1. Introduction

It is generally accepted that dietary components are involved in immune regulation. The intestinal immune system, especially, seems to be directly affected by the digestion and absorption of dietary materials. Intestinal tissues are primary sites for infection by many pathogenic microorganisms, and commensal bacteria are abundant. Thus, the intestinal immune system has to create harmonious immunological condition, and the disruption of the intestinal immune homeostasis leads to the development of allergic, inflammatory, and infectious diseases [1,2].

Dietary lipids seem to be the dietary materials most involved in the regulation of intestinal immune responses after the conversion into lipid mediators [3]. Among various lipid mediators, sphingosine-1-phosphate (S1P) is a biologically active sphingolipid that regulates cell trafficking and activation [4,5]. S1P is abundantly present in the blood and lymph, which is originated from the cell membranes from sphingomyelin and is produced mainly by platelets, erythrocytes, and endothelial cells [6]. It is degraded by S1P lyase in the lymphoid tissues [7]. This metabolic pathway establishes an S1P gradient between the blood/lymph and lymphoid tissues and mediates cell trafficking.

The S1P gradient is recognized by cells expressing S1P receptors, and these cells migrate toward high concentrations of S1P. Of the five types of S1P receptor, type-1 S1P receptors (S1P1) are preferentially expressed by lymphocytes, and they determine lymphocyte emigration from and retention in the lymphoid tissues [8]. S1P1 is highly expressed in naive lymphocytes, including single-positive thymocytes expressing either CD4 or CD8, and expression is decreased upon lymphocyte activation. S1P1 expression recovers once the activated lymphocytes are fully differentiated and this recovery leads to their emigration from the lymphoid tissues into the blood circulation [4,5]. Studies indicate that the trafficking of macrophages, dendritic cells, and natural killer cells is mediated by S1P2, S1P3, and S1P5, respectively [9–11].

Recent studies have revealed additional functions of S1P in immune regulation that are independent of cell trafficking [4]. For example, differentiation of T cells is regulated by S1P1-mediated signaling [12–14]. It has also been demonstrated that a S1P2-mediated pathway is involved in the activation of mast cells [15] and macrophages [16], and that S1P3 are involved in dendritic cell endocytosis [10]. These findings together suggested that the S1P plays critical role in the activation and differentiation of immunocompetent cells involved in the both innate and acquired phases of immune responses in addition to their function of cell trafficking.

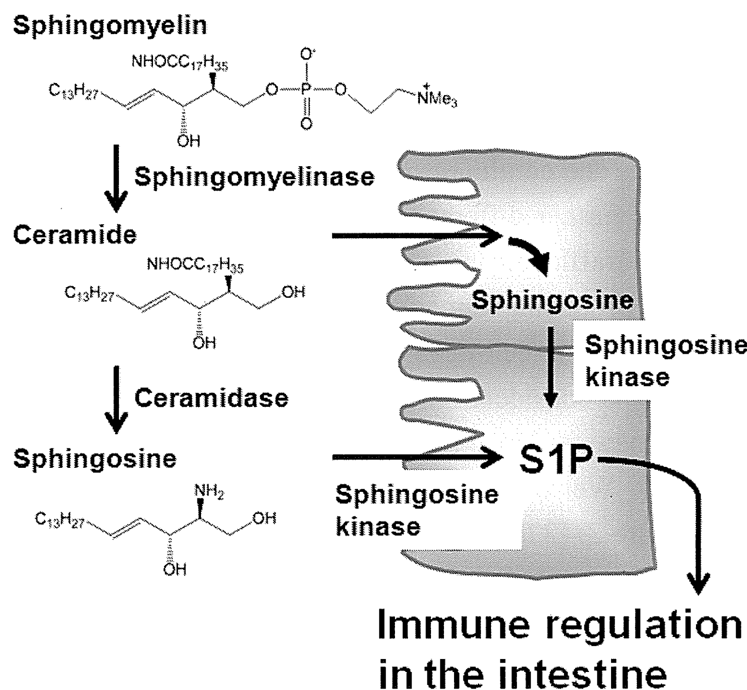
These biological and immunological functions show that S1P is involved in the maintenance of immunosurveillance as well as the development of immune diseases. In this review, we discuss the relationship between dietary materials (e.g., lipids, vitamin, and colorant) and S1P metabolism and describe the immunological functions of S1P, such as regulation of immunoglobulin A (IgA) production and intraepithelial T-lymphocyte trafficking, and its role in the development of intestinal immune diseases such as food allergy and intestinal inflammation.

2. Relationship Between S1P and Dietary Lipids

Several lines of evidence demonstrate that intestinal tissues contain higher levels of sphingolipids, including S1P, than other tissues [17]. There is no evidence of intestinal uptake of sphingolipids from

the blood, and germfree rats have comparable levels of sphingolipids in the intestine to conventional specific pathogen-free (SPF) rats [18]. Therefore, it is plausible that a source of sphingolipids in the intestine could be daily consumed diet. Adult humans ingest around 0.3 to 0.4 g sphingolipids per day, especially sphingomyelin from meat, milk, egg, and fish [19]. Dietary sphingomyelin is not directly absorbed, but is first degraded into ceramide and sphingosine [20,21] by alkaline sphingomyelinase and ceramidase, respectively, which are expressed on the apical membranes of epithelial cells [22,23]. Because epithelial cells express several key enzymes (e.g., sphingosine kinase) in the production of S1P from ceramide and sphingosine [23,24], it is possible that epithelial cells obtain ceramide and sphingosine from dietary sphingomyelin to produce S1P (Figure 1), thereby regulating intestinal immune responses and the associated intestinal immune diseases. Consistent with this, several studies showed that the incidence and severity of intestinal inflammation was changed by the uptake of dietary sphingomyelin [25,26] and the enzymatic activity of sphingomyelinase [27] and sphingosine kinase [28]. In addition, it was reported that dietary cholesterol inhibits the intestinal absorption of sphingolipids [29], implicating that cholesterol-rich Western diets may affect the availability of S1P precursors and consequently interfere with S1P-mediated intestinal immunity.

Figure 1. Dietary sphingolipids in epithelial-cell S1P production. Dietary sphingomyelin is degraded into ceramide and subsequently sphingosine by alkaline sphingomyelinase and ceramidase, respectively, which are expressed on the apical membranes of epithelial cells. In the epithelial cells, absorbed ceramide is metabolized into sphingosine. Together with absorbed sphingosine, sphingosine kinase metabolizes sphingosine into S1P, which then participates in immune regulation in the intestine.



3. Regulation of S1P Metabolism by Dietary Materials

In addition to dietary lipids, other dietary materials are also involved in the regulation of S1P metabolism. For instance, S1P lyase, a key enzyme to degrade S1P and thus keep optimal S1P low

concentration, requires vitamin B6 as a co-factor [7]. Thus, administration of vitamin B6 antagonist impaired S1P lyase activity, which consequently led to the defect of lymphocyte trafficking caused by inappropriate S1P gradient [7]. Similar effect was noted in 2-acetyl-4-tetrahydroxybutylimidazole (THI), a component of caramel food colorant III used in food products. THI inhibits S1P lyase and thus, like treatment with vitamin B6 antagonist, prevents normal lymphocyte trafficking [7]. These findings led to the use of THI for the treatment of immune diseases [30–32].

4. S1P Regulates Innate and Acquired Phases of Intestinal IgA Responses

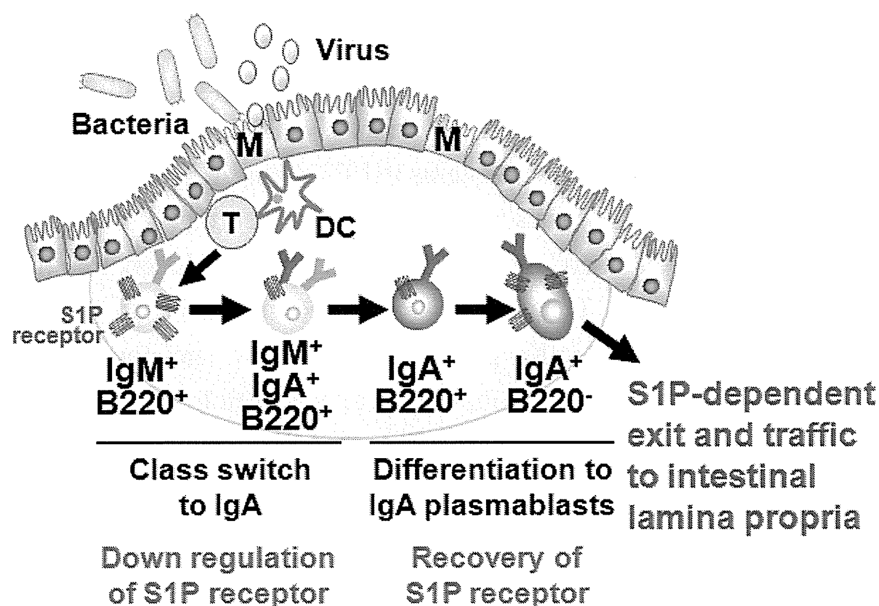
IgA is the most frequently observed antibody isotype in the intestinal compartments and provides the first line of defense against pathogenic microorganisms invading through mucosal tissues. Therefore, the induction of appropriate IgA responses is a logical strategy for the development of oral vaccines [33]. Since IgA antibody is one of the major arms of the mucosal immune system in the digestive tract, which covers a large surface area, the intestinal IgA is originated from several induction sites including Peyer's patches (PPs), isolated lymphoid follicles, and the peritoneal cavity [34].

A well characterized gut-associated lymphoid tissue (GALT) is PPs. PPs act as induction sites for the initiation of IgA responses against T-cell-dependent antigens [35]. PPs are covered with a specialized epithelium known as follicle associated epithelium (FAE) containing antigen-sampling M cells, which are responsible for the uptake and transport of antigens from the intestinal lumen to antigen-presenting cells such as dendritic cells (DCs) (Figure 2) [36]. Then, DCs capture antigens from the M cells, process and present them to T cells. It has been shown that the formation of PP DC-T cell clusters provide both cellular and molecular environment for the generation of IgA committed B cells in PPs [34]. In this pathway, some of the activated T cells differentiate into follicular helper T cells to help the antibody class switching of B cells in the germinal centers [34]. Because of the unique cytokine environment (e.g., TGF-β, IL-4, and IL-21) and continuous stimulation by commensal bacteria in the intestine, PPs have been shown to equip with efficient molecular and cellular environment for the spontaneous and continuous B cell class switching from IgM to IgA [34,35]. After class switching to IgA, B cells further differentiate into IgA plasmablasts and then migrate out from the PPs for their subsequent trafficking to the intestinal lamina propria, where they terminally differentiate into plasma cells producing dimeric (or polymeric) forms of IgA. This process mainly contributes to the development of T cell-dependent antigen-specific immune responses. Thus, the PP-mediated induction pathway is considered to be a major arm of the acquired IgA response [34].

Our investigation provided new evidence that S1P regulated the B cell trafficking in the PPs for the intestinal IgA production [37]. We initially found that S1P1 expression in B cells changes during differentiation in the PPs (Figure 2) [37]. High levels of S1P1 expression were detected in IgM^+ naive B cells, and expression was down-regulated when B cells started class switching to IgA. The low expressions of S1P1 allowed newly class-switched IgA^+ B cells to retain in the PPs for the sufficient differentiation into the IgA^+ plasmablasts. S1P1 expression was restored on the IgA^+ plasmablasts, resulting in their emigration from the PPs. Mice treated with FTY720, an immunosuppressant inducing S1P1 downregulation [38], show selective accumulation of IgA^+ plasmablasts in the PPs, leading to the disturbance of continuous delivery of IgA committed B cells from the PPs to the lamina propria of intestine. Consequently, the decrease of same population in the intestinal lamina propria was noted,

which associated with the reduction of intestinal antigen-specific IgA responses against orally immunized protein antigen [37].

Figure 2. Sequential changes in S1P1 expression during B-cell differentiation in Peyer's patches. Dendritic cells (DC) take the antigens transported by M cells from intestinal lumen and present them to T cells for their activation. Through the interaction with T cells and DCs, IgM^+ naive B cells show class-switch from IgM to IgA. During this process, S1P1 is expressed at high levels in IgM^+ naive B cells and downregulated on B cells class-switching from IgM to IgA and subsequently recovered on IgA^+ $B220^-$ plasmablasts, resulting in their emigration from the Peyer's patches and traffic into the intestinal lamina propria.



In the IgA production pathway in the gut, peritoneal B cells are an additional source of intestinal IgA [39]. A number of peritoneal B cells belong to a unique B-cell subset, termed as B1 cells, which produces antibodies against T-cell-independent antigens such as lipids and polysaccharides. Because these T-cell-independent antigens are conserved in various microorganisms, B1-cell-derived antibodies undiscriminatingly react to commensal and pathogenic bacteria and prevent their attachment and invasion into the host. This reaction is opposite to antibody responses against protein antigen mediated by PP B cells, which show rigid specificity against microorganisms. Therefore, it has been considered that B1-cell-derived IgA is categorized as to be innate-type antibodies that recognize a wide range of microorganisms in the intestine [39].

Trafficking of peritoneal B1 cells into the intestine requires S1P-mediated signaling [40]. Like B cells in the PPs, peritoneal B1 cells identically expressed S1P1. Thus, trafficking of peritoneal B cells into the intestine and consequent production of intestinal IgA are diminished by treatment with FTY720, mainly because of the inhibition of B1 cell emigration from the parathymic lymph nodes, which drain to the peritoneal cavity [40]. This impaired trafficking in FTY720-treated mice was associated with the decreased IgA responses against phosphorylcholine (a T-cell-independent antigen) induced by oral immunization with heat-killed *Streptococcal pneumoniae* [40].

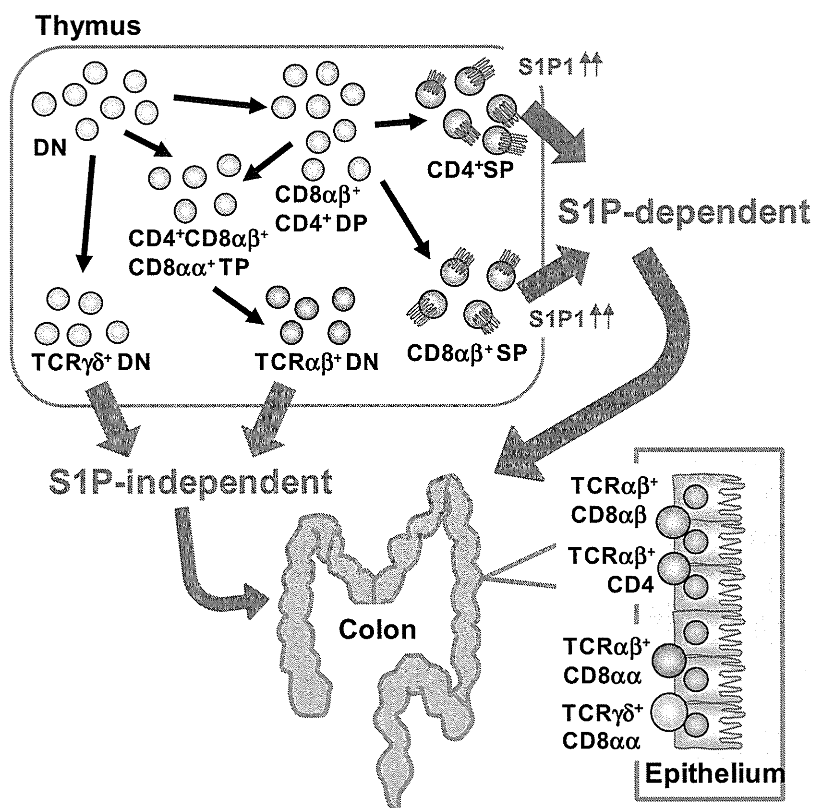
We also found that S1P-mediated regulation of peritoneal B-cell trafficking requires crosstalk with stromal cells in the peritoneal cavity [41]. This interaction mediated by adhesion molecules (e.g., ICAM-1 and VCAM-1) on stromal cells and the expression is regulated by NFκB-inducing kinase (NIK). Therefore, NIK-mutant aly/aly mice show decreased sensitivity to FTY720 in the regulation of peritoneal B-cell trafficking due to the impaired expression of adhesion molecules although peritoneal B1 cells in aly/aly mice expressed comparable levels of S1P1.

5. Distinct S1P Dependency of Trafficking of Intraepithelial T-Lymphocytes in the Gut

Large numbers of lymphocytes are also present in the intestinal epithelium and called as intraepithelial lymphocytes (IELs) [42]. IELs are mostly T cells, but unlike in conventional T cells observed in the systemic compartments (e.g., spleen) which predominantly express the αβ T-cell receptor (αβTCR), in the IEL subset there is an abundance of T cells expressing the γδ T cell receptor (γδTCR) in addition to $αβTCR^+$ T cells [42]. αβTCR recognizes peptide antigen presented via major histocompatibility complex (MHC) molecules, whereas γδTCR recognizes non-classical MHC molecules such as MHC class I chain-related proteins (MIC) A and B (MICA/B) in human and Rae-1 in mouse [43]. Unlike MHC molecules that act as ligand by presenting peptide antigen, non-classical MHC molecules act as a ligand by itself and the expression was induced by stress (e.g., infection, tumors, or chemical treatment) [44]. Thus, it is considered that αβTCR is involved in acquired immunity through the activation by specific presentation of antigenic peptides, whereas γδTCR is involved in innate immunity by the ligation of non-classical MHC molecules [42]. A distinctive pattern of CD8 expression has also been noted in IELs. Conventional $αβTCR^+$ T cells express CD8 as a heterodimer of α and β (CD8αβ). In contrast, some IELs uniquely express CD8 as a homodimer (CD8αα) [42]. A previous study identified a unique precursor of CD8αα IELs in the thymus [45]. In the thymus, $CD4^-$ $CD8^-$ double-negative thymocytes differentiate into $CD4^+$ $CD8^+$ double-positive thymocytes and then further differentiate into single-positive thymocytes expressing either CD4 or CD8. $CD8αβ^+$ IELs are derived mainly from $CD8^+$ single-positive thymocytes expressing αβTCR. $CD8αα^+$ IELs, however, originate from double-negative thymocytes expressing either αβTCR or γδTCR that have themselves differentiated from unique $CD4^+$ $CD8αα^+$ $CD8αβ^+$ triple-positive thymocytes (Figure 3) [45].

S1P has been involved in the regulation of cell trafficking of different subsets of IELs originated from thymus. We found that each type of IEL shows a different dependency on S1P in its trafficking from the thymus to the intestine, especially in the colon (Figure 3) [46]. When mice were treated with FTY720, decreased numbers of $CD8αβ^+$ IELs were observed. In contrast, the numbers of $CD8αα^+$ IELs were barely affected. These data suggest that, in the colonic epithelium, $CD8αβ^+$ IELs are S1P dependent and $CD8αα^+$ IELs are S1P independent. Consistent with this finding, $CD8^+$ single-positive thymocytes—the precursors of $CD8αβ^+$ IELs—express high levels of S1P1 [8], whereas no S1P1 expression has been noted on double-negative thymocytes, the precursors of $CD8αα^+$ IELs [46]. These findings suggest that S1P1 expression was different in different subsets of thymic precursors of IELs and provide versatile immunological pathways in the intestine.

Figure 3. Distinct dependency on S1P in T-cell trafficking into the colonic epithelium. In the thymus, $CD4^-$ $CD8^-$ double-negative (DN) thymocytes differentiate into $CD4^+$ $CD8^+$ double-positive (DP) thymocytes and then into single-positive (SP) thymocytes expressing either CD4 or CD8 and αβTCR. These SP thymocytes express high levels of S1P1 and migrate out from the thymus and into the colon in an S1P-dependent manner. DN thymocytes express TCRαβ or TCRγδ. DN thymocytes expressing TCRαβ are derived from $CD4^+$ $CD8\alpha\alpha^+$ $CD8\alpha\beta^+$ triple-positive (TP) thymocytes differentiated from DN or DP thymocytes. Little or no S1P1 expression is noted in the DN thymocytes expressing TCRαβ or TCRγδ, so traffic to the colonic epithelium proceeds in an S1P-independent manner.

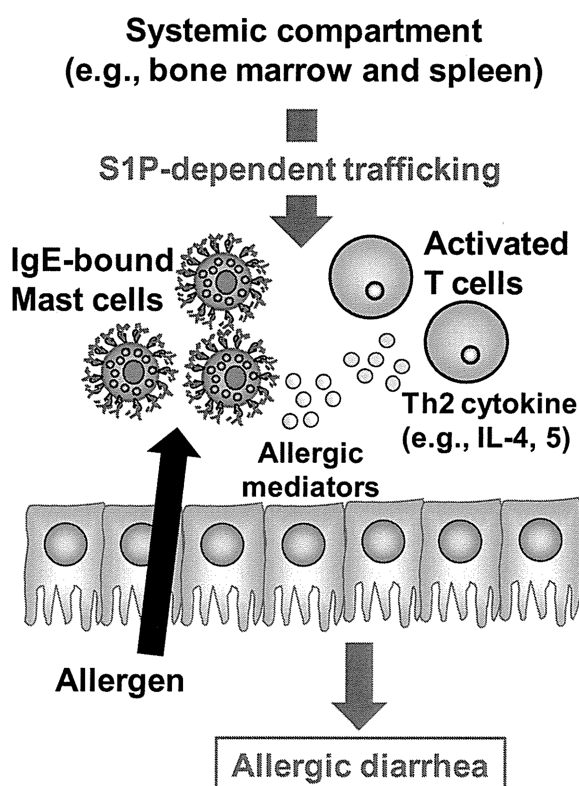


6. S1P-Mediated Regulation in the Development of Intestinal Immune Diseases

Accumulating evidence has revealed the pivotal role of S1P in the development of inflammatory diseases such as autoimmune type 1 diabetes, rheumatoid arthritis, and multiple sclerosis [5]. FTY720 prevents the egress of autoreactive lymphocytes from the lymph nodes into the peripheral circulation and subsequent across the blood–brain barrier into the central nerve system and thus has recently been approved as an oral therapy for multiple sclerosis [47]. In addition to being involved in these immune diseases at the systemic immune compartments, S1P is involved in the development of intestinal immune diseases including food allergies and intestinal inflammation [5]. The number of patients with food allergies has increased not only in children but also in adults; the development of effective preventive and therapeutic strategies for food allergies is therefore required to improve patients' quality of life. Using the ovalbumin-induced murine food-allergy model developed by our group [48], we examined the molecular and cellular mechanisms underlying the development of food allergies and found that, in allergic mice, activated T cells migrate into the colon, where they produced high

amounts of Th2 cytokines such as IL-4 and IL-5 [48]. We demonstrated that the trafficking of pathogenic T cells from the systemic compartments into the colon was mediated by S1P (Figure 4) [49]. Indeed, activated T cells in the colon of allergic mice expressed S1P1 and their infiltration into the colon and subsequent production of Th2 cytokines (e.g., IL-4 and IL-5) were inhibited by the treatment with FTY720 [49]. In addition, the infiltration of mast cells, effector cells in the development of food allergy, into the colon was also prevented in the FYT720-treated mice [49]. As a mechanism of FTY720-mediated inhibition of mast cell infiltration, it was likely that FTY720 directly and indirectly prevented the mast cell infiltration into the colon. Direct effect of FTY720 was predicted by results that mast cells expressed S1P1 and their *in vitro* migration was inhibited by FTY720 [49]. Indirect effect is mediated by activated T cells producing Th2 cytokines which enhanced the proliferation and recruitment of mast cells [50]. Thus, inhibition of activated T cell trafficking into the colon by FTY720 resulted in the reduced recruitment and/or proliferation of mast cells. Taken together, involvement of S1P in the trafficking of both pathogenic T cells and mast cells is a potential target for prevention and treatment of food allergies.

Figure 4. S1P mediates intestinal allergy by regulating pathogenic T and mast cell infiltration into the colon. In murine food allergy model, systemically sensitized T cells migrate into the colon upon the oral challenge with same allergen. This trafficking is mediated by S1P and thus treatment with FTY720 resulted in the inhibition of activated T cell trafficking into the colon. In the colon, these activated T cells produced high amounts of Th2 cytokines such as IL-4 and IL-5 for promotion of mast cell recruitment and proliferation. In addition, mast cell itself expresses S1P1. Therefore, FTY720 treatment directly and indirectly (Th2 cytokine from activated T cells) decreases the numbers of mast cells in the colon. These effects lead to the inhibition of allergic diarrhea.



Similarly, several lines of evidence have demonstrated that the FTY720 treatment prevents the development of intestinal inflammation [51–53]. For example, in a spontaneous colitis model in interleukin-10-deficient mice, administration of FTY720 suppressed the infiltration of pathogenic T cells producing interferon-γ [51]. Infiltration of the colon by pathogenic T cells was also inhibited by treatment with FTY720 in both a dextran sulfate sodium (DSS)-induced colitis model and a T-cell transfer model in mice [52,53]. Although S1P regulates the activation of several inflammatory cells via modulation of the signaling of certain innate receptors such as toll-like receptors, TNF receptor, and protease-activated receptor 1, and S1P itself is produced by activated inflammatory cells [4], collectively these findings suggest that S1P–S1P1 axis participates mainly in the development of intestinal immune diseases at the stage of pathogenic cell trafficking into the colon.

7. Conclusion

It is clear from past and current studies that S1P plays an important role in the regulation of the immune system of the gut in both healthy and disease states. In general, S1P is derived from sphingomyelin and is produced mainly by platelets, erythrocytes, and endothelial cells in the body. However, in the intestine, it is likely that epithelial cells contribute most to the production of S1P. Most importantly, S1P produced by epithelial cells seems to originate from dietary sphingolipids, especially sphingomyelin. Thus, elucidation of the complex networks established by dietary lipids will create a new era in nutrition-based mucosal immunology and should provide a new strategy against intestinal immune diseases.

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Conflict of Interest

The authors declare no conflict of interest.

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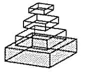

Alcaligenes is commensal bacteria habituating in the gut-associated lymphoid tissue for the regulation of intestinal IgA responses

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Secretory-immunoglobulin A (S-IgA) plays an important role in immunological defense in the intestine. It has been known for a long time that microbial stimulation is required for the development and maintenance of intestinal IgA production. Recent advances in genomic technology have made it possible to detect uncultivable commensal bacteria in the intestine and identify key bacteria in the regulation of innate and acquired mucosal immune responses. In this review, we focus on the immunological function of Peyer's patches (PPs), a major gut-associated lymphoid tissue, in the induction of intestinal IgA responses and the unique immunological interaction of PPs with commensal bacteria, especially *Alcaligenes*, a unique indigenous bacteria habituating inside PPs.

Keywords: Peyer's patch, IgA, commensal bacteria

INTRODUCTION

Secretory-immunoglobulin A (S-IgA) is predominantly observed in the intestine where it participates in immune defense (Mestecky et al., 2005; Brandtzaeg, 2010). S-IgA inhibits adherence of pathogens to host epithelial cells in the intestinal lumen and neutralizes pathogenic toxins by binding to the toxins' biologically active sites. Based on the immunological importance of S-IgA in immunosurveillance in the intestine, the development of oral vaccines has focused on the induction of antigen-specific S-IgA responses (Kunisawa et al., 2007). In addition to the immunosurveillance in the intestine, S-IgA antibody contributes to the establishment of beneficial gut commensal microbiota and thus dysfunction of S-IgA formation resulted in the alteration of normal bacterial flora (e.g., the reduction of Lactobacillus and increase of segmented filamentous bacteria, SFB; Suzuki et al., 2004).

Peyer's patches (PPs) are major gut-associated lymphoid tissue (GALT) where intestinal IgA responses are initiated and regulated by unique immunological crosstalk via cytokines [e.g., interleukin-4 (IL-4), IL-6, IL-21, and transforming growth factor-β (TGF-β)] and cell–cell interactions (e.g., via CD40/CD40 ligand interactions) among dendritic, T, and B cells (Kunisawa et al., 2008; Fagarasan et al., 2010). Thus, oral delivery of antigens to PPs is considered an important strategy for the effective induction of antigen-specific intestinal IgA responses (Kunisawa et al., 2011).

In addition to host-derived factors, microbial stimulation is also required for the maximum production of S-IgA in the intestine (Cebra et al., 2005). Indeed, germ-free (GF) mice have decreased intestinal IgA responses with immature structure of GALT when compared with mice housed under SPF or conventional conditions (Weinstein and Cebra, 1991). Although it was reported that some commensal bacteria [e.g., SFB and altered Schaedler flora (ASF), a combined eight culturable bacteria] and bacterial products (e.g., peptidoglycan, CpG oligonucleotide, and LPS) stimulated the intestinal IgA production (Michalek et al., 1983; Talham et al., 1999; Butler et al., 2005), it is obscure which bacteria is involved in this process indigenously. Because predominant commensal bacteria in the intestine is uncultivable, it was difficult to determine by culture-based method which bacteria regulated specific immune responses. However, recent advances in the genomic analysis allowed us to identify the uncultivable bacteria, which revealed key bacteria in the regulation of specific immune responses (Ivanov et al., 2009; Atarashi et al., 2011) as well as the development of immune diseases (Chow et al., 2010; Hill and Artis, 2010). Using genomic and immunological methods, we recently found that the microbial community inside PPs is different from those on the epithelium of PPs or in the intestinal lumen (Obata et al., 2010).

In this review, we discuss initially the immunological features of PPs in the induction and regulation of intestinal IgA responses. In the later part, we focus on the unique cross-communication between PPs and habitat commensal bacteria, *Alcaligenes*, a unique indigenous bacteria habituating inside PPs and regulating dendritic cells (DCs) for the efficient production of intestinal IgA.

IMMUNOLOGICAL FEATURES OF PEYER'S PATCHES

In the intestine, GALT comprise several different, organized lymphoid structures (Spencer et al., 2009; Fagarasan et al., 2010). Among them, PPs are the largest and most well-characterized sites

for the initiation of intestinal IgA responses, especially responses to T cell-dependent antigens (Kunisawa et al., 2008; Fagarasan et al., 2010). There are generally 8–10 PPs in the mouse small intestine and hundreds in the human small intestine. Each PP is composed of several B cell-rich follicles surrounded by a mesh-like structure consisting of T cells known as the interfollicular region (**Figure 1**).

Inside PPs, antigen-sampling M cells located in the follicle-associated epithelium transport luminal antigens to DCs situated in the subepithelium region (Neutra et al., 2001), which then form clusters with T-, B-, and stromal cells in the germinal centers and promote μ-to-α-class-switch recombination of B cells with the help of cytokines such as IL-4, IL-21, and TGF-β (Fagarasan et al., 2010). Upon immunoglobulin class-switching from μ to α, IgA-committed B cells (IgA^+ B cells) begin to express type 1 sphingosine-1-phosphate receptor, CCR9, and α4β7 integrin, allowing them to depart from the PPs and subsequently traffic to the intestinal lamina propria (Mora et al., 2006; Gohda et al., 2008). In the intestinal lamina propria, they further differentiate into IgA-secreting plasma cells under the influence of terminal differentiation factors (e.g., IL-6; Cerutti et al., 2011). DCs play a key role in these processes. For instance, nitric oxide, TGF-β, APRIL, and BAFF produced by TNF-α/iNOS-producing DCs (Tip-DCs) promotes IgA production (Tezuka et al., 2007). Also, DCs in the PPs metabolite vitamin A and produce retinoic acid, which induces the expression of gut-homing receptors (CCR9, and α4β7 integrin) on activated B and T cells (Iwata et al., 2004; Mora et al., 2006). Retinoic acid also induces the preferential differentiation into regulatory T (Treg) cells (Hall et al., 2011), and some of Treg cells differentiated into follicular helper T cells to promote IgA production in the PPs (Tsuji et al., 2009).

The identification of the molecular pathway of PP organogenesis allowed the establishment of PP-deficient mice through the loss of any part of this pathway (Nishikawa et al., 2003). Notably, disruption of the PP organogenesis pathway by blockade of tissue genesis cytokine receptor signaling [IL-7R and/or lymphotoxin-β receptor (LTβR)] during a limited fetus time period results in the selective loss of PPs without affecting other lymphoid tissue organogenesis (Yoshida et al., 1999). Experiments with PP-deficient mice showed that the dependency on PPs in the induction of antigen-specific IgA responses depends on the form of the antigen. For instance, the PP-deficient mice failed to develop antigen-specific IgA responses against orally administered antigens in particle form, but retained their ability to respond to soluble forms of antigens (Yamamoto et al., 2000; Kunisawa et al., 2002). It was also reported that lamina propria DCs are capable of initiating systemic IgG responses, whereas antigen transport by M cells into the PPs is required for the induction of intestinal IgA production (Martinoli et al., 2007). This is consistent with another finding that DCs in the PPs are responsible for intestinal IgA production (Fleeton et al., 2004). Therefore, PPs are considered to be one of the major sites for the initiation of intestinal antigen-specific IgA responses.

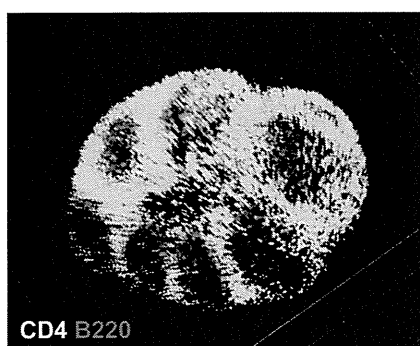

FIGURE 1 | Microarchitecture of murine Peyer's patches. Purified T cells (green) and B cells (red) were chemically labeled with carboxyfluorescein succinimidyl ester and carboxy-SNARF-1, respectively, and adoptively transferred into mice. Fifteen hours after the transfer, cell distribution in the Peyer's patches was observed at the whole-tissue level by using macro-confocal microscopy.

EFFECT OF MICROBIAL STIMULATION ON THE PRODUCTION OF INTESTINAL IgA

It is well known that microbial stimulation is required for the full production of S-IgA in the intestine. Indeed, GF mice have decreased intestinal IgA responses when compared with mice housed under SPF or conventional conditions (Cebra et al., 2005). Studies using mono-associated GF mice with SFB have demonstrated that only a minor proportion of the total intestinal IgA is reactive to mono-associated bacteria (Talham et al., 1999). In addition, bacterial products produced by commonly expressed on commensal bacteria (e.g., peptidoglycan, CpG oligonucleotide, and LPS) stimulated the intestinal IgA production (Michalek et al., 1983; Butler et al., 2005). In contrast, a recent study using reversible colonization of GF mice with genetically engineered *E. coli* showed that intestinal IgA induced in those mice bound to parent strain but not other bacteria (Hapfelmeier et al., 2010). Therefore, it remains unclear whether intestinal IgA responses induced by commensal bacteria is mediated by polyclonal stimulation and/or by B cell receptors specific for microbial antigens.

As one mechanism of impaired IgA production of GF mice, it was reported that GF mice have structurally immature GALT (e.g., PPs and ILFs) when compared with SPF mice (Weinstein and Cebra, 1991; Hamada et al., 2002). In the PPs, several key pathways for the IgA production require microbial stimulation. For example, Tip-DCs enhance the IgA production by producing nitric oxide, TGF-β, APRIL, and BAFF, which requires microbial stimulation through innate receptors (Tezuka et al., 2007). Indeed, the number of Tip-DCs was much reduced in the intestine of GF and MyD88-deficient mice (Tezuka et al., 2007). Another cell involved the microbe-dependent IgA production is non-hematopoietic follicular DCs (FDCs). It was reported that microbial stimulation of FDCs resulted in expressing chemokine CXCL13, BAFF, and TGF-β for the germinal center formation and B cell class-switching from IgM to IgA (Suzuki et al., 2010).

ALCALIGENES IS A UNIQUE INDIGENOUS BACTERIA INSIDE PPs

Recent advances in genomic technology make it possible to detect commensal bacteria in the intestine, allowing identification of key bacteria involved in the regulation of specific immune responses. For example, SFB was identified as commensal bacteria inducing

Th17 cells (Ivanov et al., 2009), whereas colonic regulatory T cells were induced by *Clostridium* clusters IV and XIV (Atarashi et al., 2011). These commensal bacteria localize at the surface of intestinal epithelium, but we supposed that the immunological crosstalk between host and commensal bacteria might establish in the regulation of intestinal IgA responses in the GALT. In this issue, we analyzed the composition of the microbial community inside PPs and identified *Alcaligenes* as a major commensal bacteria uniquely locating inside PPs (Obata et al., 2010).

By using the 16S rRNA clone library method, SFB are the predominant commensal bacteria co-habitat on FAE of PPs as like small intestinal epithelium. Although the FAE consisted with antigen-sampling M cells, SFB was not found inside of PPs. Instead, *Alcaligenes* are predominant bacteria inside PPs. The result obtained by the 16S rRNA analysis was further confirmed by fluorescence *in situ* hybridization (FISH) method and thus *Alcaligenes* are present exclusively inside PPs, not on the FAE of PPs, and intestinal villous epithelium and intestinal lamina propria (**Figure 2**). Of note, the preferential presence of *Alcaligenes* was observed not only in mouse but also in monkey and human (Obata et al., 2010). One of interesting but unresolved questions is the species specificity of *Alcaligenes*. We are now investigating whether *Alcaligenes* isolated from human or monkey colonize in the PPs to promote IgA production when they are orally fed to GF mice. Inside PPs, a proportion of the *Alcaligenes* seemed to be alive in mice. The presence and growth of *Alcaligenes* were detected in the PPs of GF mice after adoptive transfer of PP homogenates containing *Alcaligenes* from SPF mice. These findings suggest that *Alcaligenes* are indigenous bacteria ubiquitously living inside the PPs of various mammalian species.

ANTIBODY-MEDIATED RECIPROCAL INTERACTION BETWEEN *ALCALIGENES* AND THE HOST IMMUNE SYSTEM

As mentioned above, M cells located on the FAE of PPs transport luminal bacteria into DCs locating at the subepithelial region of FAE (Neutra et al., 2001). 16S rRNA clone library methods consistently revealed that DCs in the PPs predominantly contain *Alcaligenes*, whereas these bacteria are rarely detected in DCs isolated from other lymphoid tissues (e.g., spleen and mesenteric lymph nodes; Obata et al., 2010). We examined the immunological effects of *Alcaligenes* on DCs and found that the production of IgA-enhancing cytokines such as IL-6, TGF-β, and BAFF was increased when DCs isolated from the PPs of GF mice were stimulated with *Alcaligenes* (Obata et al., 2010). Several lines of evidence have revealed that immunological functions of DCs are different between intestinal and other lymphoid tissues (reviewed in Rescigno, 2010), we are now investigating whether immune stimulatory functions of *Alcaligenes* is specific for the PP DCs or not.

In agreement with the uptake of *Alcaligenes* and subsequent production of IgA-enhancing cytokines by DCs, *Alcaligenes*-specific IgA-forming cells were frequently observed in PPs, and consequent IgA production was noted in the intestinal lumen of SPF mice, but not GF mice (Obata et al., 2010). Although biological role of *Alcaligenes*-specific IgA antibody remains to be elucidated, the antibody might be involved in the creation of intra-tissue co-habitation of *Alcaligenes* in PPs. To this end, the number of *Alcaligenes* inside PPs is decreased in B cell-deficient CBA/N xid and IgA-deficient mice compared with wild-type mice (Obata et al., 2010). Therefore, it is interesting to suggest that *Alcaligenes*-specific IgA antibody mediates the uptake and presence of *Alcaligenes* in the PPs. Since M cells express IgA receptors

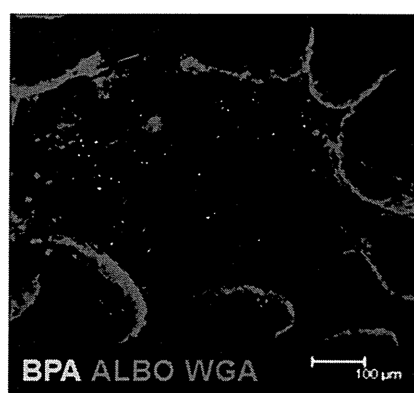

FIGURE 2 | Microarchitecture of murine Peyer's patches. Whole-mount fluorescence *in situ* hybridization was performed to visually analyze the presence of *Alcaligenes* inside PPs. Both BPA and ALBO34a were used as specific probes for *Alcaligenes*. Wheat germ agglutinin (WGA), an *N*-acetylglucosamine-specific lectin, was used to detect epithelial cells. Scale bar indicates 100 µm.

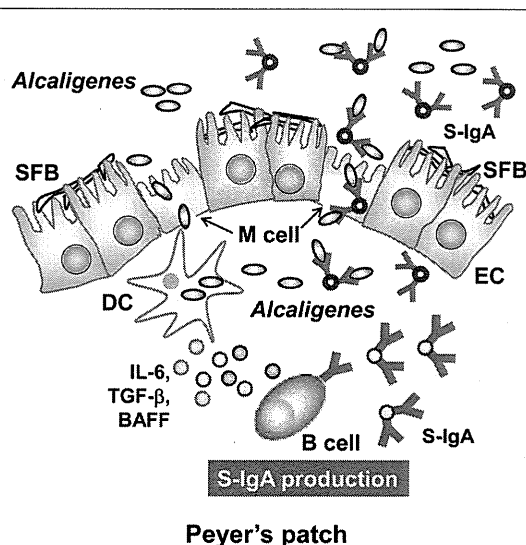

FIGURE 3 | *Alcaligenes* mediates symbiotic communication inside Peyer's patches. On the follicle-associated epithelium of PPs, segmented filamentous bacteria (SFB) is predominantly observed. In contrast, *Alcaligenes* specifically localizes inside Peyer's patches, where some are taken up by dendritic cells (DCs). Stimulation by *Alcaligenes* prompts the DCs to produce IgA-enhancing cytokines [e.g., interleukin-6 (IL-6), transforming growth factor-β (TGF-β)], and B cell activating factor (BAFF), which enhance the intestinal IgA response. The intestinal IgA includes *Alcaligenes*-specific IgA, which might mediate the preferential uptake and presence of *Alcaligenes* in the PPs. The uptake is presumably mediated by M cells.

(Mantis et al., 2002), one possibility is that *Alcaligenes* coated with the *Alcaligenes*-specific antibody are taken up into PPs through M cells. Further, the antigen-specific IgA coating on *Alcaligenes* might be beneficial for the bacteria to create the co-habitation niche since IgA antibody has been shown to non-inflammatory antibody (Mestecky et al., 2005).

CONCLUSION

In this review, we discussed a new concept of symbiotic communication in PPs that is mediated by commensal bacteria-specific IgA antibody. *Alcaligenes*-specific antibodies may mediate the uptake and the presence of *Alcaligenes* in the PPs, and the co-habitation of *Alcaligenes* within the PPs is one of the key factors to promote the intestinal IgA production by enhancing the production of IgA-enhancing cytokines from DCs (**Figure 3**). We still have various questions regarding this co-habitation of *Alcaligenes* in the PPs. For example, it remains unclear whether the presence of *Alcaligenes* inside of PPs is physiologically beneficial or harmful for the host immune system. In this issue, we are now addressing the microbial community in the PPs of mice and human patients suffering from intestinal immune diseases (e.g., intestinal inflammation and allergy). The biological roles of intra-tissue habitation of *Alcaligenes* in the PPs in the appropriate regulation of mucosal immune responses need to be elucidated. The current goal is to elucidate the mechanisms behind the co-habitation of *Alcaligenes* within PPs, and the exact contribution of *Alcaligenes* to educate and guide mucosal immunocompetent cells especially DCs in the PPs for the development, maturation and maintenance of the appropriate host immune system. These studies will provide novel molecular and cellular mechanisms of symbiotic communication with commensal bacteria in the regulation of host immunity.

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Membrane-bound human SCF/KL promotes in vivo human hematopoietic engraftment and myeloid differentiation

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In recent years, advances in the humanized mouse system have led to significantly increased levels of human hematopoietic stem cell (HSC) engraftment. The remaining limitations in human HSC engraftment and function include lymphoid-skewed differentiation and inefficient myeloid development in the recipients. Limited human HSC function may partially be attributed to the inability of the host mouse microenvironment to provide sufficient support to human hematopoiesis. To address this problem, we created membrane-bound human stem cell factor (SCF)/KIT ligand (KL)–expressing NOD/SCID/IL2rgKO (hSCF Tg NSG) mice. hSCF Tg NSG recipients of human HSCs showed higher levels of both human CD45$^+$ cell engraftment and human CD45$^+$CD33$^+$ myeloid development compared with NSG recipients. Expression of hSCF/hKL accelerated the differentiation of the human granulocyte lineage cells in the recipient bone marrow. Human mast cells were identified in bone marrow, spleen, and gastrointestinal tissues of the hSCF Tg NSG recipients. This novel in vivo humanized mouse model demonstrates the essential role of membrane-bound hSCF in human myeloid development. Moreover, the hSCF Tg NSG humanized recipients may facilitate investigation of in vivo differentiation, migration, function, and pathology of human mast cells. (*Blood*. 2012;119(12):2768-2777)

Introduction

The humanized mouse system, a xenogeneic transplantation and engraftment model for human hematopoietic stem cells (HSCs) and peripheral blood (PB) mononuclear cells (MNCs), facilitates the investigation of human hematopoietic and immune systems in vivo.[1,2] Since the pioneering work using SCID-hu[3] and Hu-PBL-SCID models,[4] investigators have attempted to better recapitulate human biology in mice across xenogeneic immunologic barriers. Recently, the introduction of targeted null mutations of immune-related genes, such as *Rag1, Rag2, Il2rg*, or *Prf1* in recipient mice, has improved engraftment levels of human CD45$^+$ leukocytes.[2,5-9] However, limitations remain in the ability of the host mouse hematopoietic microenvironment to support human hematopoiesis. The impaired development of human T-lymphoid and myeloid lineage cells compared with human B-lymphoid lineage cells in NOD/SCID and other immune-compromised mice may be the result of the lack of appropriate microenvironmental support. The recently created human leukocyte antigen (HLA) class I expressing immune-compromised NOD/SCID/IL2r γ null (NSG) mice partially addresses this issue for human T-cell development. Human CD8$^+$ T cells developing within these recipients of transplanted human HSCs exhibited cytokine production and cytotoxicity in an HLA-restricted manner.[10-12]

To create a hematopoietic microenvironment more suitable for human myeloid development, we developed a new immune-compromised mouse strain that expresses human membrane bound stem cell factor (SCF) under the control of the phosphoglycerate kinase (PGK) promoter (hSCF Tg NSG). Using hSCF Tg NSG mice as recipients of human HSCs, we aimed to clarify the role of membrane-bound form of SCF in supporting the engraftment of human hematopoietic cells and influencing the differentiation of the human myeloid lineage in the recipient mouse BM, spleen, and other organs. Here we show nearly complete human hematopoietic chimerism in the BM of hSCF Tg NSG recipients. In the BM of these recipients, human granulocytes accounted for the majority of engrafted human cells reflecting the physiologic human BM status. In addition to the development of immature and mature granulocytes, c-Kit$^+$ human mast cells differentiated efficiently in BM, spleen, and mucosal tissues. The hSCF Tg NSG mice, by supporting efficient human myeloid development including mast cells, may serve as a novel platform for in vivo investigation of human mast cell development and allergic responses.

Methods

Mice

NOD.Cg-*Prkdc^{scid} IL2rg^{tm1Wjl}* (NSG) mice and NOD.Cg-*Prkdc^{scid} IL2rg^{tm1Wjl}* Tg(PGK1-KITLG*220)441Daw/J, abbreviated as hSCF Tg NSG mice,

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were generated at The Jackson Laboratory. The human membrane-bound SCF transgene driven by the human PGK promoter was backcrossed more than 10 generations from the original C3H/HeJ strain background[13] onto the NSG strain. All the mice were bred and maintained at The Jackson Laboratory and animal facility at RIKEN RCAI under defined flora according to guidelines established and approved by the Institutional Animal Committees at each respective institution.

Purification and transplantation of human HSCs

All experiments were performed with authorization from the Institutional Review Board for Human Research at RIKEN RCAI. Cord blood (CB) samples were first processed for isolation of MNCs using LSM lymphocyte separation medium (MP Biomedicals). CB MNCs were then enriched for human $CD34^+$ cells using anti–human CD34 microbeads (Miltenyi Biotec) and sorted for $7\text{-}AAD^-$ lineage $(hCD3/hCD4/hCD8/hCD19/hCD56)^-CD34^+CD38^-$ HSCs using FACSAria (BD Biosciences). To achieve high purity of donor HSCs, doublets were excluded by analysis of forward scatter (FSC)–height/FSC-width and side scatter (SSC)-height/SSC-width. Purity of each sorted sample was higher than 95%. Newborn (within 2 days of birth) hSCF Tg and non-Tg NSG recipients received 150 cGy total body irradiation using a ^{137}Cs-source irradiator, followed by intravenous injection of 5×10^2 to 5.3×10^4 sorted HSCs via the facial vein.

Analysis of human cell engraftment by flow cytometry

The recipient PB harvested from the retro-orbital plexus was evaluated for human hematopoietic engraftment every 3 to 4 weeks starting at 4 to 6 weeks after transplantation. After lysis of erythrocytes, cells were stained with anti-hCD45, anti-msCD45, anti-hCD3, anti-hCD19, anti-hCD33, and anti-hCD56 to determine human hematopoietic chimerism and to analyze cell lineages engrafted in the recipients. At 8 to 35 weeks after transplantation, the recipients were killed and single-cell suspensions of BM and spleen were analyzed using flow cytometry. Antibodies used for flow cytometry are specified in supplemental Methods (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). The labeled cells were analyzed using FACSCantoII or FACSAria (BD Biosciences).

Morphologic analysis of cytospin specimens

Cytospin specimens of FACS-purified human myeloid cells were prepared with a Shandon Cytospin 4 cytocentrifuge (Thermo Electric) using standard procedures. To identify nuclear and cytoplasmic characteristics of each myeloid cell, cytospin specimens were stained with 100% May-Grünwald solution (Merck) for 3 minutes, followed by 50% May-Grünwald solution in phosphate buffer (Merck) for additional 5 minutes, and then with 5% Giemsa solution (Merck) in phosphate buffer for 15 minutes. All staining procedures were performed at room temperature. Light microscopy was performed with Zeiss Axiovert 200 (Carl Zeiss).

Microarray analysis

Purified $hCD45^+CD33^+c\text{-}Kit^-CD203c^-HLA\text{-}DR^-$ granulocytes and $hCD45^+CD33^+$ $c\text{-}Kit^-CD203c^-HLA\text{-}DR^+CD14^+$ monocytes from BM of 4 hSCF Tg NSG recipients and 3 non-Tg NSG recipients as well as neutrophils and monocytes from 2 healthy persons were evaluated using Human Genome U133 plus Version 2.0 GeneChips (Affymetrix). Total RNA was extracted with TRIzol (Invitrogen) from more than 10^4 sorted cells and amplified to cDNA using the Ovation Pico WTA System (Nugen). Biotinylated cDNA was synthesized with Two-Cycle Target Labeling Kit (Affymetrix). Microarray data were analyzed using the Bioconductor package (Bioconductor; http://www.bioconductor.org). The signal intensities of the probe sets were normalized using the GC-RMA program (Bioconductor). The RankProd program was used to select differentially expressed genes with a cutoff P value of less than .01 and an estimated false-positive rate of less than 0.05.[14] Gene annotation was obtained from Ingenuity Pathway Analysis and Gene Ontology Annotation databases (Ingenuity systems, http://www.ingenuity.com; Gene Ontology Annotation, http://www.ebi.ac.up/GOA). For differentially transcribed genes, GO term enrichment analysis was performed according to a method described by Draghici et al[15] with a correction of multiple testing using false discovery rate.[16] Eventually, GO terms with the false discovery rate-corrected P value $< .05$ were selected as functionally enriched terms. Raw data for microarray data are accessible at the RefDIC database (http://refdic.rcai.riken.jp) under the following accession numbers: RSM06616, RSM06617, RSM06618, RSM06620, RSM06621, RSM06622, RSM06623, RSM06633, RSM06642, RSM06648, RSM06665, RSM06667, RSM06668, RSM06669, RSM06670, RSM08241, and RSM08243. Differences in expression levels were considered significant if P is $< .05$ using Kruskal-Wallis, Wilcoxon-Mann-Whitney, or Student t test in KaleidaGraph (Synergy Software).

IHC and immunofluorescence imaging

Thin ($\sim 5\text{-}\mu m$) sections prepared from paraformaldehyde-fixed paraffin-embedded tissues were stained with H&E using standard procedures. Immunohistochemistry (IHC) and immunofluorescence labeling were performed using standard procedures. Antibodies used for IHC and immunofluorescence labeling were mouse anti–human mast cell tryptase monocolonal antibody (Dako North America, clone AA1), mouse anti–human CD45 monoclonal antibody (Dako North America, clone $2B11^+PD7/26$), rabbit anti–human CD117 monoclonal antibody (Epitomics, clone YR145), and rabbit anti–human CD14 polyclonal antibody (Atlas Antibodies). Light microscopy was performed using an Axiovert 200 (Carl Zeiss). For quantification of $tryptase^+$ cell frequency, 3 high-power fields from 3 different recipients were examined using AutoMeasure module of AxioVision software (Release 4, Carl Zeiss). Confocal microscopy was performed using a LSM710 equipped with C-APOCHROMAT $40\times/1.2$ (Carl Zeiss).

Results

Human hematopoietic repopulation is enhanced in hSCF Tg NSG recipients

The humanized mouse model system has served as a tool to investigate human hematopoiesis, immunity, and diseases in vivo. However, one of the major limitations in the system is that the microenvironment supporting human hematopoiesis and immunity is primarily of mouse origin. In the present study, we created a strain of NSG mice expressing membrane-bound human SCF to analyze the role of the BM microenvironment in human hematopoietic lineage determination and development.

c-Kit, the receptor for SCF, is expressed at lower levels in human CB $Lin^-CD34^+CD38^-$ early HSCs and at high levels in mast cells.[17-19] For reconstitution of human myeloid and lymphoid cells, 5×10^2 to 5.3×10^4 FACS-purified CB $Lin^-CD34^+CD38^-$ HSCs were transplanted into newborn sublethally irradiated (1.5 Gy) hSCF Tg NSG mice and into non-Tg NSG controls (Table 1). To determine the kinetics of human hematopoietic chimerism in the recipient circulation, we performed flow cytometric analysis of PB every 3 to 4 weeks starting at 4 to 6 weeks after transplantation. During long-term observation, all the 21 hSCF Tg NSG recipient mice became moribund at 8 to 35 weeks after transplantation. Complete blood count analysis demonstrated reduced erythrocyte hemoglobin concentration in the PB of hSCF Tg NSG recipients compared with non-Tg NSG recipients (Figure 1A). Anemia in hSCF Tg NSG recipients was not associated with abnormalities in mean corpuscular volume, mean corpuscular hemoglobin, or mean corpuscular hemoglobin concentration (supplemental Figure 1). The suppression of host erythropoiesis in the hSCF Tg NSG recipients was related to the irradiation and engraftment of the human HSCs because unmanipulated nontransplanted hSCF Tg NSG mice did not develop anemia (supplemental Figure 1).

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Table 1. Summary of hSCF Tg NSG and non-Tg NSG recipients analyzed

				CBC at time of death					% chimerism at time of death			% of CD45⁺ in BM				% of CD33⁺ in BM			% of CD45⁺ in BM			
Recipient ID	CB ID	Graft dose	Survival, wks	WBC, × 10³/L	RBC, × 10⁴/μL	Hemoglobin, g/dL	Hematocrit, %	Platelets, ×10³/μL	PB	BM	Spleen	CD33⁺	CD3⁺	CD19⁺	CD3⁻ CD56⁺	CD117⁺ CD203c⁺	HLA DR⁻	HLA DR⁺	CD117⁺ CD203c⁺	HLA DR⁻	HLA DR⁺	% chimerism of erythroid cells in BM
N1-1	1	5000	21	1.1	500	10.0	35.0	550	75.5	93.3	95.3	31.1	1.2	50.2	0.3	0.9	51.5	47.6	0.3	16.0	14.8	NA
N1-2	1	5000	16	1.4	730	13.0	44.0	1100	42.6	76.1	72.9	35.2	0.1	54.8	0.4	0.7	39.6	59.7	0.2	13.9	21.0	NA
N1-3	1	5000	24	NA	NA	NA	NA	NA	20.7	12.6	4.4	32.9	3.8	60.7	0.0	4.4	37.9	57.7	1.0	12.5	19.0	3.1
S1-1	1	5000	23	4.1	220	6.7	15.6	150	99.1	99.7	94.4	77.2	24.5	19.2	0.5	3.9	80.6	15.5	3.0	62.2	11.9	NA
S1-2	1	5000	20	6.8	220	5.0	18.0	30	83.2	99.4	97.7	75.5	2.6	28.4	0.3	6.7	68.7	24.6	5.1	51.8	18.6	36.3
S1-3	1	5000	21	0.7	320	7.0	25.0	330	76.9	99.7	95.8	70.9	1.5	16.6	0.2	14.6	50.0	35.4	10.4	35.5	25.1	0.0
S1-4	1	500	26	0.5	150	3.0	11.0	250	30.7	97.0	86.3	69.0	2.0	47.5	0.1	6.9	68.6	24.5	4.8	47.3	16.9	NA
N2-1	2	10 000	35	0.8	390	8.0	30.0	110	31.7	54.9	87.1	50.3	21.8	25.8	2.4	19.1	7.4	73.5	9.6	3.7	37.0	11.5
S2-1	2	10 000	16	1.3	186	4.1	13.2	583	90.7	96.6	99.3	18.7	3.2	40.6	1.2	34.4	18.0	47.6	6.4	3.4	8.9	NA
S3-1	3	10 000	13	0.2	215	3.9	12.6	793	52.0	97.8	90.9	54.2	0.0	42.8	0.7	19.0	44.6	36.4	10.3	24.2	19.7	NA
S3-2	3	10 000	15	2.2	161	3.1	9.8	458	93.6	99.6	98.2	61.5	4.4	20.4	1.4	7.3	60.5	32.2	4.5	37.2	19.8	NA
S4-1	4	10 000	13	0.5	97	1.9	5.1	6	98.6	100.0	98.2	52.0	1.2	39.8	0.3	2.7	81.9	15.4	1.4	42.6	8.0	27.2
N5-1	5	12 000	35	3.1	310	7.0	25.0	550	6.8	36.8	48.9	29.9	17.5	41.9	NA	22.6	7.0	70.4	6.8	2.1	21.1	5.8
N6-1	6	14 000	23	2.1	520	10.0	34.0	620	57.6	65.0	83.6	29.3	17.6	43.1	1.6	3.3	45.1	51.6	1.0	13.2	15.1	7.4
N6-2	6	14 000	21	1.6	680	12.0	40.0	660	64.6	25.3	81.5	10.4	17.2	64.4	0.5	33.9	17.5	48.6	3.5	1.8	5.1	1.4
N6-3	6	14 000	24	2.3	490	10.0	33.0	80	73.8	71.8	92.8	18.5	13.9	51.1	0.8	10.6	8.5	81.4	2.0	1.6	15.1	12.8
S6-1	6	14 000	16	2.2	236	4.5	15.5	33	70.0	88.5	94.7	39.4	5.8	43.6	0.7	68.9	8.1	23.0	27.1	3.2	9.1	33.1
S6-2	6	14 000	14	4.6	270	6.0	21.0	108	85.4	90.0	95.2	33.3	11.6	46.8	0.9	36.5	16.0	47.5	12.2	5.3	15.8	5.8
S7-1	7	15 000	16	4.9	293	6.1	19.4	132	93.6	99.7	98.8	76.5	1.4	42.3	0.6	1.6	80.1	18.3	1.2	61.3	14.0	NA
S8-1	8	16 000	13	1.0	183	3.3	9.4	78	98.6	100.0	99.7	16.2	19.9	52.1	0.9	25.1	1.0	73.9	4.1	0.2	12.0	NA
S8-2	8	16 000	11	1.0	376	5.6	18.4	568	89.5	99.6	98.5	48.6	0.2	54.5	0.6	4.9	42.0	53.1	2.4	20.4	25.8	NA
N9-1	9	18 000	20	2.7	650	13.0	42.0	130	71.6	87.2	92.0	15.0	1.7	72.4	0.4	1.4	24.7	74.0	0.2	3.7	11.1	9.8
S9-1	9	18 000	16	1.4	164	3.4	10.6	80	90.4	99.9	97.0	51.4	0.2	35.1	0.2	2.4	54.9	42.7	1.2	28.2	21.9	25.5
N10-1	10	20 000	19	1.0	660	12.0	42.0	77	38.4	46.5	87.1	25.9	44.4	24.1	0.8	3.9	6.2	89.9	1.0	1.6	23.3	12.0
N10-2	10	20 000	14	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
N10-3	10	20 000	12	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
S10-1	10	20 000	19	2.5	292	6.1	20.9	6	95.5	99.7	97.3	42.3	14.4	35.5	0.9	5.7	28.8	65.5	2.4	12.2	27.7	4.3
S10-2	10	20 000	14	1.5	170	3.0	11.0	30	95.8	99.7	89.3	27.3	28.4	28.0	5.4	18.6	19.8	61.6	5.1	5.4	16.8	17.1
N11-1	11	36 000	20	25.1	470	9.0	31.0	240	84.1	70.7	94.0	19.5	5.9	63.6	0.7	3.8	17.8	78.4	0.7	3.5	15.3	42.3
S11-1	11	36 000	10	19.2	420	8.0	27.0	410	77.5	98.0	95.3	34.0	0.2	43.5	0.5	4.5	34.6	60.9	1.5	11.8	20.7	58.5
N12-1	12	53 000	8	NA	NA	NA	NA	NA	36.9	95.6	83.3	20.3	0.0	72.2	0.2	NA	NA	NA	NA	NA	NA	NA
S12-1	12	53 000	11	NA	NA	NA	NA	NA	100.0	100.0	99.9	43.5	6.5	39.1	1.5	NA	NA	NA	NA	NA	NA	NA
S12-2	12	53 000	8	NA	NA	NA	NA	NA	93.1	100.0	NA	65.6	0.2	13.2	NA	1.6	83.7	14.7	1.0	54.9	9.6	NA
S12-3	12	53 000	13	13.6	211	3.9	10.9	165	57.0	79.5	68.3	47.5	12.5	35.7	2.2	8.8	59.9	31.3	4.2	28.5	14.9	NA
N13-1	13	17 000	20	6.4	720	13.0	45.0	470	39.2	85.0	81.8	21.8	0.0	69.0	0.2	0.4	46.1	53.5	0.1	10.0	11.7	7.6
S13-1	13	17 000	20	2.8	290	7.0	27.0	510	74.9	93.7	94.5	38.1	6.0	47.8	0.4	26.6	31.3	42.1	10.1	11.9	16.0	48.4

A total of 21 human HSC-engrafted hSCFTg NSG (S) recipients and 15 human HSC-engrafted non-Tg NSG (N) recipients were created.
WBC indicates white blood cell count; RBC, red blood cell count; and NA, not applicable.