethanol, 1 mmol/L sodium pyruvate, and 10% (v/v) FCS (Gibco, Gaithersburg, MD, USA) in the presence of recombinant mouse granulocyte macrophage colony-stimulating factor (rmGM-CSF,10 μg/mL; R&D Systems, Minneapolis, MN, USA). All cultures were incubated at 37°C with 5% humidified CO_2. Nonadherent cells would be released spontaneously from proliferating clusters after 48 hours of culture. Medium change containing rmGM-CSF was done every two days. In the sixth day, the donor antigen and recombinant mouse TNF-α (rmTNF-α, 50 μg/L; R&D Systems, Minneapolis, MN, USA) were added to the medium. In the eighth day, the buoyant cells were harvested at the concentration of 1×10^6 cells/mL. DCs were irradiated with 3000 rads γ-ray and then incubated with B7AP (10 mg/L) at 37°C for 90 min. After washing with phosphate buffered saline (PBS) once, tolegenic DCs were modulated at the concentration of 3×10^6/mL. The purity of DC preparations was routinely monitored by flow cytometric analysis using anti-CD11c monoclonal antibody (eBioscience, San Diego, CA, USA). This DC preparation protocol could enrich CD11c + cells more than 85%.

2.5. Flow Cytometry. Cell-surface phenotypic analysis of B7AP-treated DCs was done by EPICS ELITE flow cytometer (Coulter, Hialeah, FL, USA). Fluorescein-isothiocyanate-(FITC-) conjugated antimouse MHC Class II, CD80 and CD86 antibody (anti-MHC Class II-FITC, anti-CD80-FITC, anti-CD86-FITC) were used for cell staining (BD PharMingen, San Diego, CA, USA). FITC-conjugated isotype-matched irrelevant monoclonal antibodies (rat IgG2b, Armenian hamster IgG2, and rat IgG2a, resp.) were used as negative controls (Cedarlane Laboratories, Hornby Ontario, Canada).

2.6. Mixed Lymphocyte Reaction (MLR). T cells prepared from recipient splenocytes by nylon wool purification were used as responders (2.5×10^6/mL). B7AP-treated recipient DCs (0.5×10^6 from C3H/He mice) were used as stimulators and irradiated with 3000 rads before use. Untreated recipient DCs pulsed with donor antigen and recipient DCs without



(a) (b) (c)

Figure 1: BM-DCs detected by inverted microscope and scanning electron microscope. Progenitor cells were propagated in the presence of rmGM-CSF. After TNF-α and donor antigen addition, the cells show significant differentiation with typical DC appearance under inverted microscope (a) and scanning electron microscope (b) and (c).

donor antigen were included as controls. B7AP was diluted to 4×10^2 mg/L, 4×10^1 mg/L, 4×10^0 mg/L,4×10^{-1} mg/L, 4×10^{-2} mg/L, 4×10^{-3} mg/L, 4×10^{-4} mg/L, and 4×10^{-5} mg/L by serum-free RPMI 1640 and then incubated with recipient DCs (0.05 mL DCs and 0.05 mL B7AP) for 1 hour at 37°C. After that, MLR between DCs and T cells was performed. Cultures were established in triplicate in 96-well round-bottom microculture plates (200 μL per well) and maintained in complete medium for 7 days in 5% CO_2 at 37°C. ^{3}[H] TdR (1 μCi/well) was added in the final 18 hrs. Cells were harvested onto glass fiber disks using an automated system, and incorporation of ^{3}[H] TdR into DNA was assessed by Wallac 1450 Microbeta liquid scintillation counter. Data was presented as mean counts per minute (cpm) ±SD in triplicate replication.

2.7. Induction of Indirect Allogeneic-Specific Hyporesponse to Recipient T cells In Vitro . Mixed lymphocyte reaction (MLR) between recipient T cells and B7AP-pretreated recipient DCs pulsed with donor antigen was performed in complete medium for 72 hrs in 5% CO_2 at 37°C. Then PBS was added and the suspension was harvested by centrifugalization at low rotation speed (500 r/m × 15 min) to remove the precipitated dead cells. After being washed for three times by PBS, the pretreated T cells were adjusted at the concentration of 5×10^6/mL and placed in static condition for 24 hrs. For the second round of MLR, the pretreated T cells were used as responders (2.5×10^5 per well). The recipient DC pulsed with donor antigen untreated by B7AP was used as stimulators (0.5×10^5 per well). The recipient DC pulsed with the third party donor (derived from BALB/C) without B7AP treatment and the recipient DC without donor antigen were included as controls. Cell harvesting and thymidine incorporation was performed the same way as the first round of MLR.

2.8. Murine Model of Allogeneic Carotid Artery Orthotopic Transplantation. Both C3H/He-recipient mice and C57BL/6 donor mice were anesthetized with ketamine (75 mg/kg, i.p.), had their hair shaved, and then placed in a supine position with their limbs immobilized. The skin of the operative region was sterilized. Longitudinal incisions from the right mandibular angle to the middle point of the right clavicle were made for both donor and recipient surgeries. Common carotid artery of the donor was dissected, excised for a 0.5 cm segment, and then flushed and stored at 4°C Ringer's solution. After the dissection and removal of a 0.5 cm segment of recipient carotid artery, the arterial graft was anastomosed to the recipient carotid artery orthotopically using an end-to-end interrupted suture technique with 10-0 PROLENE at 40x magnification. The distal end of the carotid was first reperfused, followed by the reperfusion of the proximal end under the condition of no anastomotic bleeding. The incision was closed with layered sutures. Animals were allowed access to food and water right after surgery.

To assess the effect of the pretreated DCs on the intimal hyperplasia of the arterial graft, recipient mice ($n = 10$) were given 3×10^6 cells intravenously via the femoral vein, 7 days before transplantation in the absence of immunosuppression. Meanwhile, control group ($n = 10$) were established without any treatment. Arterial grafts were harvested for histopathological examination 2 months after transplantation.

2.9. Morphometric and Histological Analysis. Cross sections of arterial grafts were fixed in 10% formaldehyde and then embedded into paraffin and stained with hematoxylin and eosin. The area of the tunica intima and tunica media was assessed by light microscopy and computer-based Morphometric Analysis System. Only vessels that were cut orthogonally and displayed a clear internal and outer elastic lamina were accepted. The thickness of tunica intima was calculated by the following equation: Thickness of tunica intima = Area of tunica intima/(Area of tunica intima + Area of tunica media) [29].

2.10. Statistical Analysis. All data are presented as means ± SD. Statistical analysis was performed by STATA version 8.0. Homoscedasticity analysis and t test were used to

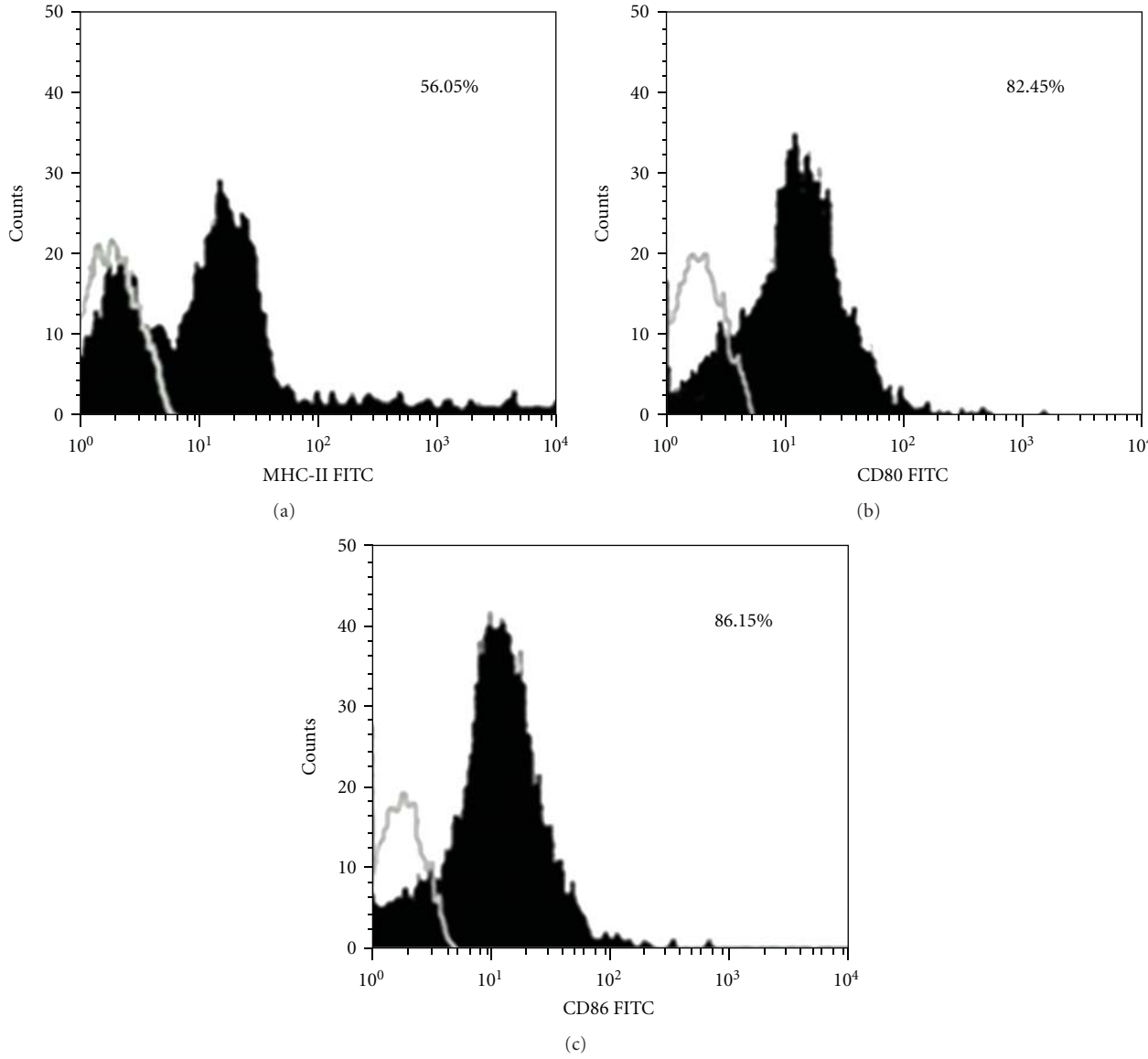


Figure 2: Expression of MHC-II (I-Ak) and B7 molecules (CD80/86) on BM-DCs. The expression of B7 or MHC-II by the indicated cell fractions is represented by filled histograms (black). The open histograms (grey) represent control staining with an isotype control antibody. Flow cytometric analysis demonstrated that over 55% of the cells express MHC-II (I-Ak) molecule (a), while 80 + % of the cells expressed B7.1 (CD80) and B7.2 (CD86) molecules (b) and (c). One representative of three independent experiments is shown.

analyze the significance of differences between groups for the proliferative response of T cells and the thickness of the arterial intima. P value less than 0.05 was considered as statistically significant.

3. Results

3.1. Propagation of Murine Recipient BM-DCs Loaded with Donor Antigen. A large number of progenitor cells were propagated in the presence of rmGM-CSF. After TNF-α was added in the medium, the cells show significant differentiation with typical DC appearance [30] (Figure 1) under inverted microscope and scanning electron microscope. Majority of the progenitor cells differentiated to DCs as MHC-II (I-Ak) molecule are expressed on 55 + % of the cells by flow cytometric analysis and 80 + % of the cells expressed B7(CD80,CD86) molecules (Figure 2).

3.2. Inhibition of T-cell Proliferative Response Using B7AP Pretreated DCs. In order to find the concentration of B7AP that can maximally block B7 molecules, DCs were incubated with B7AP at various concentrations, and then MLR between DCs and T cells was performed. B7AP could inhibit the proliferative response of T-cells compared with untreated group ($P < 0.05$, Table 1) and perform the maximal blockage at the end-point concentration of 10 mg/L (Figure 3(a)). Furthermore, to test the specificity of the inhibitory effect of B7AP, a control peptide (FTD_{10}) was synthesized and

Table 1: MLR of T cells and recipient DCs with different treatment.

Groups	Final concentration of B7AP	Cpm
B7AP-pretreated $^{C57BL/6a}$C3H/He DCs + T cells		
Subgroup 1	100 mg/L	252 ± 40
Subgroup 2	10 mg/L	236 ± 36
Subgroup 3	1 mg/L	2239 ± 316
Subgroup 4	1×10^{-1} mg/L	2197 ± 777
Subgroup 5	1×10^{-2} mg/L	2097 ± 150
Subgroup 6	1×10^{-3} mg/L	2039 ± 210
Subgroup 7	1×10^{-4} mg/L	1935 ± 79
Subgroup 8	1×10^{-5} mg/L	2070 ± 208
Untreated $^{C57BL/6}$C3H/He DCs + T cells	0	2106 ± 326
DCs without donor antigen + T cells	0	252 ± 125
T cells alone	0	124 ± 35

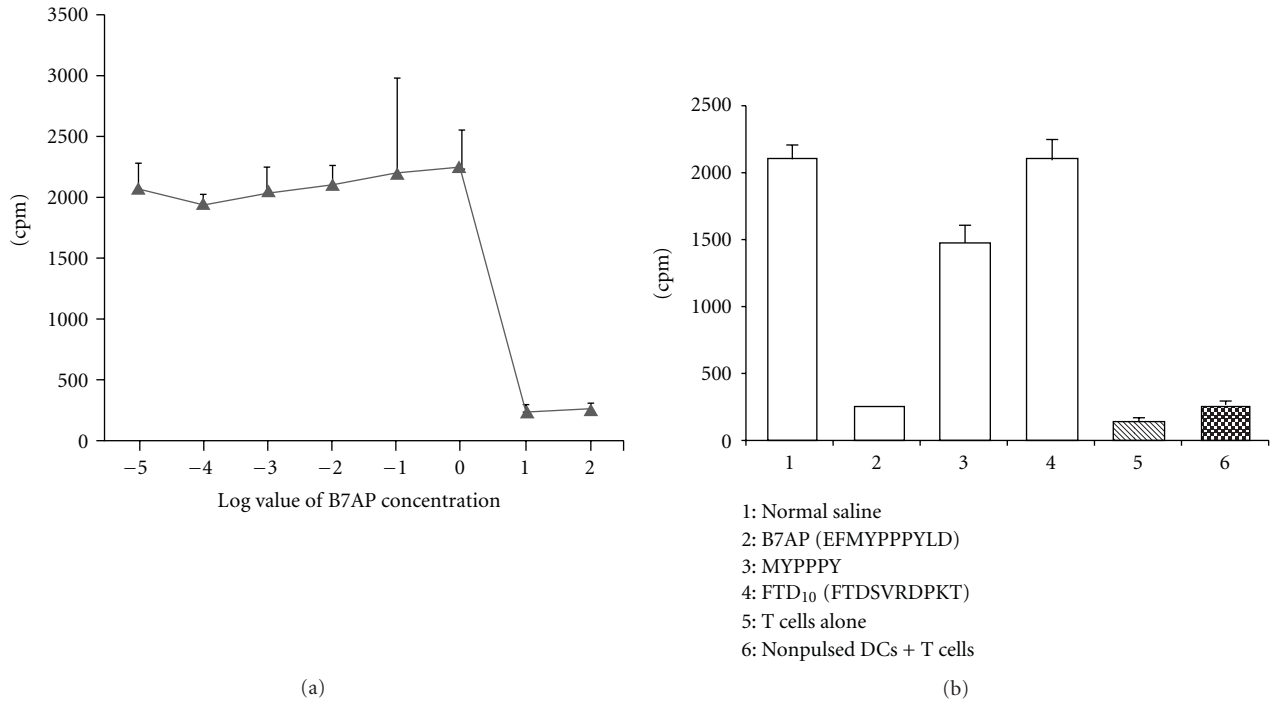
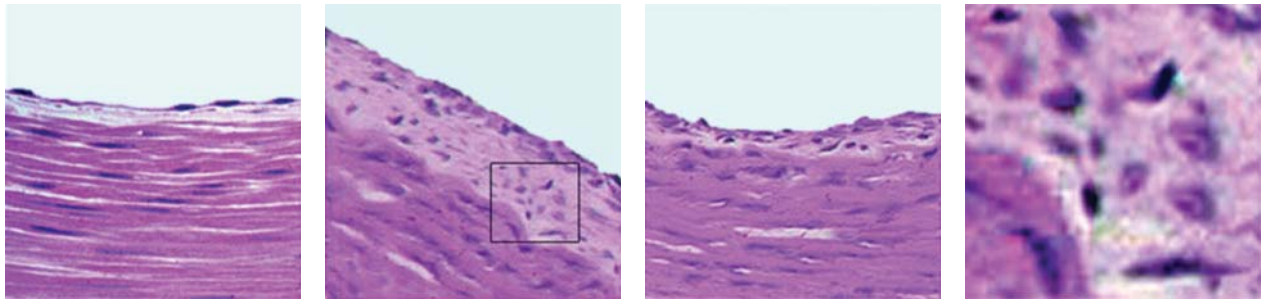


Figure 3: B7AP-pretreated DCs inhibited T-cell proliferation in MLR. Under high concentrations of B7AP (100 mg/L), T cells demonstrated a low proliferation capacity. However, proliferation of T cells surged to a rather high level when the final concentration of B7AP went down from 10 mg/L to 1 mg/L, followed by a plateau (a), indicating that B7AP final concentration of 10 mg/L could ensure maximal blockage of DC surface B7 molecules. The BM-DCs were pretreated by the B7AP and control peptide at the same concentration (10 mg/L), and the MLR was performed as described above (b). NS: normal saline group, MYPPPY group: relative peptide, FTD10 (FTDSVRDPKT) group: negative control peptide.

the MLR was performed at the same concentration of B7AP (10 mg/L). A relative peptide, MYPPPY, was also tested for its inhibitory effect in the MLR at the concentration of 10 mg/L.

As shown in Figure 3(b), all groups have significant differences compared to the B7AP group ($P < 0.05$). The FTD_{10} control group showed no significant difference compared to the normal saline (NS) group (2087 ± 150 versus 2102 ± 101, $P > 0.05$). The relative peptide MYPPPY showed a certain extent of inhibitory effect, but also with no significant difference compared to the NS group (1480 ± 130 versus 2087 ± 150, $P > 0.05$). The data demonstrated that the inhibitory effect of B7AP was specific (Figure 3(b)).

3.3. Induction of Alloantigen-Specific T-cell Hyporesponsiveness Using B7AP Pretreated DCs. The proliferative response of T cells was examined in secondary MLR, which has shown that T cells harvested from the primary MLR exhibited markedly reduced responses to alloantigen (C57BL/6) versus



(a) (b) (c) (d)

Figure 4: HE stained sections of the arterial grafts 2 months after transplant. (a) native carotid artery; (b) carotid arterial allograft (untreated control); (c) carotid arterial allograft (pretreated); (d) zoomed-in picture of the square fragment in Figure 4(b). Grafts in both DC-pretreated and control group showed diffused and concentric intimal thickening compared with normal nontransplanted artery (a), which is the main feature of chronic allograft rejection. However, the intima was significantly thinner in the pretreated group (c) than that in the control group (b). Also we could see more lymphocytes infiltrated within control group allografts (d).

Table 2: MLR of pretreated T cells and recipient DCs pulsed with different donor antigen.

Groups	Cpm values
Pretreated T cells + $^{C57BL/6}$C3H/He DC	355 ± 46
Pretreated T cells + $^{BALB/C}$C3H/He DC	2230 ± 248
Control	105 ± 22

$^{C57BL/6}$C3H/HeDC: Recipient DCs pulsed with donor antigen (derived from C57BL/6).
$^{BALB/6}$C3H/HeDC: Recipient DCs pulsed with the third party antigen (derived from BALB/C).

the third party unrelated antigen (BALB/C), indicating B7AP pretreated DCs could induce alloantigen-specific T-cell hyporesponsiveness. ($P < 0.05$, Table 2).

3.4. Inhibition of Arterial Allograft Intimal Hyperplasia. A total of 20 transplants were performed with the surgical successful rate of 100%. All mice and arterial grafts survived 2 months. The arterial grafts were examined under microscope 2 months after transplantation, and three histologic sections of every allograft were analyzed. During specimen harvesting, arterial grafts in the pretreated group presented stronger pulse compared to the control group. In all arterial grafts, histopathological examination demonstrated diffused and concentric intimal thickening compared to the normal nontransplanted artery, which involves the entire circumference of the vessel with a chronic picture at 2 month. However, the mean intimal thickness was significantly reduced in the pretreated group compared to the control group (mean intimal thickness: 0.071 ± 0.03 versus 0.179 ± 0.056, $P < 0.05$, Figure 4).

4. Discussion

In the field of organ transplantation, it is accepted that both donor and recipient DCs mediate the allograft rejection. The recognition of foreign major histocompatibility complex (MHC) molecules by recipient alloreactive T-cells via two distinct pathways, "direct" (donor DCs presenting donor MHC molecules) and "indirect" (recipient DCs presenting donor MHC molecules), is one of the major causes of different types of organ transplant rejection [2, 3]. It has been suggested that the direct pathway predominates during early acute rejection and the indirect pathway provides a continuous supply of alloantigen responsible for chronic rejection later [4, 5]. Animal trials found that blocking the direct allorecognition pathway only did not attain allograft tolerance, which indicates that maintaining long-term clinical transplantation tolerance by arresting the indirect pathway is essential [3]. In addition, administration of donor-derived dendritic cells (DCs) to prevent allograft rejection is not applicable for clinical use. We therefore attempted to explore the use of recipient-derived DCs pulsed with donor antigens via the indirect pathway.

T-cell activation is a process involving alloantigen recognition and costimulatory signaling. Besides the interaction of the TCR and MHC-antigen complex, a productive immune response and maintenance of T-cell homeostasis are determined largely by co-stimulatory molecules [31]. Co-stimulatory molecule-deficient DCs have the capacity to control immune responses and induce T anergy [25, 32]. It would result in the alloantigen-specific T hyporesponse. The most active pathway of costimulation is the interaction of CD28 receptor and B7 ligands [7].

Aortic allotransplantation in mice is a useful experimental model to study the mechanisms of chronic rejection in allotransplantation [33–35]. However, the application of the conventional aortic model is limited by a high morbidity and technically difficult to perform. So we developed a new simple method of carotid artery orthotopic allotransplantation in mice. This new procedure is easy to carry out and has a low morbidity after extensive training based on our experience.

Compelling evidence that induction of tolerance in the indirect pathway favors graft survival came from experiments in which blockade of CD28-B7 by monoclonal antibodies (MoAbs) against B7 or CTLA4-Ig could produce indefinite allograft survival [36]. Antisense peptides are short peptide sequences that specifically constitute one side of the binding sites of complementary protein pairs [24]. B7AP is a peptide

analogue of the CD28-binding region [26, 37], which can be characterized by higher affinity to B7 ligand, lower molecular weight, and lower immunogenicity and difficult to be metabolized compared with CTLA4-Ig [26, 38]. It would block the allorecognition in a longer period to take more effect. Chen et al. reported [26] that blockade of B7 molecules on donor splenocytes with B7AP could induce specific immune hyporesponse and prolong allograft survival in the recipients. Our study also confirmed that B7AP could inhibit the T-cell proliferative response stimulated by DCs. Transient blockade of B7-CD28 (using CTLA4Ig) did not abrogate the development of the intimal hyperplasia [3, 39]. It was uncertain whether more stable blockade of the indirect allorecognition would be more effective to inhibit intimal thickening in carotid artery allotransplantation. Using B7AP can lead to a stable blockade of CD28-B7 costimulation by virtue of its characters. The result found recipient BM-DCs pulsed with donor antigen and pretreated by B7AP could induce recipient T-cell-specific hyporesponse to the donor antigen *in vitro* and significantly alleviate the intimal thickening in the murine allotransplantation, which is a key manifestation of chronic rejection. It indicates that an approach to use recipient DCs as a "vaccine" strategy provides a feasible approach to inhibit the chronic rejection in organ transplantation.

5. Conclusion

Our research has demonstrated that blockade of B7/CD28 costimulatory pathway by B7AP in the indirect allorecognition could induce allogeneic-specific cross-hyporesponsiveness and inhibit the arterial allograft intimal hyperplasia due to chronic rejection, which may be involved in future strategies for human allograft chronic rejection.

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Ethical Approval

All animal surgical procedures were approved by the Institutional Animal Care and Use Committee of Fudan University.

Conflict of Interests

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this paper.

Authors' Contribution

Yu-Feng Yao and Yi-Ming Zhou contributed equally to this work and should be considered co-first authors.

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Review Article

Developing and Activated T Cell Survival Depends on Differential Signaling Pathways to Regulate Anti-Apoptotic Bcl-x_L

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Survival of T cells in both the central and peripheral immune system determines its ultimate function in the regulation of immune responses. In the thymus, developing T cells undergo positive and negative selection to generate a T cell repertoire that responds to foreign, but not self, antigens. During T cell development, the T cell receptor α chain is rearranged. However, the first round of rearrangement may fail, which triggers another round of α chain rearrangement until either successful positive selection or cell death occurs. Thus, the lifespan of double positive ($CD4^+CD8^+$; DP) thymocytes determines how many rounds of α chain rearrangement can be carried out and influences the likelihood of completing positive selection. The anti-apoptotic protein Bcl-x_L is the ultimate effector regulating the survival of $CD4^+CD8^+$ thymocytes subject to the selection process, and the deletion of Bcl-x_L leads to premature apoptosis of thymocytes prior to the completion of the developmental process. In addition to its critical function in the thymus, Bcl-x_L also regulates the survival of peripheral T cells. Upon engagement with antigens, T cells are activated and differentiated into effectors. Activated T cells upregulate Bcl-x_L to enhance their own survival. Bcl-x_L-mediated survival is required for the generation of effectors that carry out the actual immune responses. In the absence of Bcl-x_L, mature T cells undergo apoptosis prior to the completion of the differentiation process to become effector cells. Therefore, Bcl-x_L ensures the survival of both developing and peripheral T cells, which is essential for a functional immune system.

1. Introduction

Bcl-x_L is an anti-apoptotic member of the Bcl-2 family of apoptosis regulators. Proteins in this family contain at least one of the four conserved α-helical motifs known as Bcl-2 homology (BH) domains (BH1–BH4) [1]. The family members are further classified into three subgroups. The first group contains anti-apoptotic members that possess all four BH domains and includes Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, Bcl-B, and A1. The other two groups are composed of proapoptotic proteins, which are grouped into those containing three BH domains, including Bax, Bak, and Bok; and those containing BH3 only, including Bad, Bid, Bim, Bmf, Bik, Hrk, Noxa, and Puma. Anti-apoptotic Bcl-x_L and Bcl-2 possess a hydrophobic cleft, the BH-3 binding groove, which can accommodate BH3-only members of proapoptotic proteins and neutralize their function [2, 3]. In T cells, Bcl-2 expression is relatively consistent, whereas Bcl-x_L expression is induced in response to environment stimuli. In this paper, we will focus on the function and regulation of expression of Bcl-x_L in developing and activated mature T cells.

2. Bcl-x_L Function in the Development of T Cells in the Thymus

2.1. T Cell Development. T cells are critical components of adaptive immunity, as recognition of foreign antigens by T cells initiates adaptive immune responses. The goal of T cell development in thymus is to arm T cells with all necessary

machineries to, upon activation in the periphery, launch responses to foreign antigens by either direct killing ($CD8^+$ T cells) or helping other immune cells battle the antigens ($CD4^+$ T cells). Therefore, during T cell development, T cells must be educated to target only "nonself" foreign antigens, and this is accomplished by eliminating self-responsive T cells through positive and negative selection in the thymus [4]. T cell development is usually divided into three stages [5]: double negative (DN), double positive (DP), and single positive (SP). At the DN stage, thymocytes express neither CD4 nor CD8, and on the basis of their expression of CD25 and CD44, they are further divided into the DN1 ($CD25^-CD44^+$), DN2 ($CD25^+CD44^+$), DN3 ($CD25^+CD44^-$), and DN4 ($CD25^-CD44^-$) subsets. At the DN3 stage, thymocytes start rearranging the T cell receptor (TCR) β chain locus to produce the TCRβ chain, and only those thymocytes that generate a successfully rearranged TCRβ chain survive and progress in T cell development. The successfully rearranged TCRβ chain, combined with the invariant pre-TCRα chain, forms a pre-TCR, which delivers signals to stimulate the proliferation of post-β thymocytes and instruct the transition from the DN to DP stage. More than 80% of all thymocytes are DP, and only about 5% are positively selected and mature into either $CD4^+$ or $CD8^+$ SP cells if a DP thymocyte bears a TCR that interacts with an MHC-self peptide complex with sufficiently low affinity. The other DP T cells are negatively selected if the TCR is recognized by an MHC-self peptide with high affinity or die by neglect if the TCR cannot be recognized at all [6]. Once thymocytes mature into SP cells, they migrate out of the thymus into peripheral lymphoid organs, such as the lymph nodes and spleen, to mediate adaptive immunity.

2.2. Survival of DP Thymocytes Limits Positive Selection. A critical event, α chain rearrangement, is carried out in DP thymocytes. A productive α chain rearrangement generates a TCR that recognizes self-MHC, and thus delivers survival signals to allow the T cell to progress to the next stage. However, if α chain rearrangement is not productive or produces a TCR that does not recognize self-MHC, T cells can initiate another round of α chain rearrangement. DP thymocytes are able to initiate multiple rounds of TCRα chain rearrangement until they are either positively selected or die because they have reached the end of their lifespan. Thus, the lifespan of DP thymocytes limits the progression of TCRα chain rearrangement and controls the opportunity for assembling a functional TCR [7]. The longer the lifespan of DP cells, the more rounds of rearrangement they can try, and therefore the greater the opportunity for the eventually generation of a TCR that responds to foreign, but not self, antigens. Given the importance of DP thymocyte survival, it is critical that there are precise mechanisms in place to ensure their survival.

2.3. Bcl-x_L Is the Ultimate Survival Factor for DP Thymocytes. The first clue that Bcl-x_L regulates DP thymocyte survival was its unique expression pattern during T cell development [8]. During the DN to DP transition, Bcl-x_L is specifically upregulated, whereas another survival factor Bcl-2 which belongs to the same family as Bcl-x_L, is downregulated. Furthermore, Bcl-x_L is downregulated while Bcl-2 is upregulated in the following SP stage. The specific upregulation of Bcl-x_L during the DP stage strongly suggests that it functions in DP thymocyte survival. Indeed, deletion of Bcl-x_L led to accelerated apoptosis of DP but not SP thymocytes both *in vitro* and *in vivo* [8, 9], which corresponded to its expression pattern in DP cells. In contrast, overexpression of Bcl-x_L led to a significantly increased total thymocyte number due to enhanced DP cell survival [10]. Bcl-x_L, an anti-apoptotic molecule, is therefore specially upregulated in DP thymocytes to ensure their survival. This then raised the question of what signals are required to stimulate Bcl-x_L expression in DP cells. Both our work as well as that of others has demonstrated a network of transcriptional factors involved in the regulation of Bcl-x_L expression in DP thymocytes. We will discuss each of these factors in the following sections.

2.4. RORγt. RORγt is a transcription factor that belongs to the steroid nuclear receptor superfamily and was initially identified by expression cloning to screen for molecules that regulate activation-induced cell death [11]. We identified RORγt by yeast two hybrid screening for CD4 interacting proteins. However, CD4 only binds to RORγt in yeast, and not in mammalian cells. Similar to Bcl-x_L, RORγt is specifically upregulated in DP thymocytes during T cell development, whereas its expression levels are extremely low to undetectable in DN and SP cells. We created RORγt knockout mice and demonstrated that RORγt is required for DP thymocyte survival and lymph-node genesis [12], which was confirmed by an independently generated RORγt knockout mouse strain [13]. $ROR\gamma t^{-/-}$ mice have very small thymuses due to apoptosis of DP thymocytes. The accelerated DP apoptosis was accompanied by greatly reduced Bcl-x_L levels, and overexpression of Bcl-x_L rescued $ROR\gamma t^{-/-}$ thymocyte apoptosis, demonstrating that RORγt enhances DP cell survival by upregulating Bcl-x_L expression [12]. We further demonstrated that recruitment of steroid receptor coactivator (SRC) through the activation function 2 (AF2) motif of RORγt is essential for supporting thymocyte survival by RORγt [5, 14]. Thus, Bcl-x_L was identified as a downstream effector of RORγt involved in regulation of DP cell survival.

Our recent study also identified TCF-1 as the upstream signaling molecule that regulates the RORγt-Bcl-x_L pathway in DP thymocytes.

2.5. TCF-1. TCF-1 is the ultimate effector in the canonical Wnt/β-catenin pathway. The Wnt-β-catenin pathway has been shown to regulate multiple developmental processes, ranging from regeneration of stem cells to organogenesis of the kidney and reproductive systems [15]. β-catenin is usually regulated at the protein level. In the absence of Wnt signaling, several serines and threonines located at the N-terminus of β-catenin (amino acids 31–45) are phosphorylated by glycogen synthase-3β (GSK-3β) bound to the scaffolding proteins axin and adenomatous polyposis

coli (APC). The phosphorylated β-catenin is a target for ubiquitination and degradation by the 26S proteasome [16]. In addition, there are reports that β-catenin can also be degraded in a phosphorylation-independent manner [17, 18]. In the absence of β-catenin, TCF-1 is bound by corepressors such as Groucho/Transducin-like enhancer (GRG/TLE) and turns off target gene expression. Activation of Wnt signaling leads to inactivation of GSK-3β and accumulation of nonphosphorylated β-catenin in the cytoplasm. Accumulated β-catenin is then available to bind to and activate TCF-1, which turns on target gene expression.

TCF-1 is important at multiple stages of thymocyte development, including the DP stage. DP thymocytes from *TCF-1*$^{-/-}$ mice undergo rapid apoptosis during *in vitro* culture, and thymocyte survival can be restored by expression of full-length TCF-1 but not by truncated TCF-1 that lacks the domain mediating the interaction with β-catenin, suggesting that Wnt signaling mediated by β-catenin is required to support DP thymocyte survival [19]. To further establish the importance Wnt signaling in DP thymocyte survival, we established a β-catenin transgenic mouse strain (β-*cat*^{Tg}) that overexpresses constitutively active β-catenin under the control of a CD4 promoter [20]. The β-catenin transgene is not expressed until the DP stage, which ensures that thymocyte development at DN or earlier stages is not affected. As expected, the four DN subsets have normal distribution and cell numbers in these mice. However, the frequency and numbers of DP thymocytes are significantly greater in β-*cat*^{Tg} mice than in wildtype (WT). In addition, DP thymocytes from β-*cat*^{Tg} mice undergo much slower apoptosis than those of WT mice during both spontaneous and glucocorticoid-induced apoptosis. Furthermore, promotion of DP thymocyte survival by the β-catenin transgene is mediated by upregulation of Bcl-x_L. These data demonstrated that β-catenin/TCF-1 extends DP thymocyte survival by up-regulating Bcl-x_L. However, there was still the question of whether Wnt signaling mediated by β-catenin/TCF-1 directly targets Bcl-x_L or acts through other factors.

Our recent work has shed light on this by showing that enhancement of DP thymocyte survival by β-catenin/TCF-1 is mediated by RORγt. Microarray analysis revealed that RORγt was significantly downregulated in *TCF-1*$^{-/-}$ thymocytes that underwent accelerated apoptosis, whereas it was greatly up-regulated in thymocytes that had enhanced survival due to transgenic expression of β- cat^{Tg}. Both *TCF-1*$^{-/-}$ and *RORγt*$^{-/-}$ DP thymocytes underwent similar accelerated apoptosis. Forced expression of RORγt successfully rescued *TCF-1*$^{-/-}$ DP thymocytes from apoptosis, whereas ectopically expressed TCF-1 did not rescue the defective T cell development due to lack of RORγt-supported survival. Furthermore, activation of TCF-1 by stabilized β-catenin could enhance DP thymocyte survival only in the presence of RORγt, indicating that RORγt acts downstream of TCF-1 during regulation of DP thymocyte survival. Moreover, β-catenin/TCF-1 directly interacted with the RORγt promoter region and stimulated its activity. Thus, we showed that TCF-1 enhances DP thymocyte survival through transcriptional upregulation of RORγt, an essential survival molecule for DP thymocytes that acts through upregulation of Bcl-x_L [9, 14].

2.6. c-Myb. A recent paper by Yuan et al. identified another transcription factor, c-Myb, encoded by the proto-oncogene *Myb*, as an important factor for regulating DP thymocyte survival [21]. In this work, c-Myb was conditionally deleted starting at the DP stage. This deletion led to premature DP thymocyte apoptosis caused by decreased expression of Bcl-x_L . More specifically, due to an enhanced dependence on Bcl-x_L for survival, small preselection DP thymocytes underwent faster premature apoptosis than large preselection and postselection DP thymocytes. Forced expression of Bcl-x_L rescued thymocyte survival, and re-introduction of c-Myb restored both Bcl-x_L expression and the small preselection DP compartment. The defective DP thymocyte survival caused by reduced expression of Bcl-x_L was reminiscent of what has been observed in *TCF-1*$^{-/-}$ and *RORγt*$^{-/-}$ mice. However, the authors proposed that the transcriptional regulation of Bcl-x_L by c-Myb is independent of both TCF-1 and RORγt, since c-Myb expression in both TCF-1- and RORγt-deficient thymocytes was comparable to that in WT thymocytes, indicating that multiple pro-survival pathways could synergize to ensure proper survival of DP thymocytes via the Bcl-x_L pathway.

2.7. HEB. HEB is a member of the E protein family. Thymocytes from T lineage-specific HEB-deleted mice undergo rapid apoptosis and have reduced Bcl-x_L expression. In c-Myb or RORγt-deficient thymocytes, forced expression of Bcl-x_L rescued DP thymocyte survival, indicating that HEB is another transcription factor that functions upstream of Bcl-x_L to promote DP thymocyte survival. In contrast to the independence of RORγt and TCF-1 in c-Myb-mediated regulation of DP thymocyte survival, HEB regulates RORγt expression by binding to the two E-box sites present in the RORγt promoter and stimulating its transcription, which suggests that HEB could act upstream of RORγt in the same pathway to promote DP thymocyte survival. Since both TCF-1 and HEB are upstream of RORγt, the relationship between them during the regulation of DP cell survival remains to be determined.

In summary, the transcription factors discussed above work together to form a network for regulating DP thymocyte survival through upregulation of Bcl-x_L. This complicated network ensures DP thymocytes complete their development in the thymus to generate a functional immune system that responds only to foreign antigens.

3. Bcl-x_L Function during Activation of Peripheral Mature T Cells

3.1. T Cell Activation. Adaptive immunity is unique in that only antigen-specific cells are activated to mediate immune responses against specific pathogens. T cells that have just migrated out of the thymus cannot mediate immune responses and therefore are called naïve T cells. Effector T cells differentiated from naïve T cells mediate immune

responses *in vivo*. Engagement of TCR by antigen initiates TCR signals that trigger the activation and differentiation of naïve T cells into effector cells, which is an important mechanism for ensuring that only antigen-specific T cells are activated and clonally expanded to become competent effector cells. The T cell activation process is, therefore, not only preparatory to arm T cells for attacking pathogens, but also essential to ensure the adaptive nature of the immune system.

3.2. Survival of Activated T Cells Determines Immune Responses. An efficient adaptive immune system must be able to rapidly expand as well as reduce the number of immune cells. T cells meet these requirements, because they can be induced toward proliferation, anergy, or apoptosis depending on the signals received via the TCR. Naïve T cells are activated to proliferate in response to foreign antigens, which is a critical step in adaptive immunity. On the other hand, T cells will undergo apoptosis or anergy if they engage with self-antigens, which is an important mechanism for self-tolerance. Productive engagement of the TCR results in delivery of signals required for T cell proliferation as well as T cell survival. If TCR-mediated survival signals are blocked, T cells undergo apoptosis instead of proliferation upon TCR stimulation. Therefore, TCR-delivered survival signals ensure the completion of the T cell activation process required for differentiation of naïve T cells into effector cells that mediate actual immune responses *in vivo*.

3.3. Bcl-x_L Enhances the Survival of Activated T Cells. Stimulation of the TCR leads to T cell activation, resulting in cell proliferation and production of IL-2. Proliferating T cells, especially during S phase, are susceptible to apoptosis [22, 23]. Thus, TCRs deliver signals to enhance T cell survival during activation [24, 25]. Such survival signals include IL-2, which acts as an extrinsic survival factor. More importantly, activated T cells substantially up-regulate Bcl-x_L, which intrinsically increases their ability to resist apoptosis [23, 26, 27]. Without Bcl-x_L, stimulation of T cells via the TCR leads to apoptosis instead of clonal expansion. Therefore, Bcl-x_L ensures naïve T cells complete activation. This raises the question of what TCR signals stimulate the upregulation of Bcl-x_L during T cell activation.

3.4. CD28. CD28, together with its ligands B7.1 and B7.2, is a costimulatory molecule that transduces the secondary signals required for T cell activation. CD28 signaling markedly lowers the TCR signal threshold required for T cell activation, and enhances cytokine production [28]. Another way CD28 facilitates T cell activation is by enhancing intrinsic T cell survival [23, 27, 29]. CD28 costimulation augments the expression of anti-apoptotic Bcl-x_L, but not that of Bcl-2, to render T cells resistant to apoptosis induced by crosslinking of TCR and Fas, and withdrawal of IL-12 [30]. In contrast to WT T cells, survival of T cells obtained from Bcl-x_L transgenic mice is not inhibited by blocking CD28 signals, suggesting that CD28 costimulation sustains T cell survival [29] and that downstream signaling molecules of CD28 are also important for mediating the upregulation of Bcl-x_L.

3.5. PI-3 Kinase. Distinct motifs within the cytoplasmic domain of CD28 regulate T cell proliferation and induction of Bcl-x_L [31], suggesting differential signals are responsible for these two CD28-regulated biological effects. PI-3 kinase is required for CD28-mediated induction of Bcl-x_L, as upregulation of Bcl-x_L is prevented by a pharmacological inhibitor of PI-3 kinase and by mutation of the CD28 residues essential for PI-3 kinase activation [31, 32]. Further evidence supporting a role of PI3-kinase in enhancement of T cell survival is that Akt, a target of PI-3 kinase, has been shown to mediate T cell survival by regulating Bcl-x_L [33]. Therefore, the PI-3 kinase-Akt pathway mediates CD28 signals to up-regulate Bcl-x_L and enhance the survival of activated T cells.

3.6. PKC-θ. CD28 also facilitates the activation of another important signaling molecule, PKC-θ. PKC-θ mediates TCR signals essential for T cell activation [34–36] and is required to enhance the survival of activated $CD4^+$ T cells by up-regulating Bcl-x_L. In response to TCR stimulation, $CD4^+$ *PKC-*$\theta^{-/-}$ T cells failed to up-regulate Bcl-x_L and underwent accelerated apoptosis via a caspase and mitochondria-dependent pathway. Similar to these findings, siRNA-mediated knockdown of PKC-θ in Jurkat cells also resulted in apoptosis upon TCR stimulation. Forced expression of Bcl-x_L was sufficient to inhibit the apoptosis observed in PKC-θ knockdown cells. Furthermore, ectopic expression of PKC-θ stimulated a reporter gene driven by a mouse Bcl-x_L promoter, whereas the expression of an inactive form of PKC-θ or knockdown of endogenous PKC-θ led to inhibition of the Bcl-x_L reporter. Thus, PKC-θ-mediated signals may function not only in the initial activation of naïve $CD4^+$ T cells, but also in their survival during T cell activation by directly regulating Bcl-x_L. PKC-θ has a similar function in survival of $CD8^+$ T cells [37]. We further demonstrated that PKC-θ-regulated survival is essential for cardiac allograft rejection in an adoptive transfer model [38], suggesting that PKC-θ-mediated survival plays a role in immune responses *in vivo*.

3.7. NF-κB. One of the critical downstream targets of PKC-θ is NF-κB. We demonstrated that TCR-initiated NF-κB activation was lacking in *PKC-*$\theta^{-/-}$ T lymphocytes, whereas the activation of NF-κB by tumor-necrosis factor alpha and interleukin-1 was not affected in the absence of PKC-θ [36]. Similarly, PKC-θ was also found to mediate NF-κB activation in Jurkat cells [39]. There is considerable evidence that TCR-mediated activation of NF-κB extends T cell survival [32, 40], raising the question of whether NF-κB is important for Bcl-x_L upregulation. Interestingly, functional NF-κB binding sites are present on the promoter region of Bcl-x_L gene [41, 42]. We showed that PKC-θ-mediated activation of Bcl-x_L promoter was inhibited by dominant negative IKKβ, suggesting that PKC-θ mediates the signals stimulating the expression of Bcl-x_L via the NF-κB pathway. Stimulation of

the PI-3 kinase/Atk pathway, which enhances T cell survival in a similar manner as PKC-θ, leads to activation of NF-κB [40], suggesting that the two pathways may interact in some way during activation of NF-κB. Akt activation is normal in *PKC-*$\theta^{-/-}$ T cells [43, 44], which suggests that Akt is not downstream of PKC-θ during activation of NF-κB. There is also no evidence to support that PKC-θ is downstream of Akt. Therefore, the current model is that PKC-θ and Akt cooperate with each other to mediate the CD28 signals and activate NF-κB, which in turn, stimulates the expression of Bcl-x_L required to enhance the survival of activated T cells.

4. Summary

Bcl-x_L is specifically up-regulated in DP thymocytes during T cell development and in stimulated T cells during T cell activation. This upregulation is important for the completion of T cell development in the thymus as well as the differentiation of naïve T cells into effector cells in the periphery. However, the signaling pathways that regulate Bcl-x_L upregulation in the thymus and mature T cells are distinct. In the thymus, a transcription factor network that includes TCF-1, RORγt, Heb, and c-Myb, which are also important for T cell development, ensures DP thymocyte survival by up-regulating Bcl-x_L. Whereas in the periphery, CD28-mediated activation of NF-κB via PKC-θ and Akt stimulates Bcl-x_L expression. Thus, developing and mature T cells use the same factor, Bcl-x_L, to enhance their survival but through different upstream signaling pathways. Expression of Bcl-x_L, in contrast to Bcl-2, is inducible and therefore modulates T cell survival in response to environmental signals, which is an essential mechanism for maintaining a functional immune system.

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Research Article

Development of Murine Hepatic NK Cells during Ontogeny: Comparison with Spleen NK Cells

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The phenotype of developing liver NK cells ($CD3^-NK1.1^+$) was investigated during mouse ontogeny comparing with spleen NK cells. The highest percentage of hepatic $CD27^-CD11b^-$ NK cells occurred at the fetal stage. After birth, the percentage of $CD27^-CD11b^-$NK cells in both the liver and spleen gradually decreased to their lowest level at 6 weeks. More $CD27^+CD11b^-$NK cells were detected in the liver than that in spleen from week 1 to 6. Expression of NKG2A on liver NK cells was decreased but still much higher than that of spleen NK cells after 1 week. The NKG2D expression on liver NK cells increased to its highest level and was significantly higher than on spleen NK cells till 4 weeks. During mouse ontogeny, weaker expression of NKp46 and CD2 and stronger expression of CD69, CD11c, 2B4, and CD73 were observed on liver NK cells. Furthermore, neonatal liver NK cells express higher IFN-γ and perforin than adult .These results suggest that the maturation process of NK cells is unique in the livers, and liver microenvironments might play critical roles to keep NK cells in an immature status.

1. Introduction

NK cells are derived from haematopoietic stem cells (HSCs). The precursors of NK cells are generated in the bone marrow; they are committed to the NK cell lineage and develop into mature NK cells with full effector function and heterogeneous phenotypes [1, 2]. The definitive site(s) for NK cell development can only be inferred from where immature and mature NK cells have been detected. NK cell precursors (NKPs) are found in different organs, such as bone marrow, fetal thymus, lymph node (LN), liver, spleen, and peripheral blood, whereas immature NK (iNK) cells are found in the bone marrow, liver, and spleen [3]. It is unknown whether these developmental intermediates leave the bone marrow to complete their differentiation elsewhere, such as the liver and spleen.

In liver, but not spleen, a unique subset of immature NK cells constitutively express tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and low levels of mature NK cell markers, such as the Ly49 receptors and CD11b [4–8]. A subset of NK-cells highly expressing CD11c have also been found specifically in the liver [9]. Adoptive transfer of either adult or neonatal mouse liver $TRAIL^+$ NK cells results in the appearance of $TRAIL^-$ NK cells with a mature phenotype, suggesting that these $TRAIL^+$ NK cells in the liver were indeed a precursor subset [4]. Stromal cells in various organs send signals through cytokines, receptors, and transcription factors that influence the ultimate phenotypes and functions of NK cell precursors [2, 10–15], suggesting that there may be specific developmental pathways for intrahepatic NK cells. D. M. Andrews and M. J. Smyth have described differences in the accumulation of NK cell subsets in the liver, bone marrow, spleen, and lung between WT B6 mice and *Rag1*$^{-/-}$ mice during weeks 1–5 and at 8 weeks of age. Costaining of CD27 and CD11b were used to divide $NK1.1^+CD3^-$ NK cells into four subsets that were at different maturation stages [16]. The first appearance of mature $CD27^-CD11b^+$ NK cells in these organs, including bone marrow, spleen, and lung, occurs at 3 weeks of age, and maturation is complete by 8 weeks of age. Complete maturation of hepatic NK cells occurs at 2 weeks of age, with fewer $CD27^-CD11b^+$ NK cells accumulating in the adult mouse liver. These results demonstrate that the liver displays slower kinetics in the accumulation of terminally mature

$CD27^-CD11b^+$ NK cells. Furthermore, in neonatal *Rag1*$^{-/-}$ mice, NK cells are absent in bone marrow and spleen, but a precursor NK cell subset is found in the liver, and normal NK cells without functional deficiencies can be detected in adult *Rag1*$^{-/-}$ mice. It was hypothesised that liver NK cells develop independently out of the bone marrow and that Rag-1 has a significant role in NK cell development [17, 18]. These results have helped us to understand the unique development pathway of liver NK cells; however, the details of phenotypes of developing liver NK cell subsets during mouse ontogeny have not been fully elucidated.

In our study, NK cell development in liver was explored and compared with NK cell development in spleen during mouse ontogeny. We found an abundance of NKPs, but the development pathway did not occur concurrently in the liver and spleen. The $CD27^-CD11b^-$ NK cell precursors accumulated predominantly in the adult liver and not in the spleen. In the liver, more immature NK cells were present, which express a higher level of NKG2A and lower levels of Ly49 receptors. Additionally, different stimulatory receptors and adhesion molecules were expressed on NK cells in the liver and spleen during ontogeny. And the expression level of IFN-gamma and perforin were higher of neonatal liver NK cells comparing with 10-week-old liver NK cells. These results indicate that there might be a specific developmental pathway of NK cells in the liver and that the microenvironments play important roles in NK cell development and differentiation.

2. Results

2.1. Maturation of Liver NK Cells Is Different from That of Spleen NK Cells during Ontogeny. Based on the expression of CD11b and CD27, NK cells ($NK1.1^+CD3^-$) can be divided into four subsets at different maturation stages [16, 19]. The gating strategy is shown in Figure 1 of Supplementary Material available at doi 10.1155/2012/759765. As the most immature subset, $CD27^-CD11b^-$ NK cells are the precursors of the other three NK cell subsets [16]. As shown in Figure 1, the highest percentage of $CD27^-CD11b^-$ NK cells in the liver occurred at embryonic day 20 (E20) (35.38 ± 0.64%). Comparatively, the percentage of $CD27^-CD11b^-$ NK cells was much lower in the spleen of E20 mice (14.62 ± 3.19%). However, the percentage of $CD27^-CD11b^-$ NK cells increased markedly to their highest level in the spleen of neonatal mice (29.73 ± 6.50%), which was similar to that found in the liver (Figure 1). As the mice growth, the percentage of $CD27^-CD11b^-$ NK cells in the spleen and liver decreased markedly, reaching a nadir at week 6 (4.91 ± 0.74% and 8.97 ± 3.51%, resp., Figure 1), but $CD27^-CD11b^-$ NK cells increased markedly in the liver to their highest levels once more at week 8 to 10 (24.64 ± 2.66% and 31.53 ± 5.13%, resp.). These results indicate that $CD27^-CD11b^-$ NK precursor cells reside predominantly in the adult liver and not in the spleen.

Subsequently, in the liver of E20 mice, the percentage of immature $CD27^+CD11b^-$ NK cells was significantly lower than in the spleen (37.61 ± 1.51% versus 57.57 ± 0.007%, $P = 0.0029$, Figure 1). From week 1 to 6, there was higher percentage of $CD27^+CD11b^-$ NK cells in the liver compared with the spleen. At 8 weeks of age, no significant difference in the percentage of $CD27^+CD11b^-$ NK cells was found between the liver and spleen of adult mice.

$CD27^+CD11b^+$ NK cells and $CD27^-CD11b^+$ NK cells are mature NK cells [5]. In the liver, the percentage of $CD27^+CD11b^+$ NK cells remained at a steady level, while in the spleen, this NK cell subset increased to a higher percentage during ontogeny. At 6 weeks of age, the percentage of $CD27^+CD11b^+$ NK cells in the spleen of the adult mice was 41.93 ± 4.58%, but the percentage of $CD27^+CD11b^+$ NK cells in the liver was only 26.73 ± 2.28% (Figure 1), which is similar to levels found in 8- to 10-week-old mice. In 3-day-old mice, the percentage of mature $CD27^-CD11b^+$ NK cells in the liver was higher than in the spleen (21.18 ± 1.67% versus 11.48 ± 1.51%, $P = 0.0017$, Figure 1), but from weeks 1 to 4, the percentages in the liver and spleen were reversed. In the 6-week-old adult mice, the percentages of $CD27^-CD11b^+$ NK cells in the liver and spleen were similar (31.48 ± 1.86 and 34.85 ± 6.17, resp., $P = 0.4161$, Figure 1).

These results indicate that the maturation process is different in liver and spleen. In each organ of neonatal mice, there was a high percentage of NK cell precursors, but the liver NK cell development did not occur in parallel to spleen NK cells. In the liver, there were more immature NK cells.

2.2. Developing NK Cells Expresses Different Inhibitory and Stimulatory Receptors in the Livers When Compared with Spleen during Ontogeny. As shown in Figures 2(a) and 2(b), at the earliest stage of development, almost all NK cells were NKG2A-positive in the liver and spleen (95.71 ± 1.07% and 90.98 ± 1.95%, resp., Figure 2). With further development, the percentage of $NKG2A^+$ NK cells gradually decreased, but the decrease in liver was delayed compared with spleen. In 10-week-old adult mice, 59.16 ± 3.36% of NK cells were $NKG2A^+$ in liver, compared to 45.05 ± 1.11% in spleen (Figure 2). NK cells gradually acquired expression of Ly49 receptors, which are markers delineating the maturation stages of NK cells [3]. In the livers from fetal and neonatal mice, only 7.81 ± 5.31% and 6.04 ± 1.98% of NK cells, respectively, were $Ly49C/I/F/H^+$ (Figure 2). Of note, in 3-day-old mice, the percentage of $Ly49C/I/F/H^+$ NK cells in the liver rapidly increased to 17.36 ± 2.50% (Figure 2), which was significantly higher than in spleen (5.01 ± 0.61%, Figure 2). However, at 1 week the expression of Ly49 C/I/F/H on spleen NK cells rapidly increased, and after 4 weeks, the expression level in the liver was markedly lower than in the spleen. These results further indicate that the development of NK cells did not occur concurrently. In the liver, NK cells had phenotypes characteristic of immature subsets.

To further investigate the functions of NK cells at different developmental stages in the liver and spleen, a series of stimulatory receptors was detected by flow cytometry. From the mean fluorescence intensity and percentage in both liver and spleen of fetal and neonatal mice, the expression of NKG2D on NK cells was very low (Figure 3(a)). At later developmental stages, the expression of NKG2D was upregulated. In liver, the expression of NKG2D increased to its highest

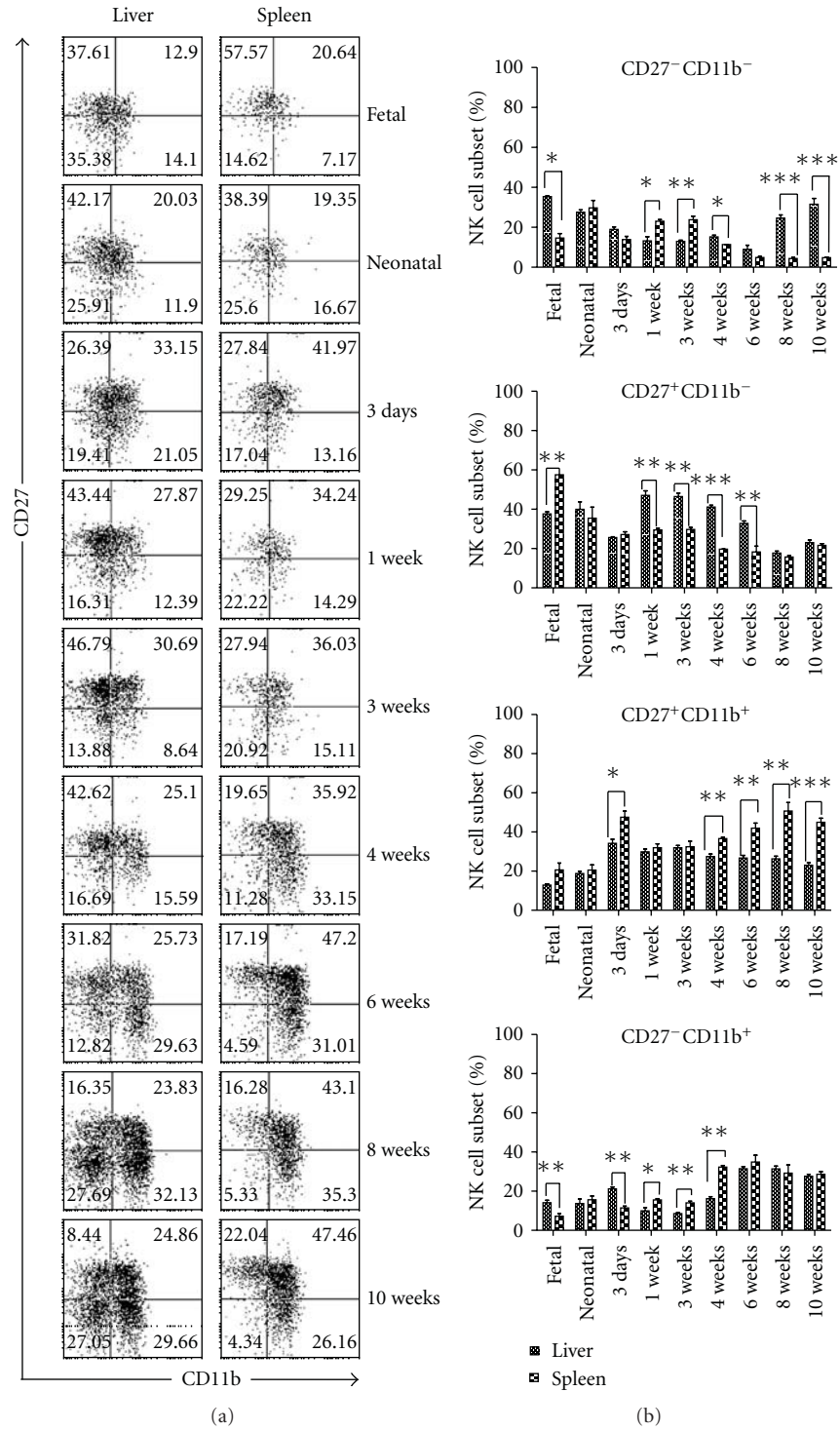


Figure 1: Different maturation stages of NK cells according to expression levels of CD27 and CD11b. Flow cytometry analysis was performed to analyse lymphocytes from the liver and spleen of B6 mice at the ages of E20, the neonatal stage, and at 3 days, 1 week, 3 weeks, 4 weeks, 6 weeks, 8 weeks, and 10 weeks, which were stained with the indicated antibodies. NK cells ($CD3^-NK1.1^+$) were gated to analyse the expression of CD27 and CD11b. Six to seven fetal mouse livers or spleens were put together to acquire enough cells to perform FACS analysis in one experiment, and three independent experiments were performed. In the other groups, there were three mice independently detected for one experiment, and three independent experiments were performed. (a) The percentages represent the net percentage (%) of cells in the appropriate quadrant. These are from a single experiment representative of three independent experiments. (b) The percentages of four NK cell subsets in the liver and spleen were calculated. Data are shown as the mean ± SEM from three mice in each group. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

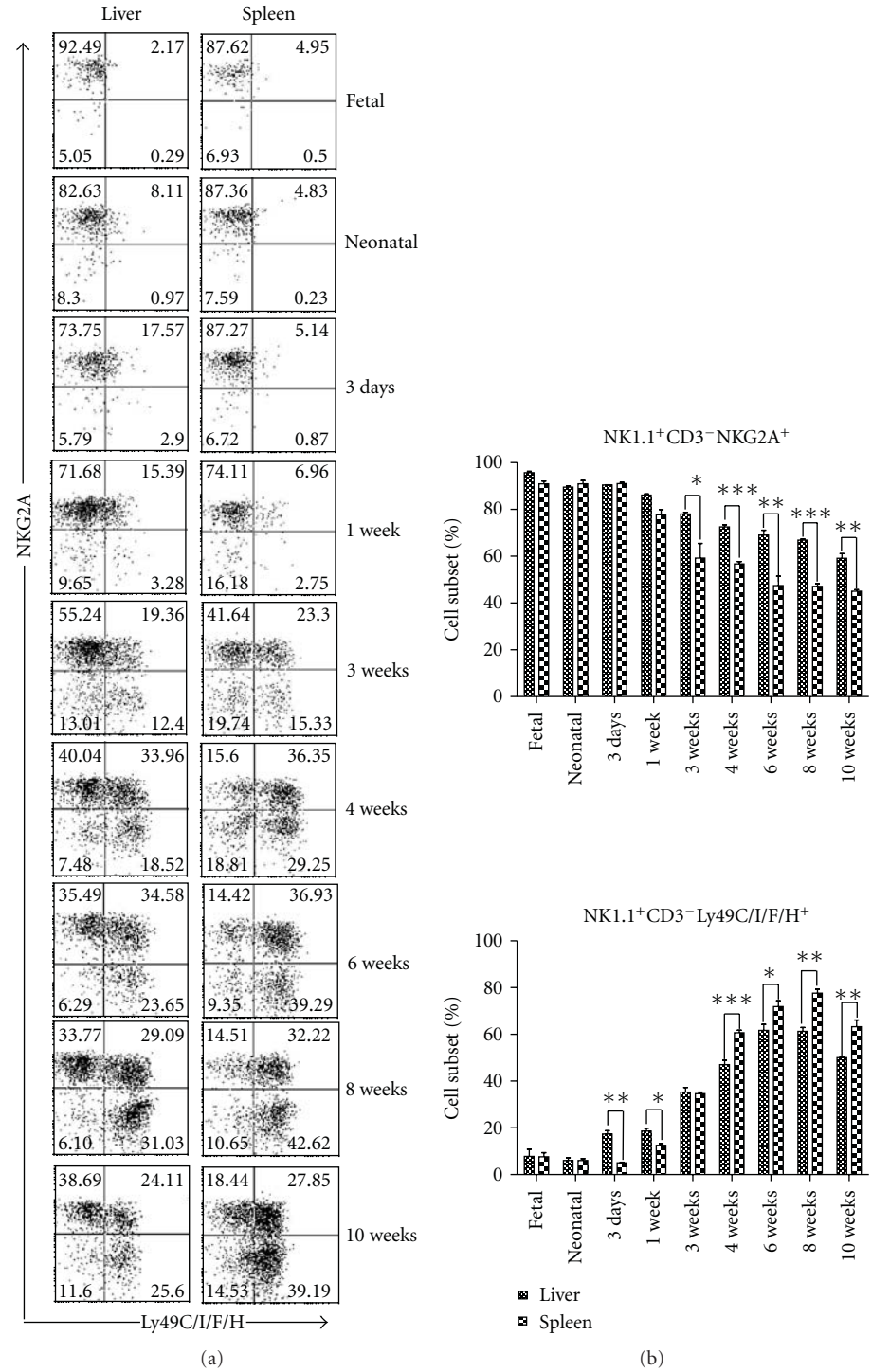
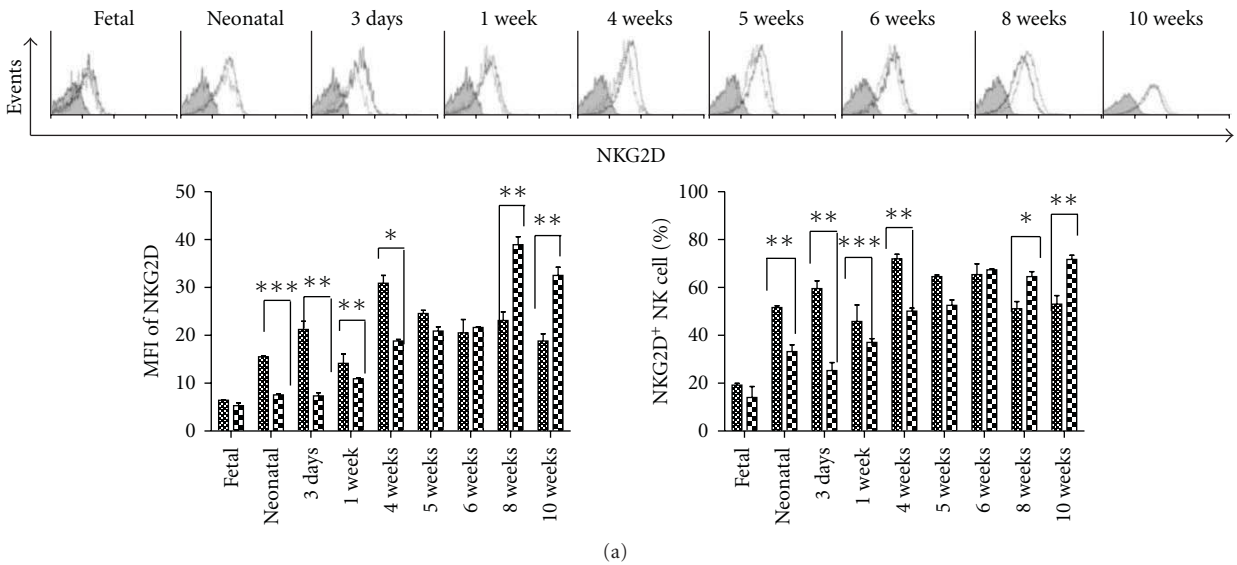
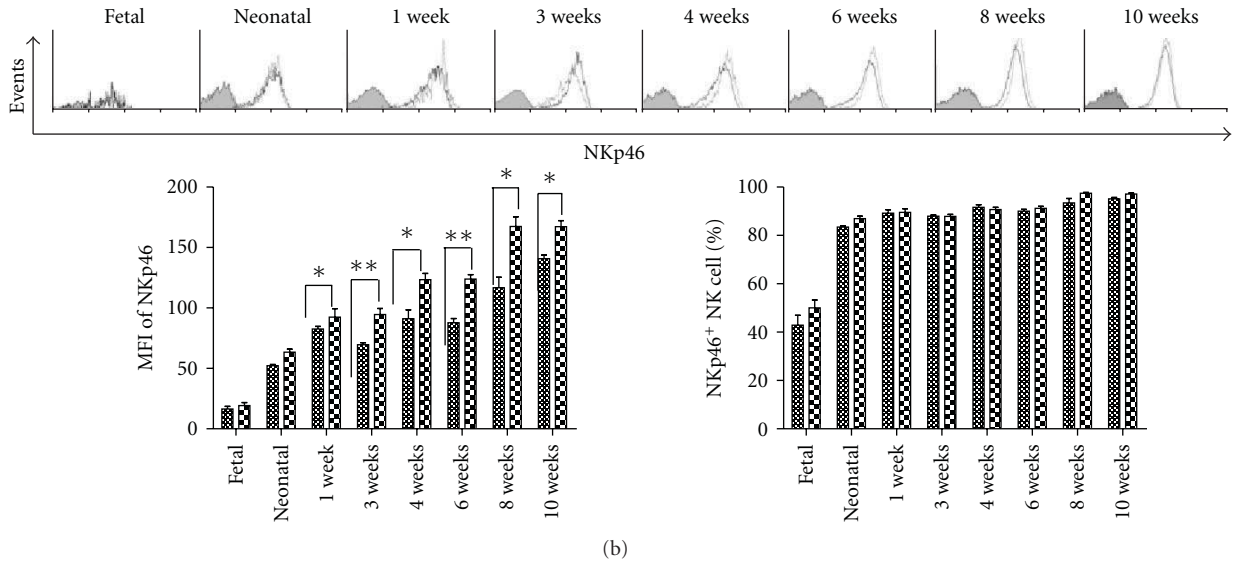


Figure 2: Different expression patterns of the NKG2A/Ly49 family of receptors on NK cells during mouse ontogeny. Flow cytometry was performed to analyse the lymphocytes stained with indicated antibodies from liver and spleen of B6 mice at E20, the neonatal stage, and at 3 days, 1 week, 3 weeks, 4 weeks, 6 weeks, 8 weeks, and 10 weeks. NK cells ($CD3^{-}NK1.1^{+}$) were gated to analyse the expression of NKG2A and Ly49. In each independent experiment, six to seven fetal mouse livers/spleens were put together to acquire enough cells to perform FACS analysis, and independent experiments were repeated for three times. (a) The percentages represent the net percentage (%) of cells in the appropriate quadrant. These are from a single experiment representative of three independent experiments. (b) The percentages of the $NKG2A^{+}$ NK cell subset (%) and the $Ly49^{+}$ NK cell subset (%) were calculated from the total number of NK cells in the liver and spleen. Data are shown as the mean ± SEM from three mice in each group. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ compared with the corresponding group.



(a)



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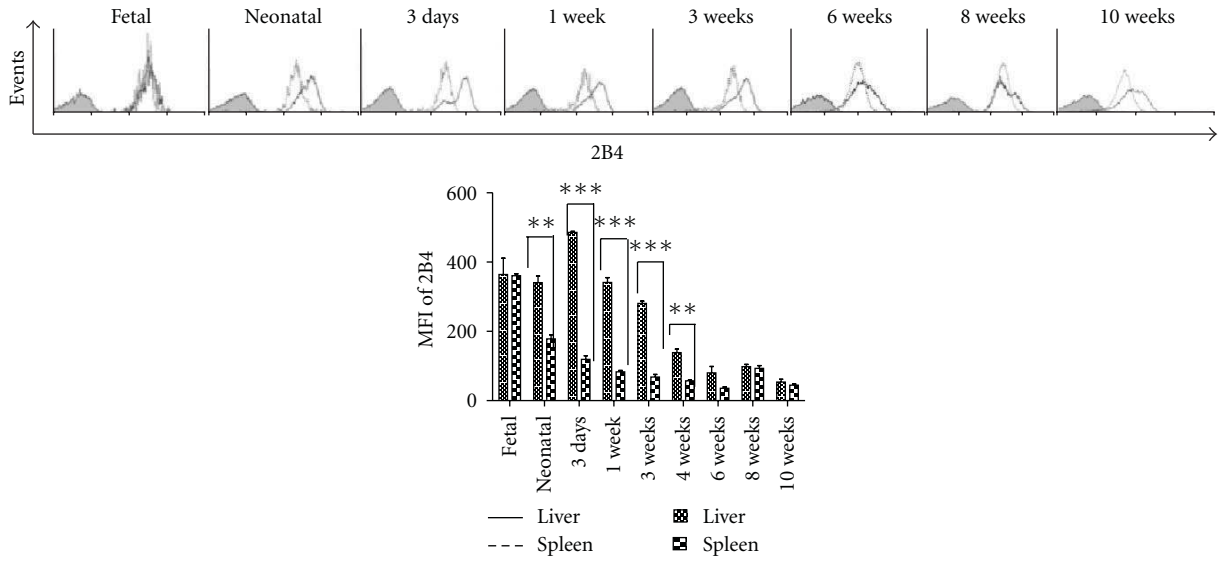

(c)

Figure 3: Continued.

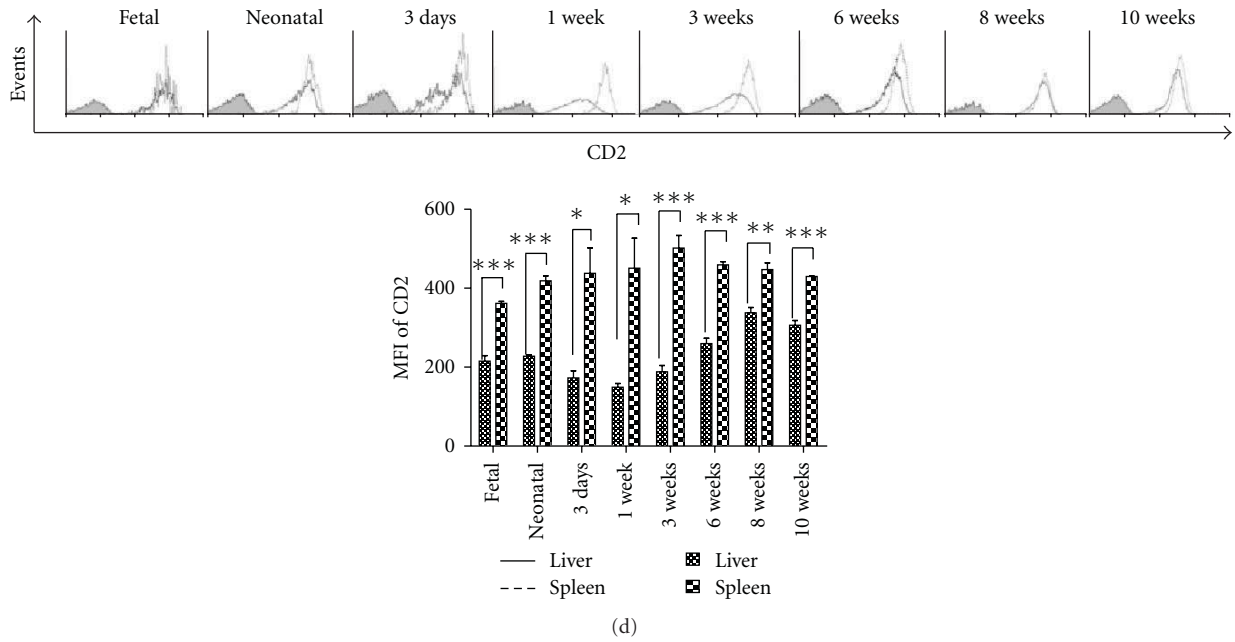

Figure 3: Stimulatory receptors expressed on NK cells during mouse ontogeny. At the indicated time points, mononuclear cells were isolated from liver and spleen and analysed by flow cytometry for the expression of stimulatory receptors. NK cells ($NK1.1^+CD3^-$) were gated, and each marker was analysed by histogram. Unstained controls are shown in grey. The expression of NKG2D (a), NKp46 (b), 2B4 (c), and CD2 (d) on liver NK cells is shown and compared with spleen NK cells during ontogeny. In each independent experiment, six to seven fetal mouse livers or spleens were put together to acquire enough cells to perform FACS analysis in one experiment, and three independent experiments were performed. In the other groups, there were three mice independently detected for one experiment, and three independent experiments were performed. The mean fluorescence intensity (MFI) and positive percentage of each marker are shown as mean ± SEM from three mice in each group. These are from a single experiment representative of three independent experiments. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ compared with the corresponding group.

level at 4 weeks and was significantly higher than in spleen, while in spleen, NKG2D increased to its highest level at 8 weeks. This result indicates that earlier expression of the stimulatory NKG2D receptor in hepatic NK cells might be associated with their specific function in the liver. At 8 to 10 weeks, the expression level of NKG2D on liver NK cells (51.14 ± 5.18% and 53.01 ± 6.2%, resp.) was significantly lower than in spleen (64.56 ± 3.58% and 71.68 ± 3.10%, resp., Figure 3(a)). As shown in Figure 3(b), there was no significant difference in the percentage of NKp46-expressing NK cells between liver and spleen. From newborn to adult mice, NKp46 was expressed on almost all NK cells in the liver and spleen, but the fluorescence intensity of NKp46 expression was much weaker in the liver compared with the spleen.

In addition, we found that all NK cells in both the liver and spleen expressed 2B4 and CD2; however, the fluorescence intensity differed between the two tissues (Figures 3(c) and 3(d)). In the earlier stage of ontogeny, from the fetal stage to 3 weeks after birth, the expression level of 2B4 on NK cells in liver was consistent with a high MFI, while 2B4 expression on NK cells in spleen decreased markedly after the neonatal stage (Figure 3(c)). At 6 weeks of age, the expression of 2B4 on NK cells in adult mice decreased to a nadir in both liver and spleen. During ontogeny, the expression of CD2 on NK cells in liver and spleen was relatively stable, and the expression of CD2 on NK cells in the liver was significantly lower than in the spleen (Figure 3(d)). Different inhibitory and stimulatory receptors were expressed on NK cells in the liver compared with the spleen during ontogeny, which may be related to the specific functions of NK cells in different organs.

2.3. Higher Expression of Function-Related Molecules on Liver NK Cells Compared with Spleen NK Cells during Ontogeny. In the foetus, liver and spleen NK cells exhibited elevated expression of CD69 (71.18 ± 3.05% and 69.75 ± 3.68%, resp., Figure 4). At later stages of development, the expression of CD69 on NK cells in the liver was upregulated to reach a maximum at 3 weeks, while in the spleen, the expression was downregulated after the neonatal stage. Although the percentage of CD69-expressing NK cells in the liver decreased to 50.07 ± 4.65% at 8 weeks of age, it remained significantly higher than in the spleen (10.33 ±1.14%); similar percentages were found in 10-week-old mice (53.23 ± 2.42% and 10.29 ± 1.62%, resp., $P < 0.0001$, Figure 4). The results obtained with dynamic MFI further confirmed higher expression of CD69 on liver NK cells during mouse ontogeny (Figure 4(b)).

Additionally, the differential expression pattern of adhesion molecules distinguished liver NK cells from spleen NK cells. CD11c was expressed more highly on liver NK cells than on spleen NK cells during mouse ontogeny, except in fetal mice (Figure 5(a)). The highest level of CD11c on liver NK cells was observed at 4 weeks, but it decreased

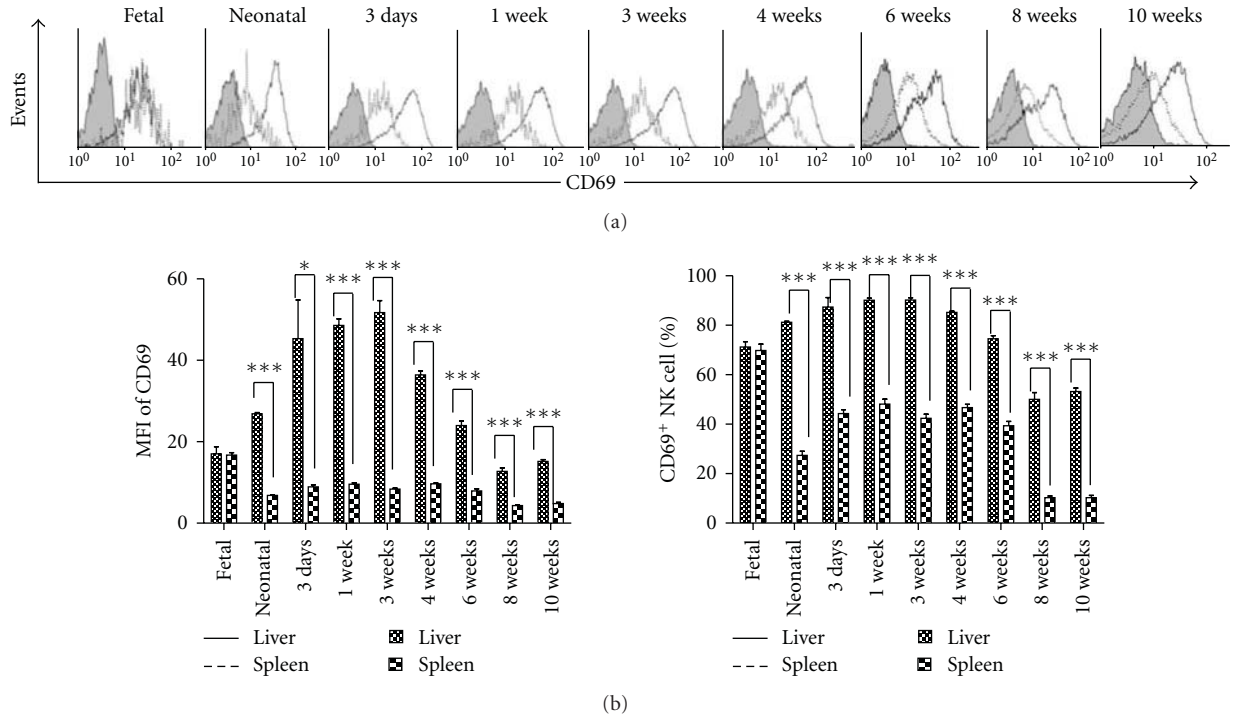

Figure 4: The expression level of CD69 on liver NK cells is higher than on spleen NK. Flow cytometry was performed to analyse lymphocytes stained with the indicated antibodies from liver and spleen of B6 mice at E20, the neonatal stage, and at 3 days, 1 week, 3 weeks, 4 weeks, 6 weeks, 8 weeks, and 10 weeks. NK cells ($CD3^-NK1.1^+$) were gated to analyse the expression of CD69. In each independent experiment, six to seven fetal mouse livers or spleens were put together to acquire enough cells to perform FACS analysis in one experiment, and three independent experiments were performed. In the other groups, there were three mice independently detected for one experiment, and three independent experiments were performed. (a) Each percentage represents the net percentage (%) of cells in the appropriate quadrant. These results are from a single experiment representative of three independent experiments. (b) The percentages of the $CD69^+$ NK cell subset (%) were calculated for the total number of NK cells in the liver and spleen. Data are shown as mean ± SEM from three mice in each group. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ compared with the corresponding group.

in adult mice (Figure 5(a)). Similarly, CD73 was expressed more highly on liver NK cells, reaching its highest level at 3 weeks (Figure 5(b)).

To further explore the functions of hepatic NK cells at different developmental stages, hepatic MNCs were stimulated with Poly I : C in vitro, and then the expressions of IFN-gamma, perforin, and Granzyme B of NK cells were tested. After stimulated with Poly I : C in vitro, there were more IFN-gamma and perforin expressed by the neonatal liver NK cells than 10-week old liver NK cells ($P = 0.0082$ and $P = 0.0009$) (Figure 6). However, there were no significant differences of the expression of GranzymeB between neonatal liver NK cells and 10-week-old liver NK cells (Figure 6). It suggested that the function of neonatal liver NK cells may stranger than adult liver NK cells.

3. Discussion

The phenotypes and functions of NK cells change with age and location [12]. There is a unique intrahepatic NK cell subset with the immature phenotype of $NKG2A^+Ly49s^-DX5^-TRAIL^+$ [4–6, 8, 17, 18]. A large body of evidence has supported the existence of a specific development pathway of NK cells in liver [4, 17, 18]. In our study, we described the development of NK cells in liver compared with spleen during mouse ontogeny.

There is a significantly higher percentage of the $CD27^-CD11b^-$ NK cell subset in liver than in spleen in adult wild-type C57BL/6 mice [18–20], which we confirmed in our study (Figure 1). Furthermore, our study is the first to describe the presence of a high percentage of the $CD27^-CD11b^-$ NK cell subset in fetal mouse liver and to demonstrate that the percentage of this NK cell subset was persistently elevated in the adult liver during ontogeny (Figure 1). Because the fetal liver is the major haematopoietic organ during embryogenesis, we speculate that the $CD27^-CD11b^-$ NK cell subset originated from the fetal liver. The NK cell population is absent in bone marrow and spleen from neonates of $Rag1^{-/-}$ mice but accumulates in bone marrow and spleen of adult mice. Additionally, an overrepresentation of $CD27^-CD11b^-$ NK cells, which are considered a precursor NK cell subset found normally in the liver, is observed in the bone marrow of $Rag1^{-/-}$ mice. This suggests that liver NK cell precursors might seed into other organs to compensate for the absence of bone marrow-derived NK cells [17]. The predominant NK cell subsets in the spleen were of the mature phenotypes, $CD27^+CD11b^+$ and $CD27^-CD11b^+$(Figure 1),

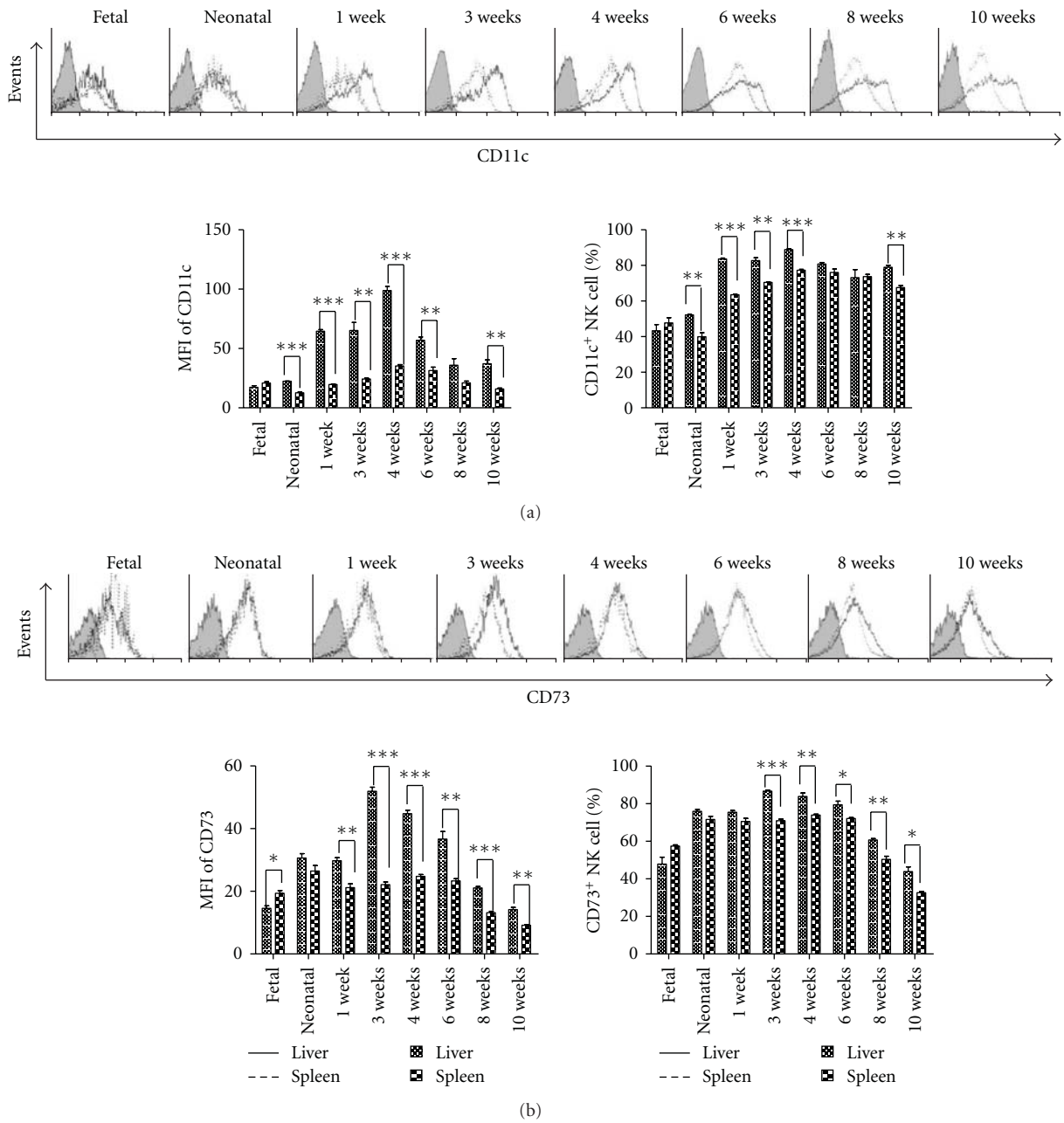

Figure 5: Adhesion molecules expressed on NK cells during mouse ontogeny. At the indicated time points, mononuclear cells were isolated from the liver and spleen and analysed by flow cytometry for the expression of adhesion molecules. NK cells ($NK1.1^+CD3^-$) were gated, and each marker was analysed by histogram. In each independent experiment, six to seven fetal mouse livers or spleens were put together to acquire enough cells to perform FACS analysis in one experiment, and three independent experiments were performed. In the other groups, there were three mice independently detected for one experiment, and three independent experiments were performed. The expression of CD11c (a) and CD73 (b) on liver NK cells is shown and compared with spleen NK cells during ontogeny. The mean fluorescence intensity (MFI) and percentage of each marker is shown as the mean ± SEM from three mice in each group. These are from a single experiment representative of three independent experiments. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ compared with the corresponding group.

which differed from liver NK cells during mouse ontogeny. The $CD27^-CD11b^-$ NK cells were the most immature NK cells which can develop into the other three subset NK cells. As reported, NK cells can develop along DN ($CD27^-CD11b^-$) → $CD27^+CD11b^-$ → DP ($CD27^+CD11b^+$) → $CD27^-CD11b^+$model [16]. However, studies of physiological functions of these four NK subsets were limited. It has been reported that $CD27^+CD11b^+$ exhibited stronger cytotoxicity and produced more IFN-γ than $CD27^-CD11b^+$ NK cells in response to cytokine stimulation [19]. In the liver, there may be a unique developmental pathway distinct from the spleen such that NK cell subsets at different maturation

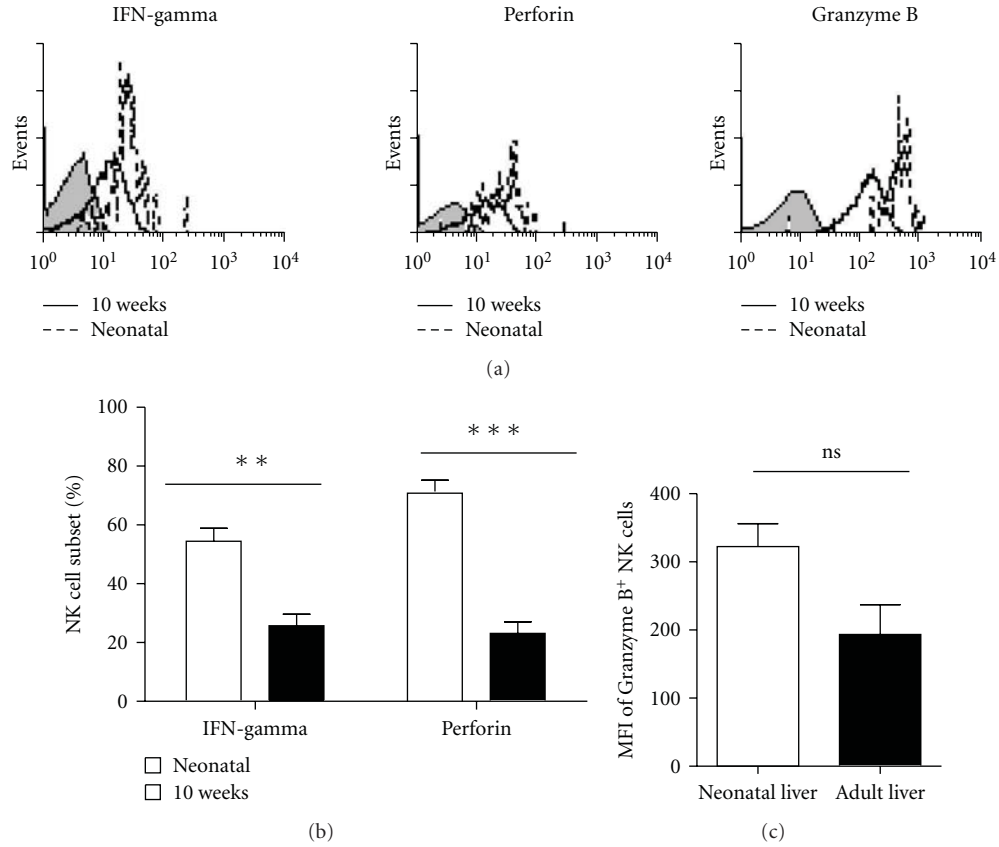

Figure 6: Neonatal liver NK cells produced more IFN-gamma, perforin, and Granzyme B than 10-week-old liver NK cells after stimulated with Poly I: C in vitro. In each independent experiment, six to seven neonatal mouse livers/spleens were put together to acquire enough cells to perform FACS analysis. NK cells ($NK1.1^+CD3^-$) were gated, and then each marker was analyzed by histogram. Unstained controls are the grey. (a) Each percentage represents the net percentage (%) of cells in the appropriate quadrant. (b) The percentages of the IFN-gamma$^+$ NK cell subset (%) were calculated for the total number of NK cells in the liver and spleen. Data are shown as mean ± SEM three mice in each group. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ compared with the corresponding group.

stages distribute specifically to liver and spleen during mouse ontogeny.

The inhibitory NKG2A and Ly49 receptors can be considered markers of the NK cell maturation stage. In fetal and neonatal spleen, almost all NK cells express NKG2A, and the percentage of $NKG2A^+$ NK cells decreases during mouse ontogeny [20–22], however, NK cells do not express Ly49C/I/F/H in early development and gradually acquire these receptors [21, 23, 24]. The same kinetics of the expression levels of NKG2A and Ly49 receptors were observed on spleen NK cells during mouse ontogeny in our study (Figure 2). Furthermore, for the first time, we demonstrated that almost no Ly49C/I/F/H was expressed on liver NK cells in fetal and neonatal mice (Figure 2) and that the decrease of $NKG2A^+$ NK cells and the increase of $Ly49C/I/F/H^+$ NK cells were much slower in the liver than in the spleen during mouse ontogeny. This resulted in a higher percentage of $NKG2A^+$ NK cells and a lower percentage of $Ly49^+$ NK cells in liver compared to spleen (Figure 2(b)). These results further indicate that the development of NK cells does not occur concurrently in the liver and spleen. In the liver, NK cells displayed predominantly immature phenotypes.

During mouse ontogeny, we observed that in the livers of 3-day-old mice, the percentage of $CD27^-CD11b^-$ NK cells was comparatively lower than in fetal and neonatal mice, while the percentage of $CD27^-CD11b^+$ NK cells was higher in the 3-day-old mice (Figure 1). Accordingly, in the liver of 3-day-old mice, NK cells had acquired the expression of Ly49 receptors, occurring earlier than in spleen NK cells (Figure 2). These results indicate that NK cells in the liver undergo a rapid progression of development and differentiation after birth. In view of special double blood supplies from the hepatic artery and the portal vein, the liver is continuously exposed to large amounts of intestinal antigens after birth [25–27]. The development of liver NK cells during early mouse ontogeny might relate to the special physiological functions of the liver. We found that the expression of NKG2D rapidly increased to its highest level at 4 weeks and that the expression of CD69 was upregulated to its highest level at 3 weeks, indicating an activated phenotype of liver NK cells. However, intrahepatic NK cells expressed lower levels of NKp46 and CD2 if compared with spleen NK cells during mouse ontogeny (Figures 3 and 4). The liver with specific blood supply from intestines

continuously encounters bacterial products and food-derived antigens. The liver must eliminate the blood toxic waste products and endotoxins or other bacterial degradation products from the gut, without eliciting an immune response in the normal condition, so the liver acts as a complex immune organ, functioning as a site of effective immune responses or of tolerance appropriately [28, 29]. The constitutive presence of non-self and microbial molecules may result in the activated statement of hepatic NK cells, which is related to the liver tolerance. It was evidenced that the liver's resident immune cells exist in a state of active tolerance and this state of tolerance may be reversed by the right combinational administration with immunostimulants. Furthermore, it has been speculated that the high content of organ-specific NK cells might be associated with liver immune tolerance. In humans, it has been proposed that the unique properties of the transferred hepatic NK cells from a donor may enable them to play a role in regulating the immunological response of the recipient against the graft and therefore contribute to liver tolerogenicity after liver transplantation [30].

The liver is a lymphoid organ with a predominantly innate immune system [31, 32]. NK cells are abundant in the normal liver, accounting for approximately one-third of intrahepatic lymphocytes, which differs from other lymphoid organs and peripheral blood [25, 33, 34]. NK cells sequentially express different integrins over the course of development and maturation [5] and alter their expression of integrins and chemotactic receptors for their redistribution from the bone marrow and lymph nodes to blood, spleen, liver, and lung [35]. In our study, we found a constantly elevated level of CD11c and CD73 expression on liver NK cells compared to spleen NK cells during mouse ontogeny (Figures 5(a) and 5(b)). These adhesion molecules may play a role in intrahepatic NK cell adherence and retention in the liver.

In this study, the development of intrahepatic NK cells was described and compared to spleen NK cells during mouse ontogeny. Our results indicate that in the liver, there might be a specific developmental pathway of NK cells and that the microenvironments play important roles in NK cell development and differentiation. Further research on the mechanisms of differentiation and activation, chemoattraction, adhesion, and functions of hepatic NK cells is warranted.

4. Materials and Methods

4.1. Animals. C57BL/6 mice were obtained from the Shanghai Experimental Animal Center (Shanghai, China) and maintained under specific pathogen-free and controlled conditions (22°C, 55% humidity, and 12-hour day/night rhythm). The animal experiments were performed in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals. All procedures were in compliance with the regulations of animal care of University of Science and Technology of China. The accreditation number of the laboratory is SYXK (Anhui) 2005-004 from Anhui Science and Technology Department. All the surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. To obtain timed pregnant mice, mice were mated for 15 hours, and then at E20 (plug date = day 0) the foetuses were acquired. Neonatal mice were 24 hours old.

The protocols regarding the use and care of animals in the research as described by the paper had been reviewed and approved by *the Intuitional Animal Care and Use Committee of University of Science and Technology of China* (the date of approval: March 15, 2009, the reference number of approval: USTCAU200900005).

4.2. Isolation of Liver Mononuclear Cells. Liver mononuclear cells (MNCs) were isolated essentially as described previously [36]. Briefly, mouse liver was removed and pressed through a 200-gauge stainless steel mesh. The liver cell suspension was collected and then centrifuged at 50 g for 1 min. Supernatants containing MNCs were collected and washed in phosphate-buffered saline (PBS). The cells were resuspended in 40% Percoll (GE Healthcare) and then gently overlaid on 70% Percoll and centrifuged at 1260 g for 30 minutes at room temperature. The interface cells between the Percoll solutions were aspirated and washed twice in PBS. Six to seven fetal mouse livers were harvested together, and the liver MNCs were isolated as described above.

4.3. Isolation of Splenocytes. Mouse spleen was removed and pressed through a 200-gauge stainless steel mesh. The cell suspension was collected and centrifuged at 890 g for 10 min, and then the cells were subjected to red blood cell lysis before incubation in PBS. Six to seven fetal mouse spleens were harvested together for splenocyte isolation.

4.4. Flow Cytometry Analysis. For the intracellular cytokine assay, MNCs were cultured in the presence of 6 uM monensin (Sigma Chemical Co., St. Louis, MO) for 4 h in humidified 5% CO_2 at 37°C. After blocking with anti-FcR, cells were subsequently stained with a saturating amount of the indicated fluorescence-labelled antibodies for 30 min at 4°C in darkness for the surface antigens. Subsequently, cells were fixed and permeabilized using 100 uL of cytofix and cytoperm solution (eBioscience, San Diego, Calif, USA), respectively, and then stained with the indicated fluorescence-labelled antibodies for 1 hour at 4°C in darkness for the intracellular antigens. Stained cells were washed twice in PBS and then acquired with an LSRII (Becton Dickinson) and analysed with WinMDI 2.9 software.

4.5. Reagents. The fluorescence-labelled antibodies used in this study included FITC-anti-CD69 (clone H12F3), FITC-anti-CD11c (clone HL3), FITC-anti-CD11b (clone M1/70), FITC-anti-AHIgG1, FITC-anti-RatIgG2b, PE-anti-CD27 (clone LG.3A10), PE-anti-NKG2D (clone CX5), PE-anti-CD244 (clone 2B4), PE-anti-CD2 (clone RM2-5), PE-anti-AHIgG1, PE-anti-RatIgG2a, PE-anti-RatIgG1, PE-anti-MsIgG2b, PE-anti-RatIgG2b, PE-anti-IFN-gamma (clone

XMG1.2), PerCP-Cy5.5-anti-NK1.1, PerCP-Cy5.5-anti-MsIgG2a, APC-anti-CD3e (clone 1452C11), APC-anti-AHIgG1, APC-anti-IgM, Alexa647-anti-RatIgG2a, APC-Cy7-anti-CD3e (clone 145-2C11), APC-Cy7-anti-AHIgG1 (BD Pharmingen, San Diego, Calif, USA), PE-anti-NKG2A (clone 16a11), FITC-anti-Ly49C/I/F/H (clone 14B11), PE-anti-CD73 (clone TY/11.8), PE-anti-perforin (clone eBioOMAK-D), PE-anti-Granzyme B (clone 16G6) and Alexa647-anti-NKp46 (eBioscience, San Diego, Calif, USA). RBC lysis buffer was purchased from Biolegend (San Diego, Calif, USA).

4.6. Statistical Analysis. The results were analysed by Student's t-test, performed with GraphPad Prism v5.00 software. All data are shown as the mean ± standard error of the mean (SEM). $P < 0.05$ was considered statistically significant.

Abbreviations

HSC: Haematopoietic stem cell
NK cell: Nature killer cell
NKP: Nature killer cell precursor
LN: Lymph node
iNK cell: Immature NK cell
TRAIL: Tumour necrosis factor-related apoptosis-inducing ligand
Rag: Recombination-activating gene
MFI: Mean fluorescence intensity
PBS: Phosphate-buffered saline
MNCs: Mononuclear cells
RBC: Red blood cells.

Conflict of Interests

The authors declare no financial or commercial conflict of interests.

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