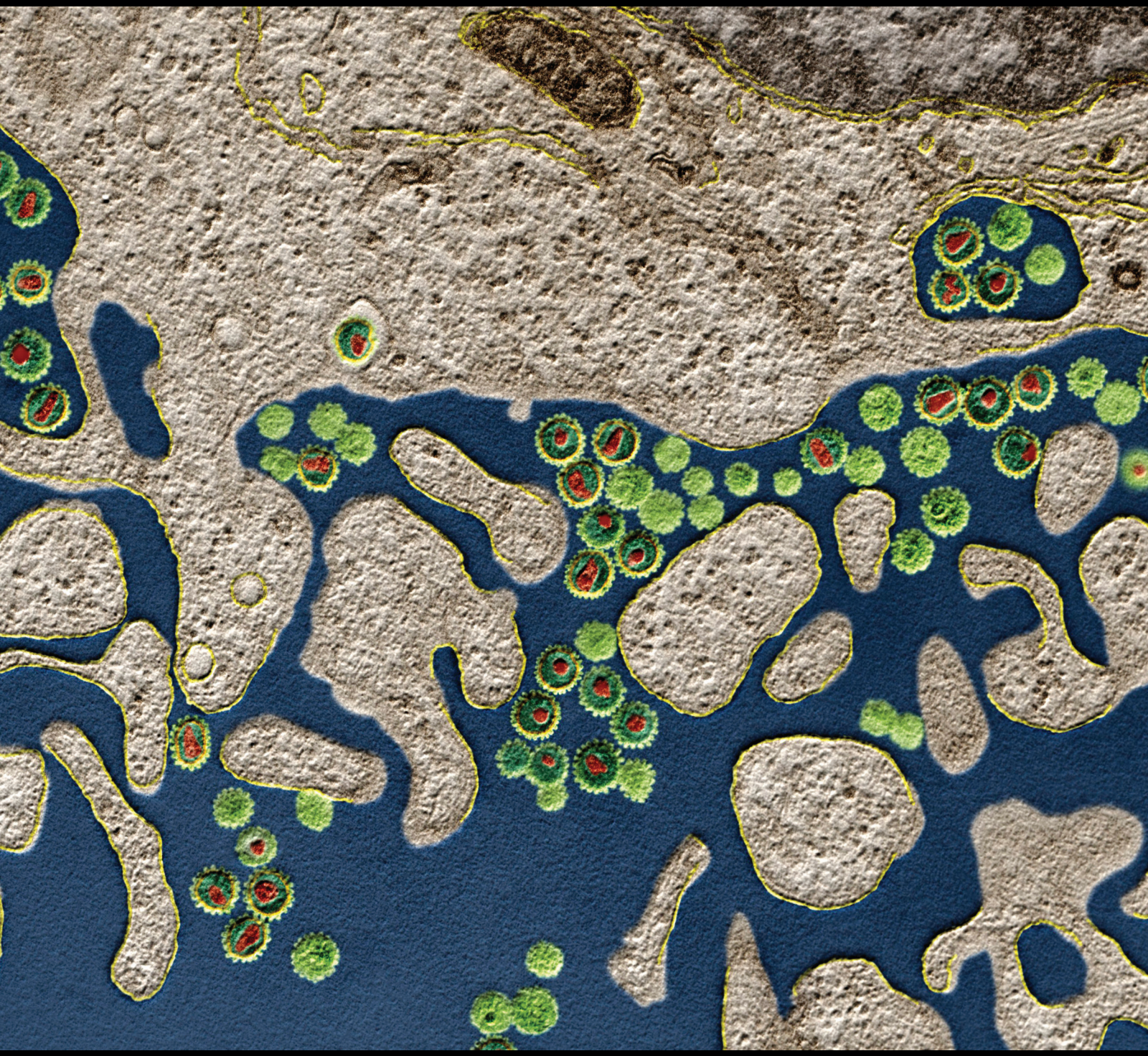
Journal of Immunology Research

Innate-Adaptive Immune Crosstalk

Guest Editors: Anil Shanker, Menaka C. Thounaojam, Manoj K. Mishra, Mikhail M. Dikov, and Roman V. Uzhachenko



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Editorial Board

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Editorial

Innate-Adaptive Immune Crosstalk

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The vertebrate immunological defense system relies upon interdependent regulatory interactions between the innate and adaptive immune compartments playing a pivotal role in various physiological and immunopathological conditions. Recent studies have demonstrated that interactions between dendritic cells and NK cells or T cells and NK cells are important for clearing various bacterial and viral infections. The importance of intratumoral crosstalk between T cells and NK cells during their antitumor immune response has also been established for efficient tumor rejection. Our work in mouse tumor models [1, 2] and others work in models of obesity [3–5], atherosclerosis [6], peritonitis [7], viral and bacterial infections [8–10], and intestinal microbiota [11] suggest a much broader bidirectional cooperativity of the adaptive and innate immune functions. It is, thus, imperative to study both these immune compartments as one functional unit. Detailed understanding of the interaction between innate and adaptive immunity can lead to new approaches aimed to improve immunotherapy for various diseases. This special issue consists of 4 review and 5 research articles.

Behçet's disease is an inflammatory disorder characterized by orogenital ulcerations, ocular manifestations, arthritis, and vasculitis. Md. S. Hasan and colleagues reviewed the role of gamma delta ($\gamma\delta$) T cells' involvement in Behçet's disease and addressed their potential interactions with neutrophils and monocytes mediated by proinflammatory cytokines TNFα and IFNγ and the chemokine CXCL8. In another review article, IFNγ priming as a mechanism affecting both innate immune cells and effector memory $CD4^+$ T cells was reviewed by H. B. da Silva et al. IFNγ is the main cytokine produced by effector memory T cells committed for the Th1 phenotype. Compiling available literature on various infectious and chronic diseases, authors make the case that IFNγ is an important point of crosstalk between innate and adaptive immunity. The review article by P. de Matos Silva and colleagues provides an update on current therapies based on tolerogenic dendritic cells and their crosstalk with T cells modulated by costimulatory blockers with the aim of reducing transplant rejection. They also highlighted challenges for allograft rejections. They review the successes and failures of clinical trials employing iNKT cell-based immunotherapy and explore the future prospects of using such strategies. A review article by J. B. Altman et al. focused on the role of a specific lineage of NKT cells and their interaction with tumor-associated macrophages influencing tumor microenvironment and antitumor immunity.

The research article collection for this special issue covers 2 clinical and 3 preclinical research articles. M. Wu et al. studied the immunoregulatory effects of human umbilical cord's mesenchymal stem cells (UC-MSCs) on IL-22 production in patients with immune thrombocytopenia (ITP). Herein, authors reported for the first time that UC-MSCs downregulate IL-22 through soluble cellular factors but not PGE2 in ITP patients. The study by M. Lima and colleagues evaluated chemokine receptor expression on normal blood $CD56^+$ NK cells in a network with other immune cells. They identify a transitional NK cell population between the $CD56^{high}$ and $CD56^{low}$ NK cell populations, which is

CXCR3/CCR5$^+$ with intermediate expression levels of CD16, CD62L, CD94, and CD122.

I. J. Kosten et al. studied crosstalk between keratinocytes, fibroblasts, and Langerhans cells and showed that the proinflammatory cytokine, IL-18, and chemokines CCL2, CCL20, and CXCL12 are mostly secreted by skin, when compared with gingiva. Furthermore, CCL27 was primarily secreted by skin whereas CCL28 was mostly released by gingiva. This suggests that the cytokines and chemokines involved in triggering and mediating Langerhans cell migration and the innate immune response are different in skin and gingiva. D. Wang et al. explored potential natural ligands of antitumor monoclonal antibody HAE3 by performing carbohydrate microarrays. Authors demonstrate that HAE3 recognizes a conserved cryptic glycoepitope of blood group precursors, which is nevertheless selectively expressed and surface-exposed in certain human breast cancer cell lines, including some triple-negative ones that lack the estrogen, progesterone, and Her2/neu receptors. Findings by X.-T. Yin et al. indicated that the interaction of Fas with FasL in the cornea restricts the development of recurrent herpetic stromal keratitis (HSK) following herpes simplex virus-1 (HSV) infection of the cornea. Authors demonstrated that infection of the cornea with HSV-1 results in increased functional expression of FasL in ocular tissues and that mice expressing mutations in Fas (lpr) and FasL (gld) display increased recurrent HSK following reactivation than do wild-type mice. This clinical disease is the result of a crosstalk of inflammatory cells, consisting of polymorphonuclear leukocytes, macrophages, and T cells (both CD4$^+$ and CD8$^+$) that are recruited to the corneas of patients with HSK.

The presented collection of papers further highlights the cross-regulatory interaction between the innate-adaptive immune networks. We hope that these articles will encourage more clinical and basic studies focused on understanding the malleable functional innate-adaptive immune crosstalk by abandoning the rigid taxonomic dichotomy of immunity.

Acknowledgment

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Review Article

Antitumor Responses of Invariant Natural Killer T Cells

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Natural killer T (NKT) cells are innate-like lymphocytes that were first described in the late 1980s. Since their initial description, numerous studies have collectively shed light on their development and effector function. These studies have highlighted the unique requirements for the activation of these lymphocytes and the functional responses that distinguish these cells from other effector lymphocyte populations such as conventional T cells and NK cells. This body of literature suggests that NKT cells play diverse nonredundant roles in a number of disease processes, including the initiation and propagation of airway hyperreactivity, protection against a variety of pathogens, development of autoimmunity, and mediation of allograft responses. In this review, however, we focus on the role of a specific lineage of NKT cells in antitumor immunity. Specifically, we describe the development of invariant NKT (iNKT) cells and the factors that are critical for their acquisition of effector function. Next, we delineate the mechanisms by which iNKT cells influence and modulate the activity of other immune cells to directly or indirectly affect tumor growth. Finally, we review the successes and failures of clinical trials employing iNKT cell-based immunotherapies and explore the future prospects for the use of such strategies.

1. Introduction

Natural killer T (NKT) cells are innate-like lymphocytes typified by coexpression of receptors characteristic of natural killer and conventional T cells [1]. As such, murine NKT cells generally bear Ly49 receptors, NKG2 family of receptors, CD94, and NK1.1 (the latter only being expressed in specific strains, including the commonly used C57BL/6). Human NKT cells often express similar surface molecules including CD56, CD161, CD94, NKG2D, and NKG2A. Both human and mouse NKT cells display a variety of stimulatory and inhibitory T cell-associated receptors and ligands (e.g., CD28 and CD154), whose expression depends on the activation status of the cell. Finally, both human and murine NKT populations include $CD4^+$ and $CD4^-CD8^-$ (double negative; DN) subpopulations; while $CD8^+$ NKT cells are found in humans, they are rare in mice [2].

The T cell receptors (TCRs) expressed by NKT cells recognize the conserved and nonpolymorphic MHC class I-like molecule, CD1d. Unlike classical MHC class I-like molecules, the expression of CD1d is largely restricted to cells of bone marrow origin including antigen presenting cells (APCs) such as dendritic cells (DCs), macrophages, and B cells. Furthermore, the CD1d molecule (via heterodimerization with β_2-microglobulin) specializes in displaying lipid moieties rather than protein polypeptides. Importantly, intact expression of CD1d is critical for the development of NKT cell populations, as *Cd1d*−/− mice are devoid of these cells [3]. NKT cells are further subclassified into Type I or II lineages, depending on the composition of their TCR and the CD1d-presented glycolipid antigens to which they respond. Type I or invariant NKT (iNKT) cells express canonical TCRα chains comprised of specific gene segments (Vα14-Jα18 in mice and Vα24-Jα18 in humans) that preferentially pair with specific TCRβ chains (Vβ8, Vβ7, or Vβ2 in mice and Vβ11 in humans). These invariant TCR$\alpha\beta$ pairings confer reactivity to CD1d and a restricted array of presented glycolipid antigens. The dependence of iNKT cells on the Vα14-Jα18-comprised TCRα is demonstrated by *Vα14* TCR transgenic mice, in which a higher frequency and number of iNKT cells are observed [4], and also *Jα18*−/− mice, in which no mature iNKT cells develop [5]. Despite the conserved use of the

invariant TCR, iNKT cell populations are phenotypically (e.g., presence or absence of CD4 expression) and functionally (e.g., preferential production of certain cytokines, such as IL-17) diverse.

The prototypical (and first discovered) iNKT cell stimulatory glycolipid, alpha-galactosylceramide (α-GalCer), was identified during a screening for compounds from marine sponges (*Agelas* species) with antitumor activity [6]. Since this initial discovery, a number of naturally occurring and synthetic lipid antigens have been described to bind CD1d and activate iNKT cells. These cells are now typically identified using CD1d tetramers loaded with α-GalCer or its synthetic analogs (e.g., PBS-57; [7]). In contrast, Type II or variant NKT (vNKT) cells bear a more diverse array of TCRα and β chains and have been shown to recognize sulfatide moieties presented by CD1d [8]. More recently, Type II NKT cells have also begun to be better characterized through development of CD1d tetramers loaded with sulfatide [9, 10], but these cells are still less well characterized than their invariant brethren. Given that far more is known regarding the antitumor activity of iNKT cells, we will predominantly focus our attention on these cells.

2. iNKT Cell Development and Acquisition of Effector Function

iNKT cells develop in the thymus, by originating from $CD4^+CD8^+$ double positive (DP) thymocytes. Positive selection of iNKT cells is mediated by homotypic interactions of DP cells and recognition of glycolipid antigen-CD1d complexes [11–14]; however, the nature of the self-antigens involved in this process remains somewhat elusive. Like conventional T cells, maturation of iNKT cells at the DP stage and beyond depends on the ability to construct a functional TCR and intact signaling. As such, iNKT cells are profoundly diminished or absent in mice lacking expression of RAG, CD3ζ, Lck, ZAP-70, SLP-76, ITK, LAT, or Vav [15–21]. Transcriptionally, development of iNKT cells at the DP stage is regulated by the transcription factor RORγt, which prolongs the survival of DP thymocytes by upregulating Bcl-X_L, to allow sufficient time for distal TCRα gene segment rearrangements to occur [22, 23]. More recent studies have shown that HEB, the E protein family of basic helix-loop-helix transcription factors, regulates iNKT cell development by regulating RORγt and Bcl-X_L mRNA [24]. Finally, the absence of the transcription factor Runx1 also blocks iNKT cell development at the earliest detectable iNKT cell-committed subset [23].

iNKT cell development at the DP stage also critically depends on the signals generated by engagement of the Signaling Lymphocyte Activation Molecule (SLAM) family of surface receptors, which are expressed on developing iNKT cells, as well as conventional DP thymocytes. SLAM family receptor signaling is transduced by the adaptor molecule SAP (SLAM-associated protein), which in turn binds to the tyrosine kinase Fyn, and results in propagation of a phosphorylation signal [25, 26]. Accordingly, iNKT cells fail to develop in mice and humans bearing mutations in the gene that encodes for SAP [27–29], in mice lacking Fyn or expressing a mutant version of SAP that cannot bind Fyn [23, 30], in mice in which both Ly108 and SLAM signaling are simultaneously disrupted [31], and in those lacking the transcription factor *cmyb* (which is necessary for appropriate expression of SAP and certain SLAM family members) [32]. Taken together, these studies establish the importance of the SLAM-SAP-Fyn signaling axis in iNKT cell development.

Following positive selection, iNKT cells undergo distinct stages of maturation that are characterized by the sequential acquisition of CD24, CD44, and NK1.1: $CD24^{hi}CD44^{lo}NK1.1^-$ (Stage 0), $CD24^{lo}CD44^{lo}NK1.1^-$ (Stage 1), $CD24^{lo}CD44^{hi}NK1.1^-$ (Stage 2), and finally $CD24^{lo}CD44^{hi}NK1.1^+$ (Stage 3) [33]. As these cells progress through these developmental stages, they begin to upregulate NK cell markers (e.g., NKG2D and Ly49 receptors), CD69, and CD122 and acquire distinct effector functions (e.g., production of IL-4, IFN-γ, perforin, and granzymes) [34]. Acquisition of these effector functions is tightly regulated by several transcription factors [35]. One of the key regulators of iNKT cell development and acquisition of an effector/memory phenotype and functions is the broad complex tramtrack bric-a-brac-zinc finger transcription factor PLZF, whose expression is highest in Stage 0 and 1 populations [36, 37]. PLZF-deficient animals exhibit a severe reduction in iNKT cell number and PLZF-deficient iNKT cells fail to cosecrete Th1 and Th2 cytokines upon stimulation [36, 37]. Recently, it was demonstrated that the lethal-7 microRNA posttranscriptionally regulate PLZF expression and iNKT cell effector functions [38].

The transcription factor T-bet is indispensable for the final maturation stages of iNKT cells [39, 40] and absence of this transcription factor results in reduced iNKT cell numbers due to developmental blockade at Stage 2. T-bet-deficient iNKT cells fail to proliferate in response to IL-15 as they lack surface expression of CD122, a component of the IL-15 receptor [40]. In addition, T-bet-deficient iNKT cells fail to produce IFN-γ in response to TCR stimulation and exhibit defective cytolytic activity [39, 40] as T-bet directly regulates the activation of genes associated with mature iNKT cell functions, such as perforin, CD178, and IFN-γ [40].

As iNKT cells progress to Stage 1, a proportion of cells downregulate CD4, giving rise to DN iNKT cells. Generation of the $CD4^+$ iNKT cell lineage and production of Th2-type cytokines is critically regulated by the transcription factor GATA-3. Similar to PLZF-deficient iNKT cells, GATA-3 deficient iNKT cells fail to produce Th1 or Th2 cytokines in response to α-GalCer [41]. Recent studies have identified a unique subpopulation of $NK1.1^-CD4^-$ iNKT cells that are transcriptionally regulated by RORγt and capable of producing large quantities of IL-17 upon stimulation [42]. As such, iNKT cells are also sometimes classified into NKT1, NKT2, and NKT17 based on their cytokine production profiles and respective expression of T-bet, GATA-3, and RORγt [43, 44]. Finally, mechanistic target of rapamycin (mTOR) signaling has also been shown to be important for iNKT cell lineage diversification and acquisition of effector functions [45–48], and loss of mTOR2 may result in loss of NKT17 cells.

Taken together, these recent studies provide new insights into the transcriptional regulation of iNKT cell maturation and functional differentiation.

3. iNKT Cells and Antitumor Immunity

The importance of iNKT cells in mediating protection against tumors is highlighted by several findings. First, a number of independent studies have shown a decrease in the number of iNKT cells in the peripheral blood of patients with a variety of cancers and even precancerous myelodysplastic syndromes [49–51]. Moreover, the iNKT cells that persist appear to have decreased proliferative and functional responses [52–54]. Interestingly, an increased frequency of peripheral blood iNKT cells in cancer patients portends a more favorable response to therapy [55, 56]. While these observations identify an association between iNKT cell numbers and/or function and development of malignancy, they do not provide a direct causal link. This link has been established in a number of mouse studies in which the biology of the host and initiation of tumors can be more systematically manipulated via gene knockouts, antibody depletion strategies, and adoptive transfer of various lymphocyte populations into cancer-predisposed or tumor-challenged hosts.

In mice that are prone to development of tumors due to loss of one allele of a tumor suppressor (*p53*+/−), absence of iNKT cells (by virtue of genetic knockout of the Jα18 gene segment or CD1d) results in earlier and more frequent development of tumors and thus shorter survival [57], when compared to iNKT-sufficient littermates. Similarly, treatment of *Cd1d*−/− and *Jα18*−/− mice with a carcinogen resulted in increased incidence and earlier onset of tumors in comparison to treated wild type mice [58]. Conversely, administration of α-GalCer to mice controlled the growth and metastasis of adoptively transferred [59, 60] or carcinogen-induced [61, 62] or spontaneous [63] tumors. Moreover, adoptive transfer of iNKT cells into *Jα18*−/− iNKT cell-deficient mice prevented the growth of subcutaneous sarcomas [62]. Finally, adoptive transfer of small numbers of purified iNKT cells into lymphocyte-deficient NOD-*Scid-IL2rγ*−/− (NSG) mice was sufficient to protect mice from challenge with a $CD1d^+$ tumor [64]. These findings collectively argue that iNKT cells play a central and nonredundant role in the response to tumors. Further studies would shed light on the mechanisms by which iNKT cells exert these antitumor effects.

3.1. Indirect Cytokine-Mediated Modulation of Antitumor Responses. Engagement of the invariant TCR by CD1d/glycolipid antigen complexes results in iNKT cell activation, an event that is typified by rapid and robust production of a variety of cytokines and chemokines, including—but not limited to—IL-2, IL-4, IL-10, IL-13 IL-17, IFN-γ, TNFα, TGFβ, GM-CSF, RANTES, eotaxin, MIP-1α, and MIP-1β [65, 66]. The nature and magnitude of the iNKT cell cytokine response depend on the glycolipid antigen; for example, α-GalCer-mediated iNKT cell activation elicits a strong IFN-γ-dominated cytokine response, while OCH (a synthetic analog of α-GalCer with a truncated lipid chain) elicits a response with significantly higher level of IL-4 production [67]. The rapidity of this cytokine response is attributed to the semiactivated state of iNKT cells and the presence of preformed cytosolic mRNA for a variety of cytokines [68]. Indeed, administration of α-GalCer to iNKT cell-sufficient, but not iNKT cell-deficient, mice results in polyclonal activation of conventional T, B, and NK cells within 3-4 hours [69] and also eventually leads to the mobilization of macrophages and neutrophils [70]. Intriguingly, it was previously believed that mammalian species are incapable of producing glycolipids (such as α-GalCer), in which the sugar moiety is attached via an O-linkage to the ceramide backbone in an alpha-anomeric configuration. Despite the absence of α-glucosyl or α-galactosyl transferases in mammals, recent findings indicate that a small percentage of the glycolipids that are constitutively presented by mammalian CD1d are indeed α-anomeric [71]. Whether the percentage of CD1d-presented α-anomeric glycolipids is altered in tumor tissues represents an interesting question that deserves further future investigation.

Nonetheless, following encounter with CD1d/antigen complexes displayed by APCs, iNKT cells not only produce cytokines but also upregulate surface expression of CD154 (see Figure 1(a)). Ligation of APC-expressed CD40 is especially important for mediating subsequent maturation and functional activation of DCs, subsequent upregulation of CD80 and CD86, and amplified production of IFN-γ [72, 73]. In addition, the ligation of the chemokine receptor CXCR6 on iNKT cells by CXCL16 expressed on APCs also provides costimulatory signals resulting in robust α-GalCer-induced iNKT cell activation [74]. Importantly, matured DCs are potent producers of IL-12, which induces sustained IFN-γ production by iNKT cells [75–77]. The importance of iNKT cells in IL-12-mediated tumor rejection was effectively demonstrated by the defective clearance of a variety of tumors in *Jα18*−/− mice [5]. Mature DCs also support the priming and activation of $CD8^+$ T cells, culminating in optimal effector and memory cell formation [72, 78]. Finally, the sustained release of IFN-γ by iNKT cells leads to activation and proliferation of NK cells and NK cell secretion of IFN-γ. The combination of cytokines (e.g., IL-2, IL-12, and IFN-γ) as a result of iNKT cell activation also leads to upregulation of death-inducing ligands (e.g., CD178 or CD253) on NK cells and $CD8^+$ T cells [79, 80]. These sequential activation events are believed to be critical for the α-GalCer-induced iNKT cell-mediated antitumor effects [76, 81, 82]. As such, iNKT cells not only bridge the activation of innate and adaptive immunity, but also indirectly potentiate the antitumor activity of other cytotoxic effector lymphocytes.

3.2. Indirect Control of Tumor Growth via Alteration of Tumor Microenvironment. Tumor establishment and growth are believed to be intricately modulated by a myriad of soluble and contact-derived signals obtained from the tumor microenvironment (TME), which consists of the tumor cells themselves, tumor-infiltrating lymphocytes (TILs), and stromal cells that communicate in a dynamic and bidirectional manner. In addition to their indirect modulation of

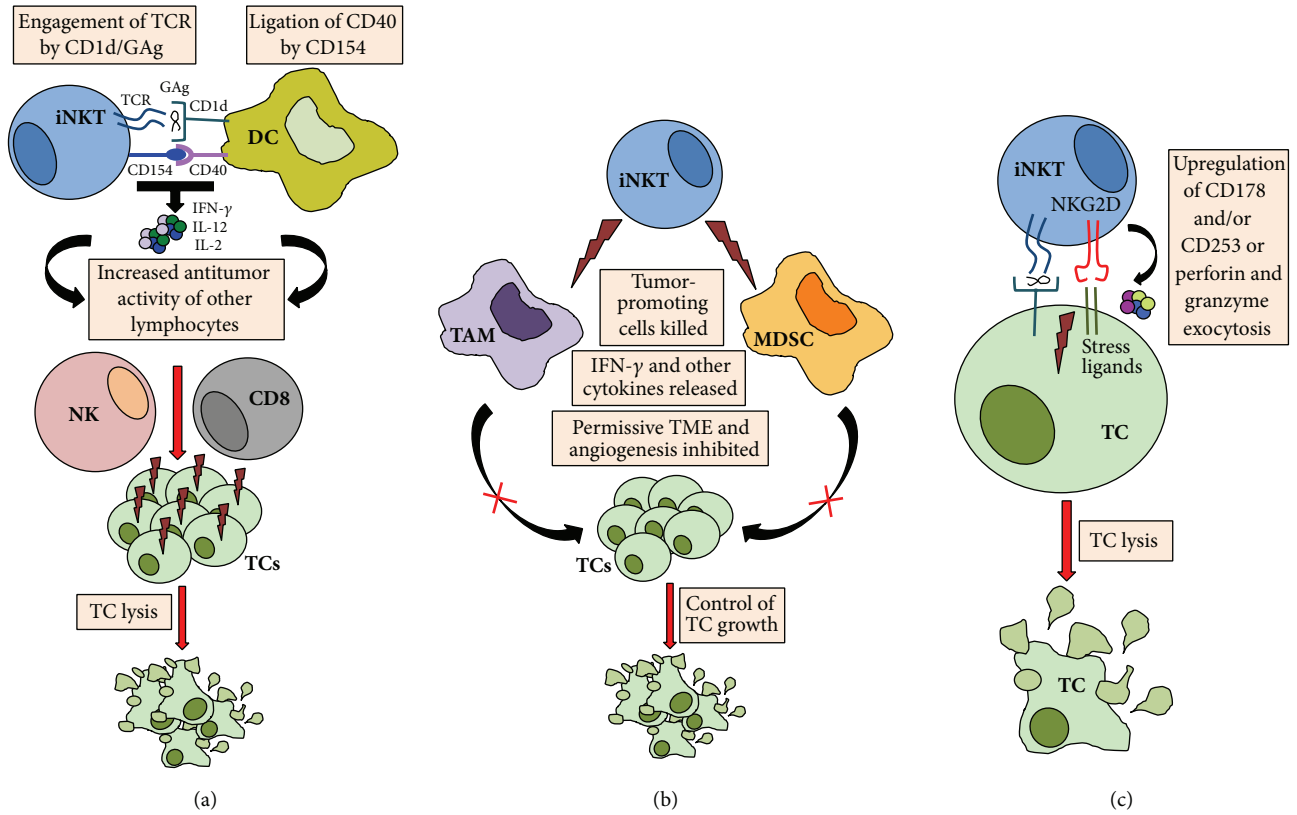

Figure 1: Possible mechanisms of iNKT cell-mediated antitumor responses. (a) Indirect cytotoxicity. iNKT cells and DCs reciprocally coactivate each other via TCR:CD1d/GAg and CD40:CD154 interactions, resulting in the release of several cytokines that secondarily activate and promote the antitumor cytotoxicity of other effector lymphocytes. (b) Modulation of the TME. iNKT cells kill tumor-supporting cells, such as TAMs and MDSCs, and also limit angiogenesis, to indirectly control tumor growth. (c) Direct cytotoxicity. iNKT cells mediate lysis of tumor targets via engagement of TCR or NKG2D. Lightning bolt: exertion of direct cytotoxicity; DC: dendritic cell; GAg: glycolipid antigen; TC: tumor cell; TAM: tumor-associated macrophage; MDSC: myeloid-derived suppressor cell; TME: tumor microenvironment.

other effector lymphocyte populations, iNKT cells may also regulate tumor growth via their effects on the TME (see Figure 1(b)). Indeed following intravenous administration, iNKT cells were shown to represent a significant percentage of the TILs in patients with head and neck carcinomas [83, 84]. Importantly, higher frequency of tumor-infiltrating iNKT cells correlated with overall and disease-free survival as an independent prognostic factor in primary colorectal cancer patients [85] and with tumor regression in head and neck carcinomas [86]. Conversely, in patients with primary hepatocellular or metastatic cancer, $CD4^+$ iNKT cells that produced high levels of Th2-type cytokines and had low cytolytic activity were enriched within the tumor and appeared to inhibit the expansion of antigen-specific $CD8^+$ T cells, suggesting that these particular iNKT cells may contribute to generate an immunosuppressive microenvironment [86].

In experimental studies, cotransfer of human monocytes and iNKT cells to tumor-bearing NOD-*Scid* mice suppressed tumor growth when compared with mice that received monocytes alone [87]. Importantly, iNKT cells can target tumor supportive cells such as tumor-associated macrophages (TAMs), a highly plastic monocyte-derived subset of inflammatory cells that can exert immunosuppressive functions, and promote tumor proliferation and matrix turnover [88, 89]. Indeed TAMs are known to produce IL-6, a cytokine that appears to promote the proliferation of many solid tumors, including neuroblastomas and breast and prostate carcinomas [87]. Consistent with the tumor-permissive capacities of TAMs, Chen et al. found that macrophage density correlated positively with microvessel counts and negatively with patient relapse-free survival [90]. Since TAMs cross-present neuroblastomaderived endogenous CD1d ligand(s), they can be specifically recognized and killed by iNKT cells in an IL-15-dependent process [87]. Other potential iNKT cell TME targets include myeloid-derived suppressor cells (MDSCs). MDSCs have been found to accumulate in the blood, lymph nodes, and bone marrow and at tumor sites in most patients and experimental animals with cancer and inhibit both adaptive and innate immunity [91]. The absence of iNKT cells in mice during influenza virus infection resulted in the expansion of MDSCs, high viral titer, and increased mortality. The adoptive transfer of iNKT cells abolished the suppressive activity of MDSCs and restored virus-specific immune responses, resulting in reduced viral titers and increased rates of host survival [92].

Thus, certain populations of iNKT cells may help alter the TME via their effects on TAMs and MDSCs, to help create a tumor-suppressive or immune-permissive milieu.

3.3. Direct Antitumor Cytotoxicity. In addition to their indirect control of tumor growth, iNKT cells can mediate direct killing of tumor targets (see Figure 1(c)). iNKT cells alone, or in combination with NK cells, have been shown to kill a variety of tumor targets *in vitro* [6, 93, 94]. While this mechanism of killing appears to be dependent on the presence of stimulatory glycolipids and CD1d [95, 96], iNKT cell cytotoxicity also appears to be triggered via ligation of NKG2D by target-expressed stress ligands [97]. NKG2D ligation can also costimulate TCR-triggered cytotoxicity [97]. It remains to be seen whether MULT1, the newly identified shed form of high affinity NKG2D ligand that triggers NK-mediated tumor rejection in mice, also activates iNKT cells [98].

Consistent with their direct cytotoxic capacity, iNKT cells express perforin and granzymes, as well as CD178 [34, 96, 99, 100]. In our hands, blockade of CD1d-mediated lipid antigen presentation, disruption of T cell receptor (TCR) signaling, or loss of perforin expression was found to significantly reduce iNKT cell killing *in vitro* [64]. Moreover, we demonstrated that iNKT cells alone were sufficient for control of the growth of a T cell lymphoma *in vivo* that preferentially relies on perforin and the adaptor protein SAP [64, 69]. Mechanistically, iNKT cells rely on SAP for formation of stable conjugates with the tumor targets as well as proper orientation of the lytic machinery at the immunological synapse [69]. Despite the majority of studies implicating iNKT cells as having an antitumor role, a limited number of studies also implicate iNKT cells as suppressing antitumor responses [101], but these paradoxic responses may be related to the level of tumor CD1d expression [102, 103]. Alternatively, these differences may stem from the fact that—contrary to the use of C57BL/6 mice in the previously discussed studies—these last two studies were performed in BALB/c mice, in which there is a predominance of IL-4-producing Th2 phenotype iNKT cells [43].

Interestingly, the antitumor responses of iNKT cells may be regulated by the activity of Type II NKT cells [104]. Terabe et al. demonstrated that Type II variant NKT (vNKT) cells were sufficient for the downregulation of tumor immunosurveillance and relapse growth of a model fibrosarcoma in an antigen-dependent manner [105], while a second study found that activation of vNKT cells with sulfatide antigen could suppress the activation of iNKT cells [106]. These suppressive vNKT cells were found to be predominantly $CD4^+$ [107]. Conversely, Type II vNKT cells were, in at least one study, suggested to promote the antitumor activity of CpG oligodeoxynucleotides [108].

iNKT cell antitumor activity is also suppressed by regulatory T (Treg) cells. This suppression appears to be mediated through a contact- and IL-10-dependent mechanism [109, 110]. Indeed, induction of Treg cells suppressed the protective effect of adoptive transfer of iNKT cells into *Jα18*−/− mice [111]. Consistent with these findings, depletion of Treg cells or short-term elimination of their suppressive activity results in enhanced iNKT cell-mediated antitumor responses and increased NK and CD8 T cell activation and IFN-γ production [112]. Interestingly, the ability of Treg cells to suppress iNKT cell proliferation depends on the degree of invariant TCR agonism, such that responses to weak (e.g., OCH), but not strong (e.g., α-GalCer), agonists were effectively suppressed [110]. When viewed collectively, these findings suggest that iNKT cells possess inherent capacity for direct cytotoxicity but their antigenic exposure may modulate whether their antitumor effects can be suppressed by Treg and vNKT cells.

4. iNKT Cell-Based Immunotherapy

Given the preponderance of evidence suggesting that the activation of iNKT cells provides protection against the growth and metastasis of a variety of tumors, safety of α-GalCer administration was examined in a Phase I trial [113]. While administration of α-GalCer was well tolerated at a range of doses, no clinical responses were observed in patients with advanced solid tumors. On the heels of this study, Nieda et al. showed that treatment of metastatic cancer patients with α-GalCer-pulsed immature monocyte-derived DCs resulted in dramatic increases in serum IFN-γ and IL-12 and activation of NK and T cells in the majority of subjects. Importantly, this Phase I trial also documented reduction in tumor biomarkers and tumor necrosis in several patients [100]. These findings were extended in a study of a small number of patients, in which the α-GalCer-pulsed DCs were matured prior to adoptive transfer. This study demonstrated a >100-fold increase in blood iNKT cell numbers in all patients, and this increase was long-lived (>6 months) [114]. A number of subsequent clinical trials, all with limited number of patients with advanced head and neck or non-small cell lung cancers, have since employed similar strategies of adoptive transfer of α-GalCer-pulsed APCs [115–118]. Collectively, these studies demonstrate increases in blood IFN-γ levels and iNKT cells in some but not all patients, stabilization of disease in a few of the subjects, and absence of severe treatment-related toxicities.

In a different approach, chemotherapy-refractory 5 lymphoma patients were treated with autologous peripheral blood mononuclear cells (PBMCs) stimulated with anti-CD3, IL-2, and IFN-γ. This *ex vivo* stimulation resulted in enrichment of NKT cells (to ~20% on average), and this cell fraction was shown to possess the highest cytotoxic capacity *in vitro*. Of the nine patients who received adoptive transfer of these cells, two showed partial responses and two others had stabilization of disease [119]. Two subsequent studies by Motohashi et al. evaluated the adoptive transfer of *ex vivo* expanded iNKT cell-enriched cells to patients with advanced cancer. In the first, 6 patients with advanced non-small cell lung cancer were treated with either a low or a high dose of *ex vivo* expanded iNKT cells. Of the 3 patients treated with the high dose, all had an increase in the frequency of IFN-γ-producing PBMCs and 2 showed expansion of iNKT cells [120]. Although no clinical responses were observed in this

study, a follow-up trial of 17 patients with advanced head and neck cancers treated with a high dose of iNKT cell-enriched autologous PBMCs showed a significant increase in IFN-γ-producing PBMCs in 10 of 17 patients. Importantly, while none of these patients displayed tumor regression, 5 had disease stabilization and the mean survival time for the subjects with higher frequencies of IFN-γ-producing PBMCs was tripled above those with low percentages of IFN-γ-producing PBMCs (29.3 versus 9.7 months) [117]. Finally, in a combinational treatment strategy, Kunii et al. administered both *in vitro* expanded iNKT cells and α-GalCer-pulsed APCs to patients with advanced head and neck squamous cell carcinomas. Treatment increased the frequencies of iNKT cells and IFN-γ-producing PBMCs, and a partial clinical response or disease stabilization was observed in 7 of 8 patients [83]. Although the responses in these studies have not been profound, it must be noted that these iNKT cell-based immunotherapies have all been conducted on patients with advanced malignancies—often those in whom standard chemotherapy, irradiation, and/or surgical excision treatments had failed. Moreover, the majority of these patients had $CD1d^-$ cancers.

Future studies of iNKT cell-based immunotherapy may be able to take advantage of two recent technologies. As mentioned previously, many malignancies are associated with a decrease in the numbers and proliferative capacity of peripheral blood iNKT cells. In order to circumvent the difficulty of being able to expand these infrequent and potentially defective cells from patients, Watarai et al. generated induced pluripotent stem (iPS) cells from mature iNKT cells and then expanded large numbers of iNKT cells from these established iPS cells. iPS-NKT cells generated in this fashion were demonstrated to be able to activate and expand antigen-specific CD8 T cell responses to limit the growth of leukemia in mice [121] without inducing graft versus host disease (GvHD) [122]. The second strategy employs chimeric antigen receptors (CARs). Recently, a report by Heczey et al. described iNKT cells engineered to express CARs bearing specificity for GD2, a highly expressed moiety on neuroblastoma cells. In their studies, they showed that iNKT cells expanded from the PBMCs of healthy human donors and transduced with retroviral CAR constructs could protect humanized NSG mice against metastatic neuroblastoma without inducing GvHD [123]. Whether these two technologies could be combined to generate functional CAR-bearing iPS-NKT cells remains to be seen.

5. Concluding Remarks

iNKT cells are innate-like effector lymphocytes that not only are directly cytotoxic, but also possess the unique ability to nucleate the antitumor responses of other effector lymphocytes and alter the cellular and angiogenic makeup of the tumor microenvironment. As such, the promise of an effective iNKT cell-based immunotherapy can only be realized by devising and evaluating strategies that simultaneously maximize each of these antitumor effector mechanisms. The challenge for the future will thus be to identify these strategies and apply them to tumors against which iNKT cells wield the most optimal responses.

Conflict of Interests

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

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Research Article

Chemokine Receptor Expression on Normal Blood CD56$^+$ NK-Cells Elucidates Cell Partners That Comigrate during the Innate and Adaptive Immune Responses and Identifies a Transitional NK-Cell Population

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Studies of chemokine receptors (CKR) in natural killer- (NK-) cells have already been published, but only a few gave detailed information on its differential expression on blood NK-cell subsets. We report on the expression of the inflammatory and homeostatic CKR on normal blood CD56^{+low} CD16$^+$ and CD56^{+high} CD16$^{-/+low}$ NK-cells. Conventional CD56^{+low} and CD56^{+high} NK-cells present in the normal PB do express CKR for inflammatory cytokines, although with different patterns CD56^{+low} NK-cells are mainly CXCR1/CXCR2$^+$ and CXCR3/CCR5$^{-/+}$, whereas mostly CD56^{+high} NK-cells are CXCR1/CXCR2$^-$ and CXCR3/CCR5$^+$. Both NK-cell subsets have variable CXCR4 expression and are CCR4$^-$ and CCR6$^-$. The CKR repertoire of the CD56^{+low} NK-cells approaches to that of neutrophils, whereas the CKR repertoire of the CD56^{+high} NK-cells mimics that of Th1$^+$ T cells, suggesting that these cells are prepared to migrate into inflamed tissues at different phases of the immune response. In addition, we describe a subpopulation of NK-cells with intermediate levels of CD56 expression, which we named CD56^{+int} NK-cells. These NK-cells are CXCR3/CCR5$^+$, they have intermediate levels of expression of CD16, CD62L, CD94, and CD122, and they are CD57$^-$ and CD158a$^-$. In view of their phenotypic features, we hypothesize that they correspond to a transitional stage, between the well-known CD56^{+high} and CD56^{+low} NK-cells populations.

1. Introduction

Natural killer- (NK-) cells were originally identified by their natural ability to kill target cells and are known for a long time as effector cells of the innate immune system, with an important role in controlling several types of tumors and infections [1]. In recent years, NK-cells have also been recognized as regulatory cells, which are able to interact with other cells of the immune system, such as dendritic cells (DC), monocytes/macrophages, and T cells, thereby influencing the innate and adaptive immune responses [2–5]. The role of their interaction with neutrophils in shaping the immune response is also being increasingly documented [6, 7].

The cytotoxic activity of the NK-cells is controlled by the balance between inhibitory and activating receptors, whose ligands are self-Major Histocompatibility Complex (MHC) class I molecules and molecules expressed on stressed, viral infected, and tumor cells. They comprise, among others, the killer cell immunoglobulin-like receptors (KIR), killer cell

lectin type receptors (KLR), and natural cytotoxic receptors (NCR) as well as immunoglobulin Fc receptors (FcR) and complement receptors [8–10].

Meanwhile, the immunoregulatory properties of the NK-cells are mediated, not only by cell-to-cell contact, but also by the soluble factors they produce, which enable them to recruit and to activate other immune cells. These include chemokines (CK), such as MIP-1α (macrophage inflammatory proteins-1 alpha, CCL3) and MIP-1β (CCL4), RANTES (regulated activation, normal T cell expressed and secreted, CCL5), and ATAC (activation-induced, T cell derived, and chemokine-related cytokine, CXCL1). They also comprise cytokines, for example, IFN-γ (interferon-gamma) and TNF-α (tumor necrosis factor alpha) and growth factors, such as GM-CSF (granulocyte-macrophage colony-stimulating factor) [11, 12].

Using adhesion molecules and chemokine receptors (CKR), NK-cells are able to circulate in the blood and to distribute throughout the body, by homing into secondary lymphoid organs (e.g., lymph nodes), localizing in specific nonlymphoid organs (e.g., liver, placenta), and migrating into acute or chronic inflamed tissues, where they participate in the immune responses [13–16]. In some organs, NK-cells exhibit specific phenotypes and functions [17, 18], for example, promoting decidualization of the endometrium, embryo implantation and placenta development [19, 20], and influencing the hematopoiesis [21, 22].

Two different subsets of mature $CD56^+$ NK-cells have been described in humans, based on the levels of CD56 and CD16 expression: $CD56^{+low}$ $CD16^+$ and $CD56^{+high}$ $CD16^{-/+low}$ NK-cells from now on designed $CD56^{+high}$ and $CD56^{+high}$, respectively [23, 24]. While the former clearly predominates in the peripheral blood (PB), where they represent around 90% of the circulating $CD56^+$ NK-cells, the latter are more represented in secondary lymphoid organs, chronically inflamed tissues and placenta [13–16, 19, 20].

Apart from the different expression of CD16, the low affinity receptor for IgG (FcγRIIIA) and CD56, the neural cell adhesion molecule (NCAM), the conventional $CD56^+$ NK-cell subsets also differ in the expression of other adhesion, homing, and costimulatory molecules as well as on the repertoires of NCR, KIR and KLR, and receptors for cytokines, chemokines, and growth factors [25–29]. In addition, these NK-cell subsets exhibit distinct sialylated forms of CD43 and posttranslational modifications of the P-selectin glycoprotein ligand-1 (PSGL-1) [30, 31].

From the functional point of view, $CD56^{+low}$ NK-cells are essentially cytotoxic, with a greater level of antibody dependent cell mediated cytotoxicity (ADCC) [32], whereas $CD56^{+high}$ NK-cells have a high proliferative response to low doses of interleukin- (IL-) 2 (IL-2) and C-kit ligand [33]. In addition, the latter display a more important immunomodulatory role associated with cytokine production in response to IL-2 and monokines [33]. More recently it became apparent that upon target cell recognition, $CD56^{+low}$ NK-cells are more prominent cytokine and chemokine producers than $CD56^{+high}$ NK-cells [34]. These diverse functional properties would suggest that $CD56^{+low}$ and $CD56^{+high}$ NK-cells could be naturally prepared to act in different sites and at different phases of the immune response.

The exact relationship between these NK-cell subsets still remains unclear. Some studies have shown that bone marrow progenitor cells give rise to $CD56^{+high}$ or $CD56^{+low}$ NK-cells depending on being cultured in the presence of IL-15 alone or in combination with IL-21, respectively [35, 36]. However, more recent data would favor a possible maturation relationship between these NK-cell subsets and suggest that $CD56^{+low}$ NK-cells originate from $CD56^{+high}$ NK-cells [37–42].

Chemokines are small proteins that control a number of biological activities, including cell development, differentiation, tissue distribution, and function [43]. They act by binding chemokine receptors (CKR), a family of seven-transmembrane proteins that are classified by structure according to the number and spacing of conserved cysteines into four major groups given the names CXCR, CCR, CX3CR, and XCR to which four groups of CK correspond: CXCL, CCL, CL, and CX3CL [44]. In addition, CXCL chemokines have been further subclassified into glutamic acid-leucine-arginine tripeptide (ELR) positive or negative, based on the presence or absence of the ELR motif N-terminal to the first cysteine. From a functional point of view, two distinct types of CK have been considered: inflammatory/inducible CK, which are regulated by proinflammatory stimuli and dictate migration to the inflamed tissues and homeostatic/constitutive CK, which are responsible for the homing of the immune cells to the lymphoid organs and tissues. Similarly, two distinct groups of CKR have been described: those that interact mainly with inflammatory/inducible CK and have overlapping specificities and those that are relatively specific for homeostatic/constitutive CK [43, 44].

To the best of our knowledge only a few studies analyzed in detail the CKR repertoire on $CD56^{+low}$ and $CD56^{+high}$ NK-cells and the results obtained were somewhat divergent [45, 46]. For instance, Campbell et al. have found that $CD56^+/CD16^+$ (primarily $CD56^{+low}$) NK-cells uniformly express high levels of CXCR1, CXCR4, and CX3CR1 and low levels of CXCR2 and CXCR3 but no CCR1–6, CCR9, CXCR5, and CXCR6; they also found that $CD56^+/CD16^-$ (primarily $CD56^{+high}$) NK-cells do express CXCR3, CXCR4, CCR5, and very low levels of CX3CR1, but no CXCR1, CXCR2, CXCR5, CCR1–4, 6, and 9 [45]. In contrast, Berahovich et al. observed that NK-cells are $CXCR1^+$, $CXCR3^+$, and $CXCR4^+$ and contain subsets expressing CCR1, CCR4, CCR5, CCR6, CCR9, CXCR5, and CXCR6 [46]; according to their work, with the exception of CCR4, these CKR are expressed at higher percentages by $CD56^{+high}$ NK-cells [46]. Additionally, both authors have found CCR7 to be restricted to $CD56^{+high}$ NK-cells, which has been proved to regulate its selective homing into the lymph nodes (LN) [47, 48], where these cells establish the link between innate and adaptive immunity [47, 48].

We have previously characterized the immunophenotype of blood $CD56^{+low}$ and $CD56^{+high}$ NK-cells [29]. In order to better understand the migration pathways and cell-interactions of these NK-cell subsets and to establish

the normal reference patterns for the study of the NK-cell lymphoproliferative disorders, we decided to investigate the expression of a number of CKR on these NK-cell subsets. At some point in our study, we found that blood $CD56^+$ NK-cells include a minor population of $CXCR3/CCR5^+$ NK-cells whose levels of CD56 expression are intermediate between those observed on $CD56^{+low}$ and $CD56^{+high}$ NK-cells, most of which are $CD16^+$. These cells, from now on referred to as $CD56^{+\,int}$ NK-cells, fail to display CD57 and KIR, and they have intermediate levels of CD62L, CD94, and CD122 expression. Based on the results presented herein and on the published data, we discuss the migration routes of the conventional $CD56^{+high}$ and $CD56^{+low}$ NK-cells and their relevance for the success of the immune response and hypothesize that $CD56^{+\,int}$ NK-cells probably represent a transitional NK-cell state.

2. Material and Methods

2.1. Subjects. We first analyzed by flow cytometry the expression of a number of CKR on the $CD56^+$ NK-cells in the PB of 15 adult healthy individuals (blood donors), 9 males and 6 females, aged from 19 to 54 years (median age of 38 years). After suspecting the existence of a subpopulation of $CD56^{+\,int}$ NK-cells, these cells were further characterized using another group of 13 adult healthy individuals (blood donors), 8 males and 5 females, aged from 20 to 64 years (median age of 40 years).

2.2. Ethical Statement. This study was approved by the Ethical Committee as part of a research project aimed to characterize the CKR on normal and neoplastic T cells and NK-cells in order to better understand the biology of the T cell and NK-cell lymphoproliferative disorders. All individuals gave informed consent to participate in the study.

2.3. Flow Cytometry Studies. Immunophenotyping was performed using a whole blood stain-lyse-and-then-wash direct immunofluorescence technique using FACS lysing solution (Becton Dickinson, San José, CA) (BD) for erythrocyte lysis and cell fixation and four-color stainings with monoclonal antibodies (mAbs) conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-Cyanine 5 (PC5) or peridinin chlorophyll protein (PerCP), and allophycocyanin (APC). These were purchased to BD, Pharmingen (PH; San Diego, CA), Beckman Coulter (BC; Miami, FL), Immunotech (IOT; Marseille, France), and CLB (Amsterdam, Netherlands). Appropriate fluorochrome-conjugated isotype matched mAbs were used as negative controls.

In order to characterize the CKR expression on the conventional $CD56^{+low}$ and $CD56^{+high}$ NK-cell subsets, APC-conjugated anti-CD3 (BD; mouse IgG1,κ; clone SK7), PC5-conjugated anti-CD56 (IOT; mouse IgG1,κ; clone N901/NKH-1), and FITC-conjugated anti-CD16 (IOT; mouse IgG1,κ; clone 3G8) mAbs were used in combination with PE-conjugated mAbs directed against the following CKR (PH): CXCR1 (CD181) (mouse IgG2b,κ; clone 5A12), CXCR2 (CD182) (mouse IgG1,κ; clone 6C6), CXCR3 (CD183) (mouse IgG1,κ; clone 1C6), CCR4 (CD194) (mouse IgG1,κ; clone 1G1), CCR5 (CD195) (mouse IgG2a,κ; clone 2D7/CCR5), and CCR6 (CD196) (mouse IgG1,κ; clone 11A9).

Subsequently, $CD56^{+\,int}$ NK-cells (which, in most of the normal PB samples, cannot be distinguished from the conventional $CD56^{+low}$ or $CD56^{+high}$ NK-cells using the staining protocol mentioned above) were further characterized using APC-conjugated anti-CD3, PC5-conjugated anti-CD56, PE-conjugated anti-CXCR3 + PE-conjugated anti-CCR5, and one of the following FITC-conjugated mAbs directed against these molecules: anti-CD16 (IOT; mouse IgG1,κ; clone 3G8), anti-CD57 (BD; mouse IgM,κ; clone HNK-1), anti-CD62L (BD; mouse IgG2a,κ; clone SK11), anti-CD94 (PH; mouse IgG1,κ; clone HP-3D9), anti-CD122 (CLB; mouse IgG2a,κ; clone MIK-b1), and anti-CD158a (BD; mouse IgM,κ; clone HP-3E4).

Data acquisition was carried out in a FACSCalibur flow cytometer (BD) equipped with a 15 mW air-cooled 488 nm argon ion laser and a 625 nm neon diode laser, using the CellQUEST software (BD). Information on a minimum of 2×10^5 events was acquired and stored as FCS 2.0 data files for each staining. For data analysis the Paint-a-Gate PRO (BD) and the Infinicyt (Cytognos, Salamanca, Spain) software programs were used.

Using the first staining protocol, NK-cells were first gated based on their $CD3^-/CD56^+$ phenotype; then, the conventional $CD56^{+low}$ and $CD56^{+high}$ NK-cell subsets were selected based on their levels of CD56 expression and on their differential positivity for CD16 and separately analyzed for the expression of CXCR1, CXCR2, CXCR3, CCR4, CCR5, and CCR6. Using the second staining protocol, in which the anti-CXCR3 and CCR5 mAbs used have the same fluorochrome, we were able to distinguish three populations of $CD56^+$ NK-cells: $CD56^{+low}$ $CXCR3/CCR5^-$, $CD56^{+\,int}$ $CXCR3/CR5^+$, and $CD56^{+high}$ $CXCR3/CR5^+$. These were separately analyzed for the expression of CD16, CD56, CD57, CD62L, CD94, CD158a, and CD122.

The percentage of positive cells, the mean fluorescence intensity (MFI, expressed as arbitrary relative linear units scaled from 0 to 10,000), and the coefficient of variation of the MFI (CV, expressed as percentage) were recorded for each molecule tested.

2.4. Statistical Analysis. For all quantitative variables under study, mean, standard deviation, median, and range values were calculated. The statistical significance of the differences observed between groups was evaluated using the Mann-Whitney U-test (SPSS 10.0, SPSS, Chicago, IL, USA). P values less than 0.05 were considered to be associated with statistical significance.

3. Results

3.1. Chemokine Receptors on Blood $CD56^{+low}$ and $CD56^{+high}$ NK-Cells. Conventional $CD56^{+low}$ and $CD56^{+high}$ NK-cells present in the normal PB have different CKR repertoires (Figure 1 and Table 1).

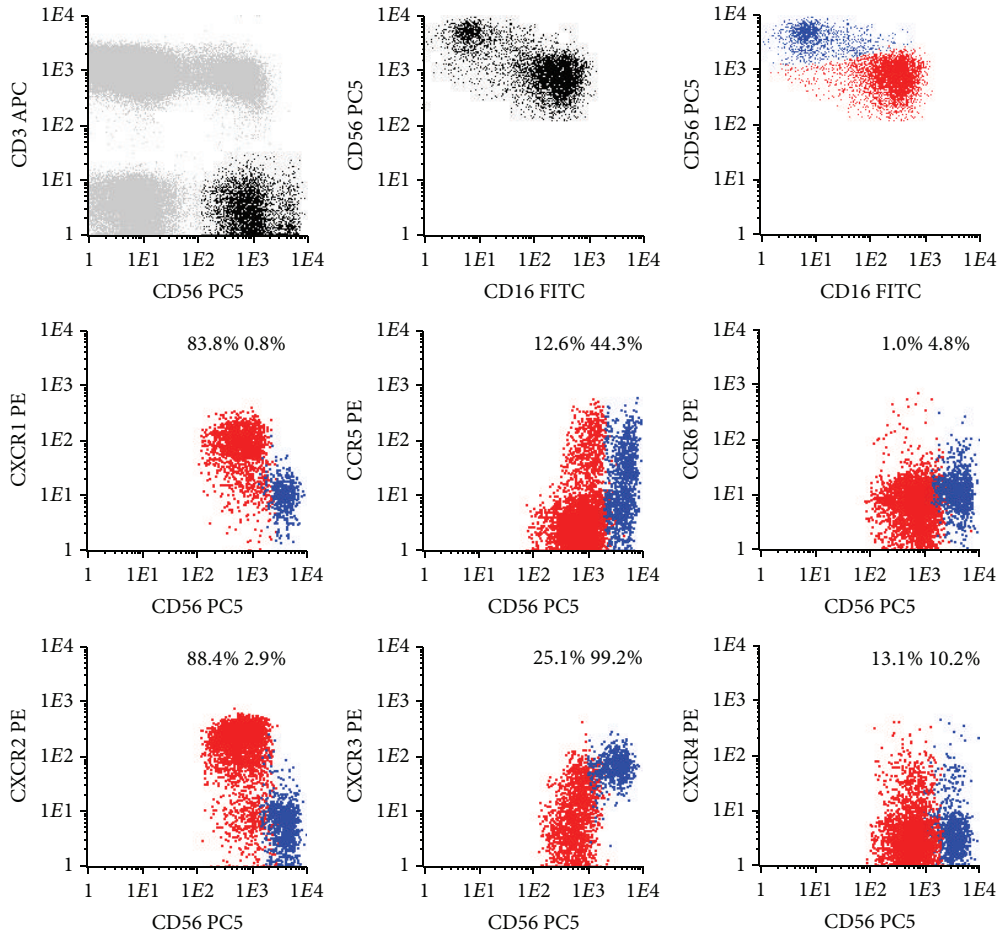


Figure 1: Representative dot plots illustrating the expression of different chemokine receptors (CKR) on the conventional $CD56^{+low}$ (red dots) and $CD56^{+high}$ (blue dots) NK-cell subsets present in the normal peripheral blood (PB). In order to obtain the dot plots showed in this figure, PB cells were stained with APC-conjugated anti-CD3, PC5-conjugated anti-CD56, PE-conjugated anti-CKR, and FITC-conjugated anti-CD16 monoclonal antibodies. Dot plots in the first row illustrate the strategy of gating. Using the CD3/CD56 dot plot, $CD56^{+}$ NK-cells were first identified based on their $CD3^{-}/CD56^{+}$ phenotype (black dots), comparatively to T ($CD3^{+}$) and B ($CD3^{-}CD56^{-}$) cells (gray dots). Then, after gating for $CD56^{+}$ NK-cells (first CD56/CD16 dot plot), the $CD56^{+low}$ (red dots) and $CD56^{+high}$ (blue dots) NK-cell populations were identified based on their typical patterns of CD56 and CD16 expression (second CD56/CD16 dot plot). Finally, these NK-cell populations were analyzed for the expression of the CKR (CKR/CD56 dot plots). The numbers above the $CD56^{+low}$ and $CD56^{+high}$ NK-cells inside the CKR/CD56 dot plots indicate the percentage of cells staining positively for the correspondent CKR and were obtained after gating separately for each NK-cell population (CKR/CD56 dot plots gated for $CD56^{+low}$ and $CD56^{+high}$ NK-cells are not shown, for simplicity).

3.1.1. Chemokine Receptors on Conventional $CD56^{+low}$ NK-Cells. Most $CD56^{+low}$ NK-cells are $CXCR1/CXCR2^{+}$; that is, the majority expresses high levels of CXCR1 (93.0 ± 4.5%) and CXCR2 (91.9 ± 3.4%), whose ligands are CXCL8 (IL-8) and other ELR motif containing chemokines involved in inflammation and angiogenesis [49] (Table 1 and Figure 1). In addition, these NK-cells are $CXCR3/CCR5^{-/+}$, which means that a variable proportion of them have low levels of CXCR3 and/or CCR5 (15.6 ± 11.1% and 13.3 ± 8.8%, resp.) (Table 1 and Figure 1). CXCR3 binds IFN-γ inducible cytokines, such as CXCL9 (monokine induced by gamma-interferon, MIG), CXCL10 (interferon-induced protein of 10 kD, IP-10), and CXCL11 (interferon-inducible T cell alpha chemoattractant, I-TAC) and mediates Ca^{++} mobilization and chemotaxis [50–52]. On the other hand, CCR5 has affinity to CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), and CCL8 (monocyte chemotactic protein-2, MCP-2) [53, 54].

Concerning the expression of constitutive/homeostatic CKR and the fraction of $CD56^{+low}$ NK-cells that expresses CXCR4, a CKR present on most hematopoietic cell types that binds to CXCL12 (stromal cell derived factor type 1, SDF-1) [55, 56] and has been shown to play a pivotal role in hematopoiesis [57] is variable (21.8 ± 8.7%) (Table 1 and Figure 1). In contrast, CCR4 is expressed in only a very small percentage of the $CD56^{+low}$ NK-cells (0.8 ± 0.4%) (Table 1 and Figure 1). This CKR has been reported to be a marker for

Table 1: Chemokine receptor expression on the well-known CD56^{+low} and CD56^{+high} NK-cells observed in blood, as identified based only on the levels of CD56 and CD16 expression.

		CD56^{+low} NK-cells		CD56^{+high} NK-cells		P values
CXCR1	% (+) cells	93.0 ± 4.5	(85.3–99.6)	4.0 ± 3.6	(0.0–12.1)	<0.001
	MFI	100.1 ± 19.4	(79.3–140.9)	7.0 ± 3.1	(2.0–14.5)	<0.001
	CV	67.1 ± 23.6	(51.5–145.3)	156.8 ± 76.2	(75.3–329.7)	<0.001
CXCR2	% (+) cells	91.9 ± 3.4	(86.1–97.0)	2.0 ± 1.4	(0.0–4.7)	<0.001
	MFI	165.7 ± 68.6	(66.9–264.1)	3.6 ± 1.5	(2.2–6.8)	<0.001
	CV	64.1 ± 8.7	(48.5–78.6)	218.1 ± 131.4	(82.7–568.9)	<0.001
CXCR3	% (+) cells	15.6 ± 11.1	(4.8–40.1)	97.0 ± 2.5	(92.5–99.6)	<0.001
	MFI	15.5 ± 10.7	(6.5–41.6)	94.5 ± 55.1	(48.7–231.1)	<0.001
	CV	209.8 ± 62.9	(145.1–403.4)	62.7 ± 8.1	(49.6–75.3)	<0.001
CXCR4	% (+) cells	21.8 ± 8.7	(8.4–43.5)	11.4 ± 4.6	(5.5–21.4)	<0.001
	MFI	11.8 ± 3.9	(5.3–20.9)	7.8 ± 3.1	(3.6–14.0)	n.s.
	CV	308.5 ± 89.8	(182.6–492.8)	318.6 ± 98.8	(184.4–581.1)	n.s.
CCR4	% (+) cells	0.8 ± 0.4	(0.2–1.5)	3.3 ± 2.9	(0.3–9.7)	<0.05
	MFI	2.2 ± 0.6	(1.3–3.0)	3.1 ± 1.1	(1.5–4.4)	n.s.
	CV	393.3 ± 195.1	(138.1–677.6)	118.7 ± 107.2	(59.1–417.3)	0.01
CCR5	% (+) cells	13.3 ± 8.8	(2.8–33.2)	50.0 ± 15.3	(24.8–78.5)	<0.001
	MFI	11.1 ± 6.0	(3.5–20.6)	27.8 ± 18.6	(7.7–79.9)	<0.01
	CV	353.5 ± 92.8	(194.8–492.5)	133.9 ± 28.1	(96.7–192.4)	<0.001
CCR6	% (+) cells	0.6 ± 0.4	(0.1–1.3)	0.9 ± 1.2	(0.0–3.0)	n.s.
	MFI	2.3 ± 0.7	(1.5–3.9)	2.1 ± 0.8	(1.4–4.0)	n.s.
	CV	214.7 ± 126.1	(61.4–432.8)	106.7 ± 73.5	(46.2–272.3)	n.s.

Data were obtained using the gating and analysis strategies described in Figure 1, where representative dot plots of these two conventional NK-cell subsets are presented.
Results are expressed as mean ± standard deviation (minimum–maximum) of the percentage of cells expressing each of the chemokine receptors analyzed within each CD56^{+} NK-cell population as well as mean ± standard deviation (minimum–maximum) of the mean fluorescence intensity (MFI) and coefficient of variation (CV) of expression.
n.s.: not statistically significant.

T helper 2 (Th2) lymphocytes [58] and promotes homing of memory T cells to inflamed skin [59] by means of interaction with CCL17 (thymus and activation-regulated chemokine, TARC) and CCL22 (macrophage-derived chemokine, MDC) [60, 61]. Similar results were obtained for CCR6, which is expressed in only 0.6 ± 0.4% of the CD56^{+low} NK-cells (Table 1). This CKR mediates responsiveness of memory T cells to CCL3 (MIP-1α) [62] and CCL20 (liver- and activation-regulated chemokine, LARC) [63] and has also been implicated in the homing of Langerhans' cells to the epidermis [64].

3.1.2. Chemokine Receptors on Conventional CD56^{+high} NK-Cells. In contrast to CD56^{+low} NK-cells, the majority of the CD56^{+high} NK-cells are CXCR1/CXCR2^{-} and CXCR3^{+}; that is, most of CD56^{+high} NK-cells express high levels of CXCR3 (96.9 ± 2.5%) whereas only a few are CXCR1^{+} (4.0 ± 3.6%) or CXCR2^{+} (2.0 ± 1.4%), and a large fraction of them (50.0 ± 15.3%) is CCR5^{+} (Table 1 and Figure 1).

Constitutive/homeostatic CKR are also present in CD56^{+high} NK-cells, with a variable fraction of them expressing CXCR4 (11.4 ± 4.6%) and only a few being CCR4^{+} (3.3 ± 2.9%) and CCR6^{+} (0.9 ± 1.2%, resp.) (Table 1 and Figure 1).

3.2. Identification of a New CD56^{+int} NK-Cell Population in the Peripheral Blood. When analyzing the conventional CD56^{+} NK-cell subsets, we observed that the percentage of CD56^{+low} NK-cells staining for CCR5 correlated positively with the percentage of CD56^{+low} NK-cells staining for CXCR3 ($r = 0.656$; $P = 0.01$). In addition, we found that CCR5^{+} and CXCR^{+} CD56^{+low} NK-cells had higher levels of CD56 and lower levels of CD16, as compared to CCR5^{-} ($P = 0.001$ and $P = 0.05$, resp.) and CXCR3^{-} ($P = 0.003$ and $P = 0.002$, resp.) CD56^{+low} counterparts. These observations allow us to investigate if CD56^{+low} cells expressing CCR5 and/or CXCR3 could represent a specific stage in NK-cell differentiation. In accordance, using another staining protocol in which anti-CXCR3 and anti-CCR5 mAbs had the same fluorochrome, we were able to identify three NK-cell populations in the normal PB, based on the expression of CD56, CD16, and the chemokine receptors CXCR3 and/or CCR5 (Figure 2): CD56^{+low} CD16^{+} CCR5/CXCR3^{-} (or simply CD56^{+low}), CD56$^{+\,int}$ CD16$^{+/-}$ CCR5/CXCR3^{+} (or simply CD56$^{+\,int}$), and CD56^{+high} CD16$^{-/+low}$ CCR5/CXCR3^{+} (or simply CD56^{+high}) NK-cells.

In the normal PB, CD56^{+low} NK-cells correspond to the majority (mean of 90 ± 4%) of CD56^{+} NK-cells, whereas the

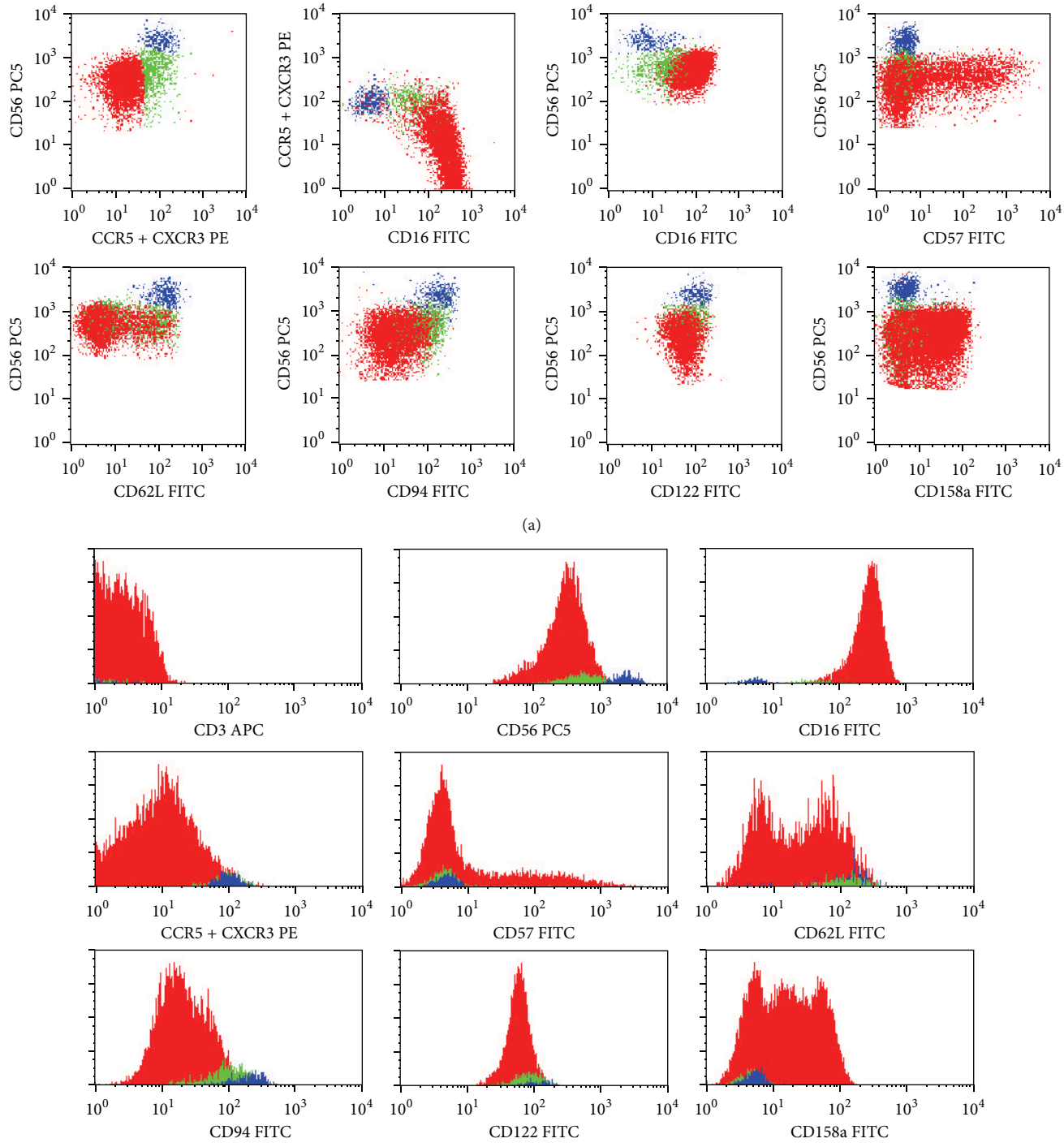


Figure 2: Representative dot plots (a) and histograms (b) illustrating the expression of the CD3, CD16, CD56, CD57, CD62L, CD94, CD122, and CD158a molecules on $CD56^{+low}$ $CXCR3/CCR5^{-}$ (red dots), $CD56^{+int}$ $CXCR3/CCR5^{+}$ (green dots), and $CD56^{+high}$ $CXCR3/CCR5^{+}$ (blue dots) NK-cells in normal peripheral blood (PB). In order to obtain the dot plots showed in this figure, PB cells were stained with APC-conjugated anti-CD3, PC5-conjugated anti-CD56, PE-conjugated anti-CXCR3 + PE-conjugated anti-CCR5, and FITC-conjugated monoclonal antibodies against CD16, CD57, CD62L, CD94, CD122, or CD158a molecules. Total $CD56^{+}$ cells were gated using the strategy illustrated in Figure 1. Then, using the CD56/CCR5 + CXCR3 dot plot (first dot plot), three different $CD56^{+}$ NK-cell populations were identified based on the levels of expression of CD56 and CXCR3/CCR5: $CD56^{+low}$ $CCR5/CXCR3^{-}$ (red dots), $CD56^{+int}$ $CCR5/CXCR3^{+}$ (green dots), and $CD56^{+high}$ $CCR5/CXCR3^{+}$ (blue dots). As it can be seen in the remaining dot plots and histograms, these NK-cell populations differ on the expression of several cell surface molecules. The percentage of cells staining positively for each molecule analyzed, as well as the mean fluorescence intensity of antigen expression and its coefficient of variation, was calculated after gating separately for each NK-cell population and is shown in Table 1 (data is not shown in the figure, for simplicity).

$CD56^{+\,int}$ and $CD56^{+high}$ NK-cells are minimally represented (mean of 6 ± 4% and 4 ± 2%, resp.) (Table 2).

Despite representing a minor NK-cell population in most normal PB samples, $CD56^{+\,int}$ NK-cells are largely expanded in some patients with chronic lymphoproliferative disorders of NK-cells (CLPD-NK) (Figure 3).

No differences were observed between these three NK-cell subsets concerning both the cell size and complexity, as evaluated by the forward (FSC) and side light scatter (SSC), respectively, except for a slightly larger size of $CD56^{+high}$ NK-cells (Table 2). Nonetheless, statistically significant differences were found concerning the expression of CD56 and CD16 (Figure 2 and Table 3) as well as of the other adhesion molecules and homing, cytokine, and killer cell receptors analyzed (Figure 2 and Table 4).

3.2.1. Phenotypic Characterization of Blood $CD56^{+int}$ NK-Cells. $CD56^{+\,int}$ NK-cells do express CD56 at levels that are intermediate between those observed on $CD56^{+low}$ and $CD56^{+high}$ NK-cells (MFI of 615 ± 149, 466 ± 108, and 2926 ± 578, resp.) (Figure 2 and Table 3). They also have intermediate percentages of $CD16^{+}$ cells (64.6 ± 23.6%), as compared to $CD56^{+low}$ and $CD56^{+high}$ NK-cells (99.9 ± 0.1% and 28.7 ± 9.9%, resp.). In addition, the levels of CD16 expression (MFI of 84 ± 55) were in between those observed on $CD56^{+low}$ and $CD56^{+high}$ NK-cells (MFI of 226 ± 107 and 47 ± 18, resp.).

These three $CD56^{+}$ NK-cell subsets also differ in the expression of other molecules (Figure 2 and Table 4).

Concerning the KLR, only a fraction of $CD56^{+low}$ (47.8 ± 13.7%) expresses dimly CD94, whereas nearly all $CD56^{+\,int}$ (91.4 ± 6.0%) and $CD56^{+high}$ (98.3 ± 1.5%) are $CD94^{+}$ (Figure 2 and Table 4). Curiously, the levels of CD94 expression on $CD56^{+\,int}$ NK-cells are in between those observed on $CD56^{+low}$ and $CD56^{+high}$ NK-cells (MFI of 129 ± 34, 71 ± 18, and 228 ± 34, resp.).

With respect to the expression of KIR, an opposite pattern is observed. Indeed, a variable fraction of $CD56^{+low}$ NK-cells is $CD158a^{+}$ (38.9 ± 30.0%), in contrast to that found in $CD56^{+\,int}$ and $CD56^{+high}$ NK-cells, which are basically $CD158a^{-}$ (mean percentage of $CD158a^{-}$ cells of 9.9 ± 9.0% and 4.1 ± 4.9%, resp.) (Figure 2 and Table 4).

Regarding cell adhesion molecules, the percentage of $CD62L^{+}$ cells is significantly lower among $CD56^{+low}$ (35.4 ± 20.4%), as compared to $CD56^{+high}$ NK-cells (97.3 ± 2.4%), intermediate values being observed in the $CD56^{+\,int}$ NK-cells (77.3 ± 19.0%). Similar results were obtained for the levels of CD62L expression (MFI of 49 ± 9, 119 ± 21, and 139 ± 30, resp.). In addition, a large fraction of $CD56^{+low}$ NK-cells (66.3 ± 15.6%) expresses variably and heterogeneously the CD57 oligosaccharide, whereas most $CD56^{+\,int}$ NK-cells fail to express this molecule and $CD56^{+high}$ NK-cells are virtually $CD57^{-}$ (mean % of $CD57^{+}$ cells of 15.3±13.7% and 1.3±1.4%, resp.). Once again, the levels of CD57 expression on $CD56^{+\,int}$ NK-cells (MFI of 355 ± 166) were in between those observed on $CD56^{+low}$ (MFI of 700±436) and $CD56^{+high}$ NK-cells (MFI of 165 ± 219).

The low affinity receptor for IL-2 and CD122, which is present in virtually all NK-cells, also exhibit intermediate levels on $CD56^{+\,int}$ cells (MFI of 77 ± 20), as compared to $CD56^{+low}$ (MFI of 46±11) and to $CD56^{+high}$ (MFI of 117±32) NK-cells (Figure 2 and Table 4).

4. Discussion

In the present study we show that $CD56^{+low}$ and $CD56^{+high}$ NK-cells that circulate in the normal blood have typical and quite different patterns of expression of receptors for inflammatory chemokines. At the same time, we identify and describe a subpopulation of $CD56^{+\,int}$ NK-cells that could represent a transitional stage in between the conventional NK-cell subsets referred to above, based on their intermediate levels of CD56 and CD16 expression and on their patterns of chemokine (CXCR3, CCR5), cytokine (CD122), and killer cell (CD94, CD158a) receptors and adhesion molecules (CD62L, CD57).

Differences on the CKR repertoires make the NK-cell subsets naturally able to circulate in the blood, to home into secondary lymphoid organs, or to migrate into inflamed tissues, in different circumstances and with different partners (Figure 4), in response to constitutive and inflammatory chemokines (Table 5).

In accordance, the majority of $CD56^{+high}$ NK-cells are $CXCR3/CCR5^{+}$, a pattern of CKR expression that is typically observed in Th1 cells [65], while $CD56^{+low}$ NK-cells do express CXCR1 and CXCR2, the only CKR specific for the ELR^{+} CXCL chemokines involved in inflammation, thus mimicking neutrophils [66, 67]. In addition, both NK-cell subsets have variable levels of CXCR4 and virtually no CCR4 and CCR6 expression.

$CD56^{+high}$ NK-cells and Th1 cells, the primary cell populations responsible for IL-2, IFN-γ, and TNF-α production in response to IL-2 or certain monokines, such as IL-12 and IL-15, are attracted together to chronically inflamed tissues in response to CCR5 (MIP-1α, MIP-1β, RANTES, and MCP-2) and CXCR3 (MIG, IP-10, and I-TAC) chemokine ligands, where they orchestrate the adaptive immune response. Some of these CK, such as RANTES and MIP-1α, also attract proinflammatory $CD14^{+low}$ $CD16^{+}$ monocytes, by acting as ligands for CCR1 and CCR4, as well as for CCR5 [68].

In agreement, $CD56^{+high}$ NK-cells accumulate within Th1-type chronic inflammatory lesions in a wide variety of pathological conditions such as rheumatoid arthritis [69], psoriasis [70], sarcoidosis [71], and allograft rejection [72] as well as in sites of intracellular bacterial infections [73], chronic viral infections [74], and tumors [75]. Inside the inflamed tissues and imbibed in the appropriate monokine environment, $CD56^{+high}$ NK-cells are able to engage with monocytes in a reciprocal fashion [76], thereby amplifying the inflammatory response and having important antitumor and antiviral effects. In the LN, they can induce the maturation of DC via IFN-γ and TNF-α release and/or cell-cell contact-dependent mechanisms [2, 3], in that way shaping the subsequent immune response. Moreover, activated NK-cells can kill immature myeloid DC, which have insufficient

Table 2: Relative representation and light scatter properties of blood $CD56^{+low}$ CCR5/$CXCR3^{-}$, $CD56^{+int}$ CCR5/$CXCR3^{+}$, and $CD56^{+high}$ CCR5/$CXCR3^{+}$ NK-cell subsets.

	A		B		C		*P* values		
	$CD56^{+low}$	CCR5/$CXCR3^{-}$	$CD56^{+int}$	CCR5/$CXCR3^{+}$	$CD56^{+high}$	CCR5/$CXCR3^{+}$	A versus B	B versus C	A versus C
% $CD56^{+}$ NK-cells	90.3 ± 3.9	(83.4–98.0)	6.1 ± 4.0	(1.2–14.6)	3.7 ± 2.3	(0.9–8.5)	—	—	—
FSC	299 ± 9	(285–315)	303 ± 10	(284–320)	311 ± 11	(291–324)	n.s.	0.044	0.041
tSSC	151 ± 7	(140–163)	156 ± 7	(141–170)	159 ± 5	(147–168)	n.s.	n.s.	n.s.

Data were obtained using the gating and analysis strategies described in Figure 2, where representative dot plots of these three NK-cell subsets are presented.
Results are expressed as mean ± standard deviation (minimum–maximum) of the percentage of each NK-cell subset within total $CD56^{+}$ NK-cells and as mean ± standard deviation (minimum–maximum) of the transformed side scatter (tSSC) and forward scatter (FSC) channel of each NK-cell subset.
n.s.: not statistically significant.

Table 3: CD56 and CD16 expression on blood $CD56^{+low}$ CCR5/$CXCR3^{-}$, $CD56^{+int}$ CCR5/$CXCR3^{+}$, and $CD56^{+high}$ CCR5/$CXCR3^{+}$ NK-cell subsets.

		A		B		C		*P* values		
		$CD56^{+low}$ CCR5/$CXCR3^{-}$		$CD56^{+int}$ CCR5/$CXCR3^{+}$		$CD56^{+high}$ CCR5/$CXCR3^{+}$		A versus B	B versus C	A versus C
CD56	% (+) cells	100.0 ± 0.0	(100.0-100.0)	100.0 ± 0.0	(100.0-100.0)	100.0 ± 0.0	(100.0-100.0)	—	—	—
	MFI	466 ± 108	(303–623)	615 ± 149	(416–999)	2926 ± 578	(2161–3993)	0.022	<0.001	<0.001
	CV	57 ± 7	(41–66)	72 ± 11	(53–93)	37 ± 5	(27–48)	0.001	<0.001	<0.001
CD16	% (+) cells	99.9 ± 0.1	(99.9–100.0)	64.6 ± 23.6	(26.8–96.2)	28.7 ± 9.9	(9.9–43.5)	<0.001	<0.001	<0.001
	MFI	226 ± 107	(55–372)	84 ± 55	(25–201)	47 ± 18	(21–47)	0.001	0.026	<0.001
	CV	45 ± 5	(38–55)	79 ± 21	(45–122)	81 ± 21	(44–118)	<0.001	n.s.	<0.001

Data were obtained using the gating and analysis strategies described in Figure 2, where representative dot plots of these three NK-cell subsets are presented.
Results are expressed as mean ± standard deviation (minimum–maximum) of the percentage of positive (+) cells within each $CD56^{+}$ NK-cell population and of the mean fluorescence intensity (MFI) and coefficient of variation (CV) of CD16 and CD56 expression.
n.s.: not statistically significant.

Table 4: CD57, CD62L, CD94, CD122, and CD158a expression on peripheral blood $CD56^{+low}$ CCR5/$CXCR3^{-}$, $CD56^{+int}$ CCR5/$CXCR3^{+}$, and $CD56^{+high}$ CCR5/$CXCR3^{+}$ NK-cell subsets.

		A		B		C		*P* values		
		$CD56^{+low}$	CCR5/$CXCR3^{-}$	$CD56^{+int}$	CCR5/$CXCR3^{+}$	$CD56^{+high}$	CCR5/$CXCR3^{+}$	A versus B	B versus C	A versus C
CD57	% (+) cells	66.3 ± 15.6	(36.7–87.4)	15.3 ± 13.7	(1.7–42.7)	1.3 ± 1.4	(0.0–4.3)	<0.001	<0.001	<0.001
	MFI	700 ± 436	(217–1557)	355 ± 166	(150–703)	165 ± 219	(9–574)	0.018	0.015	<0.001
	CV	114 ± 34	(77–194)	130 ± 35	(80–205)	85 ± 54	(6–155)	n.s.	n.s.	n.s.
CD62L	% (+) cells	35.4 ± 20.4	(9.6–73.5)	77.3 ± 19.0	(41.9–95.7)	97.3 ± 2.4	(92.8–100.0)	<0.001	<0.001	<0.001
	MFI	49 ± 9	(38–65)	119 ± 21	(77–147)	139 ± 30	(91–172)	<0.001	0.057	<0.001
	CV	83 ± 11	(72–109)	56 ± 7	(46–71)	47 ± 9	(35–69)	<0.001	0.010	<0.001
CD94	% (+) cells	47.8 ± 13.7	(34.1–74.1)	91.4 ± 6.0	(79.2–98.4)	98.3 ± 1.5	(94.5–100.0)	<0.001	<0.001	<0.001
	MFI	71 ± 18	(50–106)	129 ± 34	(77–211)	228 ± 34	(175–286)	<0.001	<0.001	<0.001
	CV	56 ± 7	(44–69)	56 ± 11	(41–85)	47 ± 8	(31–56)	n.s.	0.039	0.039
CD122	% (+) cells	100.0 ± 0.0	(100.0-100.0)	100.0 ± 0.0	(100.0-100.0)	100.0 ± 0.0	(100.0-100.0)	n.s.	n.s.	n.s.
	MFI	46 ± 11	(31–63)	77 ± 20	(49–107)	117 ± 32	(79–182)	<0.001	0.001	<0.001
	CV	50 ± 12	(35–72)	56 ± 9	(43–79)	46 ± 5	(34–54)	n.s.	<0.001	<0.001
CD158a	% (+) cells	38.9 ± 30.0	(7.9–92.9)	9.9 ± 9.0	(0.7–30.0)	4.1 ± 4.9	(0.1–19.3)	0.001	0.030	<0.001
	MFI	30 ± 8	(18–42)	35 ± 11	(22–57)	41 ± 13	(20–68)	n.s.	n.s.	n.s.
	CV	61 ± 21	(27–98)	63 ± 20	(31–96)	69 ± 29	(35–126)	n.s.	n.s.	n.s.

Data were obtained using the gating and analysis strategies described in Figure 2, where representative dot plots of these three NK-cell subsets are presented. Results are expressed as mean ± standard deviation (minimum–maximum) of the percentage of positive (+) cells within each $CD56^{+}$ NK-cell population, and as the mean fluorescence intensity (MFI) and coefficient of variation (CV) of CD57, CD62L, CD94, CD122, and CD158a expression in cells that stained positively for these antigens.
n.s.: not statistically significant.

amounts of MHC molecules to activate T cells properly [2, 3]. In addition, $CD56^{+high}$ NK-cells also predominate in placenta [77], where they are involved in maternal-fetal tolerance [78, 79].

In contrast, $CD56^{+low}$ NK-cells, which are essentially cytotoxic, and neutrophils, which are phagocytic cells by excellence, predominate in the PB and are equipped with the CXCR1 and CXCR2 chemokine receptors, making them able to comigrate into sites of acute inflammation in response to IL-8 and other ELR motif containing chemokines and to participate in the earliest phase of the innate immune response. As for the neutrophils, migration of $CD56^{+low}$ NK-cells to inflamed tissues also depends on the interaction of different forms of PSGL-1 expressed on their membrane with the selectin molecules expressed on endothelial cells [31]. Curiously, cytotoxic T lymphocytes (CTL) have also been reported to express CXCR1 [80] and PSGL-1 [81].

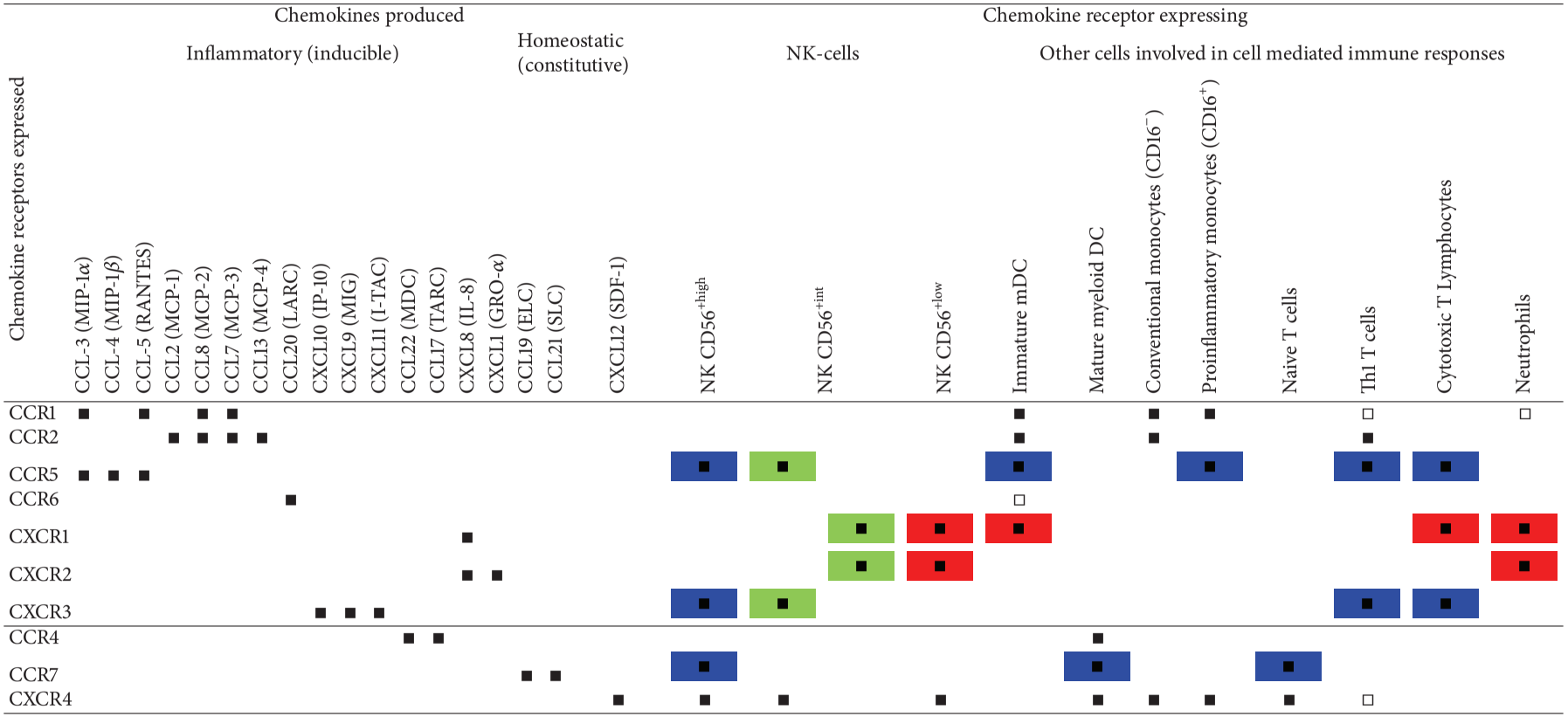
Normally, both neutrophils, which are able to neutralize efficiently the extracellular pathogens, after opsonization by antibodies, using Fc receptors for IgG and IgA and $CD56^{+low}$ NK-cells, which mediate antibody dependent cell cytotoxicity via FcγRIIIa (CD16), circulate in the blood. In that sense, it can be hypothesized that these cells are candidates to establish a bridge between the innate immune response and the antibody mediated adaptive immune response. Evidence is being accumulated in the last years for a cross-talk between neutrophils and NK-cells [6, 7]. For instance, NK-cells promote neutrophil recruitment to the inflamed tissues and several NK-cell derived cytokines and growth factors, such as GM-CSF, IFN-γ, and TNF-α, act by enhancing neutrophil survival and by modulating cell surface expression of complement and Fc receptors in neutrophils [82–84]. On the other hand, neutrophils can stimulate the production of IFN-γ by NK-cells [84].

The other anatomical sites in which $CD56^{+low}$ NK-cells and neutrophils might be concomitantly present to modulate each other's activity and its contribution to disease are not completely elucidated. Normal liver contains mainly $CD56^{+low}$ NK-cells, but these cells are different from the $CD56^{+low}$ NK-cells that circulate in the blood [85]. In addition, $CD56^{+low}$ NK-cells also infiltrate the liver of patients with primary biliary cirrhosis, an antibody mediated autoimmune disease, following the CXCR1/IL-8 axis [85]; curiously, hepatic infiltration by neutrophils is also found in these patients [86]. Moreover, $CD56^{+low}$ NK-cells and neutrophils colocalize in the skin of patients with Sweet's syndrome, an acute febrile neutrophilic dermatosis that can follow viral infections, autoimmune diseases, and hematologic malignancies [84].

Also of note, in this study we confirm previous observations about the lack of expression on both $CD56^{+low}$ and $CD56^{+high}$ NK-cells of other CKR involved in homing to nonlymphoid organs and tissues including CCR4 (skin and lung), CCR6 (intestine and liver), CCR9 (small intestine), and CCR10 (skin) [45, 87]. This suggests that, unlike memory/effector T cells, $CD56^{+}$ NK-cells may not be divided into cutaneous versus mucosal/intestinal-homing compartments, based on CKR expression [58, 88, 89].

Of special interest is also the identification of a new population of NK-cells expressing intermediate levels of CD56 that

Table 5: Homeostatic and inflammatory chemokines (CK) and chemokine receptors (CKR) involved in cell mediated immune responses and their relevance for colocalization of the NK-cell subsets with other cells of the innate-dendritic cells (DC), monocytes, neutrophils, and adaptive (T cells) immune system. Colocalization of $CD56^{+high}$, $CD56^{+int}$, and $CD56^{+high}$ NK-cells with other immune cells is signaled in blue, green, and red, respectively. Black squares inside the cells indicate positive interactions of the CK and the CKR as well as the CKR expressing cells. $CD56^{+int}$ NK-cells are transitional NK-cells, whose properties are intermediate between those of $CD56^{+high}$ and $CD56^{+low}$ NK-cells. $CD56^{+high}$ and $CD56^{+low}$ NK-cells are both able to produce cytokines and chemokines, upon stimulation with monokines and target cell recognition, respectively. The CK produced by NK-cells (e.g., MIP-1α, MIP-1β, and RANTES) not only attracted proinflammatory monocytes (via CCR1 and CCR5), immature myeloid DC (via CCR1 and CCR5), Th1 cells, and CTL (via CCR5) but also activated neutrophils (via CCR1) to the sites of inflammation. The cytokines produced by the NK-cells (e.g., IFN-γ, TNF-α) activate monocytes, DC, neutrophils, and Th1/CTL, thereby potentiating the cell mediated immune responses. For instance, activated monocytes/DC are able to produce IFN-γ inducible cytokines—CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (I-TAC), which recruit more CXCR3 expressing cells ($CD56^{+high}$ NK-cells, Th1 cells, and CTL). In addition, CXCL8 (IL-8) produced in inflamed tissues by locally resident activated cells, such as macrophages, epithelial cells, and endothelial cells, recruits neutrophils, $CD56^{+low}$ NK-cells, and CTL, via interaction with CXCL1 and CXCL2.



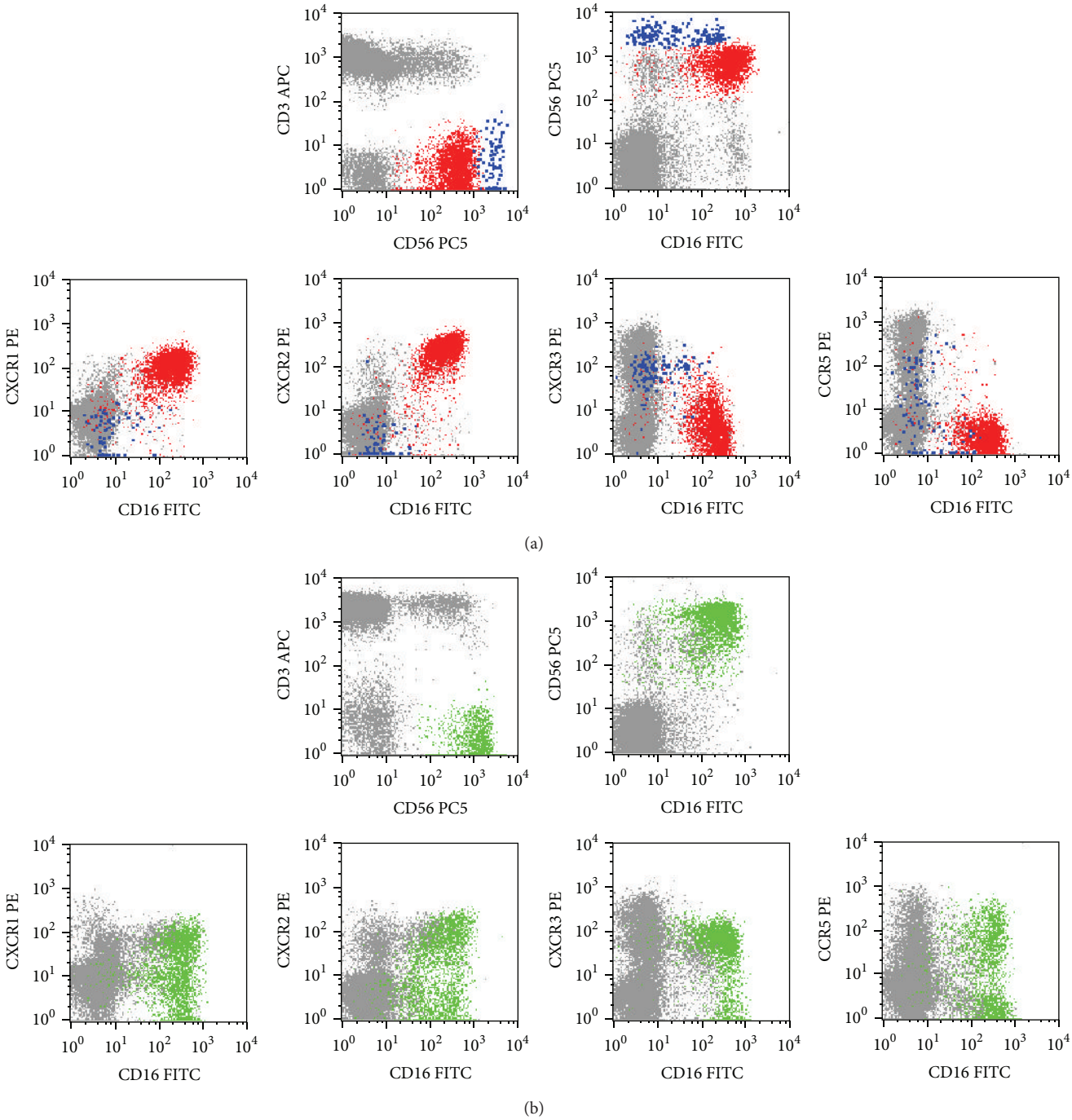


Figure 3: Illustrative dot plots showing the expression of the CXCR1, CXCR2, CXCR3, and CCR5 chemokine receptors (CKR) in normal peripheral blood (PB) (a), where only $CD56^{+low}$ (red dots) and $CD56^{+high}$ (blue dots) are observed, and in the PB of a patient with a chronic lymphoproliferative disorder of NK-cells (CLPD-NK) (b), exhibiting a transitional $CD56^{+int}$ phenotype (green dots); other lymphocytes are shown in gray. In order to obtain the dot plots showed in this figure, the PB cells were stained with APC-conjugated anti-CD3, PC5-conjugated anti-CD56, PE-conjugated anti-CKR (CXCR1, CXCR2, CXCR3, or CCR5), and FITC-conjugated anti-CD16 monoclonal antibodies. As shown in (a), in the normal PB most $CD56^{+low}$ NK-cells are $CXCR1^{+}$ and $CXCR2^{+}$, whereas only a very small fraction of cells stains positively for CXCR3 and/or CCR5; in contrast, most $CD56^{+high}$ NK-cells are $CXCR3^{+}$ whereas CCR5 is expressed in only a fraction and CXCR1 and CXCR2 are virtually negative. As it can be seen in (b), the expanded NK-cells from this patient, which have relatively high levels of CD56 expression, are $CD16^{+}$ and they express the CXCR1, CXCR2, CXCR3, and CCR5 molecules in a considerable fraction of cells.

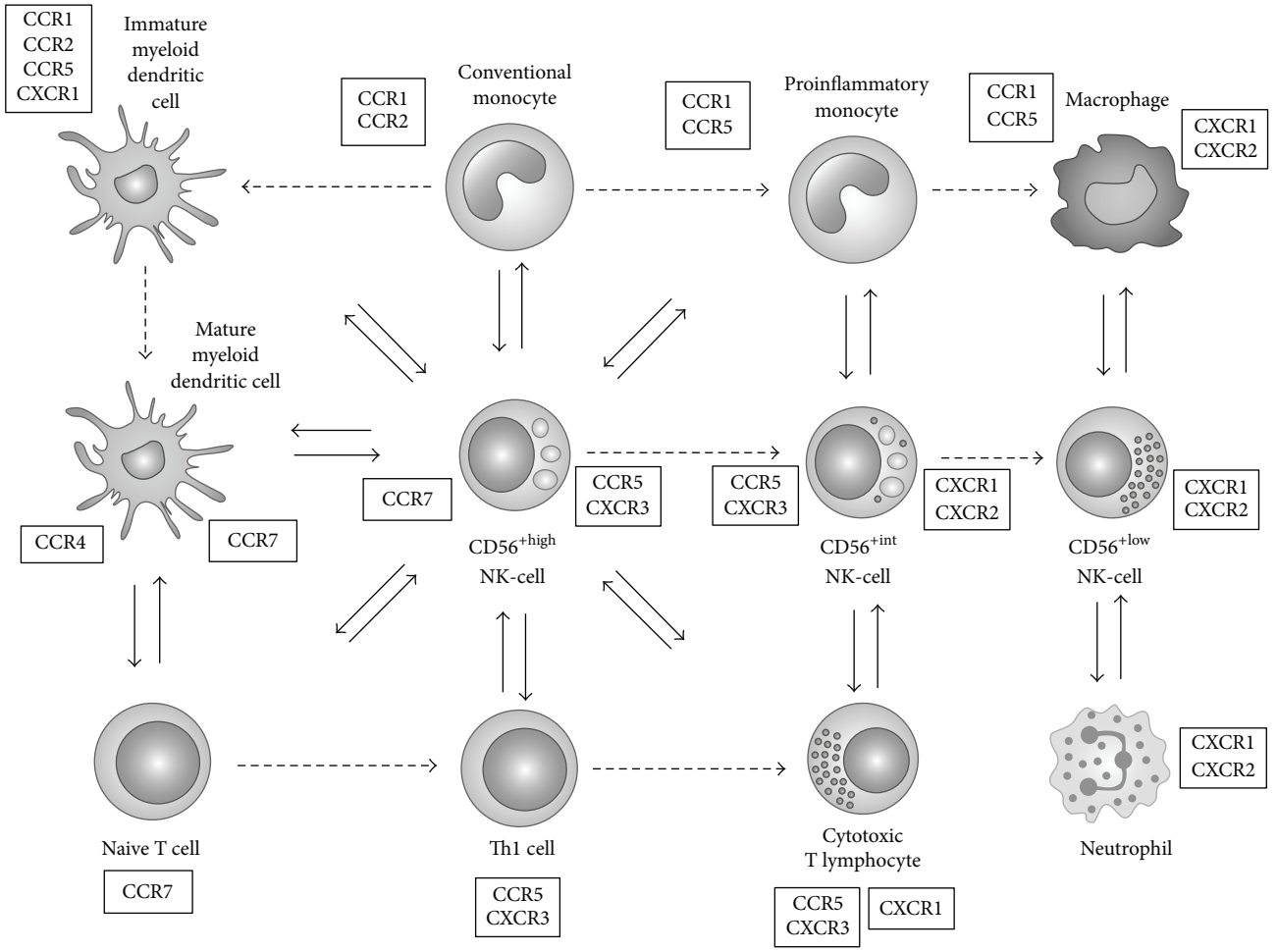


Figure 4: Diagram illustrating the complex relationship established between NK-cells and the other cells of the innate-dendritic cells (DC), monocytes, macrophages, neutrophils, and adaptive (T cells) immune system, whose homing to lymphoid organs and recruitment to inflamed tissues are mediated by the interaction of homeostatic chemokines constitutively expressed on locally resident cells and inflammatory chemokines, with the correspondent chemokine receptors. CCR7 expression on $CD56^{+high}$ NK-cells, mature DC, and naïve T cells allows these cells to migrate into the lymph nodes, in response to CCL19 (ELC) and CCL21 (SLC) produced locally. CXCR3/CCR5 expression on $CD56^{+high}$ NK-cells permits these cells to migrate into inflamed tissues, together with $CCR5^{+}$ proinflammatory monocytes, $CCR5/CXCR3^{+}$ Th1 cells, and $CCR5/CXCR3^{+}$ cytotoxic T lymphocytes (CTL). CXCR1/CXCR2 expression on $CD56^{+low}$ NK-cells, neutrophils, and CTL permits these cells to migrate into inflamed tissues in response to CXCL8 (IL-8), where they interact together and with activated macrophages. $CD56^{+int}$ NK-cells are transitional NK-cells, whose properties are intermediate between those of $CD56^{+high}$ and $CD56^{+low}$ NK-cells. Dashed arrows indicate the routes of differentiation. Full arrows indicate the cross-talk between cells mediated by cytokines and chemokines. CCR7 ligands: CCL-19 (ELC) and CCL21 (SLC); CCR5 ligands: CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), and CCL8 (MCP-2); CXCR3 ligands: CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (I-TAC); CXCL1 ligands: CXCL-8 (IL-8); CXCL2 ligands: CXCL-8 (IL-8) and other ELR motif containing CXCL chemokine; CXCR4 ligand: CXCL12 (SDF-1); CCR1 ligands: CCL3 (MIP-1α), CCL5 (RANTES), MCP-2, and MCP-3; CCR2 ligands: CCL2 (MCP-1), CCL8 (MCP-2), CCL7 (MCP-3), and CCL13 (MCP-4).

we designed as $CD56^{+int}$ NK-cells. Similar to $CD56^{+high}$ NK-cells, most of the $CD56^{+int}$ NK-cells are KIR^{-} and $CD57^{-}$; however, the majority of them display the CD16 molecule, a marker of $CD56^{+low}$ NK-cells, and they have intermediate levels of CD62L, CD94, and CD122 expression. Due to the fact that the identification of the NK-cell populations by flow cytometry is usually based only on CD56 and CD16 expression, $CD56^{+int}$ NK-cells are being considered together with $CD56^{+low}$ NK-cells on routine blood analysis.

The fact that $CD56^{+int}$ NK-cells have phenotypic features intermediate between those of conventional $CD56^{+low}$ and $CD56^{+high}$ NK-cells would suggest that they could represent a transitional NK-cell maturation stage.

In line with this hypothesis, evidence for the existence of transitional NK-cell populations with phenotypic features similar to those of the $CD56^{+int}$ NK-cells described herein has also been provided in other studies [90, 91]. In accordance, Yu et al. described a $CD56^{+low}$ $CD94^{+high}$ NK-cell subset expressing CD2, CD62L, CD56, KIR, granzymes, and perforin, producing IFN-γ in response to monokines, and exhibiting CD94-mediated redirected killing at levels intermediate between those observed in $CD56^{+low}$ $CD94^{+low}$

and $CD56^{+high}$ $CD94^{+high}$ NK-cells [90]. In addition, Juelke el al. reported on a $CD56^{+low}$ $CD62L^{+}$ NK-cell subset with the ability to produce IFN-γ and the capacity to kill [91]. Finally, when studying the differentiation of $CD56^{+high}$ CD94/$NKG2A^{+}$ into $CD56^{+low}$ CD94/$NKG2A^{-}$ NK-cells, Béziat et al. found a transitional $CD56^{+low}$ CD94/$NKG2A^{+}$ NK-cell subset, expressing intermediate levels of CD62L, granzyme-K, CD27, and CD57, among other molecules [92]. Given the immunophenotypic similarities, the $CD56^{+int}$ NK-cell population described herein, which comprises less than 10% of the $CD56^{+}$ NK-cells in the PB from normal healthy individuals, probably corresponds to a subpopulation of the $CD56^{+low}$ $CD94^{+high}$ NK-cell subset described by Yu et al., which accounts for half of the circulating $CD56^{+}$ NK-cells [90].

Another potential interest of identifying normal NK-cells with intermediate phenotypic features relies on data interpretation in clinical settings. For instance, overrepresentation of $CD56^{+int}$ NK-cells in the PB from patients with NK-cell lymphocytosis may erroneously be interpreted as phenotypically aberrant (and thus potentially neoplastic) NK-cells. Thus, the knowledge about the immunophenotype of the NK-cell populations that circulate in normal PB, as well as in the PB from patients with inflammatory and infectious conditions [93, 94], is essential to a better understanding of the phenotypic heterogeneity of the expanded NK-cell populations observed in patients with CLPD-NK [95], thereby contributing to distinguishing nonclonal from clonal NK-cell proliferations and reactive from neoplastic conditions [96, 97].

5. Conclusions

Differences in the CKR expression on $CD56^{+low}$ and $CD56^{+high}$ NK-cells may determine their ability to be recruited into inflamed tissues and colocalize with other cells at sites of inflammation, which is crucial for the success of the immune response. In addition, the phenotypic heterogeneity of the conventional $CD56^{+low}$ and $CD56^{+high}$ NK-cells may be largely due to the presence of transitional NK-cell populations, which may be preferentially expanded in some pathological conditions.

Further investigations in this area will help to better understand the terminal differentiation of the NK-cells and the maturation relationship between the NK-cell subsets, their circulation through the body, and their participation in the immune response. In addition, they will give an important contribution to establish phenotypic criteria to differentiate reactive and neoplastic NK-cell proliferations, as well as to better identify the normal cell counterparts from which the neoplastic NK-cells originate.

Abbreviations

ADCC: Antibody dependent cell cytotoxicity
APC: Allophycocyanin
BC: Beckman Coulter
BD: Becton Dickinson
BDB: Becton Dickinson Biosciences
BM: Bone marrow
CK: Chemokines
CKR: Chemokine receptors
CTL: Cytotoxic T lymphocytes
DC: Dendritic cells
ELR: Glutamic acid-leucine-arginine tripeptide motif
FcγRIIIa: Low affinity receptor for IgG Fc and alpha chain (CD16)
FCRL: Fc receptor-like
FITC: Fluorescein isothiocyanate
FSC: Forward light scatter
IFN-γ: Interferon-gamma
IL: Interleukin
ILT: Immunoglobulin-like transcript
IP-10: Interferon-induced protein of 10 kD (CXCL10)
I-TAC: Interferon-inducible T cell alpha chemoattractant (CXCL11)
KIR: Killer cell immunoglobulin-like receptors
KLR: Killer cell lectin type receptors
LARC: Liver- and activation-regulated chemokine (CCL20)
LN: Lymph nodes
MCP-2: Monocyte chemotactic protein-2 (CCL8)
MDC: Macrophage-derived chemokine (CCL22)
MHC: Major Histocompatibility Complex
MIG: Monokine induced by gamma-interferon (CXCL9)
MIP-1α: Macrophage inflammatory protein 1 alpha (CCL3)
MIP-1β: Macrophage inflammatory protein 1 beta (CCL4)
NCAM: Neural cell adhesion molecule (CD56)
NCR: Natural cytotoxicity receptors
NK: Natural killer
PB: Peripheral blood
PC5: PE-Cyanine 5
PE: Phycoerythrin
PH: Pharmingen
RANTES: Regulated upon activation, normal T cell expressed and secreted (CCL5)
SSC: Side light scatter
SDF-1: Stromal cell derived factor type 1 (CXCL12)
TARC: Thymus and activation-regulated chemokine (CCL17)
TGF-β: Transformed growth factor beta
Th: T helper
TNF-α: Tumor necrosis factor alpha.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Margarida Lima carried out concept and research question, literature review, study design, data collection and interpretation, statistical analysis, and report writing. Ana Helena Santos, Catarina Lau, João Moura, Magdalena Leander, Marlene Santos, Maria dos Anjos Teixeira, Maria Luís Queirós, Marta Gonçalves, and Sónia Fonseca were responsible for sample processing, data acquisition, and data analysis. Catarina Lau and Maria dos Anjos Teixeira were responsible for data analysis and interpretation. Alberto Orfao was responsible for scientific consulting, paper revision, and discussion. All authors read and approved the final paper.

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Review Article

Tolerogenic Dendritic Cells on Transplantation: Immunotherapy Based on Second Signal Blockage

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Dendritic cells (DCs), the most important professional antigen-presenting cells (APC), play crucial role in both immunity and tolerance. It is well known that DCs are able to mount immune responses against foreign antigens and simultaneously tolerate self-antigens. Since DCs can be modulated depending on the surrounding microenvironment, they can act as a bridge between innate and adaptive immunity. However, the mechanisms that support this dual role are not entirely clear. Recent studies have shown that DCs can be manipulated *ex vivo* in order to trigger their tolerogenic profile, what can be a tool to be used in clinical trials aiming the treatment of various diseases and the prevention of transplant rejection. In this sense, the blockage of costimulatory molecules on DC, in the attempt of inhibiting the second signal in the immunological synapse, can be considered as one of the main strategies under development. This review brings an update on current therapies using tolerogenic dendritic cells modulated with costimulatory blockers with the aim of reducing transplant rejection. However, although there are current clinical trials using tolerogenic DC to treat allograft rejection, the actual challenge is to modulate these cells in order to maintain a permanent tolerogenic profile.

1. Background

The main goal of a successful transplant is to promote immune tolerance of the transplanted organ or tissue, allowing the reestablishment of normal physiological functions, without generating damage to the recipient or to the transplanted tissue. The concept of tolerance in transplantation is understood as a state in which no pathological immune response is generated against the transplanted organ or tissue. This condition would make the graft viable while retaining the necessary immune responses against other unknown antigens [1, 2]. Thereby, the relationship between tolerance and immunity must be well balanced, since any alteration in one of the parts can cause pathophysiological modifications and, consequently, can trigger changes in the immune system that can ultimately lead to autoimmunity or graft rejection [3]. In this context, it is known that a successful transplant relies on a deep understanding of the immune system allied with the balance and maintenance of effector and regulatory immune mechanisms [1, 4].

However, even successful transplants can have severe long-term complications, which can culminate in allograft rejection. Several immunossupressor treatments have been developed in order to reduce transplant rejection. However, despite significant advances on immunosuppressive strategies, antirejection drugs still present serious side effects,

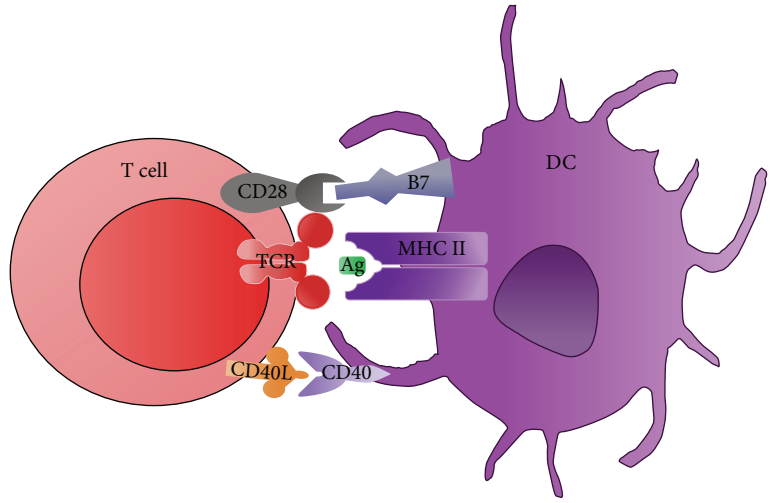

Figure 1: Schematic representation of the DC and T cell interaction: the main costimulatory molecules. Activation of T cell involves both interactions between the T cell costimulatory receptors, CD28 with their cognate ligands, CD80, and CD86 (B7 family) as well as the CD40L/CD40 pathway. Other costimulatory molecules, such as OX40/OX40-L and TIM-1 and PD-1/PD-L1, were not represented here. DC: dendritic cell; MHC II: major histocompatibility complex II; TCR: T cell receptor; CD40L: CD40 ligand.

such as high susceptibility of opportunistic infectious diseases, or even inefficient suppression of immune responses against the allograft. The knowledge acquisition about the immune regulation mechanisms, especially about the role of the antigen-presenting cells (APC) in tolerance, can help researchers propose new strategies and immunotherapies to prevent rejection [5].

Among the APC, dendritic cells (DCs) represent the first line of immune cell defense against pathogens and constitute a bridge between innate and adaptive immune response. As represented in Figure 1, DCs are the most important APC for naive T cells [5–8] and can exert either immunogenic or tolerogenic functions. Depending on the received signals, these cells can become tolerogenic, that is, can inhibit antigen-specific immune response [7, 9–13]. When TCR interacts with the peptide-MHC (pMHC) on the surface of the APC (first signal) and it is not followed by the interaction between costimulatory molecules (second signal), it can induce anergy on T cells [14]. Dendritic cells express important costimulators to T cell activation, such as the B7 family molecules: CD80 (B7-1) and CD86 (B7-2), playing an important role in either tolerogenic or immunogenic responses. Therefore, the handling of costimulatory molecules, aiming the application of DC for therapeutic purposes in immune disorders such as allergies and autoimmunities, as well as in vaccination and transplantation, has received extensive attention [15].

In this sense, in the attempt of modulating the activity of DC on the treatment of autoimmunity, hypersensibility, and transplant rejection, many researchers aim to develop therapies based on tolerogenic DC (tol-DC). Previous data has shown that DC modulated by interleukin- (IL-) 10 or transforming growth factor-beta (TGF-β) became refractory to sustain the *in vitro* proliferation of antigen-specific effector T lymphocytes [12, 13]. Additionally, adoptive transfer of DC, modulated by inhibitory cytokines such as IL-10, also leads to a reduction of *in vivo* delayed-type hypersensibility (DTH) responses [16].

Apart from DC, regulatory T (Treg) cells, particularly $CD4^+$ $CD25^+$ $Foxp3^+$ lymphocytes, play an important role in inducing and maintaining tolerance, promoted by cell to cell contact or by secreted cytokines such as IL-10 and TGF-β [17–19].

In this review, we focus our attention on current knowledge related to immunotherapeutic advances based on the use of tolerogenic DC through inhibition of the second signal, which contribute to increasing survival of transplanted organs and tissues and reducing the use of immunosuppressive drugs.

2. Innate Immune System on Graft Rejection

Even though the role of the adaptive immune system through cellular and humoral responses in transplant rejection is well known, many researchers have outlined the involvement of components of the innate immune system in the mechanisms of alloreactivity and rejection. Among these components, the most studied are the toll-like receptors (TLR), complement system, natural killer (NK) cells, DC, granulocytes, and inflammatory cytokines which perform different functions in innate immune responses [20–22].

In this regard, TLR links innate and adaptive immunity and its signaling leads to the transcription of genes involved in inflammatory responses resulting in the production of proinflammatory cytokines and chemokines, antimicrobial peptides, adhesion molecules, enhanced antigen presentation, and upregulation of costimulatory molecules in APC [20, 21]. Corroborating this fact, it was demonstrated in mouse models of graft versus host disease (GVHD) that elevated doses of radiation during pretransplant increase the epithelial damage of mucosal tissues, allowing bacterial components to pass through the barrier. These components

activate host and/or donor APC by interacting with pattern recognition receptors (PRR), such as TLR, converting these APC into an activated profile that is able to prime alloreactive donor-derived T cells, resulting in a more severe GVHD [23].

Currently, complement activation is known to occur in transplant rejection and contribute to progression of rejection. Specifically, activation of the lectin pathway of the complement system is implicated in allograft rejection. Also, the role of complement system in modulating regulatory T cells is under investigation [24–27]. Recent study suggests that C3a and C5a signaling promotes cell proliferation of activated T cells and reduces induced-Treg (iTreg) generation and stability [28].

Macrophages were considered critical components in both acute cellular allograft rejection and chronic injury [29]. However, it is already known that macrophages can play both detrimental and beneficial functions in allograft rejection. In initial stages of the transplant, the immune response can create a proinflammatory microenvironment that favors the differentiation of M1 macrophages (previously referred to as classically or alternatively activated macrophages). M1 phenotype is a proinflammatory cell type, characterized by secretion of proinflammatory cytokines, high phagocytic activity, and production of reactive oxygen species. As inflammation recedes, this may alter the milieu to favor M2 differentiation. M2 macrophages have an immunomodulatory role, since they produce IL-10, presenting reduced phagocytic activity and increased arginase production [30].

This hypothesis was supported by an assay with $CCR5^{-/-}$ mice that exhibited reduced macrophage accumulation after transplantation. In this model, M2 macrophage activation was increased while M1 macrophage activation was reduced after transplantation, in comparison with control mice [31]. However, a recent study with human biopsies showed controversial data, since a M2 macrophage infiltration was associated with inflammation, injury, and fibrosis in renal allograft [32, 33].

Additionally, NK cells are an important component of the innate immune system, helping to recognize allogeneic MHC and are capable of producing proinflammatory cytokines. NK cells can impair tolerance induction in a solid organ transplant and can also lead to acute and chronic rejection of allogeneic transplant [34–36]. It happens because NK cells can kill either directly donor cells through granzyme, perforin, Fas ligand (FasL), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) pathways [37–40] or indirectly by lysing Treg cells or by promoting $CD4^+$ T cell activation [36, 41, 42].

Controversially, NK and NKT cells have been known to be enrolled on allograft tolerance induction. This role of NK cells can be influenced by immunosuppressive therapies such as calcineurin inhibitors or steroids [35, 43, 44]. The suppressive role of NK cells has been observed on the allorecognition suppression of T cells by a perforin-dependent mechanism [45] and also by killing the donor antigen-presenting cells [46]. Also, NK cells can regulate macrophage activation in transplanted tissue or organ by a mechanism partially dependent on an activating receptor known as natural killer group 2, member D (NKG2D) [47].

Invariant NKT (iNKT) cells have regulatory functions on the Th1/Th2 imbalance by releasing Th2 cytokines such as IL-4 and IL-5 that antagonize the Th1 responses related with acute rejection [48, 49]. Also, repeated activation of NKT cells by α-galactosylceramide leads to IL-10 production [50, 51]. Therefore, the success of tolerance protocols in transplantation will require the administration of agents capable of suppressing innate immunity as well as adaptive immunity [52].

2.1. Tolerogenic Potential of Dendritic Cells on Graft Rejection. Dendritic cells are a potential tool for therapeutic applications in transplantation and strategies that promote DC tolerogenicity are under evaluation [53–56]. The discovery of DC's function is considered a landmark in the field of immunology, since it plays a relevant role in the interaction between innate and adaptive immune response. In 1973, Steinman and Cohn characterized and named DCs, a key population in naive T lymphocyte activation [57]. These studies originated from the necessity of a better understanding of how antigens could activate T cells and how this activation contributed to the effector mechanisms of the immune response. Steinman's studies have demonstrated that DC can be functionally characterized by the presence of high levels of MHC expression. Soon after, the importance of the DCs' maturation stage for their immunogenic or tolerogenic functions became clear [58]. These APC, widely distributed in lymphoid tissues, mucosal epithelium, and parenchymal organs [58, 59], are originating from myeloid or lymphoid precursors in the bone marrow and circulate in the bloodstream as immature cells before migrating to peripheral tissues [59]. In innate immunity, these cells respond to pathogen-associated molecular patterns (PAMPs) of microbes by generating and secreting inflammatory cytokines. In adaptive immunity, these cells process and present antigen to T cells that leads to their activation [60].

In humans, two major subpopulations of DC were characterized: conventional DC (cDC) and plasmacytoid DC (pDC). When stimulated by microbial antigens via TLR-2 and TLR-4, cDC produces large amounts of IL-12 that drives immune response to a Th1 profile. Conventional DC also activates cytotoxic lymphocytes (CTL) in a process known as cross-presentation. On the other hand, pDC has a lower capacity of antigen uptake. However, pDC expresses intracellular TLR 7 and TLR 9, which detect ssRNA and CpG DNA motifs, respectively. pDC's main function is to initiate antiviral responses producing large amounts of type I interferons such as IFN-α and INF-β. Together, cDC and pDC can distinctively contribute to protecting the host against pathogens [61, 62].

In an inflammatory microenvironment, DCs initiate their maturation process by undergoing changes in their morphology that facilitate the interaction with naive T cells. The hallmark of the mature stage is the upregulation of MHC and costimulatory molecules on DC surface. Another relevant factor is that DCs dramatically increase their migratory capacity due to the augmentation in chemokines expression, a

process that occurs by the upregulation of the CCR7 receptor and their interaction with two major chemokines, CCL19 and CCL21. Naive T cells also express CCR7 and, as DC, migrate to the lymph node regions, thus increasing the likelihood of interacting between APC and naive T lymphocyte [63]. The matured and activated DCs cease to recognize and process antigens, consequently preventing the presentation of self-antigens at the site of inflammation. In summary, the set of events that occur during maturation can mold DCs as highly effective inducers of proliferation and differentiation of naive T cells [63].

After exposure to antigen and crosstalk with T cells, DCs express high levels of costimulatory molecules and cytokines. In this regard, tolerogenic- (tol-) DC can be generated by altering these signals [64–66]. In order to achieve a tolerogenic profile, a DC must be immature, which means that the maturation degree of a DC can determine its tolerogenic capacity. According to this, the immature DC expresses low levels of MHC class II and low levels of costimulatory molecules, such as CD40, CD80, and CD86, and, consequently, it presents a low capacity of activating T cells, which is potentially associated with T cell anergy and increased Tregs generation. In this context, immature DC has demonstrated its ability of negatively regulating immunogenic responses to alloantigen in animal models [67, 68].

It is well known that immature cDC generated *in vitro* from bone marrow cells in the presence of the granulocyte-macrophage-colony stimulating factor (GM-CSF) administered seven days before the heterotopic cardiac transplantation graft in rats produces a significant increase in survival time of the graft. This primarily occurs because the immature DC presents a reduced expression of costimulatory molecules [69].

Regarding the secretion of cytokines, immature tol-DC can also be generated *in vitro* in the presence of specific cytokines. One of the major cytokines that contribute to the generation of tol-DC is the IL-10. DC modulated by IL-10 inhibits the expression of MHC class II and of CD80 and CD86 costimulatory molecules, acquiring the ability to induce T cell anergy. IL-2, secreted mainly by activated T $CD4^+$ lymphocytes, also plays an important role in maintaining tolerance by regulatory T cells, since these cells are highly dependent on IL-2 for their functions [59].

The literature indicates that the tolerogenic DC populations have specific markers related to their tolerogenic capacity as well as high expression and activity of indoleamine 2,3-dioxygenase (IDO), a tryptophan-catabolizing enzyme [70]. The suppressive mechanism related to IDO is associated with massive depletion of tryptophan, serotonin, and melatonin in the tissue microenvironment, producing immunoregulatory metabolites, the kynurenines [71]. Besides, other metabolites derived from tryptophan by IDO activity can also foster the generation of Treg cells, demonstrating the important immunosuppressive role of IDO, either by direct suppression of T cell activation, or expansion of Treg cells. Data shows that IDO has a greater potential to protect the tissue from damage than to prevent the activation of T cells [70, 72].

3. Adaptive Immunity on Graft Rejection

3.1. Cellular Basis of Allograft Rejection. Much of what is currently known about allograft rejection is mainly related to the understanding of the effective role of T cells in alloantigen recognition mechanisms. However, innate-adaptive immune crosstalk is fundamental in this process. The participation of T cells in the recognition of alloantigen occurs through the interaction between the receptors of T lymphocytes and allogeneic MHC expressed on APC [73], promoting the differentiation of naive T cells into effector T cells [74]. The migration of naive T cells to lymphoid organs is mediated by specific chemokines, such as intercellular adhesion molecules (ICAM) as well as chemokine (C-C motif) ligand 21 (CCL21), which allow the migration of lymphocytes through blood vessels. In lymphoid organs, naive T lymphocytes can encounter DC bearing antigen molecules. Accordingly, the naive T cells initiate their differentiation depending on the signal intensity that can then become effector cells [75].

The $CD4^+$ helper T cells promote the activation of macrophages by the production of specific cytokines, also assisting in the differentiation of plasmocytes and consequently in the production of antibodies. The helper T cells promote the expansion of memory $CD8^+$ T cells after secondary exposure to antigen [76]. Many factors, including cytokines, influence the differentiation of naive $CD4^+$ T cells. The major subsets of T helper effector cells are Th1, Th2, Th9, Th17, Th22, and follicular helper T cells [77, 78]. Among them, Th1 cells are one of the most important populations responsible for immune response evolved with the allograft rejection, while the role of Th2 cells is controversial; some data support their involvement in the activation of alloimmune responses while other data shows their contribution as a regulatory subset [79].

More recently, the Th17 population stands out as an important cell group related with inflammatory conditions. In this sense, the influence of Th17 cells in the activation of inflammatory conditions in GVHD in patients was demonstrated. These cells migrate to GVHD target organs, as skin and mucosa, and exert their proinflammatory effects, stimulating the Th1 effector cells migration to these sites [80].

It has been shown that another heterogeneous subset of $Foxp3^+$Treg cells promotes the inhibition of the activation of T lymphocytes, balancing the intensity of immune responses [75, 81]. In allogenic cardiac mouse model, the adoptive transfer of $Foxp3^+$Treg cells induced *in vitro* by exposition of naïve T lymphocytes to tolerogenic DC was able to provide long-term tolerance and allograft survival [82]. In agreement with these findings, Hu and collaborators [83] showed that infiltrating $Foxp3^+$Treg cells found in kidney allografts from mouse seem to be related with the tolerance phenomenon whereas the depletion of these cells correlates with tolerance abrogation and decreased graft survival. However, it has been demonstrated that $Foxp3^+$Treg and Th17 cells populations have a high flexibility and lineage plasticity, being able to convert from one to another by a mechanism dependent on the retinoic acid receptor-related orphan receptor γ (RORγt) [84, 85]. Supporting this, a recent investigation in a mouse cardiac transplantation model showed that the transference of

mesenchymal stem cells (MSC) before heart transplantation was able to induce Treg over Th17 development. According to the authors, the identification of IL-17A+ Foxp3+ double-positive and ex-IL-17-producing IL-17A-Foxp3+ T cells in heart and spleen of the recipients argues for direct conversion of Th17 cells into Treg cells as the underlying mechanism of immune regulation in MSC-mediated allograft survival [85].

Among immunological molecules related to allograft response, human leukocyte antigen (HLA)-G, a nonclassical class of I HLA detected in the plasma, has been associated with the reduction of acute and chronic rejection [86]. This molecule has local immunomodulatory properties, due to its limited polymorphism, contributing to the survival of allogeneic liver transplants. HLA-G1 to G4 are membrane-bound molecules while HLA-G5 to G7 are soluble molecules (sHLA-G) [87, 88]. HLA-G was previously identified as a naturally occurring tolerance-inducing molecule. Under physiological condition, HLA-G has a low tissue distribution, being mainly found in medullary thymic epithelial cells, cornea, pancreas, and mesenchymal stem cells. Their tolerogenic functions were observed during pregnancy for preventing maternal NK cytotoxicity and suppressing the activation and proliferation of CD4 and CD8 T cells [89, 90]. It is already known that low doses of sHLA can stimulate Th2 and inhibit Th1 profile [91, 92]. However, it was also demonstrated that sHLA induced proliferation and IFN-γ production by NK cells, contributing to vascular remodeling of spiral arteries and allowing successful embryo implantation in pregnancy model [92].

3.2. Humoral Basis of Allograft Rejection. Antibody-mediated immune response, described in the literature as hyperacute graft rejection, occurs mainly in highly vascularized organs transplanted into previously sensitized recipients. This phenomenon can occur in distinct conditions: when patients might have received multiple blood transfusions, when they have been pregnant, or when they could possibly have had a previous transplant treatment. All these situations would explain why they would be carrying antibodies against donor antigens (DSAs). The result is a hyperacute rejection mediated by specific antibodies due to an incompatibility between donor and recipient, manifested by a strong reaction against the donor HLA antigens in the vascular endothelial cells of the graft [93].

The interaction between graft endothelial cells and host antibodies provides rapid complement activation and subsequent graft loss. This is caused by a serious inflammatory injury in the endothelium, losing its capacity of retaining fluid within the intravascular space [94–96]. Hereupon, the evolution of therapies for reducing the impact of B cells and DSA is a goal on allograft survival.

4. Immunotherapies Targeting Allograft Rejection

The establishment of an effective standard therapy for the induction of tolerance in the prevention of graft rejection is of great complexity. Hence, the long-term graft survival is often dependent on the maintenance of immunosuppressive treatment, which generates serious side effects [97, 98]. Rather, it would be interesting to have treatments with milder side effects such as tolerance induction therapy [4, 99], highlighting the importance of creating protocols based on nonaggressive immunosuppressive drugs.

In this sense, the progression of research and immunological knowledge allowed the development of new immunosuppressive drugs and molecules based on animal model studies. The literature describes a wide range of tolerogenic therapeutics, many of which are still under experimental studies. However, some biological therapies have shown considerable success in allogeneic transplant, at least in the short term [100–102]. Thereby, therapies based on inducing cellular tolerance have become important alternatives for reducing the administration of immunosuppressive drugs in the attempt of improving the life quality of transplanted patients [4, 99]. However, more studies are necessary to investigate the risks associated with modern cellular therapies, since they can be related to an increased number of malignancy and infectious diseases [103, 104].

4.1. Immunotherapy Based on the Second Signal Blockage. As previously mentioned, the secondary signaling has a great importance to the T cell immune response and can be a relevant tool to the development of immunological tolerance. Thereby, new immunotherapies are under evaluation for the treatment of autoimmune diseases, hypersensibility, and transplantation. Several studies have focused on the generation of tol-DC by blocking the costimulatory pathways, as summarized in Table 1 [5, 98, 105].

Among costimulatory molecules, T cells express the CD28 receptor on their surface as the main responsible molecule for binding to the B7-1 and B7-2 (CD80 and CD86, resp.) receptors, present on the surfaces of APC. The interaction between these molecules promotes the differentiation and activation of T effector cells, together with the production of associated cytokines, triggering the immune response [106, 107]. Naive T cells highly express CD28 molecule which avidly interacts with B7 molecules present on APC. The interaction between CD28L/B7 induces the secretion of IL-2 and interferes with the tolerogenic property of immature DC. This occurs primarily by decreasing the induction of regulatory T cells and also by leveraging the differentiation of effector T cells [63].

On the other hand, the cytotoxic T Lymphocyte Antigen-4 (CTLA4) molecule, also known as CD152, acts as an inhibitory receptor of the immune response; that is, it blocks the binding site between CD28 and B7, providing a negative signal to T lymphocytes, thus inhibiting the immune response [148, 149]. Also, the inhibitory signals released by the interaction between CTLA4 and B7 result in an increased secretion of immunomodulatory cytokines, such as IL-10 and TGF-β, and hence the generation of Treg cells [108, 150, 151]. Suppression or anergy, induced by CTLA4, are associated with Treg functions [152]. A recent research has shown that CTLA4 molecule can also be found on DC. The cross-linking of CTLA4 can inhibit the maturation of DC, playing an inhibitory role in immune response [153].

Table 1: Immunotherapy based on the second signal blockage.

Molecule	Commercial name/clone	Target molecule	Mechanism of action	References
CTLA4-Ig	Abatacept/Belatacept	CD80/CD86	Competition for binding to B7 molecules on DC Upregulation of IDO enzyme expression High plasma levels of sHLA-G High secretion of immunomodulatory cytokines Inhibition of $CD8^+$ T cells and NK cells Generation of Treg cells Inhibition of proliferation related to p27kip1 expression	[88, 107–123]
CTLA4-KDEL	—	CD80/CD86	Retention of costimulatory molecules within the ER T cell anergy by an IDO-independent way	[72, 115]
Anti-CD40L (CD154)/anti-CD40	3A8, 4D11, ASKP1240, 7E1	CD40/CD40L	Inhibition of antibody secretion Downregulation of T cell proliferation Inhibition of cytokine secretion and costimulatory molecule synthesis Upregulation of spleen IL-10^+CD4^+; T cells and downregulation of IFN-γ^+CD4^+ T cells	[117, 123–135]
PD-L1.Ig	—	PD-1/PD-1L	Suppression of T cell activation	[136–138]
Anti-TIM-1	RMT1-10	TIM-1	Blockage of TIM-1 ligation	[139–141]
Anti-OX40-L	—	OX40-OX40L interaction	Inhibition of OX40-OX40L signaling Prevention of T memory cells Reduction of effector T cells	[128, 129, 142, 143]
LFA3-Ig	Alefacept	LFA3 (CD58)	Depletion of $CD8^+$ effector memory T cells Reduction of T cell activation	[113, 116, 144–146]

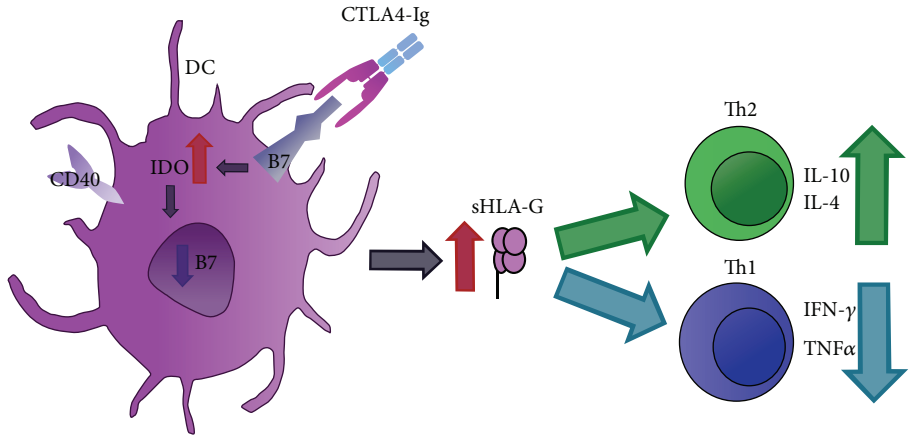

Figure 2: Mechanism of action of CTLA4-Ig on DC. CTLA4-Ig soluble molecule binds to B7 (CD80/CD86) molecules on DC. CTLA4 presents a higher affinity to B7 molecule and competes with CD28 for this ligation. This interaction induces downregulation of B7 gene transcription and upregulation of IDO as well as secretion of sHLA-G. sHLA-G can stimulate Th2 and inhibit Th1 profile. DC: dendritic cell; CTLA4-Ig: extracellular domain of human CTLA4 with a portion of the Fc region of IgG; IDO: indoleamine 2,3-dioxygenase; sHLA-G: soluble HLA-G; Th: T helper; IL: interleukin; IFN: interferon; TNF: tumor necrosis factor.

Since CD80/CD86 molecules present a higher affinity for CTLA4 rather than for CD28 molecule on the T cells, the binding of CTLA4 and B7 family molecules makes it possible to achieve tolerance to the allograft [152, 154]. It has been demonstrated that CTLA4-deficient mice exhibit severe autoimmune phenotype with early death 3 to 4 weeks after birth, resulting from the massive destruction of multiple organs, demonstrating the fundamental role of CTLA4 in the regulation of peripheral self-reactive T cells [155].

Based on this knowledge and on the attempt of modulating the immune response in allografts, recombinant molecules of CTLA4 linked to immunoglobulins (Ig) have been developed (CTLA4-Ig). These molecules, termed fusion protein antagonist CTLA4-Ig, combine the extracellular domain of human CTLA4 with a portion of the Fc region of IgG. As represented in Figure 2, the CTLA4-Ig, initially tested for the treatment of rheumatoid arthritis, has shown greater affinity for B7, acting directly on APC and optimizing the inhibition of the immune response [107, 108]. The inhibition mechanism can also be related to the fact that CTLA4-Ig-treated DC suppresses T cell proliferation through sHLA-G secretion. Additionally, CTLA4-Ig induces IDO expression in DC [88, 92, 156]. sHLA-G was associated with an increase in the number of regulatory T cells and a shift of cytokine towards Th2 [157].

In this context, Abatacept, a commercial CTLA4-Ig, selectively modulates the immune response by binding with high affinity to the B7 family present on APC. Thus, this drug inhibits the activation of T cells by competing by the binding site of CD28 receptors, preventing the secondary signal from occurring [109, 110]. In human model, the T cell hyporesponsiveness was also associated with a higher expression of a negative regulator of proliferation, named p27kip1 (cyclin-dependent kinase inhibitor 1B [CDKN1B]) [111]. Another recombinant molecule with an altered form, Belatacept, has been approved for its use in transplants. This molecule is known for having major affinity against CD86 [112–114]. A study with kidney-transplanted patients treated with Belatacept demonstrates that transplanted patients who received treatment with CTLA4-Ig had higher plasma levels of sHLA-G. It can be hypostasized that the immunosuppressive action of sHLA-G isoforms in transplants is associated with the suppression of allogeneic T cells expansion and the inhibition of the activation of both $CD8^+$ T cells and NK cells [88, 154]. In summary, the inhibitory signals carried by the blockage of the CTLA4 molecule with CTLA4-Ig can be related to the attenuation of stimulatory signal, decreasing the cell proliferation, and cell cycle progression and alteration in cytokine production of effector T cells.

As schematized in Figure 3, CTLA4 fused to the endoplasmic reticulum retention/retrieval signal sequence named KDEL (CTLA4-KDEL) is a fusion protein that targets the endoplasmic reticulum (ER). CTLA4-KDEL is confined to ER and binds to CD80/86, preventing their passage to the cell surface by interaction with receptors. APC expressing this construct retain CD80/CD86 molecules in the endoplasmic reticulum and fail to express these costimulatory molecules on their surface [158–160]. A recent study demonstrated the applicability of modulating the signal transduction in murine DC with CTLA4-KDEL in order to inhibit immune response in corneal transplantation. CTLA4-KDEL-expressing DC adopted a tolerogenic phenotype and induced anergy in alloreactive T cells, both *in vitro* and *in vivo*, resulting in a long-term survival of corneal allografts [72]. CTLA4-Ig in DC cultures showed the expected reduction in IFN-γ and IL-4 which may be associated with the upregulation of IDO in DCs, not seen in CTLA4-KDEL-transfected cells [72, 115].

The interactions between CD40 and CD40L, expressed on APC and T cells, respectively, are strictly related with allograft immune response. As represented in Figure 4, it has been shown that the blockage of the CD40-CD40L interaction improves allograft survival by preventing the occurrence of acute rejection [116, 124, 125, 161]. Therapy with anti-CD40L (CD154) MAb prolonged the survival of the corneal

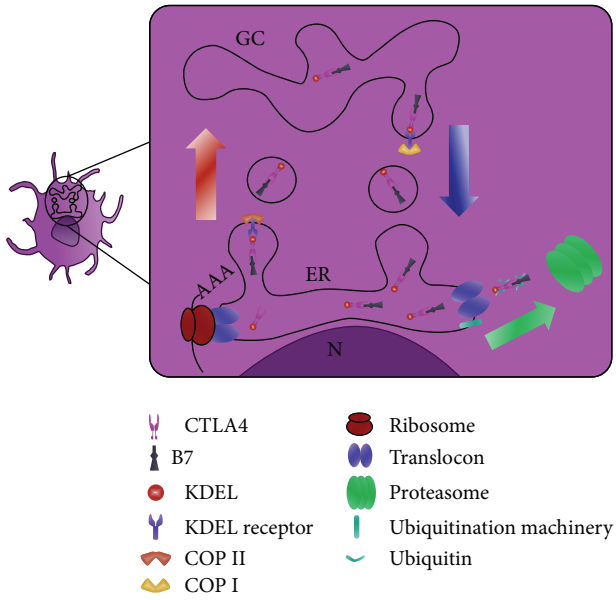

Figure 3: Hypothetical mechanism of action of CTLA4-KDEL fusion protein. Transport of proteins between the ER and Golgi apparatus is mediated by two membrane coat complexes, COPI and COPII. COPII mediates ER-to-Golgi transport and COPI mediates retrograde transport. KDEL receptor undergoes retrograde transport only after it binds its ligand [147]. On CTLA4-KDEL transfected cells, the KDEL peptide retains/retrieves proteins to the ER. CTLA4 fused to KDEL is confined to the ER where it binds CD80/86, preventing the passage of these molecules to the cell surface. CD80/CD86 molecules seem to be removed by proteasome-mediated degradation. GC: complex of Golgi; N: nucleus; ER: endoplasmatic reticulum; CTLA4-KDEL: gene construct encoding a modified CTLA4 molecule; COP: cytosolic protein coat complex.

allograft by increasing the frequency of spleen IL-10$^+$CD4$^+$ T cells and decreasing IFN-γ^+CD4$^+$ T cells. Also, the Treg/Th1 cell ratio was increased in experimental model [162]. Since therapies with anti-CD40 or anti-CD40L MAb presented several thromboembolic complications in clinical applications, further studies are ongoing to evaluate the combination of these molecules [126, 130, 161]. Accordingly, the therapy of CTLA4-Ig and a nondepleting CD40 monoclonal antibody, named 3A8, is a promising combination [126], since preliminary data showed an increased duration of graft acceptance with this immunosuppressive treatment [126, 127].

Programmed death- (PD-) 1 ligand (PD-L1 or B7-H1) and PD-L2 (B7-DC) are new B7 family members expressed on APC. PD-1 and PD-1L/PD-L2 costimulatory signals play important roles in T cell induced immune responses; PD-L1 and PD-L2 deliver inhibitory signals that regulate T cell activation and tolerance [136]. In a corneal allograft model, PD-1 prolonged transplant survival by PD-L1 interaction [137]. In this sense, dimeric PD-L1 immunoglobulin (Ig) fusion protein (PD-L1.Ig) seems to be another combinatory therapy in transplants as in corneal allograft, where PD-L1.Ig showed significant suppression on T cell activation [137, 138, 163]. However, further studies will be required to determine the therapeutic property of this molecule.

Additionally, T cell immunoglobulin domain and mucin domain (TIM) family is a newly discovered group of molecules that regulate immune cell function. TIM-1 molecule is expressed on T cells and APC [139]. The interaction between TIM-1 and TIM-4 promotes Th2 responses, and the blockage of this interaction can decrease allergic responses [140, 141]. RMT1-10, an anti-TIM-1 monoclonal antibody, was effective in blocking TIM-1 and in promoting corneal allograft survival in mice [141].

There are other coadjutant costimulatory blocking molecules that are under evaluation. Anti-OX40-L MAb therapy prevents memory T cell-mediated cardiac allograft vasculopathy in mice, suggesting a potential therapy for inhibition of OX40-OX40L signaling [128, 129, 142]. This MAb seems to act by reducing the pool of effector T cells responses, most part of these being CD8$^+$ T cells [143].

Human leukocyte function antigen-3 (LFA-3) is an adhesion and costimulation molecule, found on a variety of APC, which interacts with ligand CD2 on T cells. LFA3-Ig (Alefacept), a humanized chimeric fusion protein, comprises an extracellular CD2-binding portion of the LFA3 linked to the Fc portion of a human IgG1. LFA3-Ig promotes renal allograft survival by depleting CD8$^+$ effector memory T cells and interfering with T cell activation [113, 144, 145]. Also, this molecule can activate Fc gamma R(+) cells, such as NK cells, to induce apoptosis of sensitive CD2(+) target cells [144, 146].

4.2. Other Modulatory Agents on Tolerogenic Dendritic Cell. A new approach in the attempt of combining treatments with autologous tolerogenic DC and anti-CD3 antibodies is under development. This therapy was shown to be effective in mice with pancreatic islet allografts by providing a reduction of T cells infiltration. However, these protocols are still under clinical development [164, 165].

Alternative methods for modulating tol-DC using tolerance-inducing agents such as dexamethasone (Dexa), rapamycin (Rapa), and vitamin D3 (VitD3) are also under evaluation [54]. Dexa-treated-DC triggered by lipopolysaccharide (LPS) led to the suppression of proliferative response of primed T cells, triggering the differentiation of various populations of Tregs [166]. In another experimental study, BALB/c mice that received a corneal transplantation were treated with an analogue of resolvin D1 (RvD1). Resolvin D1 is a lipid mediator that plays an important role in resolution of acute inflammation. RvD1 modulated DC showed a significant reduction in maturation. Also, interferon-gamma-secreting T cell frequency was decreased and alloimmune sensitization was reduced after transplantation [167].

5. Concluding Remarks

Organ and tissue transplantation is still a last resource, being only considered in cases such as total organ failure. Thus, avoidance of rejection of transplanted organs is a key task. Current allograft therapies can cause many side effects; hence several alternative therapies, aiming the induction of tolerance, mainly based on infusion of DC and Tregs, have been proposed in the attempt of aiding this scenario.

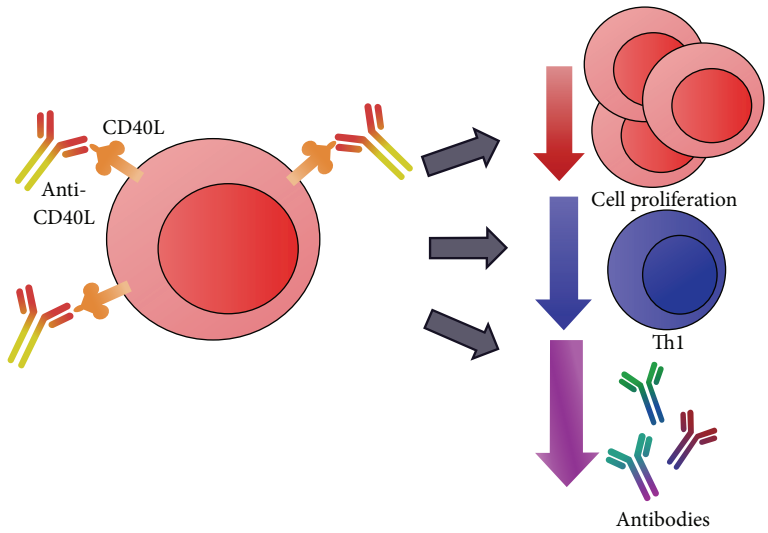

Figure 4: Anti-CD40L interaction and effects on T cells. Costimulatory molecule CD40L is primarily expressed on activated $CD4^+$ T lymphocytes. Anti-CD40L binds to the CD40L present on T cell and blocks its interaction with CD40 receptor present on APC. Therapy with anti-CD40L (CD154) or anti-CD40 MAb, alone or combined with other molecules, downregulates T cell proliferation, Th1 cytokine production, and antibody secretion. CD40L: CD40 ligand; APC: antigen-presenting cell.

Accordingly, immature DC expressing low levels of MHC and costimulatory molecules has been considered among the treatments due to its low capacity to activate T cells, thus promoting a natural immunosuppression, which reduces the need of using immunosuppressive drugs.

The modulation of DC with CTLA4-Ig has shown positive effects on suppressing the immune response. Although many studies involving fusion proteins, and even monoclonal antibodies, are in early stages, this is a very promising tool and has great clinical potential in reducing transplant rejection. Among the promising treatments, the effectiveness of using CTLA4-Ig in immune modulation and in the induction of tol-DC has been shown, even though the use of tolerogenic cells for therapeutic purposes on transplantation is still not widely available in clinical practice.

Essentially, the main challenge in these therapies is to fixate the DC phenotype, since tol-DC can only be determinate by its tolerogenic effect. Thus, the fine control of the subtle balance between immunization and tolerance by DC is necessary to allow the use of DC in clinical practice.

Despite being a very promising therapy, studies of adverse effects should be extensive, since the use of biotechnology in medical treatment, in the transplantation scenario in particular, can be very risky if not thoroughly understood.

Conflict of Interests

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

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Research Article

Carbohydrate Microarrays Identify Blood Group Precursor Cryptic Epitopes as Potential Immunological Targets of Breast Cancer

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Using carbohydrate microarrays, we explored potential natural ligands of antitumor monoclonal antibody HAE3. This antibody was raised against a murine mammary tumor antigen but was found to cross-react with a number of human epithelial tumors in tissues. Our carbohydrate microarray analysis reveals that HAE3 is specific for an *O*-glycan cryptic epitope that is normally hidden in the cores of blood group substances. Using HAE3 to screen tumor cell surface markers by flow cytometry, we found that the HAE3 glycoepitope, gp^{HAE3}, was highly expressed by a number of human breast cancer cell lines, including some triple-negative cancers that lack the estrogen, progesterone, and Her2/neu receptors. Taken together, we demonstrate that HAE3 recognizes a conserved cryptic glycoepitope of blood group precursors, which is nevertheless selectively expressed and surface-exposed in certain breast tumor cells. The potential of this class of *O*-glycan cryptic antigens in breast cancer subtyping and targeted immunotherapy warrants further investigation.

1. Introduction

Recognition of abnormal glycosylation in almost any cancer type has raised great interest in the exploration of the tumor glycome for biomarker discovery [1–3]. In this study, we explored potential glycan markers that are overexpressed on the surfaces of breast cancers. A key immunological probe of this investigation is an antitumor monoclonal antibody (mAb), HAE3. This mAb was raised against epiglycanin, the major sialomucin glycoprotein (~500 kDa) of murine mammary adenocarcinoma TA3 cells [4]. It was initially called AE3 but was later renamed HAE3 [5] to avoid confusion with a commonly used anti-cytokeratin antibody in cancer research [6, 7]. Interestingly, HAE3 was found to strongly cross-react with a number of human epithelial tumors in tissues, including lung, prostate, bladder, esophagus, and ovarian cancers [5, 8–10].

This cross-species tumor binding profile suggests the possibility that HAE3 may recognize a conserved tumor glycan marker that is coexpressed by both mouse- and human-derived epithelial cancers. HAE3 was initially suggested to resemble lectin peanut agglutinin (PNA), which recognizes the T disaccharide (Galβ1,3GalNAcα1-) linked to Ser/Thr [11]. However, the antibody differed from PNA in that the concentration of the blood group T disaccharide required for inhibition of binding to epiglycanin was 10^4 times greater than for PNA. Moreover, a T-specific mAb HH8 was found to be negative with epiglycanin in ELISA microtiter plates [12]. HH8 is specific for the T-terminal disaccharide moiety expressed by asialoglycophorin A [13]. Consistent with these observations, Palma et al. reported recently that the neoglycolipid conjugates that display a single T disaccharide epitope were negative with HAE3 [14]. Of note, mAb HAE3 was cited as AE3 in the report.

Given that HAE3 has been analyzed using a large collection of synthetic neoglycoconjugates ($n = 492$) [14], this study focused on identification of the potential natural ligands of HAE3. In essence, we produced a comprehensive carbohydrate microarray using a large collection of purified natural carbohydrate antigens for screening. These include A, B, O, Lewis$^{a/b}$, I and i, and the blood group precursors of various biological origins. As summarized below, we have revealed that HAE3 is specific for a blood group precursor cryptic epitope that is normally hidden in the cores or internal chains of blood group substances but becomes differentially expressed in human breast cancer cells.

2. Material and Methods

2.1. Antigens and Antibodies. A preparation of human carcinoma-associated antigen (HCA) (1$^{\#}$) was kindly provided by Dr. Zeqi Zhou of Egenix (Millbrook, NY). The murine hybridoma IgM antibody, HAE3, was produced by mouse immunization (C57BL/J) with asialoepiglycanin (85$^{\#}$) [4, 5]. A preparation of purified HAE3 protein was purchased from RA Biosources, Inc. (Belmont, CA). Carbohydrate antigens applied in this study are listed in Supplementary Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/510810.

2.2. Cell Lines. Cancer cell lines used include breast-derived (T-47D, SK-BR-3, MCF-7, BT-549, MD-AMB-231, and MD-AMB-468), lung-derived (A549), or prostate-derived (PC3) epithelial tumor cell lines and a skin-derived melanoma SKMEL-28. All tumor cell lines were acquired from ATCC.

2.3. Carbohydrate Microarrays. Microarray assays were performed as described [15]. In brief, a microarray robot (PIXSYS 5500C, Cartesian Technologies, Irvine, CA) was used to spot antigen preparations onto glass slides precoated with nitrocellulose polymer (FAST Slides; Schleicher & Schuell, Keene, NH). The printed microarrays were incubated at room temperature with HAE3 (IgM) antibody at 5 μg/mL in 1% (wt/vol) BSA in PBS containing 0.05% (wt/vol) NaN_3 and 0.05% (vol/vol) Tween-20. An R-phycoerythrin- (R-PE-) conjugated affinity-purified F(ab$'$) fragment of goat anti-mouse IgM secondary antibody preparation (Rockland Immunochemicals, Inc., PA) was applied at 2.0 μg/mL to reveal HAE3-specific staining signal. Fluorescence intensity values for each array spot and its background were calculated using ScanArray Express software. SAS Institute's JMP-Genomics software package (http://www.jmp.com/; Cary, NC) was used for microarray data standardization and statistical analysis. Results of the microarray assay are shown as the means of fluorescent intensities (MFIs) of triplicate detections of given antigen preparations (Figure 1(a) and Table 1).

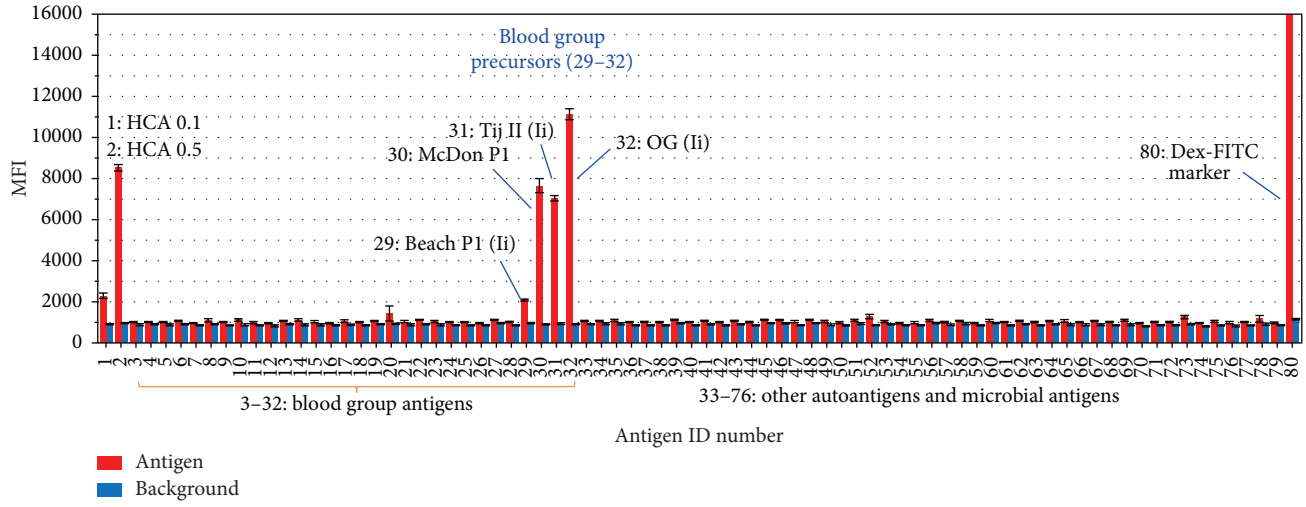
2.4. Fluorescence-Activated Cell Sorting (FACS) Analysis. HAE3 (IgM, 5.0 μg/mL) and an isotype control mAb 9.14.7 (IgM, anti-α(1,6)dextran, 5.0 ug/mL) [16] were applied to stain tumor cell lines. The R-PE-conjugated goat anti-mouse IgM antibody preparation described above was applied in the second staining step to reveal the antigen-captured IgM. FACS data were collected with LSR-II (BD Bioscience, San Jose, CA) and analyzed with FlowJo (TreeStar Inc., Ashland, OR).

2.5. Enzyme-Linked Immunosorbent Assay (ELISA) and ELISA Inhibition Assays. Carbohydrate-specific ELISA and ELISA inhibition assays were performed as described [15, 17]. In brief, glycoprotein antigen preparations were diluted in 0.1 M sodium bicarbonate buffer solution, pH 9.6, for coating on ELISA microplates (NUNC, MaxiSorp, Thermo Scientific, Rochester, NY) followed by blocking using 1% BSA and 1X Phosphate Buffered Saline Tween-20 (PBST). MAb HAE3 (IgM) (2.5 μg/mL) and biotinylated PNA (2.0 μg/mL) were diluted in 1% BSA, PBST for the ELISA binding assay. The bound HAE3 and PNA were revealed by an alkaline phosphatase- (AP-) conjugated goat anti-mouse IgM and an AP-streptavidin conjugate (Sigma Chemical Co., St. Louis, MO), respectively. ELISA inhibition assays were performed with EPGN (85$^{\#}$) (1 μU/mL) coated to display the native gp^{HAE3} epitope and a series of blood group reference antigens as potential inhibitors (25.0 μg/mL/each) to compete with the HAE3 (1.0 μg/mL) binding of EPGN. Percent inhibition was calculated as follows: % inhibition = ((standard A − blank A) − (A with inhibitor − A))/(standard A − blank A).

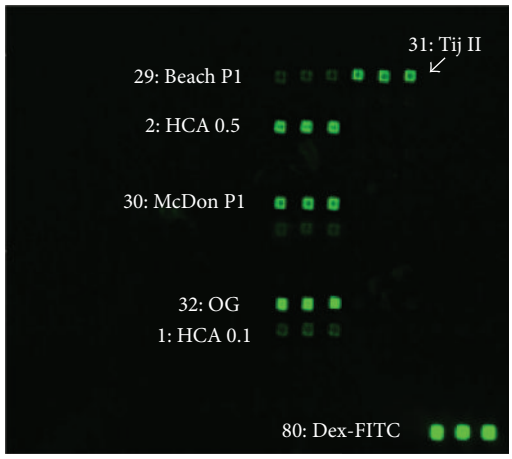
2.6. Bio-Gel Chromatography. Bio-Gel P-10 filtration was performed following the manufacture's manual (Bio-Rad Laboratories, Hercules, CA) with minor modifications. In brief, Tij II (20% from 2nd 10%) substance was fractioned in a precalibrated Bio-Gel P-10 column. The sizes of the Tij II substance were measured based on the neutral sugar elution profile with reference to the calibrated saccharide molecular weight standards as shown in Figure 4.

3. Results

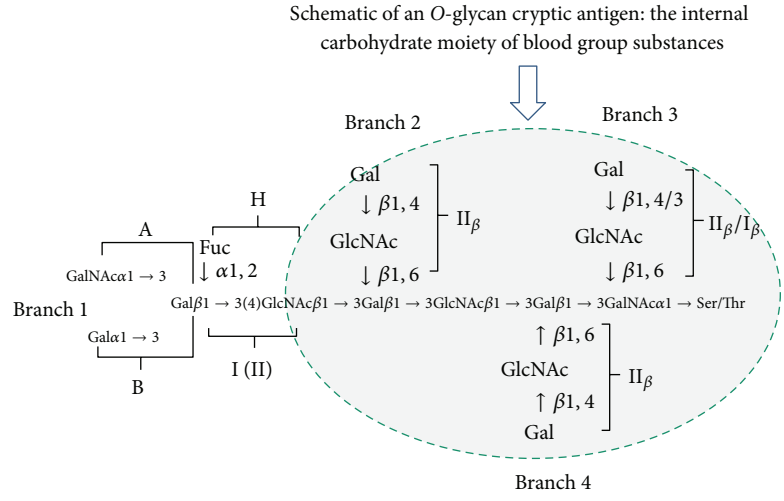
3.1. Carbohydrate Microarray Identifies Blood Group Precursors as the Natural Ligands of HAE3. As shown in Figure 1 and Table 1, an HCA preparation (ID$^{\#}$ 1 and 2) was spotted as a positive control for HAE3 activity. This HAE3^{+} glycoprotein preparation was affinity-purified from cultural supernatant of the lung cancer cell line A549 using an HAE3-agarose column (Egenix, Millbrook, NY). Given that the simple *O*-glycan core T disaccharide (Galβ1,3GalNAc-) and its peptide conjugates were found to weakly but significantly inhibit HAE3 binding to epiglycanin, we characterized a panel of blood group substances that carry *O*-glycan cores in this microarray screening. A number of blood group precursors (29$^{\#}$–32$^{\#}$) were plotted from 3$^{\#}$ to 32$^{\#}$, which was followed by other antigens from 33$^{\#}$ to 78$^{\#}$ and microarray printing and scanning calibration controls in 79$^{\#}$ and 80$^{\#}$. Blood group substance reference reagents [18] used include the following: Cyst9 and Cyst14, A active; Beach phenol insoluble, B active; Hog, H active; JS phenol insoluble, H and Le^{b} active; and N-1 20% from the second 10%, Le^{a} active. Key blood group precursor references include OG, Tij II, Beach P1, and McDon



(a)



(b)



(c)

Figure 1: Carbohydrate microarray analysis of antiepiglycanin mAb HAE3. Seventy-six glycoproteins, glycoconjugates, and polysaccharides were spotted in triplicates in 1 to 2 dilutions to yield the customized microarrays for antibody screening. (a) Microarray detections were shown as the mean fluorescent intensities (MFIs) of each microspot with antigen-binding signal in red and background reading in blue. Each error bar is constructed using one standard deviation from the mean of triplicate detections. The labeled antigens include HCA (ID# 1 and ID# 2), a number of blood group precursors ($29^{\#}$–$32^{\#}$), and a microarray spotting marker ($80^{\#}$). (b) Images of a microarray stained with HAE3 (5 μg/mL). (c) Schematic of a blood group substance structure with the conserved *O*-glycan core highlighted.

P1. These precursor substances were prepared to remove most of the α-L-fucosyl end groups that are essential for blood groups A, B, and H or Lewis active side chains but possess the internal domains or core structures of blood group substances. A large panel of other autoantigens and microbial polysaccharides were spotted in the same microarrays to examine potential polyreactivity of this IgM antibody.

Figures 1(a) and 1(b) illustrate a representative result of multiple microarray screening assays. In Figure 1(a), the MFIs of carbohydrate microarray detections of HAE3 binding signal (red column) are plotted with corresponding local background reading (blue column) as an overlay plot. Each data point represents the mean of triplicate detections; these are shown in the Figure 1(b) microarray image with the number of positive antigens labeled. Each error bar is constructed using one standard deviation from the mean. As illustrated, HAE3 is strongly positive with HCA ($1^{\#}$ and $2^{\#}$) as expected. Importantly, this antibody selectively binds to four blood group precursor antigens, Beach P1 ($29^{\#}$), McDon P1 ($30^{\#}$), Tij II ($31^{\#}$), and OG ($32^{\#}$). By contrast, HAE3 has no detectable cross-reactivity with blood group substances A, B. O, or Lewis antigens, or the large panel of other carbohydrate antigens spotted in the same array.

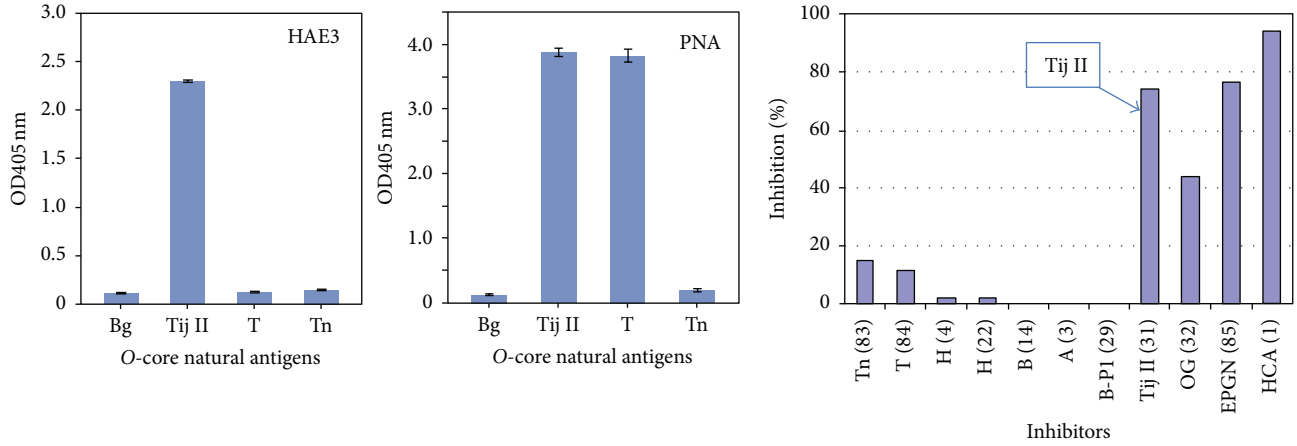
Figure 1(c) is a schematic of blood group substance structure with the common blood group precursor core structure highlighted. The four-branched structure in the circle represents the internal portion of the carbohydrate moiety of blood group substances, which was proposed based on extensive immunochemical characterization of precursor OG and other P1 fractions of blood group precursors that

Table 1: Dataset from a carbohydrate microarray analysis of mAb HAE3 (Figure 1).

*Antigen spotted		Fluorescent intensities (INT)			Microarray scores (log 2-INT)		
(ID#) antigen name	Replicates	Means	StDev	Ag/Bg	Means	StDev	**t-test (p value)
(1)HCA(A549) 1 : 5	**3**	**2306**	**214**	**2.09**	**11.167304**	**0.132563**	**6.66755E − 05**
(2) HCA (A549)	**3**	**8537**	**265**	**7.73**	**13.058996**	**0.044597**	**1.0582E − 07**
(3) Cyst9 (A)	3	1065	5	0.96	10.05708	0.0062627	0.695270476
(4) JS (A)	3	1059	2	0.96	10.048485	0.0027246	0.630836897
(5) HGM-BGS (A + H)	3	1060	12	0.96	10.049785	0.0166116	0.641720796
(6) Hog76 (A)	3	1095	6	0.99	10.096264	0.0072603	0.982754025
(7) Hog (A)	3	996	7	0.90	9.9604633	0.0096233	0.195275968
(8) MSS (A)	3	1139	134	1.03	10.147131	0.1651035	0.701231922
(9) Hog39 B2 (A)	3	1046	10	0.95	10.031084	0.0136387	0.512794031
(10) Cyst14 (A2)	3	1144	116	1.04	10.155053	0.1435773	0.6354829
(11) WG (A2)	3	1021	13	0.92	9.9952239	0.0176786	0.320320938
(12) Cyst11	3	1011	2	0.91	9.9810904	0.002181	0.261637986
(13) Cow21	3	1083	9	0.98	10.080783	0.0122256	0.886644093
(14) Beach (B)	3	1152	83	1.04	10.167059	0.1023248	0.51871479
(15) Cow28 (B)	3	1065	27	0.96	10.056322	0.0369858	0.695618692
(16) Cow43	3	1012	7	0.92	9.9824975	0.0094843	0.267256771
(17) Cow26	3	1093	56	0.99	10.092395	0.0732857	0.985749244
(18) Hog5	3	1057	4	0.96	10.046209	0.0047907	0.614466244
(19) Hog 10%	3	1076	8	0.97	10.071437	0.0104506	0.809324608
(20) Hog6 (H)	3	1462	595	1.32	10.441803	0.5435961	0.384564394
(21) Hog67	3	1067	13	0.97	10.058822	0.0176694	0.709880521
(22) Hog (H)	3	1132	10	1.02	10.144623	0.0123178	0.598292687
(23) Hog30	3	1071	40	0.97	10.064074	0.0539668	0.760857847
(24) Cow21 (I-Ma)	3	1031	7	0.93	10.009339	0.0099403	0.386688505
(25) Cow25	3	1051	24	0.95	10.036836	0.033184	0.555735986
(26) Cow26 (I)	3	1008	13	0.91	9.977683	0.017886	0.250673214
(27) N-1 10% 2X (Le^a)	3	1139	10	1.03	10.153512	0.0131909	0.537783247
(28) N-1 IO4- (Le^a)	3	1044	18	0.94	10.0273	0.0250417	0.491711028
(29) Beach P1 (Ii)	**3**	**2119**	**22**	**1.92**	**11.049118**	**0.01476**	**0.000114629**
(30) McDon P1	**3**	**7675**	**604**	**6.95**	**12.903032**	**0.113579**	**4.91829E − 08**
(31) TijII (Ii)	**3**	**7050**	**220**	**6.38**	**12.782943**	**0.044774**	**1.83548E − 07**
(32) OG (Ii)	**3**	**11166**	**433**	**10.10**	**13.446047**	**0.056595**	**2.50433E − 08**
(33) LNT-BSA (Type I)	3	1092	12	0.99	10.092703	0.0152473	0.9870917
(34) Pn XIV (Type II)	3	1108	17	1.00	10.113624	0.02265	0.838256847
(35) ASOR (Tri/m-II)	3	1128	26	1.02	10.138873	0.0330974	0.644502626
(36) AGOR (Tri/m-Gn)	3	1042	5	0.94	10.02559	0.0068188	0.477740236
(37) iAFGP	3	1024	5	0.93	10.000458	0.0070924	0.342706487
(38) Chondroitin-SO4-A	3	1038	13	0.94	10.019053	0.0179351	0.440853108
(39) Chondroitin-SO4-B	3	1128	10	1.02	10.139939	0.0128602	0.631919148
(40) Chondroitin-SO4-C	3	1047	14	0.95	10.031963	0.0189058	0.519441046
(41) Hyaluronic Acid	3	1096	9	0.99	10.098441	0.0113582	0.964347497
(79) Bg	6	1105	188	1.00	10.09423	0.219076	
(80) Dex70K-FITC	3	63225	1067	57.22	15.948071	0.0243011	8.23802E − 09

*Antigen's initial spotting concentration was 0.5 μg/μL. The positive results are emphasized with bold. Microbial antigens tested (42–78) were negative with HAE3 (Ag/Bg < 1.20; $p > 0.20$, data not shown).

**t-test: microarray scores, that is, the log 2-transformed microarray values from triplicate spots for each antigen, were applied in a t-test to examine the differences of significance between each probe and Bg (microarray reading background), which is the mean fluorescent intensity of six spots of Av-Cy3/Cy5 (ID# 79) in the FITC channel.



(a) ELISA binding assays

(b) ELISA inhibition assays

Figure 2: Carbohydrate-specific ELISA and ELISA inhibition assays validate binding specificities of HAE3. (a) An antigen-specific ELISA distinguished HAE3 binding specificity from the T-antigen-specific PNA. ELISA plates were coated with *O*-core antigen, Tij II (31$^{\#}$), T (81$^{\#}$), and Tn (82$^{\#}$) at 10 μg/mL to react with either HAE3 (5.0 μg/mL) or PNA (1.0 μg/mL). (b) ELISA inhibition assays with a series of carbohydrate antigens as competitors (25.0 μg/mL) to inhibit interaction between EPGN (1.0 μU/mL) and anti-HCA (HAE3, 1.0 μg/mL). These antigens include HCA (1$^{\#}$), EPGN (85$^{\#}$), which is the immunogen of HAE3, and blood group substances Tn (83$^{\#}$), T (84$^{\#}$), H (4$^{\#}$ or 22$^{\#}$), A (3$^{\#}$), B (14$^{\#}$), Beach P1 (29$^{\#}$), Tij II (31$^{\#}$), and OG (32$^{\#}$). Results are shown as percent inhibition in the presence of an inhibitor.

were isolated from ovarian cancer cyst fluids [19–22]. Selective detection of these blood group precursors from a large panel of blood group substances by HAE3 illustrated that this antibody is specific for a shared cryptic glycoepitope of these precursor substances.

3.2. Carbohydrate-Specific ELISA and ELISA Inhibition Assays Validate Binding Specificity of HAE3. We further examined whether the observed HAE3 binding reactivity can be attributed to cross-reactivity with T/Tn glycoepitopes that are often expressed as components of blood group precursor substances. For this purpose, we tested ELISA binding of HAE3 or T-specific lectin PNA with blood group precursor Tij II (31$^{\#}$) and two T/Tn-positive glycoconjugates, asialo-PSM (T) (81$^{\#}$) and asialo-OSM (Tn) (82$^{\#}$). Unlike PNA, which binds to both Tij II and asialo-PSM (T), HAE3 specifically binds to Tij II without cross-reacting with asialo-PSM (T) or asialo-OSM (Tn) (Figure 2(a)). Thus, HAE3 binding of Tij II is irrelevant to the native T/Tn-glyco-epitopes expressed by these glycoconjugates. ELISA inhibition assays further demonstrated that blood group precursors Tij II (31$^{\#}$) and OG (32$^{\#}$), but not other blood group antigens, including A (3$^{\#}$), B (14$^{\#}$), H (22$^{\#}$), T (84$^{\#}$), or Tn (83$^{\#}$), significantly inhibited HAE3 binding to the immunogen asialoepiglycanin (85$^{\#}$) (Figure 2(b)).

3.3. FACS Analysis Detects Tumor Cell Surface Expression of gp^{HAE3}. We examined whether the HAE3^{+} glycoepitopes were expressed as cell surface tumor markers. In the first set of experiments, we screened a panel of four tumor cell lines by cell surface staining in flow cytometry. These include (a) a breast cancer line, T-47D, which was selected owing to the fact that breast cancer patients were found to produce substances in circulation that are highly effective in inhibiting HAE3 binding of epiglycanin [12, 23], (b) a lung cancer line, A549, which is known to produce an HAE3-positive substance in cell culture, (c) a prostate cancer line, PC3, which is found to express a blood group B-related F77 glycoepitope [24, 25], and (d) a melanoma cell line SKMEL-28, which is derived from skin but not epithelial tissue. As shown in Figure 3(a), melanoma SKMEL-28 and prostate cancer PC3 were negative for HAE3. The A549 lung cancer cell line was weakly positive. By contrast, the breast cancer cell line T-47D was strongly positive in HAE3-cell surface staining.

Given these results, we extended the FACS analysis to a panel of seven human breast cancer cell lines, including two ER^{+}PR^{+} lines (T-47D and MCF-7), one ER^{+} (SK-BR-3), and four triple-negative cancers (BT-549, Hs 578T, MDA-MB-231, and MDA-MB-468). Figure 3(b) shows that two ER^{+}PR^{+} lines, T-47D and MCF-7, and two triple-negative lines, BT-549 and MDA-MB-468, are gp^{HAE3} strongly positive. SK-BR-3 is gp^{HAE3} intermediately positive. By contrast, the two remaining triple-negative cell lines, Hs578T and MDA-MB-231, were HAE3 negative.

4. Discussion

Expression of gp^{HAE3} by human breast cancer has been examined in this study. FACS analyses (Figure 3) revealed that five of the seven breast cancer cell lines are HAE3 positive. These are T-47D, MCF-7, SK-BR-3, BT-549, and MDA-MB-468. BT-549 cells are triple-negative/basal-B mammary carcinoma; MDA-MB-468 cells are known as triple-negative/basal-A mammary carcinoma. The two remaining gp^{HAE3-} triple-negative cell lines, Hs578T and MDA-MB-231, were basal-B mammary carcinoma. It is important to extend this investigation to a cohort of breast cancer patients to examine whether this marker was significantly associated with metastatic

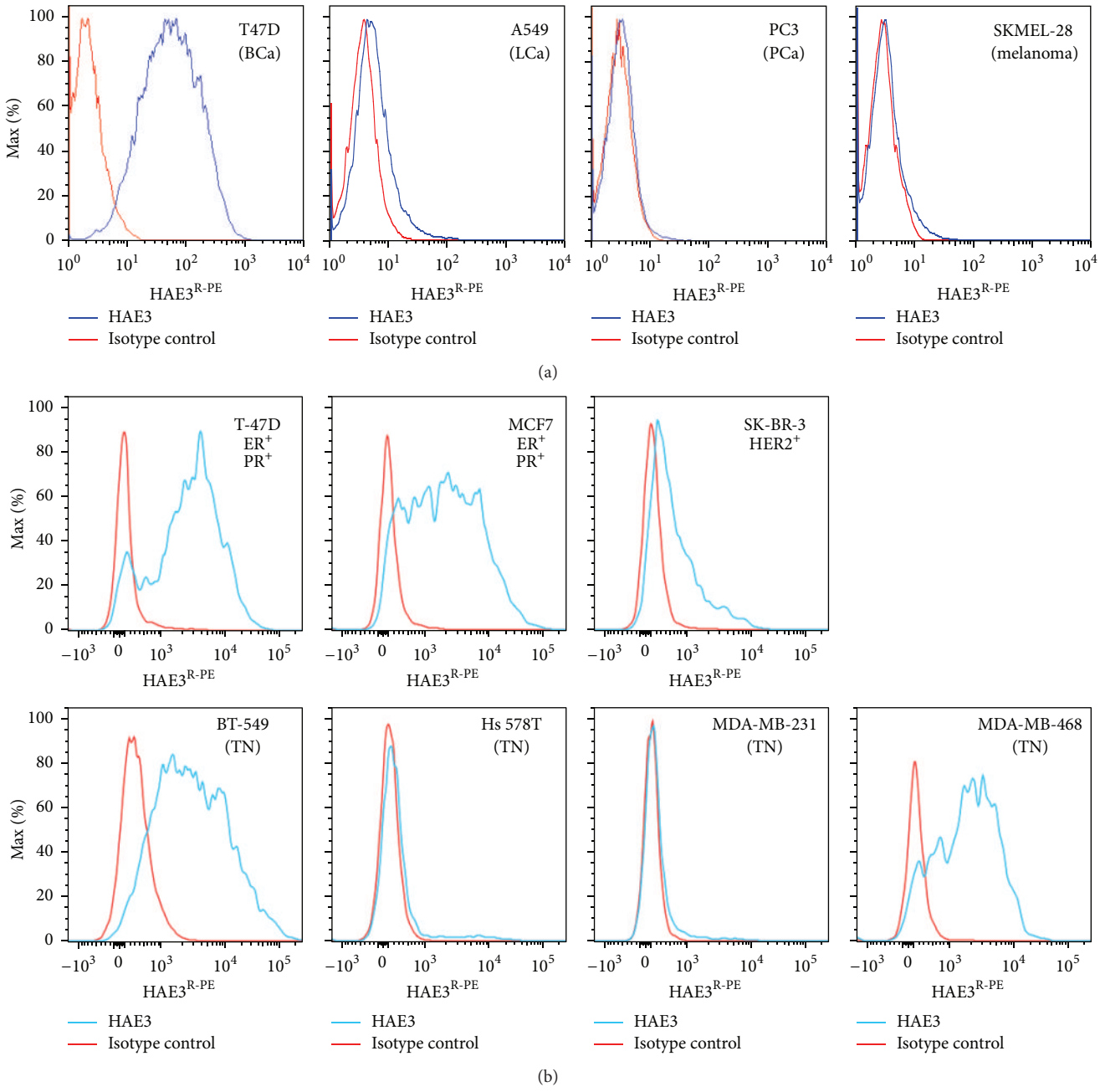


FIGURE 3: HAE3 cell surface staining detected selective expression of the HAE3-cryptic glycan markers in human cancer cell lines. (a) Four tumor cell lines, T-47D, A549, PC3, and SKMEL-28, were stained with the C1 preparation of HAE3 (IgM) at 1 : 6 dilution or with an isotype control IgM, 9.14.7 (5.0 μg/mL). (b) Seven breast cancer cell lines were stained with purified mAb HAE3 (5.0 μg/mL) or 9.14.7 (5.0 μg/mL). These cell lines are T-47D, MCF-7, SK-BR-3, BT-549, Hs578T, MDA-MB-231, and MDA-MB-468. An R-PE-conjugated goat anti-mouse IgM antibody was applied to quantify the cell surface-captured IgM antibodies. *Blue line*: HAE3 stain; *Red line*: 9.14.7 IgM isotype control.

breast cancer and breast circulating tumor cells, especially in patients with triple-negative cancer cells that lack specific surface biomarkers.

Our carbohydrate microarray analysis has identified blood group precursor substances as the natural ligands of antibody HAE3. As shown in Figure 1, four well-characterized blood group precursor reference antigens, Beach P1 (29$^{\#}$), McDon P1 (30$^{\#}$), Tij II (31$^{\#}$), and OG (32$^{\#}$), were HAE3 positive. OG [19] and Tij II [26] antigens were prepared by pepsin digestion, ethanol precipitation, and solubilization in 90% phenol, followed by fractional ethanol precipitation from phenol. Beach P1 [27] and McDon P1 [28] were obtained as the nondialyzable *O*-cores from partially hydrolyzed blood group antigen, Beach B and McDon A1, respectively. Thus, these blood group precursor substances were prepared to eliminate peripheral glycoepitopes, such as A, B, H, or Lewis antigen-specific epitopes, but preserve their *O*-glycan core structures, leaving a number of cryptic

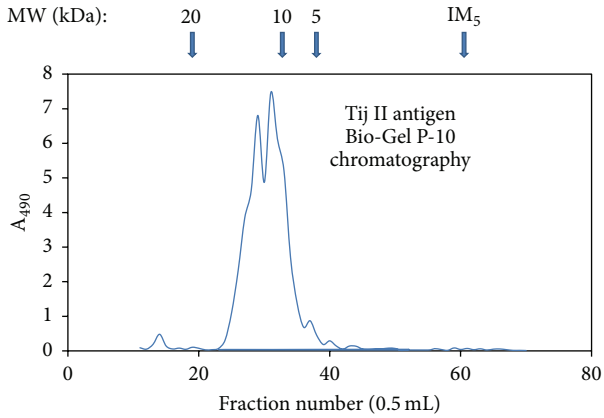


Figure 4: A Bio-Gel P-10 plot of Tij II antigen. Tij II (20% from 2nd 10%) substance was fractioned in a precalibrated Bio-Gel P-10 column at 0.5 mL per fraction. The neutral sugar content in each fraction was determined by phenol-sulfuric acid color reaction and quantitatively measured at OD490 nm. The sizes of the Tij II substance were measured based on the neutral sugar elution profile with reference to the calibrated saccharide molecular weight standards as indicated in the plot. IM_5 stands for isomaltopentaose.

O-core epitopes exposed for antibody recognition. Selective detection of these blood group precursors from a large panel of blood group substances by HAE3 demonstrated that this antibody is specific for a shared cryptic glycoepitope of these precursor substances.

The native blood group precursor substances are often more complex in carbohydrate structures than the most known model *O*-cores [29]. As determined by Bio-Gel P-10 filtration, the sizes of the Tij II blood group precursor substance were distributed in a range of molecular weights from 7 KDa to 15 KDa, approximately (Figure 4). Since this antigen contains only approximately 2% peptide sequences, its mass is apparently made up predominately of carbohydrates [26, 30, 31]. Figure 1(c) is a postulated blood group precursor core structure, which was proposed based on extensive glycan structural analyses and immunochemical studies of blood group substances [19–22]. The four-branched structure in the circle represents the internal portion of the carbohydrate moiety of blood group substances.

Chemical synthesis of such complex blood group precursors is technically challenging. However, it is not impossible to rationally design and produce HAE3-positive compounds by stabilizing relatively simple *O*-cores via specific structural modifications. For example, a recent microarray screening revealed an unpredicted binding of this antibody to a sulfated glycolipid SM1a, Galβ1-3GalNAcβ1-4(3-O-sulfate)Galβ1-4GlcCer [14], which can be viewed as an *O*-core derivative. SM1a naturally occurs in small amounts in normal kidney [32], but such a carbohydrate sequence has not been described in tumor glycome.

Tumor-associated overexpression of blood group-related autoantigens is not limited to breast cancer. Gao et al. recently reported that the natural ligand of a prostate cancer-specific mAb F77 is in fact blood group H, which is built on a 6-linked branch of a poly-N-acetyllactosamine backbone [24, 25]. Overexpression of gp^{F77} in prostate cancers may reflect increased blood group H expression together with upregulated expression of branching enzymes. As illustrated in Figures 1–3, HAE3 differs from F77 in glycan binding specificities and tumor binding profiles. Unlike F77, which is blood group H-specific and stains prostate cancer cell line PC3, HAE3 has neither reactivity with blood group H nor the cell surface targets of PC3.

Both HAE3 and F77 studies call our attention to epithelial tumor expression of blood group substance-related autoantigens. It is noteworthy that blood group substance antigens may also serve as the natural ligands of C-type lectin DC-SIGN, one of the key glycan-binding receptors of the conserved innate immune system [33–35]. Our preliminary data indicates that the HAE3-positive TijII antigen is likely a DC-SIGN ligand (data not shown). Potential of this class of tumor glycoantigens as costimulators of the immune cells in both innate and acquired immune systems for tumor vaccine development and targeted immunotherapy is yet to be explored.

Conflict of Interests

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

Disclaimer

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Research Article

Gingiva Equivalents Secrete Negligible Amounts of Key Chemokines Involved in Langerhans Cell Migration Compared to Skin Equivalents

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Both oral mucosa and skin have the capacity to maintain immune homeostasis or regulate immune responses upon environmental assault. Whereas much is known about key innate immune events in skin, little is known about oral mucosa. Comparative studies are limited due to the scarce supply of oral mucosa for ex vivo studies. Therefore, we used organotypic tissue equivalents (reconstructed epithelium on fibroblast-populated collagen hydrogel) to study cross talk between cells. Oral mucosa and skin equivalents were compared regarding secretion of cytokines and chemokines involved in LC migration and general inflammation. Basal secretion, representative of homeostasis, and also secretion after stimulation with TNFα, an allergen (cinnamaldehyde), or an irritant (SDS) were assessed. We found that proinflammatory IL-18 and chemokines CCL2, CCL20, and CXCL12, all involved in LC migration, were predominantly secreted by skin as compared to gingiva. Furthermore, CCL27 was predominantly secreted by skin whereas CCL28 was predominantly secreted by gingiva. In contrast, general inflammatory cytokines IL-6 and CXCL8 were secreted similarly by skin and gingiva. These results indicate that the cytokines and chemokines triggering innate immunity and LC migration are different in skin and gingiva. This differential regulation should be figured into novel therapy or vaccination strategies in the context of skin versus mucosa.

1. Introduction

Both oral mucosa and skin have the capacity to maintain immune homeostasis and regulate immune responses upon environmental assault. They both provide an important barrier and a first line of defence against, amongst others, toxic substances (allergens, irritants), pathogen invasion, and trauma. Whereas much is known about key innate and adaptive immune events in skin [1–3], relatively little is known in this respect about oral mucosa [4, 5].

Allergic and irritant contact dermatitis (ACD and ICD) are common pathological conditions arising in both the oral mucosa and skin and can be caused by an adverse reaction to chemicals [6–9]. The key biological events leading to skin sensitization and elicitation of ACD as well as the more general inflammatory events underlying ICD are well documented [6, 10–12]. ICD involves an innate immune response whereas sensitization and ACD-reactions involve an innate immune response which triggers an adaptive immune response. Both are caused by chemicals penetrating the outermost layer of the skin (stratum corneum) in order to reach the underlying viable epidermal cells. Keratinocytes (KC) are subsequently activated and release danger signals in the form of proinflammatory cytokines (e.g., TNFα, IL-1α, IL-18, and CCL27) [3, 6, 13–17]. These proinflammatory cytokines diffuse into the underlying dermis where they in

turn stimulate fibroblasts to secrete inflammatory mediators. Cytokines such as IL-6 and CXCL8 create a general inflammatory response stimulating the dermal infiltration of, for example, T cells, monocytes, macrophages, and neutrophils. Importantly, a chemotactic gradient is created which will enable allergen-exposed, maturing, $CXCR4^{hi}$ LC to migrate from the epidermis towards CXCL12 in the dermis, whereas irritant-exposed, nonmaturing, $CXCR4^{lo}$ LC will migrate towards CCL2/CCL5 in the dermis [18, 19]. Replenishment of immature LC into the skin is further regulated by CCL5 and CCL20 [20]. Thus, extensive cross talk between skin KC and fibroblasts and the differential chemokine receptor expression on maturing versus immature LC enable (a) LC migration from the epidermis into the dermis after allergen or irritant exposure and (b) subsequent replenishment of LC through recruitment of precursors to the epidermis [21].

Upon chemical allergen exposure, LC take up haptens, possibly complexed with proteins, and migrate via the dermis to the draining lymph nodes where they can initiate an adaptive immune response by presenting the hapten to T cells. Upon a second exposure to the same allergen, an adverse outcome in the form of ACD may be elicited [12]. If the chemical is an irritant however, LC migrating into the dermis can undergo a phenotypic change into a CD14/CD68 macrophage-like cell and remain in the dermis [22, 23]. Clearly skin KC and fibroblasts play key regulatory roles in the innate immune events of both ACD and ICD. In the oral cavity, allergen and irritant mediated inflammation can also occur and is known as contact stomatitis. Contact stomatitis can be caused by exposure to chemicals in toothpaste and mouth wash (e.g., the sensitizer cinnamaldehyde or the irritant SDS) or in dental medical devices (e.g., metals: palladium, nickel, and gold) [8, 24, 25]. However, very little is known about the innate immunological events that trigger these oral diseases. For instance, the cytokines and chemokines pivotal for LC activation and migration in oral mucosa are currently unknown. Evidence suggests that skin and oral mucosal tissues react differently to environmental insults. For example, CCL27, a general (pro)inflammatory chemokine, is predominantly secreted by skin KC [26] whereas its homologue CCL28 is predominantly secreted by mucosa KC [27, 28]. Both share the same receptor CCR10 which mediates T cell homing whereas CCL28 also binds to CCR3, which can mediate eosinophil and Th1 and Th2 recruitment [29]. Whereas CCL27 is implicated in inflammatory skin disease such as psoriasis [15], CCL28 has been shown to mediate migration of antibody secreting plasma cells to mucosal tissues [27, 28].

Notably, very clear differences are also found in the histology between skin and oral mucosa, of the epithelial tissue in particular. Gingiva epithelium demonstrates a highly proliferative, thicker epithelium compared to skin epithelium and also has different epithelial localization of keratins, SKALP, loricrin, and involucrin [29, 30]. With such distinct (immuno)histological differences between the two tissues, it can be expected that differences also exist between their innate responses to environment assault. The study in three-dimensional tissue explants of the differential regulation of innate immune responses and LC migration in homeostasis and disease between skin and oral mucosa is complicated by the scarcity of available oral mucosa samples as these are generally infected or inflamed and are only available as very small pieces (3–6 mm diameter biopsies). This means that extensive studies using excised oral mucosa cannot be carried out as we have done in the past, for example, the study of LC migration in skin [18–20, 23]. Furthermore, the use of intact biopsies, which contain many different cell types, makes it difficult to dissect specific cross talk between particular cell types.

In this study, in order to overcome the abovementioned limitations, we have employed tissue engineered oral mucosa and skin equivalents, consisting of a fully differentiated epithelium on a fibroblast-populated connective tissue matrix, to carry out comparative analyses of skin and oral gingival-derived cytokines and chemokines involved in LC migration, both in the steady state and after stimulation with proinflammatory cytokine TNFα, or upon topical exposure to a known allergen and irritant [17, 30–32].

2. Materials and Methods

2.1. Cell Culture. Human adult skin and gingiva were obtained after informed consent from patients undergoing abdominal dermolipectomy or wisdom tooth extraction, respectively, and used in an anonymous fashion in accordance with the "Code for Proper Use of Human Tissues" as formulated by the Dutch Federation of Medical Scientific Organizations (http://www.fmwv.nl/), following procedures approved by the Institutional Review Board of the VU University Medical Center. Skin and gingiva samples were not donor matched.

Epithelial KC. Adult skin and gingiva KC, isolated from 3–6 mm punch biopsies, were cultured under similar conditions essentially as described earlier [33]. KC were cultured at 37°C, 7.5% CO_2 in KC medium consisting of Dulbecco's Modified Eagle Medium (DMEM) (Lonza, Basel, Switzerland)/Ham's F-12 (Gibco, Grand Island, USA) (3 : 1), 1% Ultroser G (BioSepra S.A. Cergy-Saint-Christophe, France), 1% penicillin-streptomycin (Gibco), 1 μmol/L hydrocortisone, 1 μmol/L isoproterenol, and 0.1 μmol/L insulin and containing 1 ng/mL keratinocyte growth factor (KGF) for skin keratinocytes or epidermal growth factor (EGF) for gingival keratinocytes. Keratinocytes grew in colonies of proliferating and differentiating cells and were passaged when 90% confluent, using 0.5 mM EDTA/0.05% trypsin (Gibco), and used for experiments at passage 2. Importantly, the KC were kept in culture for the same period of time (10–12 days) to eliminate confounding culture aging effects.

Fibroblasts. Adult skin and gingiva fibroblasts were isolated and cultured under identical conditions. In short, fibroblasts were enzymatically isolated from 3–6 mm punch biopsies and were cultured in DMEM containing 1% Ultroser G, 1% penicillin-streptomycin at 37°C, and 5% CO_2 essentially as described previously [34]. Cultures were passaged when 90% confluent and used for experiments at passage 3. These fibroblast cultures are >99% CD90 positive (flow cytometry). Importantly, the skin and gingiva fibroblasts were cultured

for the same period of time (28–35 days) to eliminate confounding culture aging effects.

Skin Equivalent (SE) and Gingiva Equivalent (GE) Culture. Reconstruction of SE and GE was achieved by seeding KC (0.5×10^6 cells) onto fibroblast-populated collagen gels as previously described [32]. Cells were submerged for 3 days in KC medium (see above) containing 1 ng/mL EGF. To induce epithelial differentiation, the constructs were lifted to air-liquid interface and cultured for 14 days in KC medium containing 0.2% Ultroser G, 1×10^{-5} M L-carnitine, 1×10^{-2} M L-serine, 50 μg/mL ascorbic acid, and 2 ng/mL EGF. Unless otherwise stated, all additives were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Chemical Exposure. Finn Chamber filter paper discs of 11 mm diameter (Epitest, Oy, Finland) for SE or 03-150/38 gauze filters of 12 mm diameter (Sefar Nitex, Heiden, Switzerland) for GE were impregnated with the allergen cinnamaldehyde (CA) in 1% DMSO in water v/v or irritant SDS in water (Sigma Chemical Co.). The chemicals or vehicle (control) impregnated discs or gauzes were applied topically to the cultures for 24 h at 37°C, 7.5% CO_2 at nontoxic concentrations (>70% viability). Viability was determined by MTT assay as described previously [16].

2.2. Cytokine Exposure Experiments

Cell Monolayer Exposure to rhTNFα or rhIL-1α. Subconfluent fibroblast and KC monolayer cultures grown in 6-well plates were exposed to serial dilutions of rhTNFα or rhIL-1α (0, 100, or 200 International Units/mL) (Strathmann Biotech, Hamburg, Germany) for 4 h in 1.5 mL medium, after which the cells were washed with PBS and fresh culture medium (see above) was added. After 24 h the culture supernatant was harvested and stored at −20°C for ELISA analysis.

Tissue Equivalent Exposure to rhTNFα. SE and GE cultures were exposed to serial dilutions of rhTNFα or rhIL-1α (0, 100, or 200 International Units/mL) for 24 h in 1.5 mL medium. After 24 h the culture supernatant was harvested and stored at −20°C for ELISA analysis.

2.3. Immunohistochemical Staining. All procedures for paraffin embedded sections and immunohistochemical staining were performed as previously described [30, 31]. Primary monoclonal antibodies directed against keratin 10 (clone DE K10, ICN Biomedicals, Zoetermeer, The Netherlands) and loricrin (AF 62, BioLegend, San Diego, CA, USA) were used. All sections were counterstained with Mayer's haematoxylin. Negative controls were prepared by substituting the primary antibody with an isotype control antibody. For morphological analysis, haematoxylin and eosin staining was used. The sections were embedded in Aquatex.

2.4. ELISA. Duoset CCL2, CCL5, CCL20, CCL27, CCL28, IL-18, or CXCL12 development systems (R&D Systems), PeliKine compact kits IL-1α, IL-1β, IL-6, or CXCL8 development systems (Sanquin, Amsterdam, The Netherlands), and a TNFα high sensitive detection set were all used as described by the suppliers (R&D Systems).

2.5. Statistical Analysis. Statistical significance of differences between the unexposed and exposed equivalents, KC, or fibroblasts was calculated using a paired t-test. For comparisons between skin and gingiva, an unpaired t-test was used. Both tests used 2-way ANOVA followed by Dunnett's multiple comparison using GraphPad version 6.0.

3. Results

3.1. Tissue Engineered Full Thickness Skin and Gingiva Equivalents. In order to be able to study cytokine secretion in human skin and gingiva in a physiologically relevant model, full thickness skin and gingiva equivalents were constructed (Figure 1). Both skin and gingiva models consisted of a reconstructed epithelium (skin- or gingiva-derived KC) on a fibroblast- (skin- or gingiva-derived) populated collagen hydrogel, which served as the connective tissue matrix. The characteristic intrinsic properties of skin and gingiva are illustrated by the phenotypic differences observed in the epithelium. The SE epidermis consisted of a compact basal layer of KC, a spinous layer, a stratum granulosum, and a stratum corneum. Keratin 10 was strongly expressed in all suprabasal KC and loricrin was strongly expressed only in the stratum granulosum. In contrast, the GE epithelium showed a thicker multilayer of KC with increasing differentiation (flattening) of the KC away from the basal layer. The epithelium lacked a clearly defined stratum granulosum and a stratum corneum. Keratin 10 and loricrin were only intermittently expressed in the suprabasal KC. These results correlated closely to our previous published results for native skin and gingiva [30].

3.2. Basal Cytokine Secretion by Skin and Gingiva Keratinocytes and Fibroblasts. Homeostatic migration of immune cells including LC and their progenitors is regulated by basal secretion of cytokines and chemokines. Therefore, we first investigated basal secretion of proinflammatory mediators secreted by epithelial KC and tissue equivalents derived from skin and gingiva (Figure 2(a)). Skin and gingiva showed similar levels of secretion of IL-1α and IL-18. However, CCL27 secretion was more than 20-fold greater in SE compared to GE (with a similar trend for KC cultures), and CCL28 secretion was more than 10-fold greater in gingiva KC compared to skin KC. CCL28 secretion was below the detection limit of the ELISA in both SE and GE cultures.

Next we determined the basal secretion of inflammatory chemokines by skin and gingiva fibroblasts and tissue equivalents (Figure 2(b)). Notably the chemokine CXCL12 which is pivotal in mediating epidermis-to-dermis migration of maturing LC [18] was clearly secreted at greater levels by skin fibroblasts and SE as compared to gingiva fibroblasts and GE (more than a 7-fold difference between the tissue equivalents), whereas other chemokines involved in irritant induced immature LC migration (CCL2, CCL5 [19]) and LC

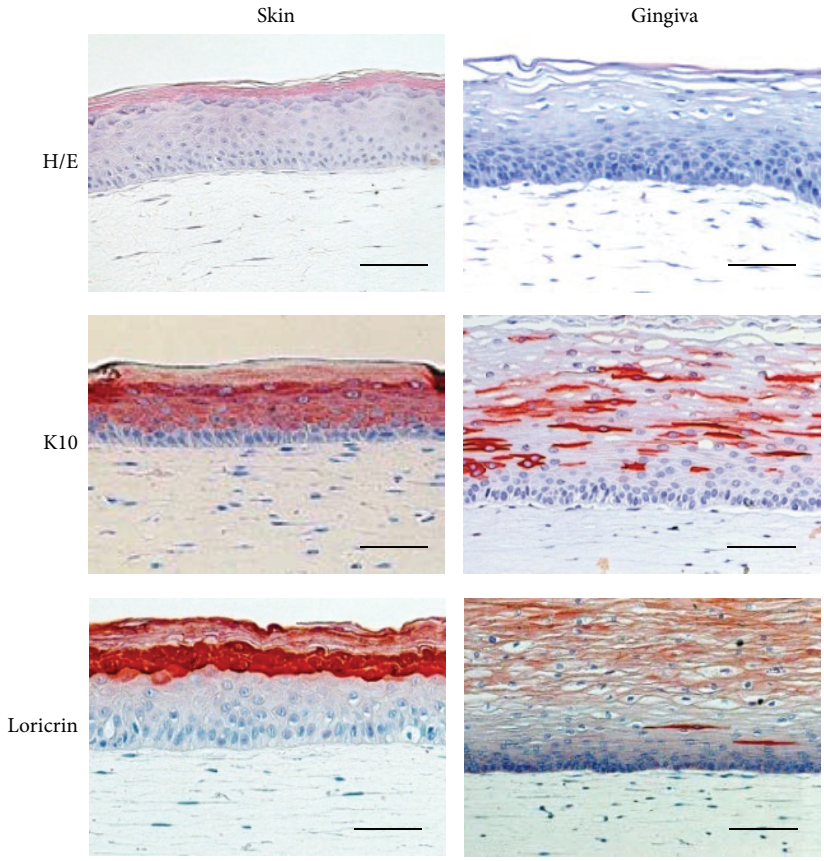


Figure 1: Full thickness tissue engineered skin and gingiva. Haematoxylin and eosin (H/E) staining and immunohistochemical analysis with keratin 10 (K10) and loricrin in tissue sections illustrate the different representative characteristics of skin and gingiva equivalents. Scale bar: 50 μm.

precursor immigration to the epidermis (CCL20 [20]) were secreted at similar levels by skin and gingiva. In contrast, the general inflammatory cytokines CXCL8 and IL-6 were secreted in larger amounts by gingiva fibroblasts than by skin fibroblasts (15-fold and 3-fold, resp.) although this difference was no longer apparent when comparing the tissue equivalents. Cytokines IL-1β and TNFα were not detectable in any of the culture models. These results indicate that skin and gingiva cells have different intrinsic capacities for basal steady-state secretion of (pro)inflammatory cytokines.

3.3. Differential Cytokine Secretion by Skin and Gingival Keratinocytes and Fibroblasts in Response to TNFα. TNFα is a major proinflammatory cytokine described to initiate LC migration and inflammatory responses [35]. Therefore, we next determined how rhTNFα influenced the (pro)inflammatory cytokine secretion by skin- and gingival-derived KC and fibroblasts. Supplementation of KC culture medium with rhTNFα only slightly increased secretion of IL-18 by skin KC and IL-1α and CCL28 by gingiva KC. Secretion of IL-18 and CCL27 remained higher from skin KC, whereas CCL28 secretion remained higher from gingiva KC (Figure 3(a)).

In contrast to KC, rhTNFα clearly caused a dose dependent increase in secretion of all six cytokines (CCL2, CCL5, CCL20, CXCL12, CXCL8, and IL-6) from both skin- and gingiva-derived fibroblasts (Figure 3(b)). Notably, in response to rhTNFα, three of the four chemokines reported to play a role in LC migration in skin were secreted more abundantly by skin fibroblasts than by gingiva fibroblasts (CCL2, CCL20, and CXCL12 but not CCL5). In contrast, the general inflammatory cytokines CXCL8 and IL-6 were secreted in similar amounts by both skin and gingiva fibroblasts in response to rhTNFα. Similar results were obtained when cultures were supplemented with rhIL-1-α (data not shown).

Taken together, these results clearly show differential proinflammatory cytokine-induced secretion of keratinocyte- (IL-18, CCL27, and CCL28) and fibroblast-derived chemokines (CCL2, CCL20, and CXCL12) between skin and gingiva.

3.4. Differential Cytokine Secretion by Skin and Gingiva Tissue Equivalents in Response to TNFα, Allergen, and Irritant. Upon topical exposure of the skin to a chemical irritant or allergen, a rhTNFα mediated innate inflammatory response is initiated [17]. In order to investigate this in more detail and importantly to determine similarities and differences between skin and gingiva in the context of cellular cross talk in a 3D tissue environment, tissue engineered full thickness equivalents were exposed to rhTNFα (in culture medium),

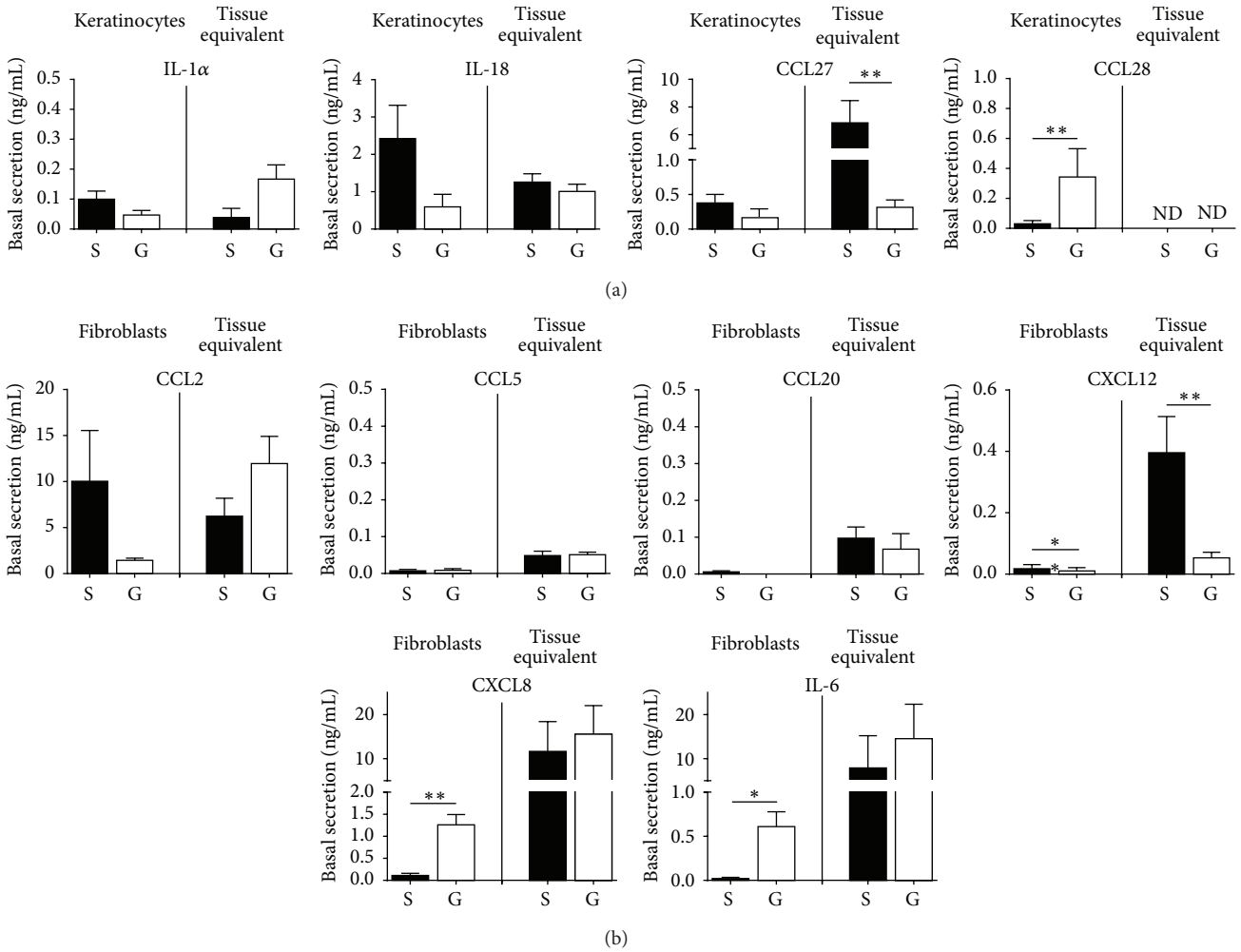


Figure 2: Basal cytokine and chemokine secretion by keratinocytes, fibroblasts, and tissue equivalents. (a) Cytokine/chemokine secretion by skin (S) and gingiva (G) keratinocytes and tissue equivalents. (b) Cytokine/chemokine secretion by skin and gingiva fibroblasts and tissue equivalents. Culture supernatants were collected over 24 h and analysed by ELISA. Data represent the average of at least 8 individual experiments ± SEM; $^{*}p < 0.05$; $^{**}p < 0.01$ (unpaired Student's t-test). ND = not detectable.

the irritant SDS (topical), or the allergen cinnamaldehyde (CA; topical) (Figure 4; Table 1). Notable differences were observed in the keratinocyte-derived proinflammatory cytokine and chemokine secretion profiles between skin and gingiva (Figure 4(a)). IL-18 showed a dose dependent increase in secretion only in response to the allergen CA in SE. This was not observed in GE, nor was it observed in SE or GE in response to rhTNFα or SDS. Chemokine CCL27, on the other hand, showed a dose dependent increase in secretion from SE in response to TNFα, SDS, and CA, but not from GE. IL-1α and CCL28 secretion were below the detection limit of the ELISA in all experimental conditions.

Next, fibroblast-derived chemokines described to play key roles in LC migration and recruitment were studied (Figure 4(b)). An increase in secretion of CCL2, CCL5, CCL20, and CXCL12 was observed in response to rhTNFα in both SE and GE. Notably CCL2, CCL20, and CXCL12 were secreted at higher levels by SE than by GE. Similarly, in response to noncytotoxic concentrations (>80% viable) of SDS, SE but not GE showed an increase in secretion of CCL20. Neither exposure to SDS nor exposure to CA resulted in an increase in secretion of CCL2, CCL5, or CXCL12 from the SE or GE. CXCL12 secretion (similarly to secretion of CCL20) was higher in SE as compared to GE. Cytokines involved in general inflammation (CXCL8, IL-6) showed a similar increase in secretion in response to rhTNFα in both SE and GE. However, secretion of these two cytokines was only increased by GE and not by SE when exposed to SDS or CA (Figure 4(c)). This increase resulted in a trend difference ($p < 0.06$) in IL-6 secretion between the two tissue types when exposed to CA.

Taken together, these results indicate that clear differences exist between skin and gingiva with regard to innate immune regulation by KC- and fibroblast-derived cytokines and chemokines. In particular, IL-18, CCL2, CCL20, and CXCL12, which mediate LC migration, are predominantly secreted by SE exposed to rhTNFα, the irritant SDS, or the allergen CA.

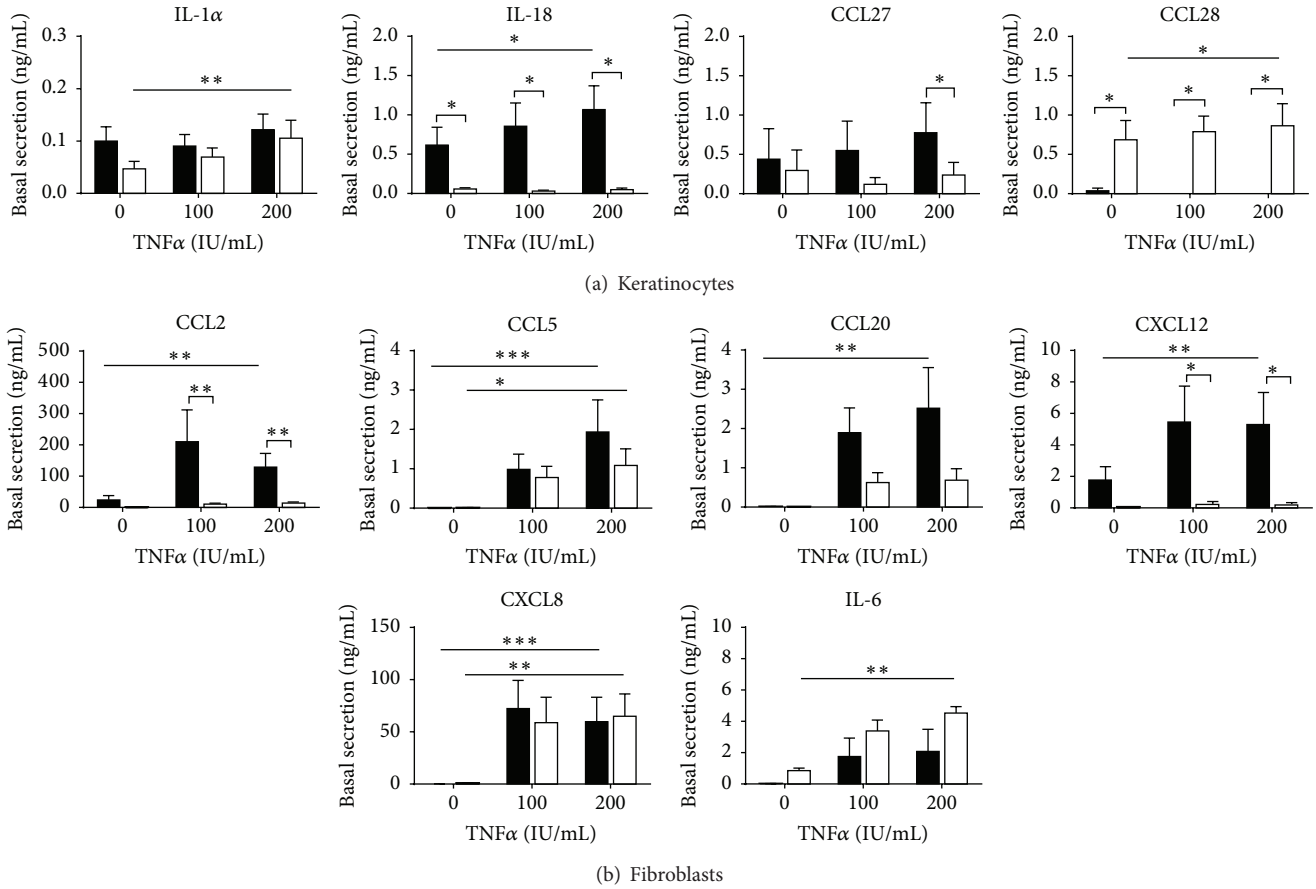


Figure 3: Differential cytokine and chemokine secretion by keratinocytes and fibroblasts in response to rhTNFα. (a) Skin compared to gingiva keratinocytes; (b) skin compared to gingiva fibroblasts. Cultures were unexposed or exposed to rhTNFα (100 and 200 IU/mL) for 24 h in the culture medium. Culture supernatants were collected and analysed by ELISA. Data represent the average of at least 3 individual experiments $\pm$ SEM; $^{*}p < 0.05$; $^{**}p < 0.01$; paired Student's t-test for intraskin and intragingiva comparisons, unpaired Student's t-test for skin versus gingival comparisons.

4. Discussion

In this study, we made an extensive comparison of oral mucosa and skin with regard to release of cytokines and chemokines involved in LC migration, as well as general inflammation. In order to do this, we used in addition to conventional KC and fibroblast cultures physiologically relevant 3D tissue engineered skin and gingiva equivalents. Environmental assault was mimicked by exposing cultures to the proinflammatory cytokine TNFα via the culture medium, or by topically exposing the epithelium to a chemical allergen (CA) and a chemical irritant (SDS). We found that key chemokines described to be responsible for LC migration in skin, that is, IL-18, CCL2, CCL20, and, most notably, CXCL12, were clearly secreted at higher levels by skin as compared to gingiva, suggesting that different and as yet unknown innate mechanisms are involved in mediating and controlling LC migration in gingiva.

Proinflammatory IL-18 together with IL-1β and TNFα is necessary for skin LC to migrate from the epidermis [13, 14]. IL-18 has been shown to play a vital and early role in the induction of allergic contact sensitization [13, 36]. Indeed, IL-18 is now an accepted biomarker in *in vitro* assays to identify and discriminate contact allergens from respiratory sensitizers and irritants [16, 37]. Here we show in line with these findings that IL-18 secretion is only increased in SE exposed to the allergen CA and is not increased by the irritant SDS. However, remarkably, we show that this cytokine is hardly secreted by gingiva in our study. In all our experimental conditions, a very low, noninducible amount was detected in gingiva culture supernatants compared to skin, suggesting that IL-18 is not required to mobilize LC in gingiva. Indeed endogenous IL-18 in experimentally induced asthma was found to be irrelevant for clinical symptoms, and therefore our finding that IL-18 may not be required to mobilize gingiva LC may possibly refer to other mucosal tissues as well [38]. In contrast to our results, it was previously shown that bioactive IL-18 was detected in the supernatant of human oral epithelial cells upon combined stimulation with neutrophil proteinase 3 (PR3) and LPS after IFNγ-priming [39]. This would suggest that IL-18 may be inducible by pathogenic but not by chemical allergen stimuli in the oral cavity.

Table 1: Summary of results obtained after exposing skin and gingiva tissue equivalents to TNFα, an allergen (CA), or an irritant (SDS).

Experimental condition	Dose response within tissue[a] Skin	Gingiva	Dose response between tissues SE > GE[b]	2 comparisons between tissues[c]
		Proinflammatory cytokines		
IL-18				
TNFα	ns	ns	ns	ns
SDS	ns	ns	ns	ns
CA	**	ns	**	**SE > GE
CCL27				
TNFα	*	ns	ns	ns
SDS	ns	ns	*	*SE > GE
CA	ns[d]	ns	*	*SE > GE
		LC migration chemokines		
CCL2				
TNFα	*	*	ns	*SE > GE
SDS	ns	ns	ns	ns
CA	ns	ns	ns	ns
CCL5				
TNFα	ns	ns	ns	ns
SDS	ns	ns	ns	ns
CA	ns	ns	ns	ns
CCL20				
TNFα	*	ns	ns	*SE > GE
SDS	**	ns	***	**SE > GE
CA	ns	ns	ns	ns
CXCL12				
TNFα	*	ns	*	*SE > GE
SDS	ns	ns	*	*SE > GE
CA	ns	ns	*	*SE > GE
		General inflammatory mediators		
CXCL8				
TNFα	***	***	ns	ns
SDS	ns	*	ns	ns
CA	**ns**	ns	ns	ns
IL-6				
TNFα	**	*	ns	ns
SDS	ns	*	ns	ns
CA	ns	*	ns	ns (0.06 GE > SE)

[a]Dose response within tissue: statistical significance of differences between the unexposed and exposed equivalents, KC, or fibroblasts was calculated using a paired t-test.

[b]Dose response between tissues: SE > GE. For comparisons between dose responses of skin and gingiva, an unpaired t-test was used.

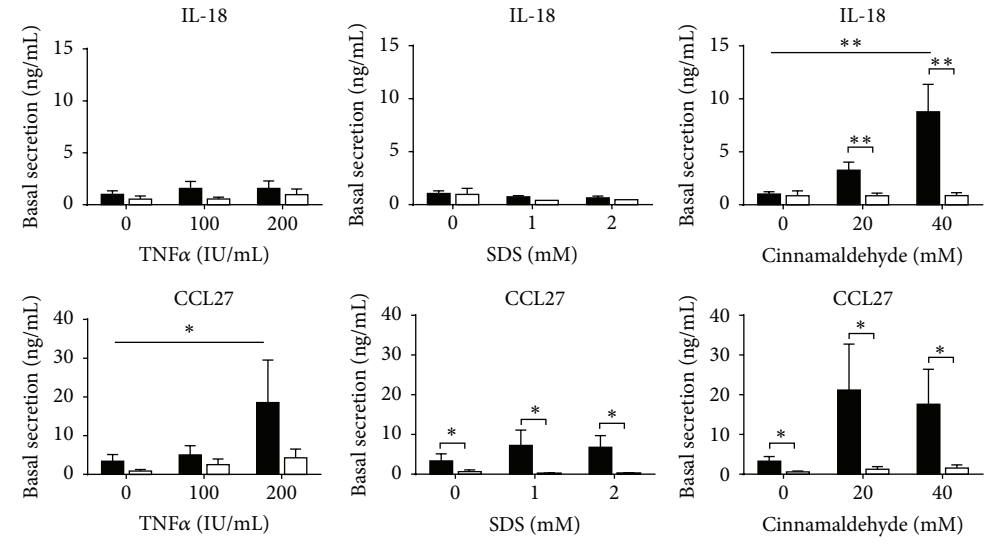
[c]Two single comparisons between tissues (unpaired t-test).

[d]Skin/CCL27: significant difference between 0 and 20 mM CA ($p < 0.05$; paired t-test) but not in entire dose response.

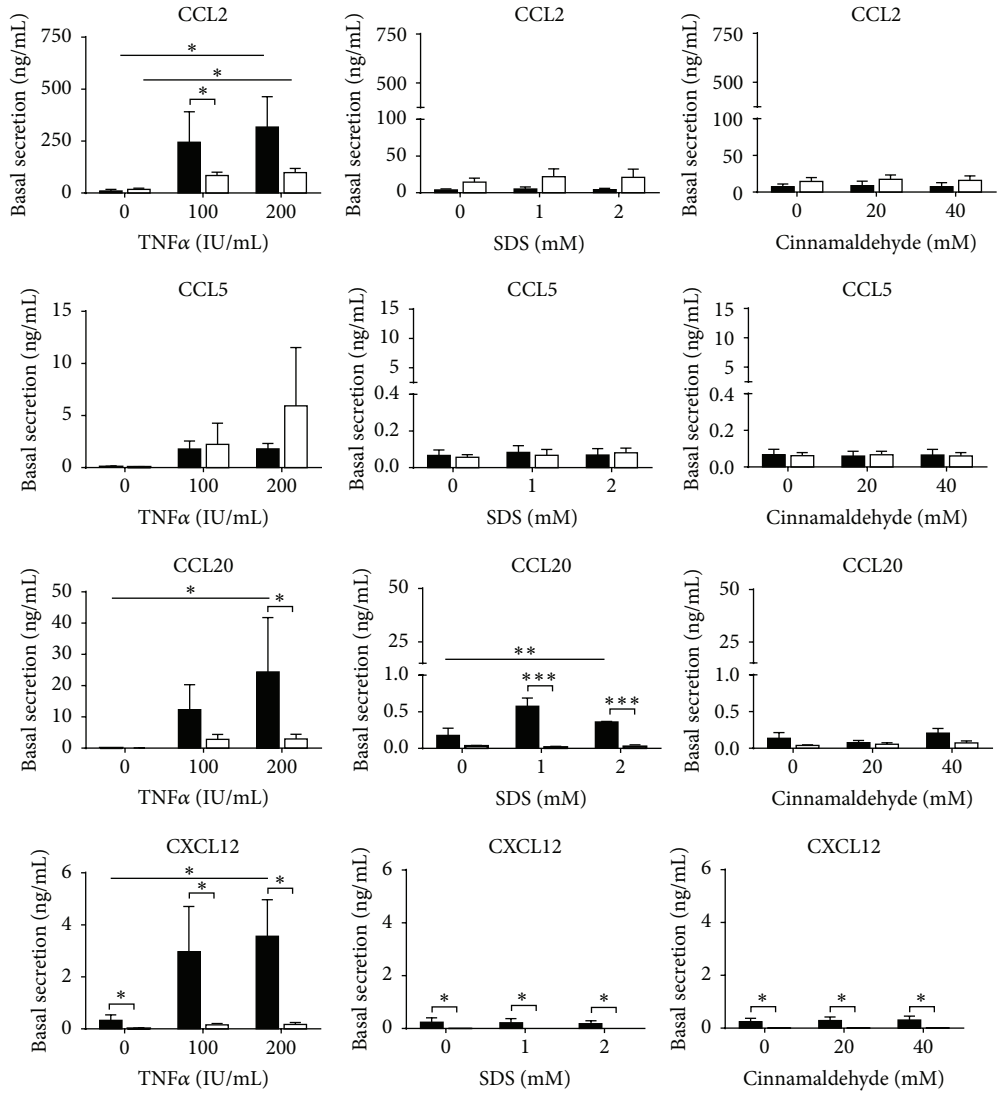
Tests used 2-way ANOVA followed by Dunnett's multiple comparison using GraphPad version 6.0, Data represent the average of at least 3 individual experiments ± SEM $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Previously we have shown that CXCL12 is a key chemokine in mediating migration of maturing LC from epidermis to dermis [18], CCL2 and CCL5 are key chemokines in mediating LC migration after irritant exposure [19], and CCL5 and CCL20 are involved in LC replenishment in the epidermis [20]. Our finding that CCL2 and especially CXCL12 are predominantly secreted by skin as compared to gingiva in response to rhTNFα (and for CXCL12 also in response to chemical exposure) indicates a clear difference in mechanisms regulating LC migration from the epithelium to connective tissue in skin as compared to gingiva. This difference in chemokines regulating LC migration is further supported by the finding that CCL20 was also significantly higher in skin after rhTNFα and SDS exposure. Only CCL5 was secreted at similar levels in skin and gingiva. Although it is clear that oral LC do have the ability to migrate from the epithelium into the lamina propria upon environmental assault [4, 40, 41], the underlying mechanisms are still unknown and our results suggest that they may differ considerably from those found in skin.

The tissue engineered skin and gingiva equivalents, and in particular the reconstructed epithelium, used in this study, closely represented their native counterparts. Not only the histology but also keratin and loricrin expression mimicked

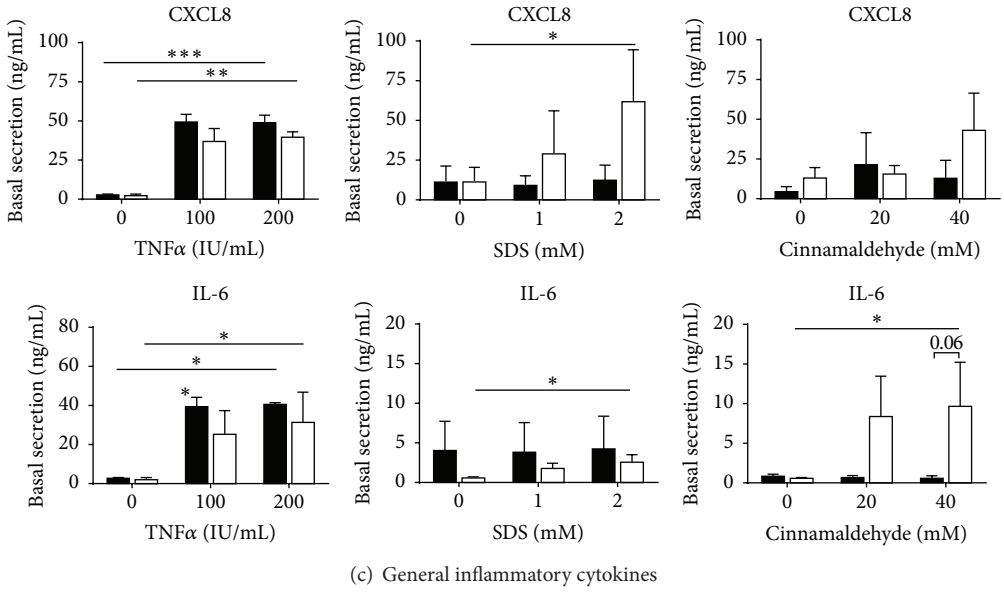


(a) Proinflammatory cytokines



(b) LC migration chemokines

Figure 4: Continued.



(c) General inflammatory cytokines

Figure 4: Differential cytokine and chemokine secretion by skin and gingiva equivalents in response to rhTNFα, irritant SDS, and allergenic cinnamaldehyde. (a) Proinflammatory cytokine secretion; (b) Langerhans cell (LC) migration related chemokine secretion; (c) general inflammatory cytokine secretion. Cultures were unexposed or exposed to rhTNFα (culture medium exposure; 100 and 200 IU/mL), SDS (topical exposure; 1 and 2 mM), or cinnamaldehyde (topical exposure; 20 and 40 mM) for 24 h. Cytotoxicity as assessed by MTT assay was <20% at the highest chemical concentration. Culture supernatants were collected and analysed by ELISA. Data represent the average of at least 3 individual experiments ± SEM; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; paired Student's t-test for intraskin and intragingiva comparisons, unpaired Student's t-test for skin versus gingival comparisons.

the different types of epithelium in line with our previous studies [30, 31]. Our current results show that basal cytokine secretion is also different in these two tissues. CCL27 was predominantly secreted by skin KC whereas CCL28 was predominantly secreted by gingiva KC in line with studies using patient derived biopsies and biological samples [26–28, 42]. Notably, CCL28 was only detected in gingiva KC cultures and not in GE, even when stimulated with rhTNFα, indicating that CCL28 may possibly be directly involved in cross talk between gingiva keratinocytes and fibroblasts, with possible consumption by fibroblasts in the GE accounting for the observed lack of detectable levels therein. Indeed, previously we have shown that the skin homologue CCL27 has proinflammatory properties and that it can stimulate adipose derived stromal cells to secrete VEGF, CXCL1, CXCL8, and IL-6 [26].

Whereas differential secretion was observed for cytokines involved in LC migration, this was not the case for the general inflammatory mediators CXCL8 and IL-6 which were upregulated by rhTNFα in skin and gingiva to a similar extent. CA and SDS did however result in a dose dependent increase only in GE. This indicates that mechanisms controlling general inflammation are different from those controlling LC migration and require further investigation.

In conclusion, our results indicate that the cytokines and chemokines involved in triggering and mediating LC migration and the innate immune response are different in skin and gingiva. Since extensive cross talk between keratinocytes, fibroblasts, and LC may direct and control LC migration, in future studies physiologically relevant immune competent skin and gingiva models with integrated LC may be used to investigate this further in a fully defined and standardized manner [32, 43].

Abbreviations

CA: Cinnamaldehyde
GE: Gingiva equivalent
KC: Keratinocyte
SDS: Sodium dodecyl sulfate
SE: Skin equivalent.

Conflict of Interests

Susan Gibbs is cofounder of A-Skin^{BV} which is a university spin-off company (SME) of the VU University Medical Center. The other authors have no conflict of interests.

Acknowledgment

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Review Article

Gamma Delta ($\gamma\delta$) T Cells and Their Involvement in Behçet's Disease

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Behçet's disease (BD) is a multisystem inflammatory disorder characterized by orogenital ulcerations, ocular manifestations, arthritis, and vasculitis. The disease follows a relapsing-remitting course and its pathogenesis is unknown. Genetic predisposition and immune-dysregulation involving gamma delta ($\gamma\delta$) T cells are reported to have a role. $\gamma\delta$ T cells are atypical T cells, which represent a small proportion of total lymphocytes. They have features of both innate and adaptive immunity and express characteristics of conventional T cells, natural killer cells, and myeloid antigen presenting cells. These unconventional T cells are found in the inflammatory BD lesions and have been suggested to be responsible for inducing and/or maintaining the proinflammatory environment characteristic of the disease. Over the last 20 years there has been much interest in the role of $\gamma\delta$ T cells in BD. We review the literature and discuss the roles that $\gamma\delta$ T cells may play in BD pathogenesis.

1. Behçet's Disease

Behçet's disease (BD) is a multisystem inflammatory disorder characterised by relapsing episodes of orogenital ulceration, ocular inflammation, and skin and joint lesions in association with other manifestations including vascular, gastrointestinal, and neurological involvement [1, 2]. BD occurs most frequently across the ancient trading (silk) route stretching between the Mediterranean, Middle East, and far East-Asia [2, 3]. The diagnosis is clinically supported by International Study Group for Behçet's Disease (ICBD) criteria, 1990 [4], and recently revised 2014 criteria [5]. Treatment is based on a combination of topical and systemic immunomodulatory agents [6], but they are by no means a cure.

1.1. Pathogenesis of Behçet's Disease. Aetiology of BD is thought to be a combination of several factors. The current consensus suggests that the pathogenesis may be triggered by an environmental agent in a genetically susceptible host [7, 8]. There is a strong association between HLA-B∗51 and BD suggesting a genetic predisposition. Recent GWAS studies indicated new susceptibility loci for BD, namely, CCR1-CCR3, STAT4, KLRK1-KLRC4, and ERAP1 [9]. Early hypotheses suggested a trigger mechanism focusing on infectious aetiology with bacterial/viral infections and molecular mimicry via heat shock proteins (see below) [10, 11], while current studies focus on immunodysregulation. Here we have reviewed the role of gamma delta T cells in BD.

2. Gamma Delta T Cells

Gamma delta ($\gamma\delta$) T cells are a minor population (~0.5–5% of total blood) of T cells expressing TCR γ and δ chain. These cells play a significant contribution to overall T cell function [12]. They have roles in the first line of defence against several microbial infections including malaria and Tuberculosis (TB), immune-surveillance of cancer, and immunoregulation. The $\gamma\delta$ T cell functions which may be relevant to the pathogenesis of BD are their ability to recognise qualitatively distinct antigens, to protect different sites of body, and their ability to mediate and modulate responses to specific pathogens. This functional diversity and plasticity make them important in diseases including Behçet's where different bodily compartments are affected.

2.1. Unique Characteristics of Gamma Delta T Cells. Human $\gamma\delta$ T cells are generally divided into Vδ1 and Vδ2 subset. Vδ1 is the predominant tissue resident cells whereas Vδ2 is the major subset in peripheral blood [13] which are not found in mice [14]. These subsets are almost exclusively coexpressed with TCR Vγ9 chain and commonly called Vγ9Vδ2. They are unique in their recognition of low-molecular-weight nonpeptide phosphoantigens, for example, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), an intermediate metabolite of mevalonate pathway, and expand rapidly in response to a wide variety of pathogens. The intermediate isopentenyl pyrophosphate (IPP) also selectively activates these cells. Unlike conventional $\alpha\beta$ T cells, these atypical prototypes have demonstrated characteristics of T cells, natural killer (NK) cells, and myeloid antigen presenting cells. *In vitro* studies have suggested that phosphoantigen activated Vδ2 T cells expressed a repertoire of antigen presentation and costimulatory molecules including HLA-DR, CD80, CD86, CD40, and CD54. Such antigen presenting phenotypes could in turn prime $\alpha\beta$ T cells to induce strong adaptive responses [12, 15]. These cells interact with dendritic cells (DCs) to regulate their function and mutually promote each other's maturation. Activated $\gamma\delta$ T cells can also produce high levels of IFNγ, TNFα, Granzymes, and IL17 reflecting their role in the effector phase of immune response and also have a regulatory role. Furthermore, pattern recognition receptors (PRRs) such as Toll Like Receptors (TLRs) can enhance their function either directly or via DC activation [16].

Migration properties of $\gamma\delta$ T cells also differ significantly from $\alpha\beta$ T cells [17]. More than 80% of Vγ9Vδ2 cells are excluded from secondary lymphoid tissues such as lymph nodes (LNs) and Peyer's patches lacking CCR7 which is exclusively expressed by $\alpha\beta$ T cells. These cells however display inflammatory migration profile instead and this is a characteristic shared by granulocytes, monocytes, immature DCs, and NK cells. Above all, they are highly efficient in providing help for B cells for antibody production including IgM, IgG, and IgA [18]. They express costimulatory molecules such as inducible T cell costimulatory molecule (ICOS), CD40, secrete IL-2, IL-4, and IL-10, and thereby have potential roles in autoimmune and chronic inflammatory diseases apart from their anti-infection and antitumour effects. However, their role in BD pathogenesis is still inconclusive.

3. Gamma Delta T Cells and Behçet's Disease

3.1. Increased Gamma Delta T Cells in Behçet's Disease. The relationship between $\gamma\delta$ T cells and BD was first documented in early 1990s when a cohort of BD patients were noted to have higher levels of $\gamma\delta$ T cells in the peripheral blood mononuclear cells (PBMCs) [19, 20]. However, these findings were not solely specific to BD as similar observations were reported in Systemic Lupus Erythematosus (SLE) but were important enough to suggest a potential role of these cells in the disease. Fortune et al. also noted that a significant increase of $\gamma\delta$ cells was confined to BD patients with inflammatory arthritis but not the ocular and mucocutaneous group of patients. Later, it was suggested that per-cell activity of $\gamma\delta$ T cells rather than total number is an important factor in BD mechanism [21]. An increased percentage of these cells, in an activated state, were found capable of secreting cytokines such as IFNγ and TNFα and thereby might induce the proinflammatory environment observed in the clinical disease [22].

There are at least eight functional Vγ genes and Vδ transcripts; however Vγ9Vδ2 are reported to be the main $\gamma\delta$ subtype in human peripheral blood [23, 24]. Increased frequency of this subset has been found in PBMCs of BD patients [25, 26], whereas in another study, the highest restriction of Vδ3 usage was found [27]. An increase in Vδ1 T cells in cerebrospinal fluid of BD patients with active neurological disease has also been demonstrated [28].

Vδ1 is the second major subset of human $\gamma\delta$ T cells which are mainly located in the epithelia and interact with cells expressing MHC class I polypeptide-related sequences A and B (MICA and MICB) through natural killer group 2 member D (NKG2D) activating receptors [29]. $\gamma\delta$ T cells work as part of the innate immune response to invading microorganisms by recognizing these invariant molecules. They are thought to influence the nature of the adaptive immune response by secreting IL-4 or IFNγ, thus regulating the preferential emergence of Th2 and Th1 $CD4^+$ T cells, respectively. In addition, they secrete growth factors essential in maintaining mucosal homeostasis. In this regard, a surprising high frequency of Vδ1 in the peripheral blood as well as in the mucosal disease group has been observed [23]. Presence of all three Vδ chains within BD oral lesions indicates that usage of Vδ chains may vary amongst BD patients and is suggestive of a polyclonal activation rather than oligoclonal one, which further suggests that these unique cells might be responding to a wide variety of antigenic and/or nonantigenic stimuli in BD. In addition, the variability of $\gamma\delta$ subset distribution may support the notion that different subsets of $\gamma\delta$ T cells might have different roles to play in disease pathogenesis but very little is known to date [30].

3.2. Conflicting Data. There is however conflicting data regarding the presence of this atypical cell population in peripheral blood of BD patients. While some groups [19, 20, 22, 28, 31] reported increased $\gamma\delta$ T cell number in BD, others [26, 32, 33] presented data with no significant increase. A recent study investigating a relatively higher number of BD patients ($n = 70$) has noted that $\gamma\delta$ T cells were only slightly increased in the blood with no statistical significance compared to healthy controls [34]. Yamashita et al., 1996, also observed an insignificant increase but it is perplexing that no further explanation is evident in the literature regarding these findings. Similarly, an increase in $\gamma\delta$ T cell expansion has been observed by some groups in active BD compared to inactive BD [20, 31, 32, 35] but there are reports that have found no differences [26, 31, 34]. On further examination of the proportion of individual subsets such as Vδ2, similar conflicting data was noted. All these discrepancies might be due to the activation status of the disease, as a reflection of local tissue inflammation compared to peripheral blood $\gamma\delta$ T cells. Such variation might be dependent on several other factors including disease severity, usage of medications such as immunomodulatory agents, and perhaps other variables,

namely, age, gender, ethnicity, and/or environmental factors which have already been found to influence the phenotypic and functional differences of peripheral $\gamma\delta$ T cells [13].

BD patients are most commonly treated with combinations of various immunomodulatory agents including corticosteroids, azathioprine, methotrexate, mycophenolate mofetil, colchicine, and pentoxifylline and biologics such as tumour necrosis factor alpha inhibitors (TNFα-inhibitors; Infliximab) were also found useful [36]. The effect of these immunomodulatory agents including pentoxifylline and Infliximab on $\gamma\delta$ T cells has been studied in BD patients and studies on pentoxifylline indicated that this medication can inhibit cell expansion, downregulate TNF receptor expression, and also inhibit perforin expression [37]. Infliximab also showed similar effects on $\gamma\delta$ T cells where it suppressed the production of IFNγ, perforin, and Granzyme A (GrA) *in vitro* [36]. Azathioprine was reported to ablate $\gamma\delta$ T cells (Vδ2 subtype) in Crohn's disease [38] but very little data is available from BD patients regarding the effect of this agent along with others. However, available data clearly suggest that medications influence $\gamma\delta$ T cells which may result in the discrepancies observed in BD studies. Vγ9Vδ2 subsets can express both activating (NKG2C/D) and inhibitory (CD94/NKG2A complex) MHC class I receptor along with CD16 which has significant functional implications including cellular proliferation and cytokine secretion [39]. In active BD, an increase of the activating receptor NKG2C and CD16 were observed; however another activating receptor, NKG2D, was found decreased [34]. Moreover, $\gamma\delta$ T cell expansion ratio showed conflicting data as restimulation failed to proliferate these cells which was reported earlier [26]. This suggests that, within BD patients, $\gamma\delta$ T cells are not a homogeneous population but are of heterogeneous spectrum. This is also supported by the phenotypic analysis of these cells, although very few reports are available, where greater variability was noted [25, 26, 35] and often the study becomes challenging with limited number of cells present.

Patients with BD can develop neurological manifestations [25] and it was found that an increased proportion of $\gamma\delta$ T cells is not linked to how long these patients have suffered from the disease. Similar findings by Ergun et al., 2001, showed comparable peripheral blood $\gamma\delta$ T cells count in BD and control groups but significantly increased $\gamma\delta$ T cells in the skin lesions of patients with BD [33]. It is indeed surprising that active and inactive BD do not always show significantly different proportions of $\gamma\delta$ T cells suggesting a qualitative rather than quantitative difference which may trigger the $\gamma\delta$ T cells in BD and those subpopulations may have different roles as suggested by the Yamashita group.

3.3. Triggers in Behçet's Disease and Gamma Delta T Cells. $\gamma\delta$ T cells respond to a wide variety of antigens [40] binding to several nonpeptides. It is possible that $\gamma\delta$ T cells undergo activation resulting in proliferation in response to the products of microorganisms present in BD patients' oral mucosal ulcers [31]. It has been postulated that, in BD patients, the flora of active oral ulcers, at least in part, drives the expansion of $\gamma\delta$ T cells. The oral microbiota of these patients is significantly populated by pathogenic *Streptococcus* strains including *S. sanguinis* and *S. mitis* [41, 42]. Interestingly, the expansion induced by oral ulcer microbial products involved Vδ2 subtype, not the Vδ1, which supports the findings of increased Vδ2 cells in BD patients. However, it has been argued that $\gamma\delta$ T cells in normal healthy individuals also expand in response to bacterial antigens, and thus the trigger for $\gamma\delta$ T cells in Behçet's to initiate the disease process remains inconclusive. However, other ligands for $\gamma\delta$ TCR such as heat shock proteins (HSP) could possibly be a trigger for initiating the disease process.

HSP are self-determinants expressed on proteins induced by stress. Cross reactivity between oral mucosal and microbial antigens has received considerable attention [43] where microbial heat shock proteins (mHSPs) showing sequence homology with human heat shock proteins (hHSPs) suggest that they may act as a trigger for inducing proinflammatory cytokine profile, characteristic of the disease [7]. Patients with BD respond to four HSP peptides including HSP65 related to *S. sanguinis* reactively present in BD patients sera and mucosal ulcers underpin a role for HSP in BD pathogenesis and a candidate ligand for $\gamma\delta$ T cells [16, 44]. Moreover, Stanford et al. demonstrated that an induction of tolerance against HSP was capable of ameliorating BD [41]. A BD specific peptide, p336–351, present within the hHSP60 initiated uveitis in rats and following tolerization, both animal model and human trial showed decreased expression of CCR5, CXCR3, CCR7, and costimulatory molecules including CD28 and CD40 by Th1 cells with little or no IFNγ and TNFα production and thereby preventing the initiation of BD uveitis. However, in contrast to these data, Vδ2 cells recovered from intraocular fluid of BD uveitis patients failed to demonstrate HSP65 reactivity but responded to nonpeptide antigens, IPP, which are released by damaged cells following infections including *Herpes simplex virus* (HSV) [32]. This again underlines the greater diversity of these cells in antigen recognition. Above all, compared to healthy individuals, BD cells responded to a significantly greater extent indicating previously primed cell population. Bank et al. also postulated that *in vivo* a second encounter with bacterial products or cross reactive autoantigens may lead to inappropriate activation of $\gamma\delta$ T cells, which after previous activation may have subsequently migrated in the PBMCs or lymphatic system [31]. Conversely Vδ2 cells in the periphery may have migrated to the mucosa in response to an antigenic exposure in the mouth and then priming other inflammatory cells at distant sites. This phenomenon is evident in Crohn's patients [45] giving rise to the question of whether BD is an *inside out or outside in* phenomena.

BD can be exacerbated following dental treatment and tonsillitis, suggesting abnormal mucosal immunity in these patients [25]. The distribution of $\gamma\delta$ T cells suggests that they play a pivotal role in mucosal immunity and thereby a major part in the first line of host defence [46]. This coincides well with that of the organ involvement of BD since ~90% of these patients firstly present oral ulceration which may precede the onset of other symptoms by many years [2]. Vγ9Vδ2 is the most studied subset of $\gamma\delta$ T cells that readily respond to infections and are found to be upregulated in patients with active disease. This may explain the clinical observations that

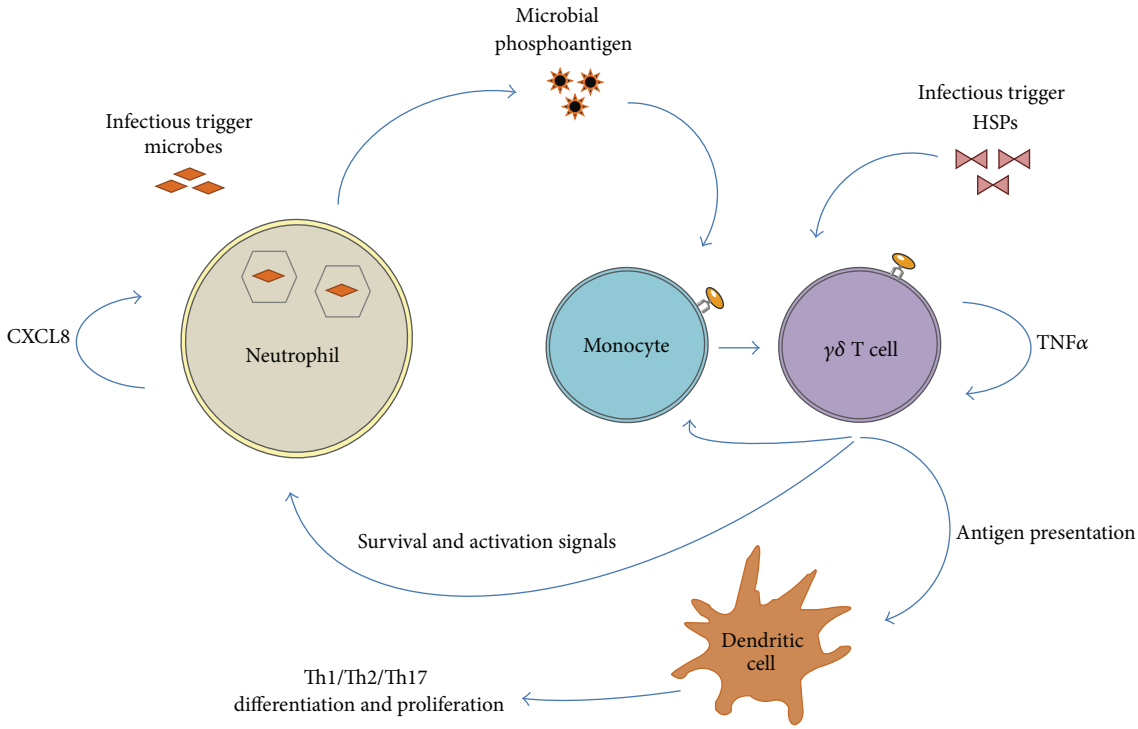

FIGURE 1: Schematic diagram of the potential interaction of neutrophils, monocytes, and DCs with γδ T cells in BD. An infectious trigger (e.g., microbes) results in extravasation of neutrophils and following phagocytosis of the invading microbes, neutrophils release traces of HMB-PP into the microenvironment where γδ T cells sense it. Monocytes then might take up or bind this soluble HMB-PP and present it to γδ T cells. This interaction triggers TNFα secretion, a proinflammatory cytokine along with other similar cytokines including IFNγ which promotes γδ T cell expansion and drive local chemokine (CXCL8) production that further recruits new neutrophils and monocytes to the site of infection. In addition, activated γδ T cells keep providing survival and activation signals to the newly recruited neutrophils and monocytes by secreting TNFα. Furthermore, activated γδ T cells present antigen to DCs and thus initiate Th1, Th2, and Th17 differentiation and proliferation. Even if the infectious trigger is in the form of a non-HMB-PP source such as HSP60/65, γδ T cells can again respond by expanding and keep the interaction active with the neighbouring cells.

BD activity is often triggered by infection. However, *in vivo* activation of Vδ1 subset was greater than that of the Vδ2 subset in HLA-B51-positive patients [47]. This finding again indicates that more than one γδ T cell subset may be responsible for disease activation suggesting a far more complex pathological mechanism. Indeed, several disease conditions including Rheumatoid Arthritis (RA), inflammatory bowel disease, psoriasis, and airway inflammation demonstrated that different subsets play different roles such as Vγ4 and Vγ1 subsets contributed towards these pathologies whereas they suppressed the development of diabetes in NOD mice and Experimental Autoimmune Encephalomyelitis (EAE) [30].

3.4. Immunodysregulation, Gamma Delta T Cells, and Behçet's Disease. γδ T cells have been shown to be a strong Th1 and Th17 inducers in experimental models [48] and the percentages of Th17 cells and IL17 have been found to be increased in BD [49]. There is now evidence that the cross talk between lymphocytes and neutrophils might be influenced by the IL17 axis [50]. It was also demonstrated that γδ T cells are able to establish effective interaction with neutrophils and monocytes in acute microbial infection responding to bacterial phosphoantigens [51]. Following phagocytosis of pathogenic microbes by neutrophils, γδ T cells recognize the bacterial end-product (HMB-PP), establish contact with monocytes, and produce proinflammatory cytokines including TNFα. As a result, local γδ T cells expand and release chemokines such as CXCL8 (IL8) which then recruits further neutrophils to the site of infection. Activated γδ T cells play a pivotal role in this interaction by providing survival and activation signals to newly recruited neutrophils and monocytes. This interaction between these cells may explain the persistent inflammatory symptoms of BD (Figure 1).

In addition, γδ T cells express Toll Like Receptors (TLRs 2, 3, 4, 7, 8, and 9) which can prime them to enhance their function [16] and importantly, BD patients have higher TLR expression [52, 53] with TLRs 2 and 4 were upregulated in both monocytes and buccal mucosal cells. In addition, novel splice variants were also expressed which influence the ability of cells to signal the presence of pathogen-associated molecular patterns (PAMPs). It is conceivable that this also occurs in γδ T cells and might represent a failure of the negative feedback loop that terminates the inflammatory process. Furthermore, activated TLRs were found on BD neutrophils following exposure to both HSPs and microbial antigens [54]. This indicates the possibility that neutrophils with activated TLRs may provide additional stimulatory signals to γδ T cells thus establishing a strong interaction with each other. In

addition suppressor of cytokine signalling (SOCS) proteins which negatively regulate the JAK-STAT signalling pathway of cytokine induction appears to be dysregulated in BD [55].

The term "autoinflammatory" disease [56] fits with BD more than "autoimmunity" as there seem to be apparently unprovoked recurrent inflammatory attacks with overexpression of proinflammatory cytokines and no significant autoantibodies. Neutrophils, a key initiator of classical autoinflammation, can go beyond their typical autoinflammatory roles to link the innate immune system with adaptive responses in BD by generating chemotactic signals (e.g., IL8/TNFα), expressing costimulatory molecules and releasing proinflammatory cytokines (e.g., IFNγ) [57]. Importantly, BD neutrophils were found preactivated [58] and thus might be initiating the intercell cross talk leading to persistent inflammatory response. Moreover, recognition of microbes by $\gamma\delta$ T cells may require the uptake of whole bacteria by monocytes, neutrophils, or DCs [59]. But the link between these cells with $\gamma\delta$ in BD has not been studied together in detail. The interplay of different subsets of $\gamma\delta$ T cells with associated innate and adaptive immune cells during different phases of the disease might be an important clue about the complexity of BD pathogenesis.

4. Conclusion

BD seems to be a far more complex disease than often anticipated. An abnormality in the innate immune response along with dysregulated adaptive immunity is likely to be triggering the disease process suggesting a complex interplay of the factors involved. There is evidence to suggest that $\gamma\delta$ T cells may play a crucial role in this process. The relationship of $\gamma\delta$ T cells and its surrounding milieu in BD patients may contribute to understanding the pathogenesis of this complex multisystem disease.

Conflict of Interests

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

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Review Article

IFN-γ Priming Effects on the Maintenance of Effector Memory CD4$^+$ T Cells and on Phagocyte Function: Evidences from Infectious Diseases

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Although it has been established that effector memory CD4$^+$ T cells play an important role in the protective immunity against chronic infections, little is known about the exact mechanisms responsible for their functioning and maintenance, as well as their effects on innate immune cells. Here we review recent data on the role of IFN-γ priming as a mechanism affecting both innate immune cells and effector memory CD4$^+$ T cells. Suboptimal concentrations of IFN-γ are seemingly crucial for the optimization of innate immune cell functions (including phagocytosis and destruction of reminiscent pathogens), as well as for the survival and functioning of effector memory CD4$^+$ T cells. Thus, IFN-γ priming can thus be considered an important bridge between innate and adaptive immunity.

1. Introduction

The immune system is continually exposed to a great diversity of pathogens. Among them, viruses, bacteria, protozoan parasites, and fungi present unique challenges for the host's immune system. In response to microorganisms, the adaptive immune system develops effector cells and functions capable of counteracting those threats. Among these effector cells, memory CD4$^+$ T (T_M) cells are considered a crucial population for the protective immunity against bacterial infections [1], viral infections [2], and protozoan infections such as malaria [3]. CD4$^+$ T_M cells participate in the responses against secondary infections by potentiating antipathogen effector mechanisms of innate immunity [4], antibody production, and CD8$^+$ T cell cytotoxicity [2].

In the past decades, however, it has become increasingly clear that the T_M population size is not a reliable marker of protective immunity per se. Zinkernagel and Hengartner previously argued that T_M cells could not provide protection against fast-dividing pathogens without the maintenance of highly responsive antigen-stimulated lymphocytes [5]. It was suggested that immunity, especially to chronic infection, is the combination of resting memory cells and activated effectors. The description of two distinct T_M cell subsets by Sallusto et al. [6] provides an additional basis for this hypothesis. Central memory T (T_{CM}) cells and effector memory T (T_{EM}) cells are classified based on their phenotype and their functional and trafficking characteristics [6, 7]. T_{CM} cells are defined by surface expression of CD62L and CCR7 molecules that allow these cells to circulate between secondary lymphoid tissues, entering the T cell zones [8]. In a T helper 1 (Th1) response, these cells produce IL-2 upon antigen reencounter and, later on, effector cytokines such as IFN-γ. T_{EM} cells, in contrast, have low expression of CD62L and

CCR7 and migrate and localize into nonlymphoid, antigen-targeted tissues, where they are capable to quickly produce effector cytokines such as IFN-γ upon antigen reexposure [9, 10].

T_{EM} cells have been considered the predominant population elicited by chronic infections [1, 10]. Therefore, the knowledge about the T_{EM} cell origin, function, and survival is critical for vaccine development. In some infections, T_{EM} cells maintain increased effector function; however, this may require the continued presence of antigen, which can also lead to T cell exhaustion. Alternatively, in the absence of antigen, the T_{CM} population may remain expanded but without prompt functionality [11]. Among the possible mechanisms by which antigen persistence can drive the functioning of T_{EM} cells, the effects of IFN-γ cannot be underestimated. This cytokine, as cited above, is one of the main products secreted by T_{EM} cells in response to secondary antigen encounter [9], and its effects on both T_{EM} cells and the effector branch of the immune system are still to be completely understood. In this review, we describe recent data on the role of IFN-γ on the protective immunity to infectious diseases with a special focus on the importance of the IFN-γ priming.

2. The Concept of IFN Priming and Its Effects on Acute Infectious Diseases

The effects of IFN-γ on the immune system are diverse, and the importance of this cytokine on the functioning of innate immune cells has been previously discussed [19]. Dendritic cells and macrophages are tightly regulated by cytokines to rapidly respond to infections and also to avoid the undesirable effects of excessive activation. Suboptimal concentrations of IFN-γ do not actually activate these cells but make them prepared for a subsequent response to stimuli, which in excess can eventually cause deleterious consequences. This effect is denominated as IFN-γ priming and has been increasingly implicated in the immune response to several infectious diseases such as viral [20, 21], bacterial [15, 22], and parasitical [15] infections. The underlying molecular mechanism for IFN-γ-priming effect involves a complex network of IFN-inducible genes, mostly from the innate immune system [22], whose understanding is still limited [17]. It is presumed that IFN-γ priming induces posttranscriptional and/or epigenetic changes, which are responsible for subsequent Toll-like receptor (TLR) ligand-triggered inflammatory response and classical macrophage activation [20, 21, 23, 24]. Recently, it has been shown that IFN-γ priming downregulates the expression of miR-3473b, a microRNA that suppresses macrophage activation and inflammatory response through directly targeting phosphatase and tensin homolog (PTEN) and promoting IL-10 production [25]. Of note, IL-10 has been shown to prevent the development of immunopathology during acute malaria [26, 27], as well as in *Toxoplasma gondii* [28] and *Trypanosoma cruzi* [29] infections. However, IL-10 promotes pathogen survival by downregulating protective immune responses during infections with *Mycobacterium tuberculosis* [30], *Bordetella pertussis* [31], and human immunodeficiency virus (HIV) [32]. The dual role of IL-10 is exemplified in *Leishmania major* infection, where IL-10 from effector Th1 cells is required to control excessive inflammatory response during acute infection [33], but IL-10 from regulatory T cells contributes to parasite persistence by suppressing effector Th1 cells during chronic infection [34, 35].

The IFN-γ priming seems to be particularly involved in several aspects of the immune response to malaria. McCall et al. (2007) showed that *Plasmodium falciparum* induces enhanced responses to TLR agonists in peripheral blood mononuclear cells [36]. This notion was further corroborated by findings on human subjects and mice, both acutely infected with *P. falciparum* and *Plasmodium chabaudi*, respectively [37], which showed an increased innate immune response to unrelated pathogens, in a TLR- and IFN-γ-dependent manner. Besides the effect of IFN-γ priming on TLR signaling, TLR engagement seems to be necessary for the initial IFN-γ production, as described for rodent malaria [38, 39]. Thus, it is likely that TLR signaling mediates initial pathogen recognition, which in turn initiates early IFN-γ production that further boosts the innate response through TLR induction. This mechanism is supported by results with malaria—as previously described [37]—and with several other infections by pathogens such as *Listeria monocytogenes* [40], *L. major* [41], *Chlamydia pneumonia* [42], *T. cruzi* [43], and *Legionella pneumophila* [44].

In acute infectious diseases, the augmented gene expression of TLR-related molecules induced by IFN-γ likely favors the pathogen recognition by phagocytic cells. Thus, a primed innate immune system can be of utmost importance to prevent or limit aggressive infections, contributing to the host survival. In contrast, a possible deleterious effect of this hypersensitivity can be inferred from the enhanced susceptibility of *P. chabaudi*-infected mice to LPS treatment [37, 45]. This was in fact demonstrated by the enhanced susceptibility of IFN-γ-primed mice to bacterial sepsis, which showed increased TNF production upon LPS stimulation [46]. Higher sensitivity to secondary infections by bacteria, such as *Salmonella*, has also been observed in human malaria [47]. Moreover, this hyperactivation of the immune system may contribute for the posterior state of immune paralysis observed in septic patients [48].

$CD4^+$ and $CD8^+$ T cells are also responsive and can be primed by type I IFNs, IFN-α/β, which are produced virtually by any cell type after stimulation [49]. Type I IFNs can be produced in large amounts by myeloid cells upon bacterial infection [50, 51], or by plasmacytoid dendritic cells upon viral stimulation [52, 53]. Production of these cytokines occurs following pathogen recognition by Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), and a growing family of intracellular DNA receptors, several of which promote signaling through stimulator of IFN genes (STING) [54, 55]. Similarly to IFN-γ, type I IFNs can drive preferentially the $CD4^+$ T cell differentiation to Th1 phenotype by activating Signal Transducer and Activator of Transcription (STAT) proteins that increase the T cell response to IL-2 [56]. These cytokines also inhibit the Th2 development by epigenetic silencing of GATA3 gene regulatory regions [57]. Interestingly, type I IFN

signaling inhibits IL-12 production [58, 59], which contrasts with the type I IFN effects on Th1 cell development. However, type I IFNs themselves also act as signal 3 cytokines during T cell activation—by promoting proliferation, survival, and effector cell differentiation [54]. Different from IFN-γ-induced priming that is always proinflammatory, the effects of type I IFNs on T cell function can be inhibitory in certain cases, especially when cytokine signaling precedes T cell receptor (TCR) engagement on T cells [54]. Optimal cross-priming of $CD8^+$ T cells is seemingly dependent on type I IFN stimulation [60]. Furthermore, type I IFNs activate T cells and sensitize them to *Listeria*-induced apoptosis [61].

3. IFN-γ Priming in Chronic Infections: Implications for Protective Immunity

IFN-γ is the main cytokine produced by T_{EM} cells committed for the Th1 phenotype ($Th1_{EM}$ cells) upon infection by microorganisms, and it is believed to play a major role in the activation of innate immune response [62]. Thus, it is reasonable to imagine that these T_{EM} cells are a major source of the IFN-γ responsible for priming the innate immune cells during chronic infections, making it an important point of crosstalk between innate and adaptive immunity. Supporting this possibility, we have recently described that the presence of $Th1_{EM}$ cells correlates with the continuous IFN-γ priming of innate immune cells during chronic malaria in mice [14]. This process is crucial for the protective immunity against reinfection with a heterologous strain of the parasite, which is not fully controlled by antibodies generated during primary infection with a different parasite clone. These findings help to explain why the immunity against *Plasmodium* is rapidly lost when the parasites are eliminated from the hosts, providing a molecular basis from strain-transcending immunity in human malaria [14, 63].

The innate immune effector mechanisms enhanced by IFN-γ priming are diverse, as pointed out by the high number of IFN-inducible genes upregulated in macrophages after *in vitro* IFN-γ priming [17, 64] or in mouse splenocytes during acute and chronic malaria [14, 37]. The biological significance of this priming is inferred from the genes expressed [14]. For instance, the upregulation of TLR-related and scavenger genes (such as CD36) possibly translates into an enhanced ability of innate immune cells to recognize and phagocytize circulating parasites, leading to an effective control of the disease [12, 45, 65]. It is important to note that an enhanced expression of TLRs facilitates the induction of the phagocytic program in innate immune cells. This was shown in chronic bacterial infections in which TLR3 and TLR9 expression leads to bacterial uptake by macrophages [13]. It is likely that IFN-γ secreted by $Th1_{EM}$ cells also primes the innate immune cells during viral infections in a manner similar to that observed in malaria. A potent $Th1_{EM}$ response is observed during infection with virus such as influenza [2, 66–68]. The IFN-γ priming of innate immune cells may ensure a rapid induction of the inflammatory response, as well as a state of refractoriness against viral proliferation in the surrounding tissues, which are important antiviral effector mechanisms [22, 69].

Besides busting proinflammatory responses, the IFN-γ priming associated with $Th1_{EM}$ cells may trigger feedback inhibitory loops, such as those mediated by IL-10, STAT3, and Suppressor of Cytokine Signaling 1 (SOCS1) [17]. The increased transcription of *stat3* gene, another IFN-inducible gene, in mouse splenocytes from chronic malaria indicates a tight control of the innate immune system during continuous IFN-γ priming [14]. The STAT3 is a transcriptional activator of *Il10* gene, and a consequent effect of IL-10 production is induction of tolerance mediated by antigen-presenting cells (APCs) [70, 71]. This fine-tuned process seems to be a common feature of TLR-mediated immune responses, since TLR agonists induce a state of late immune tolerance through the inhibition of the corresponding signaling pathways [72, 73]. Of note, mice with chronic malaria display $Th1_M$ cells coexpressing IFN-γ and IL-10, which are crucial for both the protective immunity to parasites and the protection against clinical manifestations of the disease [26]. A similar trend appears to happen in human malaria, since tolerance is often observed in patients from holoendemic areas [74]. The IFN-γ priming induced by $Th1_{EM}$ cells, thus, appears to be a fundamental mechanism for an efficient, though tightly regulated, protective immunity against chronic infections.

4. IFN-γ Priming Effects on $Th1_{EM}$ Cells

The population of $Th1_{EM}$ cells declines with time after infection in various experimental models of diseases caused by pathogens, such as *Plasmodium* [3], *Listeria* [75], and lymphocytic choriomeningitis virus (LCMV) [76, 77]. This observation suggests that the presence of prosurvival cytokines, such as IL-7 and IL-15, is not sufficient to maintain these cells [1]. On the other hand, large populations of specific $Th1_{EM}$ cells usually persist for long periods of time during phagosomal infections, such as those caused by *Salmonella enterica* [78], *M. tuberculosis* [79, 80], and *L. major* [81]. Likewise, the presence of pathogens ensures the perpetuation of $Th1_{EM}$ cells during polyomavirus infection [82] and malaria [3, 14]. Actually, the decline in the population of $Th1_{EM}$ cells along with chronic malaria is related to the progressive control of residual parasitemia [3]. It has been shown that $CD4^+$ T_{EM} cells have a rapid turnover in both human and mice [18, 83]. Thus, the continuous replenishment of this population may be induced by chronic infection, where pathogen antigens are available together with damage signals from injured tissues. In resume, the molecular signaling that is required for long-term persistence of $Th1_{EM}$ cells seems to be present during active infection and rapidly disappear after its resolution. It is possible that antigen persistence and, consequently, TCR: MHC- (major histocompatibility complex-) peptide complex interactions play a role by itself in the maintenance of $CD4^+$ T_M cells, and this has been a subject of interest for malaria [84] as well as for other infections [85, 86]. An interesting study on *Salmonella* infection in mice showed that peptide: MHC interaction in secondary lymphoid organs harboring bacteria for over 1 year after infection maintained the $CD4^+$ T_M cell population stable [78]. IFN-γ priming might further potentiate these interactions—of note, the increased $CD4^+$ T cell proliferation

in response to *Plasmodium* parasites and parasite antigens indicates that IFN-γ priming enhanced antigen presentation during chronic malaria [14]. However, whether this effect was due to higher MHC class II (or costimulatory molecule) expression on APCs was not directly addressed and is still a matter of discussion.

The generation and maintenance of $CD4^+$ and $CD8^+$ T_{EM} cells are facilitated by strong TCR engagement [87, 88], but other signaling pathways may be implicated in these processes. The kinase mammalian target of rapamycin (mTOR) induces in $CD8^+$ T cells a bias toward the glycolytic metabolism and the differentiation to effector functions [89, 90]. The STAT5-mediated IL-2 signaling pathway, a potent inhibitor of the Bcl-6 transcriptional factor and follicular T helper cell differentiation [91], promotes the expression of T-bet transcriptional factor in $CD4^+$ T cells [75]. At this respect, it has been shown that $Th1_{EM}$ cells have sustained expression of T-bet, both in humans [92] and mice [75]. T-bet upregulates IFN-γ production but is also an important target of IFN-γ signaling [93]. Therefore, IFN-γ is believed to induce—in conjunct with IL-12—IFN-γ production by Th1 cells. Thus, it is reasonable to hypothesize that IFN-γ plays a role in the generation and/or maintenance of $Th1_{EM}$ cells during chronic infections.

We have recently addressed the effects of IFN-γ priming on $Th1_{EM}$ cells in mice cured from chronic malaria in which the $Th1_{EM}$ cell response rapidly declines [14]. In these cured mice, administration of suboptimal doses of IFN-γ leads to a shift from T_{CM} cells to T_{EM} cells and restores the proliferative and IFN-γ responses to parasites and TLR agonists. This effect could result from the rescue of cross-reactive T_{CM} cells driven by *Plasmodium*-unrelated antigens, a phenomenon previously described in human malaria [94]. However, in our study, the shift to T_{EM} cells was specifically observed in previously infected mice, pointing out to a preferential activity of IFN-γ priming on malaria-specific cells [14]. The exact pathways involved in the IFN-γ priming effects on the generation and/or maintenance of $Th1_{EM}$ cells are still not well understood. It is likely that indirect signals derived from IFN-γ-primed innate immune cells play at least a partial role, and this is supported by the observation that TLR signaling is crucial for the maintenance of $Th1_{EM}$ cells [14]. However, a direct effect of IFN-γ priming on $Th1_{EM}$ cells cannot be excluded. $Th1_{EM}$ cells express the IFN receptor (IFNR) on their surface, and IFN-γ signaling helps to maintain the $Th1_{EM}$ phenotype [16]. The direct role of IFN-γ priming on $Th1_{EM}$ cells induced during chronic malaria is currently under investigation by our research group. Preliminary results showed a requirement of IFNR expression on $Th1_{EM}$ cells for their generation and maintenance (Borges da Silva, unpublished data).

Another infectious disease in which IFN-γ priming might be crucial for $Th1_{EM}$ cells is tuberculosis. Evidences from human disease and experimental mouse models show that IFN-γ produced by $CD4^+$ T cells is fundamental for *M. tuberculosis* control [95]. Importantly, expanded and sustained Th1 responses in the lungs are seemingly crucial for controlling chronic infection [96], making continuous IFN-γ priming possibly beneficial for bacterial clearance. However, in newborns vaccinated with Bacillus Calmette-Guérin (BCG) the IFN-γ production by Th1 cells did not correlate with disease protection [97].

Table 1: Effects of IFN-γ priming on innate immune cells and T_{EM} cells. The table summarizes the effects of IFN-γ priming on innate immune cells and on T_{EM} cells. The references relative to each function induced by priming are in parenthesis.

Innate immune cells	T_{EM} cells
↑ Phagocytosis [12, 13]	↑ Ag-specific proliferation [14]
↑ Antigen presentation [14, 15]	↑ T-bet expression [16]
↑ TLRs expression [14, 15, 17]	↑ Ag-driven IFN-γ production [14]
↓ Anergy [14, 15, 17]	↑ Population maintenance [14, 18]

In several infectious diseases, the pathogen persistence maintains short-lived effector T (T_{EFF}) cells alongside T_{EM} cells. This seems to be particularly true for Th1-driving infections. Of note, T-bet^+Ly6C^+ T_{EFF} cells present during chronic *L. major* cutaneous infection seem to be crucial for the protective immunity against reinfection and are proposed as major contributors for the state of concomitant immunity observed during chronic infections [98]. It is especially important to consider that the expression of Ly6C in T_{EFF} cells is directly under the control of T-bet, which, as explained above, can be driven by IFN-γ [76, 93]. Thus, it would not be surprising if IFN-γ priming acts also directly on the maintenance of the T_{EFF} cells as well, especially considering the need for infection persistence for their survival [98].

5. Concluding Remarks

The interplay between innate immune cells and $Th1_{EM}$ cells during chronic infections is seemingly complex, involving a crosstalk between innate immune cells and $Th1_{EM}$ cells. In this scenario, IFN-γ seems to play a crucial role as inducers of immune effector mechanisms in both sides, as exemplified in Table 1. Considering our current knowledge, the immune response to chronic infections might be defined as a circuit, where the two arms of the immune system (innate and acquired) constantly communicate with each other in order to achieve a tightly regulated, yet at most cases efficient, control of parasite load (Figure 1). To understand completely this relation, there is still the need to determine what all the "pieces in the puzzle" are, that is, to describe precisely all the aspects of the role of IFN-γ priming communication between T_M cells and the innate immune system. In the case of malaria, it will also be crucial to evaluate whether the observations in mouse models also hold true for humans, which are exposed to different degrees of reinfection with heterogeneous parasites. It is likely, though, that the importance of IFN-γ priming in strain-transcending immunity to malaria is a great starting point to explain, among other things, why it is so hard to achieve sterile immunity against *Plasmodium*; lowering the threshold for the activation of the host immune system could be a promising strategy for the improvement in the protective immunity against this parasite.

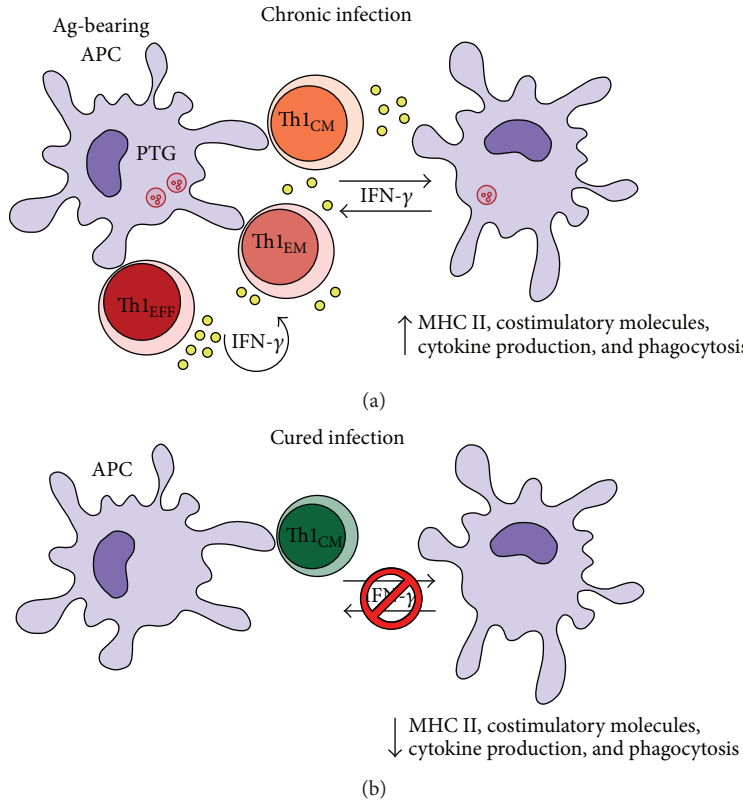

Figure 1: Schematic illustration to explain the IFN-γ priming effects on APCs and $Th1_{EFF}/Th1_{EM}$ cells during chronic infections. This figure explains how IFN-γ produced by $Th1_{EM}$ cells act on APCs (usually DCs) and directly on $CD4^+$ T cells during chronic infections. (a) When the pathogen is still present, antigen- (Ag-) bearing APCs activate $CD4^+$ T cells that produce small amounts of IFN-γ. These small amounts of IFN-γ are enough to maintain APCs poised for function, for example, phagocytosis, cytokine production, and antigen presentation. At the same time, IFN-γ acts directly on $CD4^+$ T cells and maintains the pool of $Th1_{EFF}/Th1_{EM}$ cells. Both effects culminate in enhanced immune system activation, cytokine production, and pathogen clearance. (b) After complete pathogen elimination, the IFN-γ priming on APCs and $Th1_{EFF}/Th1_{EM}$ cells ceases and, in consequence, these effector populations rapidly decline. The remaining $Th1_{CM}$ cells are important to control a secondary infection. However, in some infectious diseases such as malaria, continuous IFN-γ priming, and persistence of $Th1_{EFF}/Th1_{EM}$ cells seem to be required to protect against reinfection.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper. The funders had no role in the preparation of the paper.

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Research Article

Mesenchymal Stem Cells Immunosuppressed IL-22 in Patients with Immune Thrombocytopenia via Soluble Cellular Factors

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Mesenchymal stem cells are immunoregulation cells. IL-22 plays an important role in the pathogenesis of immune thrombocytopenia. However, the effects of mesenchymal stem cells on IL-22 production in patients with immune thrombocytopenia remain unclear. Flow cytometry analyzed immunophenotypes of mesenchymal stem cells; differentiation of mesenchymal stem cells was observed by oil red O and Alizarin red S staining; cell proliferation suppression was measured with MTS; IL-22 levels of cell-free supernatants were determined by ELISA. Mesenchymal stem cells inhibited the proliferation of activated $CD4^+$T cells; moreover, mesenchymal stem cells immunosuppressed IL-22 by soluble cellular factors but not PGE2. These results suggest that mesenchymal stem cells may be a therapeutic strategy for patients with immune thrombocytopenia.

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent cells and are able to differentiate into mature mesenchymal cells such as osteoblasts, adipocytes, and chondroblasts [1]. MSCs can be obtained from many tissues including adult bone marrow (BM), adipose tissue (AD), muscle, term placental chorionic villi (CV), cord blood, and umbilical cord (UC) [2–7]. But UC-MSCs are a more promising source [8]. Due to their stronger immunoregulation, MSCs have been widely applied for treatment of all kinds of diseases, for example, graft-versus-host disease (GVHD) [9], experimental autoimmune encephalomyelitis (EAE) [10], Crohn's disease (CD) [11], and rheumatoid arthritis (RA) [12].

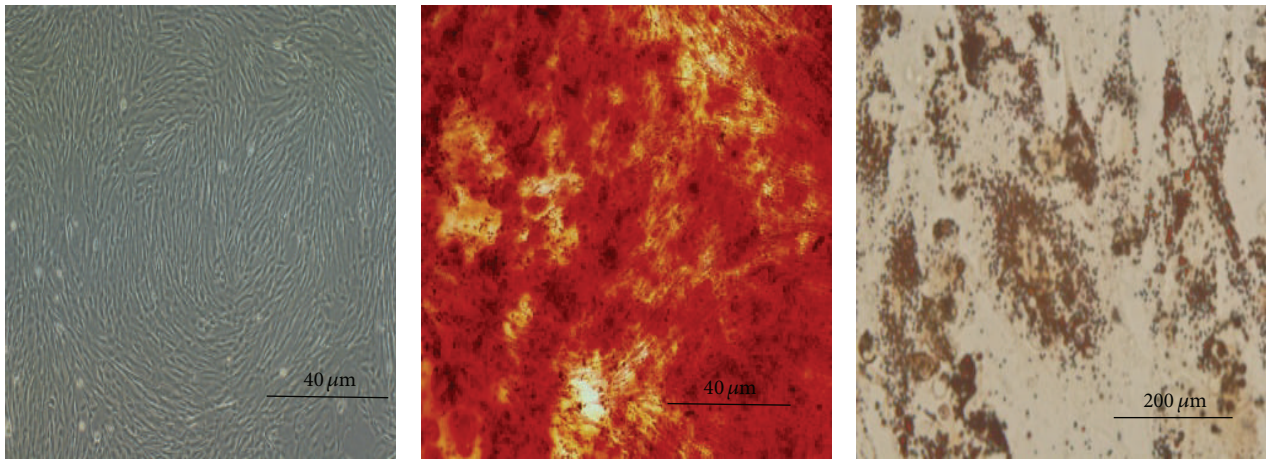
Immune thrombocytopenia (ITP), also known as idiopathic thrombocytopenic purpura, is an autoimmune disease. Because of antiplatelet autoantibodies in patients, platelets are destroyed in large numbers and platelet count is lower obviously in peripheral blood. The etiology of ITP is not clear. Therefore, the diagnosis of ITP is exclusive, and there are no specific or sensitive laboratory methods used to detect these antibodies in clinic [13]. It is known that T cells abnormalities play an important role in the pathogenesis of ITP. T cells related cytokine abnormalities are one of the T cells abnormalities [14, 15]. Many studies found that the concentration of IL-22 produced by T cell subsets increased significantly in ITP patients [16–18]. However, the effect of UC-MSCs on ITP patients remains unclear.

In the present study, our data suggest that UC-MSCs inhibited the proliferation of $CD4^+$T cells and immunosuppressed the production of IL-22 in ITP patients through soluble cellular factors.

2. Materials and Methods

2.1. The Isolation of UC-MSCs. Umbilical cords were obtained from our hospital's obstetrical department with informed consent. Human tissue collection for research was approved by the Medical Ethics Committee of Anhui Province in China. Isolation of human umbilical cord mesenchymal stem cells (UC-MSCs) was performed as described [7].

2.2. Immunophenotype Analysis by Flow Cytometry. UC-MSCs were stained with PE-conjugated antibody against



(a) (b) (c)

Figure 1: The characteristics of UC-MSCs. (a) Morphology of UC-MSCs; (b) the osteogenic differentiation of UC-MSCs; (c) adipogenic differentiation of UC-MSCs.

CD11b, CD29, CD44, CD45, CD54, CD73, CD80, CD86, CD90, CD105, CD106, HLA-DR, nestin, and sox-2 or FITC-conjugated antibody against CD19, CD31, CD34, and HLA-ABC. The IgG1-PE and IgG1-FITC were used as negative controls. Antibodies (BD Pharmingen) were used according to manufacturer's protocol and were analyzed by flow cytometry.

2.3. The Differentiation and Staining Assays of UC-MSCs. 2×10^4 UC-MSCs were cultured by DMEM/F12 media containing 10% fetal bovine serum (FBS) in 24-well plates for 24 hours. Then, the media were changed with osteogenic or adipogenic induction media for 3 weeks; cells were observed by Alizarin red S or oil red O solution, respectively.

2.4. Preparation of Human $CD4^+$ T Cells. Human mononuclear cells from patients with ITP were isolated by Ficoll-Paque (Axis-Shield). Then, $CD4^+$T cells were obtained with magnetic MicroBead kits (Miltenyi Biotec). The purity of $CD4^+$T cells was more than 95% (data not shown).

2.5. Coculture Experiment of UC-MSCs and $CD4^+$ T Cells. UC-MSCs irradiated by 30 Gy were preplated and were allowed to adhere for 5 h at 37°C; $CD4^+$T cells were added at a ratio of 1 : 10 for 72 h.

2.6. Cell Proliferation Assay. Cell proliferation was measured with an MTS kit (Promega) according to manufacturer's protocol. Absorbance was detected at 490 nm on BioTek reader (BIO-RAD).

2.7. Total RNA Extraction, Reverse Transcription, and Real-Time PCR Analysis. $CD4^+$T cell was collected. RNA of $CD4^+$T cell was extracted with E.Z.N.A. Total RNA Kit I (OMEGA). cDNA synthesis was done with the MLV RT kit (Invitrogen). Polymerase chain reaction analyses were performed by Platinum SYBR Green qPCR SuperMix-UDG w/ROX on an Applied Biosystems 7300 Real-Time PCR System. The IL-22 mRNA was expressed with ΔΔCt values. The primer of human IL-22 is 5′-ACAACACAGACGTTCGTCTCATTG-3′ and 5′-GAA CAGCACTTCTTCAAGGGTGA-3′.

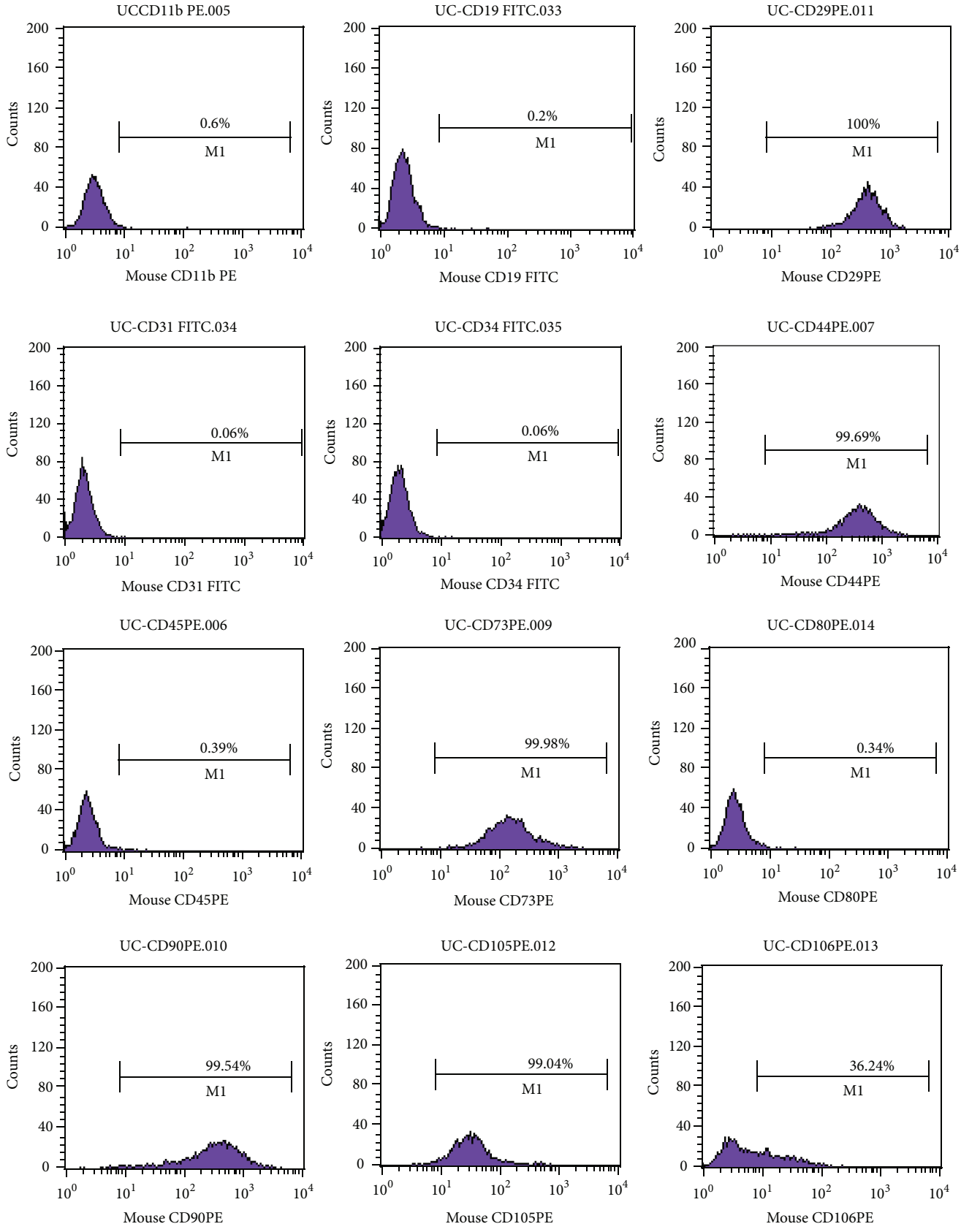
2.8. Enzyme-Linked Immunosorbent Assay (ELISA) Measured IL-22 Concentration. IL-22 concentration of cell-free supernatants was tested by Human IL-22 ELISA assay kits (Peprotech) according to manufacturer's protocol.

2.9. Statistical Analysis. The SPSS 17.0 software package analyzed data. Data are presented as mean ± SD. Comparisons were performed by one-way ANOVA. $P < 0.05$ was considered significant.

3. Results

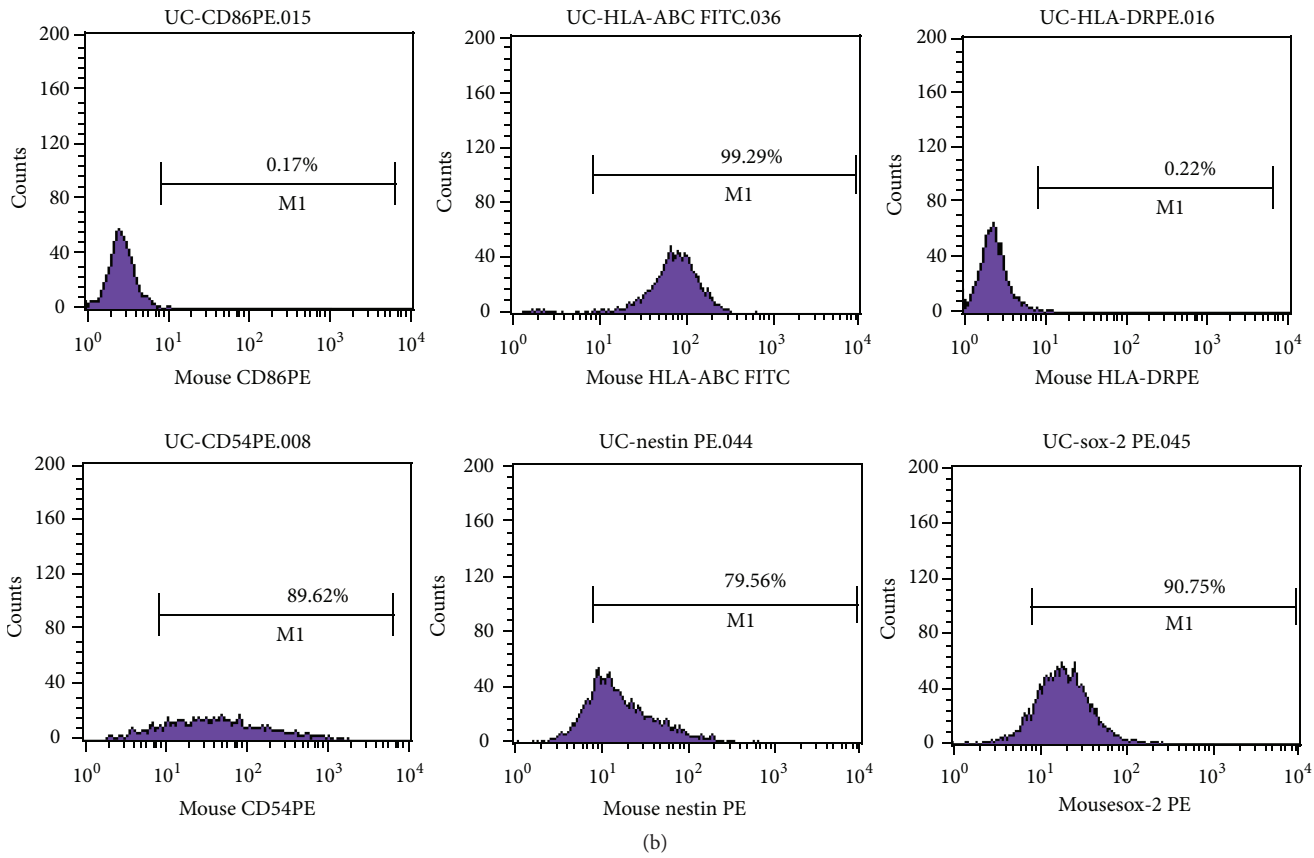
3.1. The Characteristics of UC-MSCs. As shown in Figure 1(a), UC-MSCs isolated from umbilical cord were fibroblast-like cells. They were induced successfully into osteoblasts and adipocytes observed by Alizarin red S and oil red O staining in specific medias (Figures 1(b) and 1(c)). Furthermore, flow cytometry showed that UC-MSCs were positive for CD29, CD44, CD54, CD73, CD90, CD105, CD106, HLA-ABC, nestin, and sox-2 and negative for CD11b, CD31, CD19, CD34, CD45, CD80, CD86, and HLA-DR (Figure 2, Table 1).

3.2. UC-MSCs Suppressed the Proliferation of $CD4^+$ T Cells. To examine the effect of UC-MSCs on $CD4^+$T cells from ITP patients, we treated $CD4^+$T cells with UC-MSCs in the absence or presence of stimuli (CD3/CD28) and found that $CD4^+$T cells could not proliferate culturing with UC-MSCs or in the absence of stimuli (Figures 3(a) and 3(c)). However, $CD4^+$T cells appeared to cluster in the presence of stimuli (Figure 3(b)). Most important, when cocultured with UC-MSCs, activated $CD4^+$T cells grew in a spreading



(a)

Figure 2: Continued.



(b)

Figure 2: Immunophenotypes of UC-MSCs. UC-MSCs expressed CD29, CD44, CD54, CD73, CD90, CD105, CD106, HLA-ABC, nestin, and sox-2 but did not express CD11b, CD14, CD19, CD34, CD45, CD80, CD86, and HLA-DR. This figure shows one of the three independent experiments' results.

pattern (Figure 3(d)). MTS was used to evaluate further the proliferation of $CD4^+$T cells (Figure 3(e)); the result of MTS was consistent with the proliferation of $CD4^+$T cells alone or culture with UC-MSC in the absence or presence of stimuli.

3.3. UC-MSCs Inhibited $CD4^+$T Cells Secreting IL-22. We measured the production and mRNA of IL-22 to investigate the immunomodulation of UC-MSCs on IL-22. As shown in Figure 4(a), nonactivated $CD4^+$T cells or $CD4^+$T cells cocultured with UC-MSCs without stimuli produced low level of IL-22. But the concentration of IL-22 was increased enormously in activated $CD4^+$T cells. When activated $CD4^+$T cells were cocultured with UC-MSCs, higher levels of IL-22 were reduced again ($P < 0.001$). Furthermore, this phenomenon was also observed in expression of IL-22 mRNA. Thus, UC-MSCs had strong immunosuppression in $CD4^+$T cells secreting IL-22.

3.4. UC-MSCs Immunomodulated IL-22 by Soluble Cellular Factors. It is known that MSCs play their immunosuppressive effects by cell-cell contact or soluble cellular factors. To examine it, we performed coculture experiments using the Transwell system. Transwell physically separated $CD4^+$T cells from UC-MSCs; it only allows for soluble cellular factors to

Table 1: Immunophenotypes of UC-MSCs.

Surface marker	Positive rate
CD11b	–
CD31	–
CD73	++++
CD90	++++
CD80	–
CD106	++
HLA-ABC	++++
HLA-DR	–
Sox-2	++++
CD19	–
CD44	++++
CD34	–
CD54	++++
CD45	–
CD105	++++
CD86	–
Nestin	++++

– negative, +~++++ positive, + 1–25%, ++ 25–50%, +++ 50–75%, and ++++ >75%.

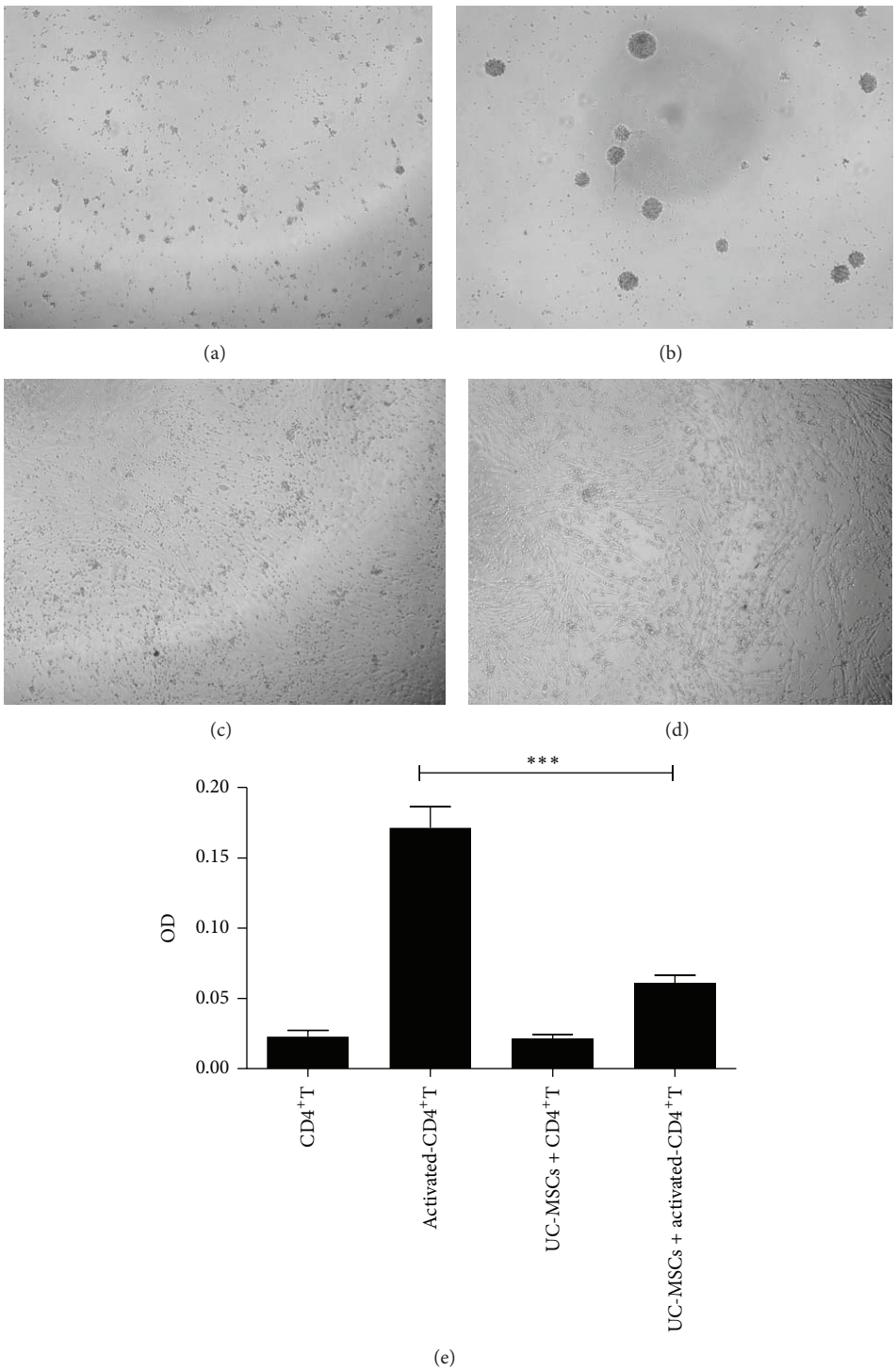


Figure 3: UC-MSCs suppressed proliferation of $CD4^+$T cells. (a) Nonactivated $CD4^+$T cells; (b) activated $CD4^+$T cells; (c) cocultured nonactivated $CD4^+$T cells with UC-MSCs; (d) cocultured activated $CD4^+$T cells with UC-MSCs; magnification: 40x; (e) proliferation was evaluated by MTS. Data represent one of the three independent experiments, each performed in triplicate. $^{***}P < 0.001$.

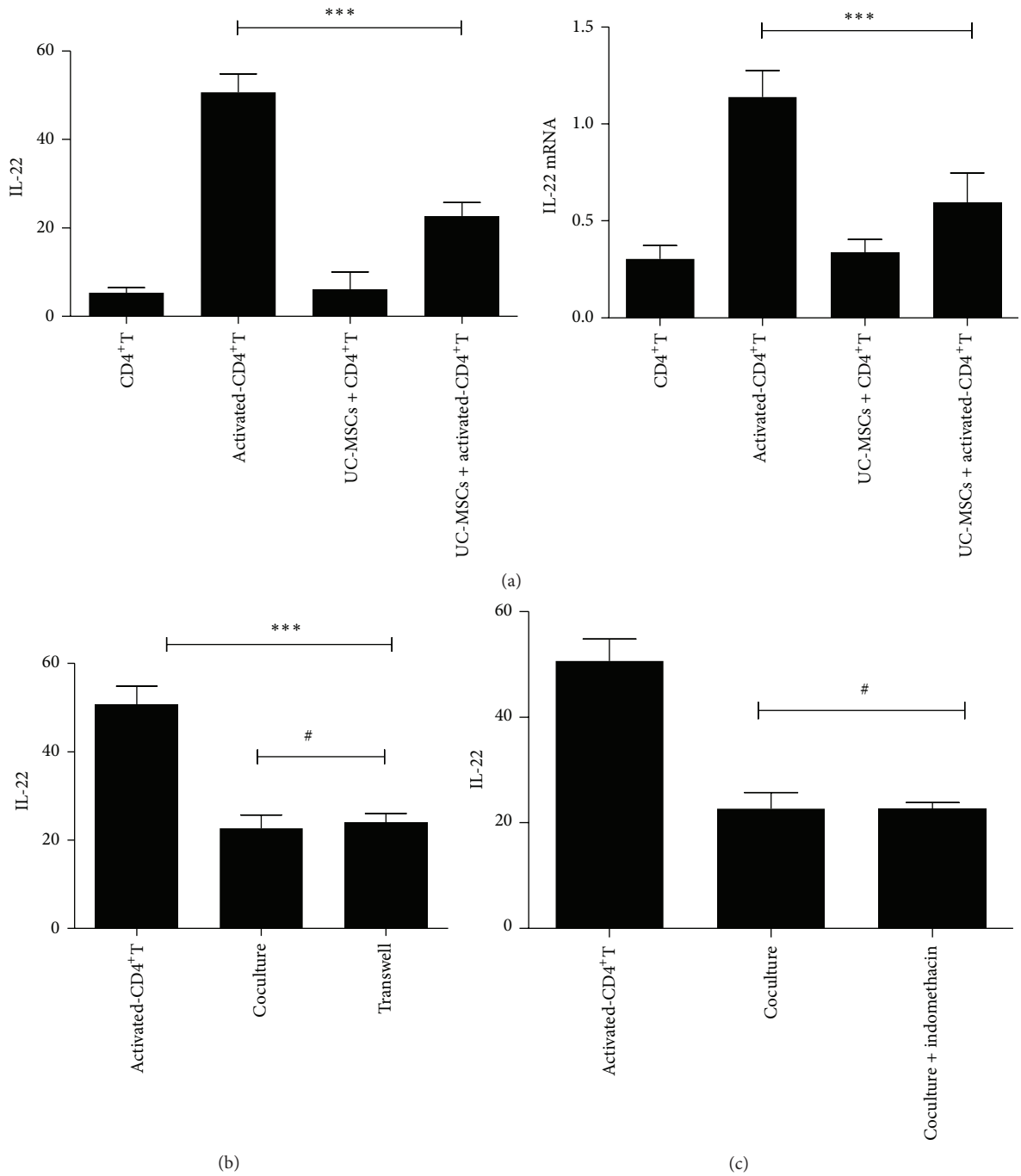


Figure 4: UC-MSCs immunosuppressed IL-22 by soluble cellular factors but not PGE2. (a) UC-MSCs inhibited $CD4^+T$ cells secreting IL-22 and the expression of IL-22 mRNA; (b) the immunoregulation of UC-MSCs on IL-22 was mediated by soluble cellular factors; (c) PGE2 did not involve in the immunoregulation of UC-MSCs on IL-22. Data represent one of the three independent experiments, each performed in triplicate. $^{***}P < 0.001$ and $^{\#}P > 0.05$.

permeate. We found that UC-MSCs were also able to inhibit dramatically the secretion of IL-22 without cell-cell contact ($P < 0.001$). Furthermore, the degree of IL-22 inhibition by UC-MSCs in coculture separated by Transwell was not different significantly from those in coculture which was cell-cell contact ($P > 0.05$), indicating that the immunoregulation of UC-MSCs on IL-22 was mediated by soluble cellular factors (Figure 4(b)).

3.5. PGE2 Did Not Mediate the Immunomodulation of UC-MSCs on IL-22. Prostaglandin E2 (PGE2) is one of the soluble cellular factors mediating the immunoregulation of MSCs. Therefore, we performed coculture experiments with indometacin (10 mM), the inhibitors of PGE2. We found that the level of IL-22 decreased by UC-MSCs was not improved in coculture with indometacin compared to group without indometacin ($P > 0.05$). Together, this data suggests that

UC-MSCs immunoregulated IL-22 via soluble cellular factors but not PGE2 (Figure 4(c)).

4. Discussion

In this present study, we have successfully demonstrated a previously uncharacterized fact that UC-MSCs possessed strong immunosuppressive capacity on IL-22 in patients with ITP.

Immunoregulation is one of the biological characteristics of MSCs; they can modulate the function of different immune cells such as T cells, B cells, neutrophils, natural killer (NK) cells, and dendritic cells (DC) [19–23]. MSCs block the division of T cells, preventing irreversible G0/G1 phase arrest and reducing the secretion of proinflammatory cytokines such as IFN-γ and TNF-α [24]. The immunomodulatory activity of the MSCs is also exerted through the inhibition of DC differentiation and maturation of antigen-presenting cells [25]. In addition, we had proved in our previous study that UC-MSCs suppressed significantly the mRNA expression of TNF-α, IL-21, IL-22, and IL-26 in $CD4^+$T cells [8].

To our knowledge, IL-22, a newly defined cytokine, is one member of the IL-10 cytokine family, produced by CD4 cells, Th22 cells, Th17 cells, and NK cells. IL-22 can combine with its counterpart receptor complex which is composed of the IL-22R1 and IL-10R2, and its signal intracellularly is mediated by transcription factor JAK/STAT [26]. In different circumstance, IL-22 may play a protective or a pathogenic role. For instance, Liang and colleagues reported that IL-22 inhibited the development of bleomycin-induced pulmonary fibrosis [27]. However, IL-22 plays a pathogenic role in ITP [18]. The effects of MSCs on IL-22 in patients with ITP are unclear. In this study, we found that UC-MSCs had ability to immunoregulate IL-22 in patients with ITP. They decreased the IL-22 level of cell-free supernatants *in vitro*. In general, MSCs exert immunomodulatory effects through cell-cell contact or soluble cellular factors. We also found that UC-MSCs downregulated IL-22 when UC-MSCs were separated from $CD4^+$T cells by Transwell. We come to conclusion that UC-MSCs suppressed the secretion of IL-22 by soluble cellular factors. Soluble cellular factors include NO, TGF-β1, PGE2, IDO1, HGF, IL-6, IL-10, and HLA-G [11, 28–34]. PGE2 is derived from the cyclooxygenase metabolism of arachidonic acid and is generated in large quantities by both macrophages and neighboring epithelial cells [35, 36]. PGE2 as an important regulator of the immune response shifts the balance towards a T helper type 2 response and promotes memory cell formation [37]. Our colleagues demonstrated that PGE2 is involved in the immunoregulation effect of MSCs [29, 38]. However, we added indomethacin which is the blocker of PGE2 into the group of UC-MSCs cocultured with $CD4^+$T cells and found that indomethacin did not reverse the immunosuppressive effect of UC-MSCs on IL-22.

In summary, this study reports for the first time that UC-MSCs downregulate IL-22 of ITP patients through soluble cellular factors but not PGE2.

Conflict of Interests

The authors have no competing financial interests to declare.

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Research Article

Impaired Fas-Fas Ligand Interactions Result in Greater Recurrent Herpetic Stromal Keratitis in Mice

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Herpes simplex virus-1 (HSV-1) infection of the cornea leads to a potentially blinding condition termed herpetic stromal keratitis (HSK). Clinical studies have indicated that disease is primarily associated with recurrent HSK following reactivation of a latent viral infection of the trigeminal ganglia. One of the key factors that limit inflammation of the cornea is the expression of Fas ligand (FasL). We demonstrate that infection of the cornea with HSV-1 results in increased functional expression of FasL and that mice expressing mutations in Fas (*lpr*) and FasL (*gld*) display increased recurrent HSK following reactivation compared to wild-type mice. Furthermore, both *gld* and *lpr* mice took longer to clear their corneas of infectious virus and the reactivation rate for these strains was significantly greater than that seen with wild-type mice. Collectively, these findings indicate that the interaction of Fas with FasL in the cornea restricts the development of recurrent HSK.

1. Introduction

Herpetic stromal keratitis (HSK) is a potentially blinding corneal inflammation that accompanies herpes simplex virus (HSV) infection of the eye. The disease course in HSK begins with a primary infection by HSV followed by a period during which the virus enters latency in sensory and autonomic ganglia. Many studies have shown that clinical disease is the result of a cocktail of inflammatory cells, consisting of PMNs, macrophages, and T cells (both $CD4^+$ and $CD8^+$) that are recruited to the corneas of patients with HSK [1–4].

In the face of this potentially blinding inflammatory attack, the cornea has the ability to reduce inflammation. This includes the presence of immunosuppressive factors such as TGF-β [5], lack of vascularization [6, 7], and the presence of Fas ligand (FasL) [8–14].

Studies from our laboratory as well as the laboratories of others have demonstrated that the presence of FasL in the eye is an important barrier to both inflammatory cells [8, 9, 12] and new blood vessels [10, 11, 13, 14]. In fact, we know that control of inflammation is required for the immune privilege of the eye [8, 9]. FasL expressed on ocular tissues induces apoptosis in Fas^+ lymphoid cells that invade the eye in response to viral infection [8] or corneal grafting [11, 12, 14]. FasL expressed in the retina and the cornea also controls new vessel growth beneath the retina and in the cornea by inducing apoptosis of Fas-expressing vascular endothelial cells [15–17]. These studies clearly indicate that the presence of FasL in ocular tissues restricts inflammatory responses.

Recently we published that the interaction of Fas with FasL is an important factor in controlling HSK during acute infection of the cornea [18]. We demonstrated that mice expressing mutations in Fas (*lpr*) or FasL (*gld*) experience significantly worse ocular disease than do wild-type mice regardless of mouse or viral strain [18]. However, since acute infection rarely leads to clinical disease in humans and factors important in acute infection do not display an analogous role during recurrent infection [19], we thought it is very important to address the role that Fas and FasL play during recurrent disease when the virus is reactivated from latency. In order to address the role of Fas-FasL interactions during recurrent HSK, we have evaluated this interaction in a mouse

model of induced recurrent HSK. We report here that mice that are defective in either Fas or FasL experience increased recurrent HSK disease following infection with HSV-1.

2. Materials and Methods

2.1. Virus and Cells. The virus used in these studies was the McKrae strain of HSV-1. A plaque-purified stock was grown and assayed on Vero cells in minimum essential medium with Earle's balanced salts (MEM-EBS) containing 5% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin [20]. Virus titers in eye swabs were determined by standard plaque assay [20].

2.2. Mice. Investigations with mice conformed to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. C57BL/6 (B6) and BALB/c mice were purchased from NCI. The B6Smn.C3-*Tnfsf6*^{gld}/J and B6.MRL-*Tnfsf6*^{lpr}/J mice were purchased from Jackson Labs and maintained in our colony. For the purposes of this paper we will refer to these mice as B6-*gld* and B6-*lpr*, respectively. We also bred the B6-*gld* and B6-*lpr* mice to BALB/c mice for a minimum of 12 generations. The resultant strains designation will be C.B6-*Tnfsf6*^{gld} and C.B6-*Tnfsf6*^{lpr} [21, 22]. However, we will refer to them as BALB-*gld* and BALB-*lpr*, respectively. In order to assure that these mice retain their mutations, tail DNA is isolated from individual mice and PCR tested for either the *gld* or the *lpr* mutation.

2.3. Infection of Mice. 6–12-week-old mice were infected on the scarified cornea with 10^6 PFU HSV-1 McKrae strain as previously described [23]. Each mouse received an intraperitoneal (IP) injection of 0.5 mL pooled human serum (Sigma Chemicals, St. Louis MO; ED50 for virus neutralization = 1 : 1600) concurrent with infection. Administration of pooled human serum which is the source of anti-HSV antibodies at the time of ocular infection has been shown to protect mice from death and corneal disease during primary infection, while allowing for the establishment of latency and subsequent reactivation of virus after corneal UV-B exposure. These human antibodies are undetectable at the time of UV-B irradiation 5 weeks after primary infection. HSV positive eye swabs obtained three days after application of virus confirm primary infection.

2.4. UV-B Irradiation and Virus Reactivation. Mice were reactivated from latency as previously described [24]. Briefly, the eyes of all latently infected mice were examined for corneal opacity before irradiation, and only animals with clear corneas were used. At least 5 weeks after primary infection, at which time human antibodies cannot be detected, the eyes of latently infected and control mock-infected mice were exposed to 250 mJ/cm^2 of UV-B light using a TM20 Chromato-Vue transilluminator (UVP, Inc., San Gabriel, CA), which emits UV-B at a peak wavelength of 302 nm. Irradiated mice were swabbed with sterile cotton applicators from day 0 to day 7, unless otherwise indicated. The swab material was cultured on Vero cells, as described above, in order to detect recurrent virus shedding from the cornea. Reactivation was defined as the finding of any HSV positive eye swab on any days after UV-B exposure, with day 0 swabs serving as a control.

2.5. Clinical Evaluation. On the designated days after viral infection or UV-B reactivation, a masked observer examined mouse eyes through a binocular-dissecting microscope in order to score clinical disease. Stromal opacification was rated on a scale of 0 to 4, where 0 indicates clear stroma, 1 indicates mild stromal opacification, 2 indicates moderate opacity with discernible iris features, 3 indicates dense opacity with loss of defined iris detail except pupil margins, and 4 indicates total opacity with no posterior view. Corneal neovascularization was evaluated as described [20, 21] using a scale of 0–8, where each of four quadrants of the eye is evaluated for the amount of vessels that have grown into them. Periocular disease was measured in a masked fashion on a semiquantitative scale as previously described [25].

2.6. Viral Tittering from Tissues. Eye swab material was collected and assayed for virus by standard plaque assay as previously described [20]. Trigeminal ganglia and 6 mm biopsy punches of periocular skin were removed and placed in preweighed tubes containing 1 mm glass beads and 1 mL of medium. Trigeminal ganglia and periocular skin homogenates were prepared by freezing and thawing the samples, mechanically disrupting in a Mini-Beadbeater-8 (Biospec Products, Bartlesville, Oklahoma), and sonicating. Homogenates were assayed for virus by standard plaque assay, and the amount of virus was expressed as PFU per milliliter of tissue homogenate.

2.7. Assays of Antibody Titers. Serum was collected from mice at weekly intervals following infection and examined for HSV-specific antibody content as previously described [26]. Briefly, for enzyme linked immunosorbent assays (ELISA), serial fourfold dilutions of mouse serum were incubated for 2 hours in duplicate wells of a 96-well plate coated with purified HSV-1 glycoprotein. Biotinylated goat anti-mouse IgG was subsequently used in a colorimetric assay to determine specific IgG amounts based on comparison to a standard curve generated as previously described [26].

2.8. Real-Time PCR Analysis for Herpes Genome. DNA was isolated using a DNeasy tissue preparation kit (Qiagen, Valencia, CA). The number of latent genomes per trigeminal ganglion was determine by real-time PCR essentially as described [27]. Briefly, a 70 bp fragment of the thymidine kinase (tk) gene was amplified from trigeminal ganglia DNA and 10-fold dilutions of purified HSV-1 chromosome DNA. HSV-1 DNA was used to generate a standard curve to determine the number of genome copies per trigeminal ganglion models the episomal, latent genome. To control for total DNA content of each sample, the single-copy mouse adipsin gene was amplified in each sample along with dilutions of mouse genomic DNA to generate a standard curve. The values for tk

copy number were normalized to the lowest value of mouse adipsin copy number to yield the normalized genome copy per ganglion, which were expressed on a log scale.

2.9. Flow Cytometric Analysis. Cells were isolated from corneas as previously described [18]. Briefly, corneas were excised at 18 and 23 dpi and incubated in PBS-EDTA at 37°C for 15 minutes at 37°C. Stromas were separated from overlying epithelium and digested in 84 U collagenase type 1 (Sigma-Aldrich, St. Louis, MO) per cornea for 2 hours at 37°C and then were triturated to form a single-cell suspension. Suspensions were filtered through a 40-μm cell strainer cap (BD Labware, Bedford, MA) and washed and then stained. Suspensions were stained with PerCP-conjugated anti-CD45 (30-F11) and Alexa Fluor700-Gr-1 (RB6-8C5) (from BioLegend, San Diego, CA); FITC conjugated anti-CD4 (RM4-5), PE-conjugated anti-CD8α (53–6.7), PE-Cy7-conjugated anti-CD11c (HL3) (all BD PharMingen); eFluor450-conjugated CD11b (M1/70) (from eBiosciences, San Diego, CA). Cells were then analyzed on a flow cytometer (FACSAria with FACSDIVA data analysis software; BD Biosciences).

2.10. In Vitro Killing of L1210 Cells Transfected with Human Fas by Mouse Corneas. The use of whole corneas in vitro to induce Fas-mediated killing has been described [10, 14]. Mouse corneas were placed in 24-well plates with either the endothelium or epithelium facing up. These corneas were infected with the KOS strain of HSV-1 or not. To them were added L1210 cells, which express Fas (2×10^5/mL were labeled with 5 μCi/mL ^{3}H-thymidine at 37°C in complete DMEM for 2 hours). After washing twice they were incubated (2×10^4 cells/determination) with corneas overnight in a 96-well plate overnight at 37°C. The L1210-Fas target cells were harvested onto microfiber filters and radioactivity counted using a microplate scintillation counter (TopCount, PerkinElmer Life Sciences, Boston, MA). Because fragmented DNA associated with apoptosis does not bind to the filters, the counts associated with the filters reflect nonapoptotic cell DNA only. The percentage of cells undergoing apoptosis is therefore defined as

$$\begin{aligned} &\%\ \text{DNA Fragmentation} \\ &\quad = \text{CPM L1210-Fas incubated alone} \\ &\qquad - \frac{\text{CPM L1210-Fas incubated with cornea}}{\text{CPM L1210-Fas incubated alone}} \times 100. \end{aligned} \tag{1}$$

To confirm that cell killing is due to Fas-FasL interactions, we added the competitive inhibitor Fas-Fc or an inhibitor of TNF-mediated killing, TNFR1-Fc, at 10 μg/mL (both from R&D Systems, Minneapolis, MN) as previously described [10, 14].

2.11. Statistical Analysis. All statistical analyses were performed with the aid of Sigma Stat for Windows, version 2.0 (Jandel, Corte Madera, CA). The Rank Sum test was used to compare corneal disease scores. Student's unpaired t-test was used to compare virus titer and antibody titer data. Fisher's exact X^2 tests were used to compare limiting dilution assay data.

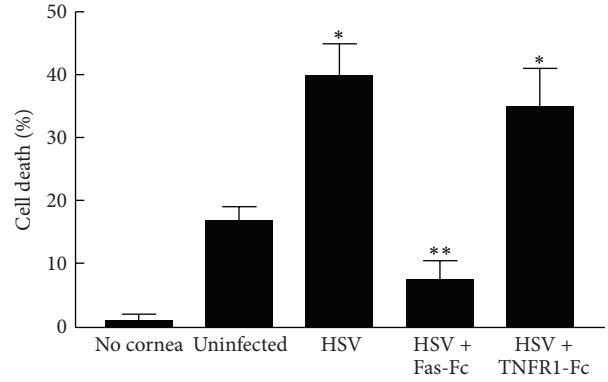

Figure 1: Infection with HSV-1 induces functional Fas ligand. Corneas were infected with HSV-1, KOS strain, for 24 hours at which point [^{3}H]Thymidine labeled L1210-Fas cells (2×10^4) were cultured in contact with the corneal epithelium in vitro for 20 h at 37°C. In some cultures the chimeric protein Fas-Fc (10 μg/mL) or TNFR1-Fc (10 μg/mL) was included for the entire incubation period. The amount of apoptotic cell death was determined by calculating percent of DNA fragmentation. Each value represents the mean of three replicate cultures ± SEM, and each culture condition was performed at least three times. * Infected corneas displayed significantly greater cell death than did uninfected corneas ($P < 0.02$). ** Addition of Fas-Fc significantly reduced cell death when compared to either HSV alone ($P < 0.01$) or HSV + TNFR1-Fc ($P < 0.02$).

3. Results

3.1. Infection of Murine Corneas Results in Increased Fas Ligand Expression. The first thing that we wished to determine was whether infection with HSV-1 had any effect on the expression of FasL on the cornea. Recent reports have demonstrated that FasL can be induced when cells are exposed to cytokines and stress [28, 29]. In order to do this we performed a functional assay to determine the ability of mouse corneas to kill Fas-expressing target cells. Thus we infected isolated mouse corneas with HSV-1 and then compared the ability of infected corneas to kill Fas targets versus uninfected corneas. As demonstrated in Figure 1, infected corneas were able to kill significantly more Fas-expressing target cells than uninfected corneas. We further demonstrated that this killing was due to increased FasL expression as Fas-Fc, which specifically interacts with FasL blocked killing while TNF-Fc did not (Figure 1). Since these isolated corneas do not contain significant numbers of CD45$^+$ cells at the time of infection, increased FasL expression will primarily be on the resident epithelial cells. Thus it is clear that one way that the cornea attempts to limit inflammation following HSV-1 infection is by increasing FasL expression which will more efficiently control the entrance of Fas-expressing inflammatory cells.

3.2. Mice with Mutations in Fas or FasL Have Worse Corneal Disease. We next compared recurrent HSK between BALB-*lpr*, BALB-*gld*, and parental BALB/c mice. The mice were infected with the McKrae strain of HSV-1 and latency was established. The mice were reactivated 8 weeks following primary infection. As can be observed in Figure 2, both

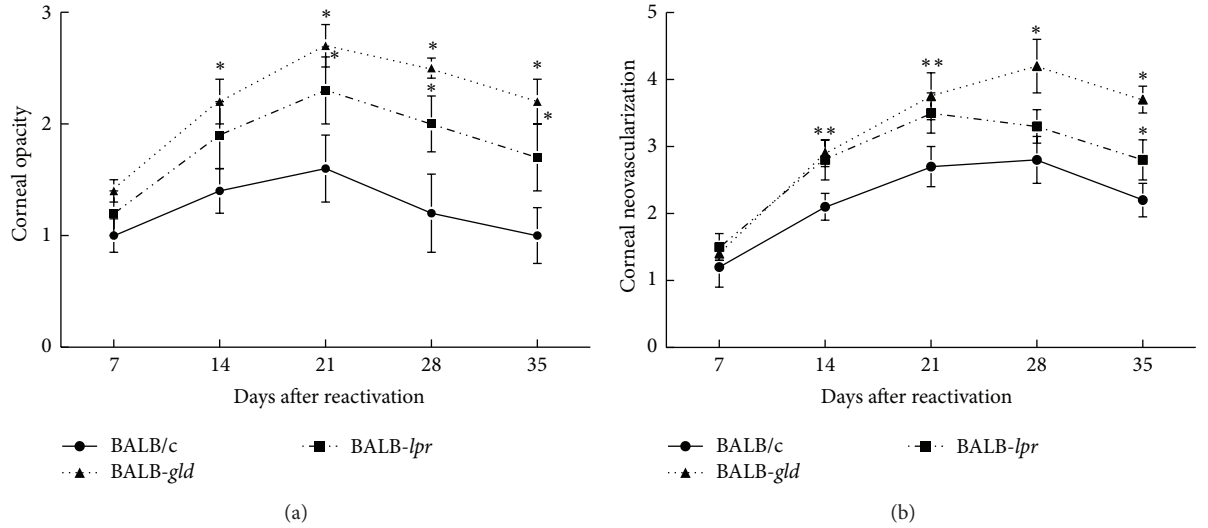

Figure 2: Defective expression of both FasL and to a lesser extent Fas results in increased recurrent HSK following UV-reactivation of a latent infection with HSV-1, McKrae strain. Eyes of BALB/c wild-type ($n = 20$), BALB-*lpr* ($n = 20$), and BALB-*gld* ($n = 20$) mice were infected with 10^6 pfu of HSV-1, McKrae strain. Six weeks following infection mice were irradiated with UV-B to reactivate the latent infection. Corneal opacity (a) and corneal neovascularization (b) were measured and compared between these strains of mice. *Significant virus-induced corneal opacity was observed for BALB-*gld* at days 14–28 time points when compared to BALB/c controls ($P < 0.01$–0.001). BALB-*lpr* mice displayed significantly more opacity than did BALB/c controls at days 21–35 ($P < 0.05$–0.01). BALB-*gld* displayed significantly greater neovascularization at days 14–35 ($P < 0.05$–0.001) and BALB-*lpr* mice had greater neovascularization at days 14, 21, and 35 ($P < 0.05$–0.01) than did BALB/c controls.

BALB-*lpr* and BALB-*gld* mice experienced significantly worse disease than did wild-type BALB/c mice. This was consistent with results of primary disease [18], though mice carrying the *gld* mutation tended to display consistently greater disease scores than did mice with the *lpr* mutation. A similar pattern of disease was seen in B6 mice that express mutations in Fas and FasL (Figure 3).

3.3. BALB/c Mice with Mutations in Fas and FasL Have Increased Mortality. In another significant departure from our previous report, mortality was much greater in those mice carrying either the *lpr* or the *gld* mutation (Figure 4). It should be noted that the previous report compared C57BL/6 with mutations in Fas and FasL with their parental B6, but it was the same strain of HSV-1, namely, McKrae. The reason for this discrepancy is not known at this time; we suspect that BALB/c mice, which are more susceptible to both corneal disease and developing a lethal infection, are more prone to lethal infection in the absence of Fas-FasL interactions because there is greater influx of inflammatory cells into the brain (data not shown).

3.4. The Magnitude of the Inflammatory Infiltrate in Mice with Mutations in Fas and FasL Is Greater Than Wild-Type Mice but the Composition of the Infiltrate Is the Same. We also compared the influx of inflammatory cells into the cornea of these strains of mice and as expected there were consistently more $CD45^+$ cells infiltrating the corneas of *lpr* and *gld* mice than in wild-type mice following UV-induced reactivation (Figure 5(a)). We believe that is due to better control of the inflammatory infiltrate in wild-type mice that have an intact Fas-FasL interaction. However, since there are no differences in the phenotype of the inflammatory infiltrate (Figure 5(b)), there does not appear to be a differential

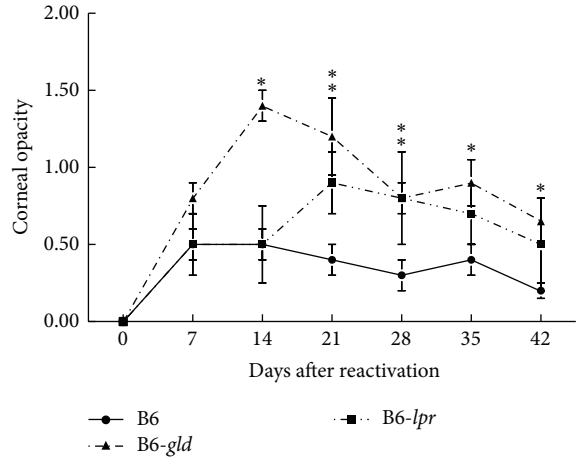

Figure 3: Similar patterns of disease were seen in C57BL/6 (B6) mice carrying the *gld* and *lpr* mutations following UV-B reactivation. Eyes of B6 wild-type ($n = 20$), B6-*lpr* ($n = 15$), and B6-*gld* ($n = 15$) mice were infected with 10^6 pfu of HSV-1, McKrae strain. Six weeks following infection mice were irradiated with UV-B to reactivate the latent infection. Corneal opacity was measured and compared between these strains of mice. *Significant virus-induced corneal opacity was observed for B6-*gld* ($P < 0.01$–0.001) at days 14–42 when compared to B6 controls. B6-*lpr* mice displayed significantly more opacity than did B6 controls at days 21 and 28 ($P < 0.05$–0.01).

Table 1: HSV-1 shedding following UV-B reactivation.

	Normal BALB/c	BALB-*gld*	BALB-*lpr*
EYES			
% of positive swabs[@]	11%	17%	23%
Total shedding days[#]	7	22	41
Days of shedding/mouse[†]	1.2 ± 0.3	2.8 ± 0.25	2.9 + 0.6
Reactivation rate[‡]	33% (6/18 total)	57% (8/14 total)	54% (14/26 total)
Final day of shedding[‖]	Day 5	Day 10	Day 10
Trigeminal ganglia			
Genome copies × 10^2 (n)[**]	6.5 ± 0.3 (10)	10.4 ± 0.4 (5)	11.1 + 0.3 (5)
Titer day 3 after react. (n)[††]	845 ± 217 (4)	1186 ± 159 (4)	1075 + 245 (4)

[@]The percent of positive swabs is the percentage of virus-positive eye swabs (140 to 286 eye swabs per group) over the 10-day period following UV-B irradiation. ($P < 0.01$ for both *gld* and *lpr* mice.)

[#]Total shedding days: number of days of positive swab. ($P < 0.01$ for both *gld* and *lpr* mice.)

[†]Days of shedding/mouse are the number of days that a positive mouse shed virus. ($P < 0.005$ for both *gld* and *lpr* mice.)

[‡]Percentage of reactivation rate is the percentage of mice that were reactivated. ($P < 0.02$ for both *gld* and *lpr* mice.)

[‖]Final day of shedding was the last day that a mouse was positive for a particular group.

[**]Mean number of genome copies from real-time PCR analysis. Statistical analysis did not indicate significant differences ($P > 0.05$).

[††]Titer of virus at day 3 after reactivation.

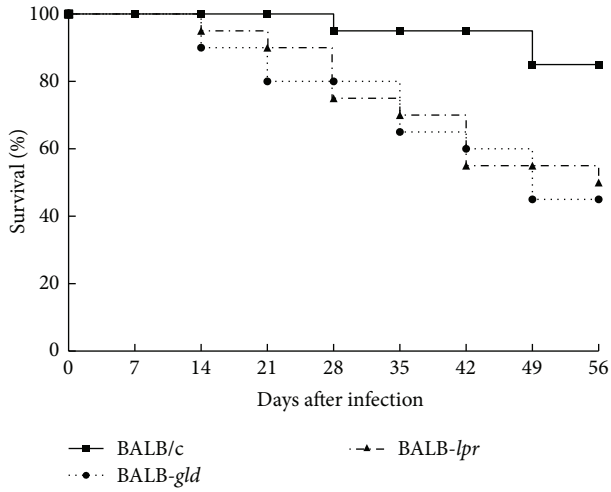

Figure 4: Mice expressing mutations in Fas and FasL display significantly greater mortality than wild-type BALB/c mice. A latent infection of BALB/c wild-type (n = 20 mice), BALB-*lpr* (n = 20 mice), and BALB-*gld* (n = 20 mice) mice was established as described and mice were observed for mortality for 10 weeks. Data displayed were compiled from two independent studies. Mortality for both BALB-*lpr* and BALB-*gld* was significantly greater than that for BALB/c wild-type mice ($P < 0.002$).

sensitivity to Fas-FasL-mediated killing among the different types of cells that make up that infiltrate. Furthermore, those mice that display significant recurrent disease demonstrate a predominance of neutrophils regardless of the strain of mouse (Figure 5(b)). Thus, it would appear that once significant inflammation begins, the make-up of the infiltrate will be relatively the same regardless of any mutations to Fas or FasL. It should also be pointed out that serum HSV-1 specific antibody titers were indistinguishable between these groups of mice (data not shown).

3.5. Mice with Mutations in Fas and FasL Have Increased Reactivation and Virus at the Cornea but Do Not Show Differences in Infection of the Trigeminal Ganglia. Interestingly, when these mice were compared for their ability to shed virus following UV-induced reactivation, wild-type BALB/c mice displayed reduced rates of shedding, total number of days of shedding, duration of shedding, and number of days of shedding/mouse for positive mice when compared to BALB-*lpr* and BALB-*gld* mice (Table 1). Furthermore, mice carrying the *lpr* and *gld* mutation consistently shed virus longer than did wild-type BALB/c mice (Figure 6). In contrast, the number of viral genomes in trigeminal ganglia was very similar for all strains (Table 1). Likewise the viral titers at day 3 after reactivation did not display significant differences between wild-type BALB/c mice and those carrying either the *lpr* or *gld* mutation (Table 1).

4. Discussion

Herpetic stromal keratitis in the human is primarily a disease that results from reactivation of HSV-1 from latently infected trigeminal ganglia neurons [1, 19, 30]. This disease is also characterized by an immunopathologic attack on the cornea following such reactivations [19, 30]. The good news is that most individuals harboring a latent infection of the trigeminal ganglia probably never exhibit overt clinical disease. Thus it is clear that should virus be reactivated in these clinically silent individuals their immune response does not result in a damaging inflammatory response to the cornea. This is likely the result of several factors, some of which are driven by the immune response towards HSV-1 that develops in the infected individual and some due to intrinsic factors within the cornea that strive to limit strong inflammatory responses. One of the prime mechanisms the eye uses to protect itself from T cell-mediated immunopathologic response is the presence of FasL which induces apoptosis in Fas^+ lymphoid cells [8, 9, 11, 12]. Consequently, those factors

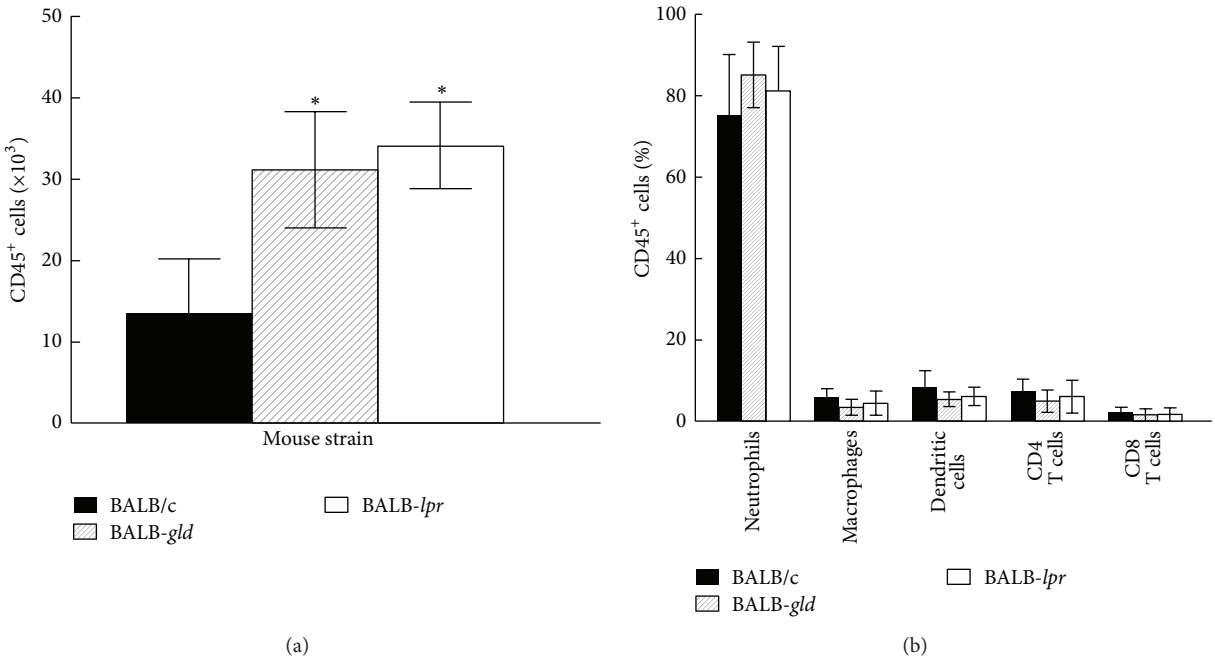

Figure 5: Inflammatory infiltrate in the corneas of BALB-*gld* and BALB-*lpr* mice displays significant increased $CD45^+$ cells, though there are no qualitative differences in subpopulations of $CD45^+$ cells. Mice were infected in one eye with HSV-1 and reactivated 8 weeks later by UV-B irradiation. The HSV-infected corneas were removed at days 17 and 23 after irradiation from mice with severe HSK disease and disaggregated into single-cell suspensions and stained with anti-CD45 (a). The $CD45^+$ cells were gated and further analyzed for staining with anti-CD4, CD8α, Gr-1, CD11b, CD11c, and F4/80 mAb (b). Cells were analyzed by flow cytometry. Data represents 4 to 6 corneas per group. Significant differences were seen in $CD45^+$ cells (a) ($P < 0.05$–0.02), but not for the percentages of $CD45^+$ subsets (b) ($P > 0.05$).

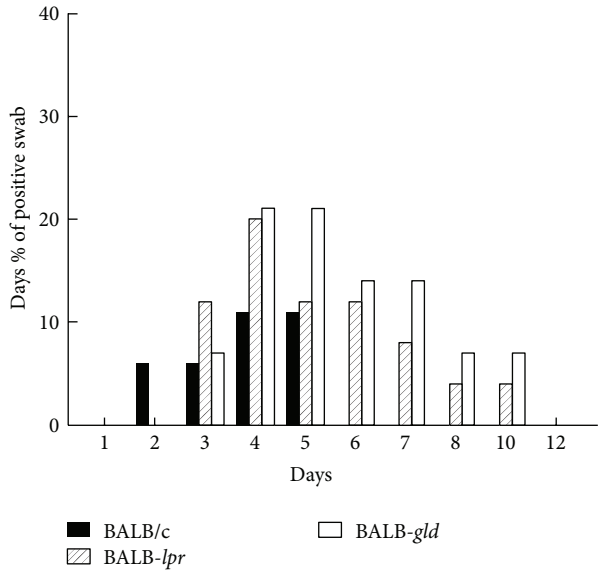

Figure 6: Daily percent of mice that were shedding virus following UV-B reactivation. Mice were reactivated by UV-B irradiation and swabbed daily and the presence of virus for each sample tested. Mice containing mutations in Fas (*lpr*) and FasL (*gld*) shed virus significantly longer than wild-type BALB/c mice ($P < 0.01$).

that either directly or indirectly lead to changes in FasL expression would greatly affect the cornea's ability to restrict inflammatory infiltrates. It is known that some factors, such as inhibitors of matrix metalloproteases, stabilize FasL expression which results in increased surface expression of FasL and a concomitant increase in the ability of the cornea or the choroid to control inflammatory [31] and angiogenic invasion [32]. Likewise the production of IL-18 has also been recently shown to induce FasL expression [29]. We report here that ex vivo infection of corneas with HSV-1 also increases the functional expression of FasL. Thus one might speculate that one way that the cornea attempts to control damaging inflammatory invasion is by increasing FasL expression following exposure to infectious agents.

We and others have reported that lack of functional Fas-FasL-mediated apoptotic ability in the eye most often leads to increased inflammatory responses [8, 9, 12], increased corneal allograft rejection [12, 33], increased acute HSK [18], increased neovascularization [10, 13, 14], and the inability to develop systemic tolerance following injection of antigen into the anterior chamber [8]. Thus it is not surprising that mice that are defective in Fas-FasL display increased recurrent HSK.

This observation is partially consistent with what was observed in these mice during acute HSK [18]. Namely, they experience significantly increased disease when compared to wild-type mice. In spite of the general similarity in results there were some potentially important differences. First, recurrent disease was more pronounced in *gld* mice than that observed in *lpr* mice. This difference, while not statistically significant, was consistent for mice on both the BALB/c and the B6 backgrounds. The reason for this difference has not

been determined and will be the subject of future studies. None the less it might relate to the fact that *gld* mice do not control neovascularization as well as *lpr* mice do [14]. It was determined that this was a consequence of the leakiness of the *lpr* mutation in terms of vascular endothelium's expression of Fas [14]. Consequently, while both *lpr* and *gld* mice would demonstrate a similar lack of control of inflammatory cell infiltrate, *gld* mice would have more neovascularization which could result in overall greater disease. In addition, since infection with HSV-1 increases the expression of FasL on the cornea, one might expect the *gld* phenotype to have a more pronounced effect on controlling any population of Fas-expressing cells to enter the infected cornea.

In addition to the minor differences in corneal disease between *lpr* and *gld* mice was the remarkable difference in mortality seen between BALB/c and both BALB-*lpr* and BALB-*gld* mice when they were infected with the neurovirulent McKrae strain of HSV-1. What is even more surprising was that this difference in mortality occurs despite the fact that all strains of mice were provided with neutralizing antibody. We had previously reported that *lpr* and *gld* mice on the B6 background that were acutely infected with the McKrae strain of HSV-1 were more resistant than wild-type B6 mice [18]. This intriguing strain-associated difference could be a clue to how different genetic backgrounds can greatly alter the ability of the HSV-1 for traffic within the infected host. We had believed that the relative resistance seen in B6-*lpr* and B6-*gld* mice was due to the lack of apoptotic cell death that might be inducing tolerance towards HSV-1 as has been reported for other types of infections [34, 35]. This does not appear to be the case with Fas/FasL mutations on the BALB/c background. Therefore, it is entirely possible that the increased inflammation seen in the cornea may also occur wherever the virus spreads and thus play a role in compromising the blood-brain barrier allowing increased viral penetration of the brain followed by entrance of inflammatory cells leading to death by encephalitis. At present we do not have direct data indicating that this is the case, but we are currently planning studies to better understand the increased mortality associated with BALB/c mice expressing mutations in Fas or FasL.

In addition to these anti-inflammatory responses that are specific to the eye, it is also well established that host T cells eliminate viral infected cells either by the perforin-granzyme pathway [36] or via apoptosis mediated by the interaction of FasL on effector cells with Fas expressed by virally infected cells [37, 38]. Thus it is also possible that mice defective in killing via Fas-FasL would display reduced ability to kill virally infected targets. This could be one of the reasons why virus persists in the corneas of both *gld* and *lpr* mice. It might also provide another explanation for why *gld* and *lpr* mice have increased mortality when infected with the McKrae strain of HSV-1. That said, lack of a functional Fas-FasL interaction does not prevent these mice from clearing infectious virus from the cornea as evidenced by the fact that infectious virus is not detectible in the corneas of reactivated mice after 10 days following reactivation.

It should also be pointed out that characterization of the inflammatory infiltrate in mice suffering from significant corneal disease illustrates two concepts. The first is that, overall, the corneas of wild-type BALB/c mice have quantitatively fewer $CD45^+$ cells than do those mice with mutations in Fas or FasL. Secondly, when mice with significant disease are analyzed qualitatively there are no significant qualitative differences between wild-type and mutant mice (Figure 5). All mice with significant disease, regardless of their genotype, have infiltrates that consist of large numbers of neutrophils and much lower numbers of T cells, macrophages, and dendritic cells. Thus, once again, it appears that corneal disease is best associated with the presence of large numbers of neutrophils that infiltrate the cornea [4, 18].

Previous work from this and other investigators have shown that development of an antibody response against HSV-1 can protect mice from the development of severe HSK [20, 39, 40]. As a consequence we tested antibody responses in BALB/c, BALB-*lpr,* and BALB-*gld* mice to determine if *lpr* or *gld* mice had impaired anti-HSV-1 responses. However, no differences were observed between these strains (data not shown), indicating that the ability to develop an anti-HSV-1 antibody response was not involved.

Taken together, these studies document that mice with impaired Fas-FasL interactions develop significantly increased HSK following UV-B induced reactivation. This response is slightly more pronounced in mice with mutations in FasL than in Fas. The mechanism responsible for increased disease is likely due to increased inflammation of the cornea not by the qualitative nature of that inflammation. This Increased inflammation is likely driven by two factors: one is the reduced control of infiltrating inflammatory cells and second a prolonged presence of infectious virus in the cornea.

Conflict of Interests

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

Acknowledgment

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