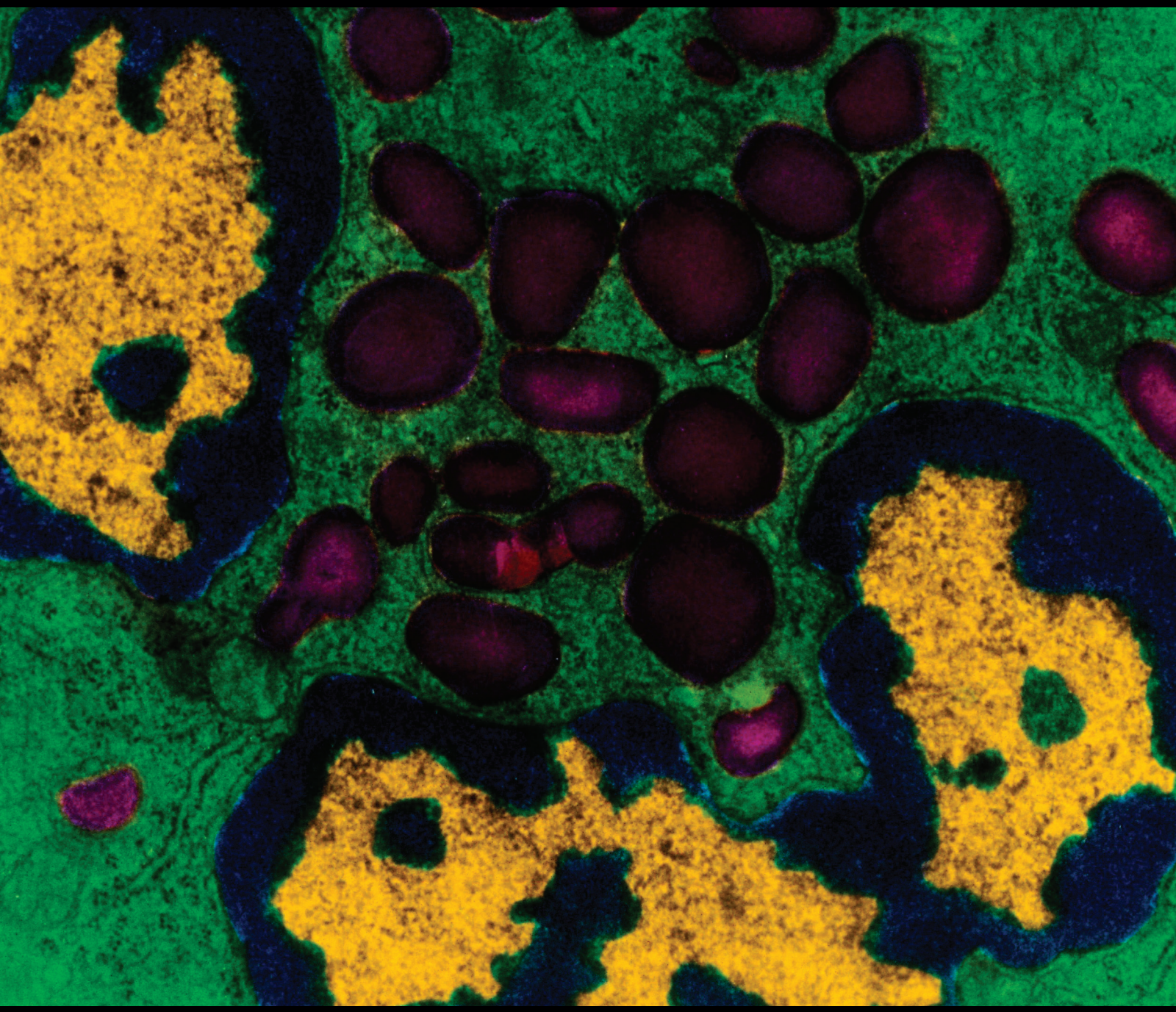
Mediators of Inflammation

Sphingolipids in Inflammation: From Bench to Bedside

Guest Editors: Kazuyuki Kitatani, Kazuhisa Iwabuchi, Ashley Snider, and Laura Riboni



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Editorial Board

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Editorial

Sphingolipids in Inflammation: From Bench to Bedside

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Sphingolipids have been appreciated as bioactive lipids that regulate a diverse range of cellular responses [1, 2]. In recent years many efforts of researchers were made to improve our knowledge of sphingolipids in pathophysiological inflammation. Different studies demonstrated that cellular signaling in inflammatory processes is controlled by ceramide [3], sphingosine-1-phosphate (S1P) [4], ceramide-1-phosphate [5], and glycosphingolipids (such as lactosylceramide and GM3) [6]. The molecular mechanisms underlying this signaling have been extensively studied.

This special issue is composed of ten articles including three research articles and seven review articles. These contributions review important discoveries and provide novel findings that support the multifaceted role of sphingolipids in inflammation.

Dysregulated formation of several sphingolipids including S1P and ceramide has been implicated in inflammatory bowel disease (IBD). L. Abdel Hadi et al. describe sphingolipid metabolism and signaling in IBD and discuss the potential of sphingolipid-targeted molecules as therapeutic strategies for this disease.

Metabolic disease, such as obesity and type 2 diabetes, is emerging as a major health crisis in many countries. High fat diet is a primary contributing factor for obesity and its related diseases. S. Choi and A. J. Snider review the evidence for sphingolipid metabolism and pathobiology in models of high fat diet.

Glycosphingolipids cluster with sphingomyelin and cholesterol in plasma membranes, forming lipid microdomains (lipid rafts) considered as platforms for signal transduction. K. Iwabuchi et al. review the evidence for biological significance of lactosylceramide-enriched microdomains in immunological and inflammatory responses of neutrophils. They also overview the significance of ceramide species and its metabolites in biological functions. A. M. Bryan et al. discuss the findings pointing to the importance of sphingolipids in immune responses of macrophages and neutrophils to fungal infections. R. Ghidoni et al. review roles of sphingolipid in the pathobiology of lung inflammation.

Three research articles discuss novel findings for S1P and its receptors. E. Moon et al. discover an involvement of S1P in stroke damage in initial and recurrent stroke models. A. Chumanevich et al. reveal that S1P/S1P receptor 2 axis promotes mast cell angiogenic potential. C. Zhao et al. demonstrate that the sphingolipid pathway controlling S1P levels is dysregulated in rheumatoid arthritis synovial fibroblasts. In addition, M. Aoki et al. and S. Mahajan-Thakur et al. review the evidence pointing to roles of S1P and its receptors in immune system and blood coagulation system.

This special issue discusses the topics associated with sphingolipid metabolism and pathobiology in inflammation. The articles in this special issue not only provide novel

findings in sphingolipid pathobiology, but also discuss the evidence collected from a large number of research articles, giving insight into drug discovery for inflammation-associated diseases.

Acknowledgment

We are most grateful to many people who contributed to this special issue, whether by writing, advising, or generating the inspiring contributions discussed in the special issue.

Kazuyuki Kitatani
Kazuhisa Iwabuchi
Ashley Snider
Laura Riboni

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Review Article

Sphingosine-1-Phosphate Signaling in Immune Cells and Inflammation: Roles and Therapeutic Potential

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Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid metabolite involved in many critical cell processes. It is produced by the phosphorylation of sphingosine by sphingosine kinases (SphKs) and exported out of cells via transporters such as spinster homolog 2 (Spns2). S1P regulates diverse physiological processes by binding to specific G protein-binding receptors, S1P receptors (S1PRs) 1–5, through a process coined as "inside-out signaling." The S1P concentration gradient between various tissues promotes S1PR1-dependent migration of T cells from secondary lymphoid organs into the lymphatic and blood circulation. S1P suppresses T cell egress from and promotes retention in inflamed peripheral tissues. S1PR1 in T and B cells as well as Spns2 in endothelial cells contributes to lymphocyte trafficking. FTY720 (Fingolimod) is a functional antagonist of S1PRs that induces systemic lymphopenia by suppression of lymphocyte egress from lymphoid organs. In this review, we summarize previous findings and new discoveries about the importance of S1P and S1PR signaling in the recruitment of immune cells and lymphocyte retention in inflamed tissues. We also discuss the role of S1P-S1PR1 axis in inflammatory diseases and wound healing.

1. Introduction

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid mediator involved in many physiological processes including angiogenesis and immune responses [1, 2]. S1P signaling has been found to be essential for vascular development, neurogenesis, and lymphocyte trafficking [3–5], as well as a second messenger during inflammation [6, 7]. Many of the actions of S1P in innate and adaptive immunity are mediated by its binding to five specific G protein-coupled receptors, S1P receptors (S1PRs) 1–5. To date, a number of S1P receptor modifying compounds have been developed [8]. FTY720 (Fingolimod, Gilenya, Novartis) is a functional antagonist of S1PR and was originally discovered by chemical modification of a natural product, myriocin. FTY720 and other S1PR modifying compounds have clarified that S1P is important for the recruitment of various types of inflammatory cells [9, 10]. In this review, we summarize current research findings on the functions of S1P in the recruitment of immune cells into inflamed tissues and discuss its role in inflammatory diseases and wound healing.

2. Sphingosine Kinases (SphKs) and S1P Signaling

S1P is a pleiotropic, bioactive, lipid metabolite of ceramide. Ceramide is the basic unit of sphingolipids and consists of a sphingosine attached to a long-chain fatty acyl group via its amino group. Whereas ceramide and sphingosine are associated with cellular growth arrest and apoptosis, S1P is associated with cellular survival and suppression of apoptosis [11]. Ceramide is broken down by ceramidases to sphingosine, which in turn is phosphorylated by one of two

SphKs, SphK1 and SphK2, to generate S1P [12]. S1P can then either be dephosphorylated by two S1P-specific phosphatases (SPP1 and SPP2) or irreversibly degraded by S1P lyase (SPL) to phosphoethanolamine and hexadecenal [6, 12]. SphK1 is located close to the cell membrane, where it can be activated by numerous stimuli, including proinflammatory cytokines, to generate S1P [6]. Ceramide is also phosphorylated in the Golgi apparatus by ceramide kinase to produce ceramide-1-phosphate (C1P). These sphingolipid metabolites, ceramide, C1P, and S1P, are bioactive molecules which are important in inflammation. S1P is particularly important in immune cell trafficking [13]. There has been extensive investigation into the extracellular signaling of S1P, particularly its role in innate and adaptive immunity. We have learned much less about the intracellular targets and signaling of S1P.

It has been proposed that S1P formed by SphK1 in response to tumor-necrosis factor (TNF) binds to the TNF receptor-associated factor 2 (TRAF2) and enhances its E3 ligase activity. This leads to lysine-63-linked polyubiquitination of receptor interacting protein 1 (RIP1) and eventually NF-κB activation [14]. TRAF-interacting protein (TRIP) suppresses the TRAF2 ubiquitin-dependent pathway by modulating the TRAF2-S1P interaction [15]. Within sites of sterile inflammation, S1P formed by SphK1 binds to the cellular inhibitor of apoptosis 2 (cIAP2) in response to interleukin-1 (IL-1) and enhances its lysine-63-linked polyubiquitination activities [16]. In response to IL-1, SphK1 and cIAP2 form a complex with interferon-regulatory factor 1 (IRF1), leading to its polyubiquitylation and activation. Consequently, IRF1 enhances expression of the chemokines CXCL10 and CCL5, which recruit mononuclear cells into sites of sterile inflammation [16]. Despite these findings, SphKs are not indispensable for the inflammatory response by macrophages [17]. This suggests that the role of SphKs as mediators in inflammatory cytokine signaling may be system or disease specific and not an essential part of the inflammatory cascade.

In contrast to the prosurvival SphK1, SphK2 inhibits cell growth and enhances apoptosis [18]. Furthermore, S1P formed in the nucleus by SphK2, or by inhibition of SPL, binds and inhibits the histone deacetylases HDAC1 and HDAC2, linking sphingolipid metabolism to inflammatory and metabolic gene expression [19, 20]. Interestingly, S1P produced in the mitochondria by SphK2 binds with high affinity and specificity to prohibitin 2 (PHB2), a highly conserved protein that regulates mitochondrial assembly and function [21]. Conjugated bile acids also bind to S1PR2 in hepatocytes [22] and the SphK2 generated S1P regulates hepatic lipid metabolism via histone deacetylase inhibition in the nucleus. This provides evidence for the role of S1P in the development of nonalcoholic fatty liver disease [23]. On the other hand, SphK1 was also reported to possess potential anti-inflammatory function by activation of p38 MAPK that suppress chemokine levels, and, in this system, activation of NF-κB is separated from SphK1 [24]. Further, the neuroinflammatory response was significantly upregulated in LPS-induced brain injury in SphK1$^{-/-}$ mice [25]. The function of SphKs and S1PR signaling in inflammation is still unclear and may be more complex than the current dogma.

Following transport out of cells, S1P binds to its ligand, consisting of a family of five specific G protein-coupled receptors in a paracrine and/or autocrine manner, known as "inside-out signaling" [1, 2, 11, 14, 16]. The crystal structure of S1PR1 suggests that extracellular access to the binding pocket by S1P occurs by sliding in the plane of membrane [26]. S1P regulates lymphocyte trafficking in immunity and allergy by attracting the lymphocytes to migrate via various receptors [27]. S1PR1 induces chemotaxis and membrane ruffling in phosphoinositide (PI) 3-kinase- and Rac-dependent manners, which induces a biphasic increase in the amount of the GTP-bound Rac. This causes the formation of the stress fibers and cytoskeletal rearrangement that decreases vascular permeability. S1PR1 and S1PR3 induce a migratory response in various types of immune cells. S1PR2 has been thought to possess function opposite of S1PR1 and S1PR3. As a G protein-coupled receptor, S1PR2 couples to Gi/o, Gq, and G12, and G13, as opposed to S1PR1, which couples solely to Gi/o. Activation of G12 and G13 leads to activation of Rho. S1PR2 has been associated with abolishment of IGF 1-directed chemotaxis and membrane ruffling, thus increasing vascular permeability in a manner dependent on the concentration gradient of S1P [28].

Recently, bile acids were found to bind to S1PR2 and regulate lipid metabolism in hepatocytes [23]. S1PR3 signaling in endothelial cells contributes to vasorelaxation. On the other hand, S1PR3 signaling in vascular smooth muscle cells contributes to vasopressor effect. Through such mechanisms, S1P and its analogues can influence heart rate via S1PR3 [29]. S1PR4 and S1PR5 have limited, specialized function in inflammation. S1PR4 is related to the migration of neutrophils from blood to tissue [30]. S1PR5 is expressed predominantly by oligodendrocytes and/or fibrous astrocytes in the rat brain and couples with Gi/oα proteins for migration and survival of those cells [31–33]. Patrolling monocytes also express high levels of S1PR5 similar to Natural Killer (NK) cells; however, it is suggested that S1PR5 in monocytes regulate their trafficking via a mechanism independent of S1P gradients [34]. S1P transport and extracellular signaling are an area of active research as they have implications for the tumor microenvironment in cancer and immune cell trafficking [2].

3. Role of S1P and S1PRs in the Regulation of Immune Cell Trafficking

S1P signaling via S1PRs is involved in various aspects of inflammatory cell function. T and B lymphocytes, as well as endothelial cells, express distinctive profiles of S1PRs. These S1PR profiles are major regulators of development, recirculation, tissue homing patterns, and chemotactic responses to chemokines of B and T cells [35]. S1PR signaling is also involved in modulation of circulating monocytes similar to lymphocytes and affects monocyte activation through CD40 expression and TNF-α production [36]. Notably, S1P regulates migration and endocytosis of mature dendritic cells via S1PR3, but not S1PR1 [37]. S1P increases macrophage homing, lymphocyte contact, and endothelial junctional complex formation in lymph nodes (LN) [38].

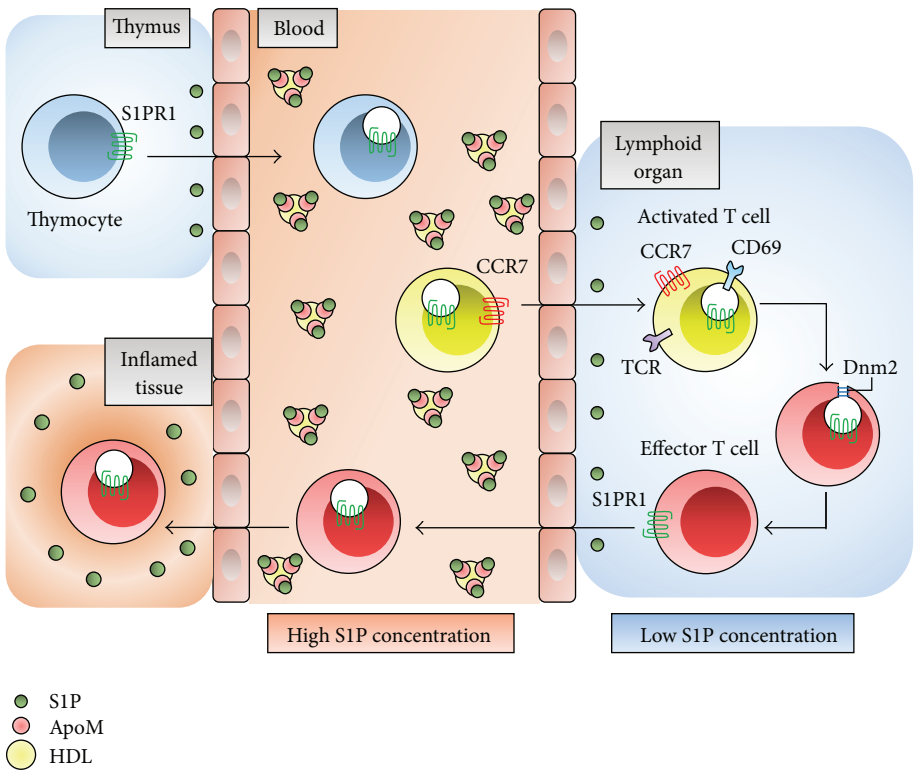

Figure 1: The role of S1P-S1PR1 axis in T cell trafficking. S1P is maintained at low concentration in the thymus and lymphoid organs and is in high concentration in blood, binding to ApoM in HDL particles. S1PR1 expression in T cells is downregulated in blood, and T cells are shifted into lymphoid organs with CCR7 signaling. CD69 forms a complex with S1PR1 and downregulates S1PR1 to promote retention in activated T cells. Because Dnm2 enables continuous S1PR1 signaling in lymphocytes, effector T cells can sense low S1P condition and egress from lymphoid organs. ApoM: apolipoprotein M; HDL: high-density lipoprotein; CCR7: CC-chemokine receptor 7; TCR: T cell receptor; Dnm2: dynamin 2.

S1P mediates chemotaxis of macrophages *in vitro* and *in vivo* via S1PR3 and causes atherosclerosis by promoting inflammatory macrophage recruitment and altering smooth muscle cell behavior [10]. S1P is also involved in mast cell and eosinophil and dendritic cell recruitment in asthma [39].

Both the S1P gradient between the bone marrow and blood and the expression of S1PR1 are essential for optimal hematopoietic stem cell mobilization and trafficking during steady-state hematopoiesis [40]. During the inflammatory process, both S1PR expression on lymphocytes and endothelial cells and S1P levels in various immune compartments are modified. This results in transient arrest of lymphocytes in secondary lymphoid tissues, which is crucial for the generation of adaptive immunity and subsequent promotion of lymphocyte recruitment to sites of inflammation [29].

3.1. S1P-S1PR1 Axis in Lymphocyte Trafficking and Retention in Inflamed Tissue. Separate sources provide S1P to blood and lymphatic fluid [41]. Circulating blood S1P is believed to be mainly hematopoietic in origin, with erythrocytes as a major contributor, whereas lymphatic fluid S1P is from lymphatic endothelial cells. Recent studies clarified that hepatic apolipoprotein M (ApoM) produced by the liver increases S1P biosynthesis in hepatocytes and also influences plasma S1P levels [42, 43]. The majority of plasma S1P binds to ApoM in high-density lipoprotein (HDL). In spite of the fact that ApoM-S1P is not essential for lymphocyte trafficking, it inhibits lymphopoiesis through S1PR1 signaling in bone marrow lymphocyte progenitors [44].

The differential S1P concentration gradient facilitates egress of lymphocytes from lymphoid organs into blood and lymphatic fluid [13, 45]. In addition to the S1P gradient, S1PR1 is also essential for lymphocyte egress from the thymus and secondary lymphoid organs [46]. The positive gradient of S1P concentration between secondary lymphoid organs and lymphatic fluid presumably promotes S1PR1-dependent movement of T cells from secondary lymphoid organs back into the lymphatic circulation and then into blood [47]. Dynamin 2 is essential for S1PR1 internalization in low S1P concentrations and enables uninterrupted S1PR1 signaling and promotes S1P egress from both the thymus and LN. This function may be involved in the mechanism by which T cells sense low S1P concentrations and egress into circulatory fluids [48] (Figure 1).

Multiple S1PRs have been shown to be associated with lymphocyte biology, recirculation, and determination of T cell phenotypes. The expression of S1PR1 on T cells regulates their egress from the thymus and entry into the blood [49]. Lymphocyte S1PR1 expression is downregulated in the blood, upregulated in lymphoid organs, and downregulated again in

the lymphatic fluid. This ligand-induced modulation of S1PR1 in circulating lymphocytes contributes to establishing their lymphoid organ transit time [50].

T cell activation and proliferation are mediated by the T cell antigen receptor (TCR), which translocates plasma membrane S1PR1 to the nuclear envelope membranes to facilitate association with Gi/o, Erk1/2, and other proteins [51]. T cells switch to a state favoring egress over retention by simultaneously upregulating S1PR1 and downregulating CCR7. LN retention of naïve lymphocytes depends on fibroblastic reticular cells (FRCs) of LN, while activated T cells remain in LN because of downregulated S1PR1 and are independent in FRCs [52]. CD69 can additionally form a complex with S1PR1 and downregulate S1PR1 through downstream IFN-α/IFN-β, and possibly other activating stimuli, to promote lymphocyte retention in lymphoid organs [53]. On the other hand, the S1P/S1PR2 axis inhibits early airway T cell recruitment in mast cell-dependent acute allergic responses in mice [54].

The increased S1P present in inflamed peripheral tissues may induce T cell retention. T cell migration from blood into tissue is induced by chemokines CXCL9–CXCL11 presented on the endothelial surface, which activates b1- and b2-integrin adhesion molecules and surface expression of S1PR1 and S1PR4 on T cells [55]. S1PR1 agonism inhibits migration of tissue T cells into afferent lymphatics in homeostatic and inflammatory conditions and causes the arrest of egress into inflamed tissues from the blood. This is mediated at least partially by interactions of the integrin LFA-1 with its ligand ICAM-1, and the integrin VLA-4 with its ligand VCAM-1 at the basal surface of lymphatic endothelium [56]. Heterotrimeric guanine nucleotide-binding protein-coupled receptor kinase-2 (GRK2) has been shown to function in downregulating S1PR1 on blood-exposed lymphocytes, allowing them to be retained in inflamed tissues [57]. According to the latest findings, regulation of KLF2 and S1PR1 transcription is associated with early CD69 expression and dictates whether $CD8^+$ T cell recirculates or resides in the tissue [58] (Figure 2). CD69 interferes with S1PR function and regulates T cell retention and local memory formation [59]. On endothelial cells, B cell-derived peptide (PEPITEM) binds cadherin-15, promoting synthesis and release of S1P, thereby regulating T cell trafficking during inflammation and in response to adiponectin [55].

Activity of SPL, which metabolizes S1P, has been demonstrated to partially regulate S1P gradient-mediated lymphocyte trafficking [60, 61]. $CD68^+$ cells on the parenchymal side of marginal reticular cells express SPL in human LN [62]. Inhibition of SPL by caramel food colorant, 2-acetyl-4-tetrahydroxybutylimidazole (THI), also prevents T cell egress from the thymus and secondary lymphoid organs under conditions of vitamin B6 deficiency [63].

B lymphocyte egress from secondary lymphoid organs also requires S1P and S1PR1. S1PR1 provides necessary signals for the transfer of newly generated immature B cells from the bone marrow to the blood [64, 65]. Marginal zone B cell localization to the marginal zone is regulated by response to the blood S1P, with S1PR1 signaling overcoming the recruiting activity of CXCL13 [66]. Marginal zone B cells migrate continually between the marginal zone and

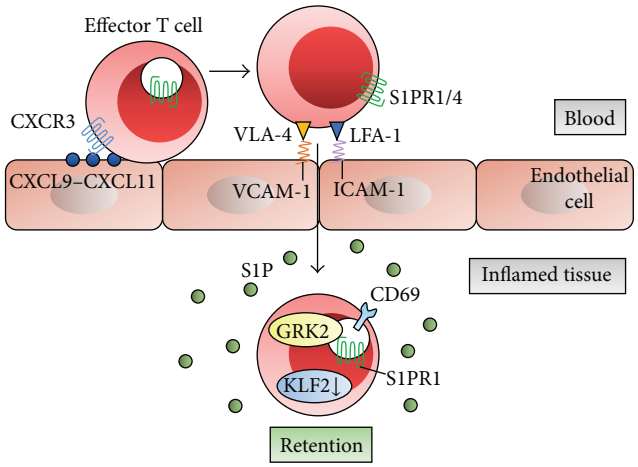

Figure 2: S1P-S1PR1 axis and lymphocyte retention in inflamed tissue. Surface expression of S1PR1 and S1PR4 on T cells is induced by chemokines CXCL9–CXCL11 presented on the endothelial surface and results in T cell migration into inflamed tissue. This is mediated at least partially by interactions of adhesion molecules LFA-1/ICAM-1 and VLA-4/VCAM-1. GRK2 downregulates S1PR1, allowing them to be retained in inflamed tissues. Downregulation of transcription factor KLF2 and S1PR1 transcription provides T cell retention in tissue, which is associated by early CD69 expression. CXCL: C-X-C chemokine ligand; CXCR3; C-X-C chemokine receptor 3; LFA-1: lymphocyte function-associated antigen-1; ICAM-1: intercellular adhesion molecule-1; VLA-4: very late antigen-4; VCAM-1: vascular cell adhesion molecule-1; GRK2: guanine nucleotide-binding protein-coupled receptor kinase-2; KLF2: Krüppel-like factor 2.

follicles, establishing the marginal zone as a site of S1PR1-dependent B cell egress from the follicles [67]. On the other hand, S1PR1 antagonism blocks passage through the cortical lymphatic endothelium and argues against a functional role for S1P gradient chemotaxis in B lymphocyte egress [68]. Overexpression of S1PR2 promotes the centering of activated B cells in the follicle and inhibits germinal center B cell responses to follicular chemoattractants and helps confine it to the germinal center [69]. S1PR2 suppresses growth and promotes local confinement of germinal center B cells through the Gα13-dependent pathway [70]. Combinations of S1P receptors are different in various B cell populations and regulate the circulation of human B cell subsets. In human B cells, S1PR1-induced signaling is transmitted through β-arrestin 2, LPS-responsive beige-like anchor protein, dedicator of cytokinesis 8, and Wiskott-Aldrich syndrome protein [71].

3.2. S1P-S1PR5 Axis and Recruitment of NK Cells. Messenger RNA for S1PR1, S1PR4, and S1PR5, but not S1PR3, are expressed in NK cells [72]. S1P-deficient mice exhibit increased NK cell retention with inhibition of egress, indicating that while NK cells can develop within the thymus without S1PR1 expression, they are not retained in the peripheral tissue [73]. S1PR5 has also been shown to be required for NK cell egress from LN and bone marrow [74], and S1PR5-deficient mice have been reported to have aberrant

NK cell homing during steady-state conditions. S1PR5 is also required for the mobilization of NK cells to inflamed tissues [75]. $CD56^{bright}$ NK cells, a minority population of NK cells, express CCR7, and S1P influences the population, phenotype, and function of NK cells in peripheral circulation [76].

3.3. Contribution of Spns2 to Lymphocyte Trafficking. Spns2, which is a member of the major facilitator superfamily of non-ATP-dependent transporters, has been identified as a transporter of S1P in some cell types [77, 78]. S1P cannot spontaneously traverse the cell membrane lipid bilayer due to its polar head group and is secreted by either Spns2 or promiscuous ABC transporters [2, 79]. In breast cancer, multidrug resistant proteins ATP-binding cassette transporters, ABCC1 and ABCG2, export S1P after estrogen stimulation of breast cancer cells [79]. Spns2 is involved in angiogenesis, lymphangiogenesis, and the generation of the lymphatic network in LN during development [80]. Although it was initially assumed that the S1P gradient between the thymus and blood is the primary determinant of egress of mature T cells from the thymus, blood S1P level alone is insufficient to promote the egress [41, 80–82]. Spns2 plays a role in the regulation of S1P levels not only in the blood, but also in LN and lymphatic fluid, thus influencing lymphocyte trafficking and development of the lymphatic vessel network [80]. The immunological phenotype of Spns2 knockout mice closely mimics the phenotype of partial S1P deficiency, including impaired S1P-dependent lymphocyte trafficking, depletion of lymphocytes in the circulation, an increase in mature single-positive T cells in the thymus, and a selective reduction in mature B cells in the spleen and bone marrow, resulting in redistribution of lymphocytes from the spleen to LN [83]. This is consistent with the notion that normal egress from the spleen is due to blood S1P gradient, and blocked egress from LN is due to lymphatic fluid S1P gradient. Spns2 is needed in endothelial cells to supply lymphatic fluid S1P and support lymphocyte circulation [84]. Spns2 is currently believed to contribute to the S1P gradient required for T and B cells to egress from their respective primary lymphoid organs into lymphatic endothelial cells [85] (Figure 2). In agreement with this notion, we have recently found that Spns2-mediated S1P transport plays a significant role in the initiation and development of adaptive immune-related disorders and autoimmune diseases, such as asthma, colitis, multiple sclerosis, and arthritis in animal models [86].

3.4. FTY720 and Lymphopenia. FTY720 is a prodrug that acts as an immunomodulator after activation [4]. FTY720 was discovered by the chemical modification of the natural product, myriocin (ISP-1), which is a metabolite of the fungus *Isaria sinclairii*. Later, FTY720 was found to be a structural analogue of sphingosine and a functional antagonist of S1PRs [87]. Use of FTY720 has revealed that S1P is involved in lymphocyte egress from the thymus and secondary lymph organs into the circulation [88]. FTY720 can be administered orally and is approved by the United States Food and Drug Administration as a new treatment for multiple sclerosis, the most common inflammatory disorder of the central nervous system [89].

FTY720 is phosphorylated *in vivo* by SphKs to generate phosphorylated-FTY720 (p-FTY720), S1P mimetic which acts as a ligand for all of the S1PRs except S1PR2. p-FTY720 modulates chemotactic responses and lymphocyte trafficking by internalization of the S1PRs [6], thus strongly suppressing lymphocyte egress from the thymus and secondary lymphoid organs [90]. As S1P mimetic, p-FTY720 is also transported by Spns2 through the same pathway as S1P [91]. S1PR1 activated by p-FTY720 maintains signaling activity for several hours despite quantitative internalization. This sustained intracellular agonism may be an important mechanism that distinguishes FTY720 from other S1PR antagonists and contributes to the therapeutic potential of FTY720 [92]. p-FTY720 causes continued cAMP signaling that is not dependent on S1P1R redistribution and induces functional antagonism of Ca^{2+} signaling after transient stimulation [93].

After binding to S1PR1 and internalization into cells, S1P returns to the plasma membrane and is recycled within several hours. However, S1PR1 internalized by p-FTY720 does not lead to receptor recycling, and p-FTY720 strongly induces subsequent polyubiquitination and proteasomal degradation of the S1PR1 [94, 95]. The mechanism of S1PR1 internalization and modulation of autoimmune inflammation remains unclear. It was recently reported that incomplete S1PR1 phosphorylation worsens Th17-mediated autoimmune neuroinflammation, and this mechanism may be related to the pathogenesis of multiple sclerosis [96]. FTY720-induced S1PR1 internalization in T cells is caused by clathrin-mediated endocytosis and is regulated by moesin, an ezrin-radixin-moesin (ERM) family member [97].

S1PR1 suppression by FTY720 correlates with reduced numbers of lymphocytes and monocytes in experimental autoimmune encephalomyelitis in mice and rats independent of S1PR3 [36]. The percentages of central memory T cells (T_{CM}) and naïve T cells decrease, while those of effector memory T cells (T_{EM}) and suppressor precursor T cells (T_{SP}) increase in both $CD4^+$ T and $CD8^+$ T cells with FTY720 therapy. The percentages of regulatory T cells (T_{reg}) in $CD4^+$ T cells and T_{EM} in $CD8^+$ T cells also increase [98]. FTY720 can impair $CD8^+$ T cell function independently of S1P pathway [99]. On the other hand, absolute numbers of NK cells are unchanged in FTY720-treated multiple sclerosis patients. However, relative proportions of NK cells within the whole circulating lymphoid population are increased. FTY720 causes a relative decrease in $CD56^{bright}$ NK cells expressing CCR7, increased sensitivity to chemokine ligand, and promotes movement into LN [76]. In addition, FTY720 has nonimmunological mechanisms in astrocytes, which present S1P signaling pathways within the central nervous system as targets for multiple sclerosis therapies [100]. Finally, we have recently reported that p-FTY720 is a histone deacetylase inhibitor that reactivates estrogen receptor expression in breast cancer both *in vitro* and *in vivo*, suggesting that FTY720 may possess functions more than those that have previously been published [101]. More elucidation of the differences in functional mechanism between FTY720 and other S1P/S1PR modifying compounds will contribute to the investigation of S1P and the therapeutic potential of such compounds.

4. Role of S1P and S1PR1 in Lymphocyte Differentiation

In addition to trafficking, S1PR1 is also involved in lymphocyte differentiation. S1PR1 delivers intrinsic negative feedback to decrease thymic production and suppress activity of $CD4^+CD25^+$ T_{reg}. S1PR1 blocks the differentiation of thymic T_{reg} precursors and inhibits the function of mature T_{reg} cells, thereby regulating T_{reg} cell-mediated immune tolerance [102]. S1PR1 signaling in T cells promotes tumor growth by inducing T_{reg} accumulation in tumors via STAT3 and inhibiting $CD8^+$ T cell recruitment and activation [103]. FTY720 induces a decrease in circulating $CD4^+$ T cells and $CD19^+$ B cells while $CD39^+$ T_{reg} cells increase in multiple sclerosis patients [104]. FTY720 directly potentiates recruitment and function of myeloid-derived suppresser cells (MDSCs) and controls the differentiation of Th1 cells to T_{reg} by targeting S1PR1 [105]. The effect of S1P in lymphocyte differentiation is related to the immune response against cancer and pathogenesis of autoimmune diseases. Further investigation and therapeutic application are expected in the near future.

5. Therapeutic Potential through Targeting Local S1P/S1PR Function in Inflamed Tissues

5.1. Asthma. ORM- (yeast-) like protein isoform 3 (ORMDL3), which is identified as a gene associated with susceptibility to asthma, promotes eosinophil trafficking, recruitment, and activation [106] and regulates sphingolipid and ceramide homeostasis [107]. Intranasal application of FTY720 was shown to decrease ORMDL3 expression and is effective for reducing airway inflammation and hyperreactivity and mucus hypersecretion in house dust mite-challenged mice [108]. On the other hand, it has been reported that prolonged FTY720 treatment induces life-threatening asthma attacks and deterioration [109]. Further investigations of therapeutic effects of FTY720 or other S1P/S1PR related-compounds for asthma diseases are expected.

5.2. Allergic Rhinitis. Allergic rhinitis and asthma are the two most common allergic diseases [110]. Intranasal FTY720 treatment significantly decreases eosinophils, mast cells, and dendritic cells in the nasal mucosa of animal allergic rhinitis models with decreased levels of IL-4, IL-5, IL-10, and IL-13 in LN of FTY720-treated animals. The mechanism includes impairment of Th2 differentiation and proliferation, inhibition of eosinophilia, and induction of apoptosis in mast cells [111].

5.3. Allergic Skin Diseases and Psoriasis. S1P controls several fundamental functions of keratinocytes and skin dendritic cells. S1P suppresses proliferation and promotes differentiation of keratinocytes. Antigen uptake, migration, and cytokine production in dendritic cells are regulated by sphingolipids. Dysregulation of sphingolipid metabolism is involved in inflammatory skin diseases such as atopic dermatitis [112]. Topical administration of S1P or FTY720 inhibits dendritic cell migration and regulates Langerhans cell migration from skin to LN and is an effective treatment for allergic skin diseases such as contact hypersensitivity and atopic dermatitis [113]. Although genetic factors, epithelial disorders, and environmental factors are involved in the pathogenesis of psoriasis, inflammation is also implicated in the progression of psoriasis. Topical administration of S1P and FTY720 has been reported to be effective for psoriasis [114]. Ponesimod, a selective S1PR1 modulator, is a functional antagonist of S1PR1, and its oral administration is undergoing clinical trial for psoriasis [115]. Considering that there are various clinical phenotypes of psoriasis, topical therapies targeting S1P/S1PR function might be a new option for the control of mild-to-moderate psoriasis lesions.

5.4. Corneal Allograft. Corneal transplantation is the most common and successful solid organ transplantation. Despite the fact that HLA matching and systemic immunosuppression are not regularly utilized, 90% of first-time corneal allografts succeed [116]. However, in order to achieve even better outcomes, there remains the option of topical administration of immunosuppressive medication. Treatment with FTY720 eye-drops can effectively prolong allogeneic corneal graft survival in mice. Topical application of FTY720 increases the percentage of $CD4^+$ T cells and T_{reg} in cervical LN, increases TGF-β1 mRNA expression, and decreases infiltration of $CD4^+$ T cells in corneal allografts [117]. Corneal graft survival is prolonged by topical application of S1PR1, and S1PR1 selective agonist may be effective in the inhibition of corneal allograft rejection [118, 119].

5.5. Wound Healing. Wound healing is one of the most fundamental research topics in surgery, since every surgical intervention creates wounds. The stages of wound healing are classified into three phases: inflammatory, proliferative, and remodeling phases [120]. The inflammatory phase is the first process of wound healing during which purification of the wound and production of cytokines and chemokines by inflammatory cells occur. The inflammatory phase strongly influences the following phases, as discovered through complications such as intractable wounds and abnormal scars, termed hypertrophic scars and keloid formation. Thus, strengthening of the inflammatory reaction by activation of S1P signaling is expected to promote wound healing. In addition, S1P promotes formation of fibronectin matrix at the dermal-epidermal junction, and keratinocyte migration, which is expected to promote wound healing. Further, in response to injury, thrombin promotes the activation of S1P, which promotes angiogenesis for wound healing [121]. Direct SphK1 plasmid application to wounds was shown to accelerate wound closure in diabetic rats [122]. This warrants further detailed analysis and investigation as human wounds heal differently from other mammals. For instance, wound healing takes longer in humans and often results in hypertrophic scar or keloid, which is rarely observed in other mammals. Treatments to promote wound healing, which are currently limited to modifying nutrition and circulation, are expected to have a large potential impact on all phases

of health recovery. S1P may be an ideal target molecule to promote wound healing.

6. Conclusion

S1P is a bioactive lipid mediator that is increasingly recognized as an important regulator of immune function. S1PR expression and S1P concentration gradient have been implicated in immune cell development, differentiation, and recruitment during both acute and chronic inflammation. Currently, numerous studies are in progress to investigate the possibility of new therapies targeting S1P signaling, including FTY720, which may have great potential as a therapeutic target for many types of diseases such as autoimmune diseases, allergy, infection, and chronic inflammation. A large number of positive results thus far support the development of S1P signaling targeted therapies to treat such conditions.

Conflict of Interests

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

Authors' Contribution

Masayo Aoki, Hiroaki Aoki, Nitai C. Hait, and Rajesh Ramanathan contributed to literature search and paper preparation. Kazuaki Takabe contributed to literature search, paper preparation, and critical review.

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Research Article

Sphingosine-1-Phosphate/Sphingosine-1-Phosphate Receptor 2 Axis Can Promote Mouse and Human Primary Mast Cell Angiogenic Potential through Upregulation of Vascular Endothelial Growth Factor-A and Matrix Metalloproteinase-2

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Mast cells (MC) are present in most vascularized tissues around the vasculature likely exerting immunomodulatory functions. Endowed with diverse mediators, resident MC represent first-line fine-tuners of local microenvironment. Sphingosine-1-phosphate (S1P) functions as a pluripotent signaling sphingolipid metabolite in health and disease. S1P formation occurs at low levels in resting MC and is upregulated upon activation. Its export can result in type 2 S1P receptor- (S1PR2-) mediated stimulation of MC, further fueling inflammation. However, the role of S1PR2 ligation in proangiogenic vascular endothelial growth factor- (VEGF-) A and matrix metalloproteinase- (MMP-) 2 release from MC is unknown. Using a preclinical MC-dependent model of acute allergic responses and *in vitro* stimulated primary mouse bone marrow-derived MC (BMMC) or human primary skin MC, we report that S1P signaling resulted in substantial amount of VEGF-A release. Similar experiments using *S1pr2*-deficient mice or BMMC or selective S1P receptor agonists or antagonists demonstrated that S1P/S1PR2 ligation on MC is important for VEGF-A secretion. Further, we show that S1P stimulation triggered transcriptional upregulation of VEGF-A and MMP-2 mRNA in human but not in mouse MC. S1P exposure also triggered MMP-2 secretion from human MC. These studies identify a novel proangiogenic axis encompassing MC/S1P/S1PR2 likely relevant to inflammation.

1. Introduction

Mast cells (MC) convey immunomodulatory functions through their unique ability to release, after activation, many mediators including cytokines, chemokines, and enzymes, some of which are prestored in cytoplasmic granules at homeostasis [1]. MC are *bona fide* sentinels present in most vascularized tissues prior to trauma, equipped with a vast selection of surface receptors, therefore sensing and quickly responding to local alterations [2]. Notorious for their key contributions to allergic responses, MC influence the course and chronicity of many inflammatory disorders [3–5].

We discovered that immunoglobulin E- (IgE-) dependent MC activation releases sphingosine-1-phosphate (S1P), a bioactive sphingolipid mediator produced by sphingosine kinases that serves to further propagate MC-mediated inflammatory response [3, 4, 6]. S1P exerts its pleiotropic actions imparted by ligation to five G-protein-coupled receptors (GPCRs), S1PR1–S1PR5, with subtype-specific distinct repertoire of heterotrimeric G protein coupling, in combination with tissue- and cell-type-specific receptor expression patterns [7]. Our recent studies established MC as critical to the onset of acute pulmonary allergic response [3] through autocrine/paracrine binding of S1P on MC surface S1P receptor type 2 (S1PR2). This interaction led to MC-derived T-cell-attracting chemokine release partly through signal transducer and activator of transcription 3 (Stat3) signaling [4]. Suppression of MC S1PR2 signaling by S1PR2 genetic ablation or pharmacological antagonism significantly impaired T-cell recruitment through decreased release

of T-cell chemoattractants in activated MC supernatants. Moreover, eliminating extracellular S1P with a monoclonal antibody (mAb) that binds and neutralizes S1P mitigated *ex vivo* and *in vivo* allergic MC activation, yielding significant inhibition of inflammatory infiltration and chemokine detection [4]. Of note, this mAb was shown to be effectively antiangiogenic in mouse xenograft and allograft tumor models [8, 9] as well as in a mouse model of wet age-related macular degeneration, that is, choroidal neovascularization [10].

Angiogenesis, or the formation and maintenance of blood vessel structures, is essential for the physiological functions of tissues and for the progression of diseases such as cancer and inflammation [11, 12]. S1P stimulates endothelial cell proliferation and survival, migration, and capillary-like tube formation via S1PR1 and S1PR3 *in vitro*, which is indicative of its angiogenic activity [13, 14]. S1P also maintains endothelial barrier function via S1PR1 [15, 16].

In addition to histamine, a potent vasoactive amine triggering vascular leakage and edema, and S1P, MC can generate an array of angiogenic factors, including vascular endothelial growth factor-A (VEGF-A), upon cross-linking of surface bound IgE on their high-affinity receptors (FcεRI) with allergen (Ag) and other stimuli [17–20]. VEGF-A regulates angiogenesis and vascular permeability by activating 2 receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk1 in mice) [21]. Interestingly, S1P can activate VEGFR-2 in the absence of its conventional ligand VEGF by receptor cross talk [22]. Ligation of S1PR2 on the cell membrane can induce ERK1/2 activation through activation of Gi protein. Activation of Gi can lead to Src activation that results in the phosphorylation of VEGFR-2. This transactivation of VEGFR-2 also contributes to vascular remodeling [23].

In the present study, we further investigated the role of S1P signaling through S1PR2 on MC and show for the first time that S1P stimulation of primary mouse and human MC results in the release of substantial amounts of VEGF-A. The relevance of these findings was substantiated using genetically S1PR2-deficient and congenic wild-type mouse model of acute allergic response [3, 4], as well as selective pharmacological receptor agonist and antagonist [3, 4, 24]. Moreover, our results provide new molecular insights pertaining to S1P-regulated VEGF-A release from primary human MC. Finally we report that S1P-dependent MC activation induces the release of matrix metalloproteinase- (MMP-) 2, endowed with extracellular and connective tissue matrix degrading properties [25], therefore potentially also contributing to neovascularization in inflammatory and cancer settings.

2. Materials and Methods

2.1. Mice. Age- and gender-matched C57BL6/J mice were obtained from the National Institutes of Health (NIH) National Cancer Institute (Frederick, MD, USA). S1PR2 knockout mice (Taconic Biosciences, Inc., Hudson, NY) and corresponding wild-type (WT) mice were on a mixed 129/SvEV-C57BL/6 background. All mice were maintained in a pathogen-free facility. Studies were performed in accordance with institutional animal care and use committee guidelines.

2.2. Reagents. Dinitrophenyl- (DNP-) specific mouse IgE was a generous gift from Dr. Daniel Conrad (VCU, Richmond, VA). DNP-human serum albumin (HSA) and ionomycin were obtained from Sigma-Aldrich (St Louis, MO). S1P was purchase from Enzo Life Sciences (Farmingdale, NY). JTE-013 and CYM-5442 were purchased from Tocris/Bio-Techne (Minneapolis, MN).

2.3. Human Skin and Mouse Bone Marrow-Derived Mast Cells. Human skin MC and mouse bone marrow-derived mast cells (BMMC) were isolated and cultured essentially as previously described [26, 27] and were more than 98% pure. Human MC and mouse BMMC were sensitized overnight with 1 μg/mL DNP-specific mouse IgE, washed to remove excess unbound IgE, and stimulated with 30 or 20 ng/mL DNP-HSA (Ag), respectively, for 24 hours. Ionomycin (1 μM), a receptor-independent stimulus, and S1P (100 nM) were also applied for 24 hours. Of note, vehicle consisted of DMSO/PBS-4 mg/mL fatty acid-free bovine serum albumin. All supernatants were collected after 24 hours of stimulation. Each experiment was performed at least three times, with triplicate determinations. For mRNA analysis, 3–5 $\times$ 10^6 human skin MC from 5 different donors were incubated for the indicated times in the presence or absence (vehicle, or culture medium alone) of S1P (100 nM).

2.4. Acute Mast Cell-Dependent Allergic Response. All injections were performed intraperitoneally in a final volume of 100 μL, as previously described [3, 4]. Briefly, mice were injected with DNP-specific IgE and 12 hours later with Ag in PBS. Mice were euthanized 2 hours later and blood was immediately collected by cardiac puncture for serum analysis.

2.5. VEGF-A and MMP-2 Measurements. Human and mouse angiogenic factors were measured by ELISA, according to manufacturer's instructions (Bio-Techne, Minneapolis, MN).

2.6. Quantitative PCR Analysis. Total RNA was isolated and purified with the miRNeasy Kit (Qiagen, Valencia, CA), following the manufacturer's procedure. The iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) was used according to the manufacturer's specifications to reverse-transcribe cDNA. QPCR was performed on a CFX Connect (Bio-Rad) with SensiFAST SYBR No-ROX Kit (Bioline). The following primers were used for real-time PCR amplification: human VEGF-A, forward primer 5′-AGGCCAGCACATAGGAGA-3′ and reverse primer 5′-ACCGCCTCGGCTTGTCACAT-3′; human MMP-2, forward primer 5′-TACAGGATCATTGGCTACACACC-3′ and reverse primer 5′-GGTCACATCGCTCCAGACT-3′; human GAPDH, forward primer 5′-TTGAGGTCAATGAAGGGGTC-3′ and reverse primer 5′-GAAGGTGAAGGTCGGAGTCA-3′; mouse VEGF-A, forward primer 5′-GGCCTCCGAAACCATGAACT-3′ and reverse primer 5′-CTGGGACCACTTGGCATGG-3′; mouse MMP-2, forward primer 5′-ACCTGAACACTTTCTATGGCTG-3′ and reverse primer 5′-CTTCCGCATGGTCTCGATG-3′; and mouse GAPDH, forward primer 5′-CAGAAGGGGGCGGAGATGA-3′ and reverse primer 5′-AGGCCGGTGCTGCTGAGTATGTC-3′. The real-time PCR conditions were as follows: initial step at 95°C for 10 min and cycles (n = 40)

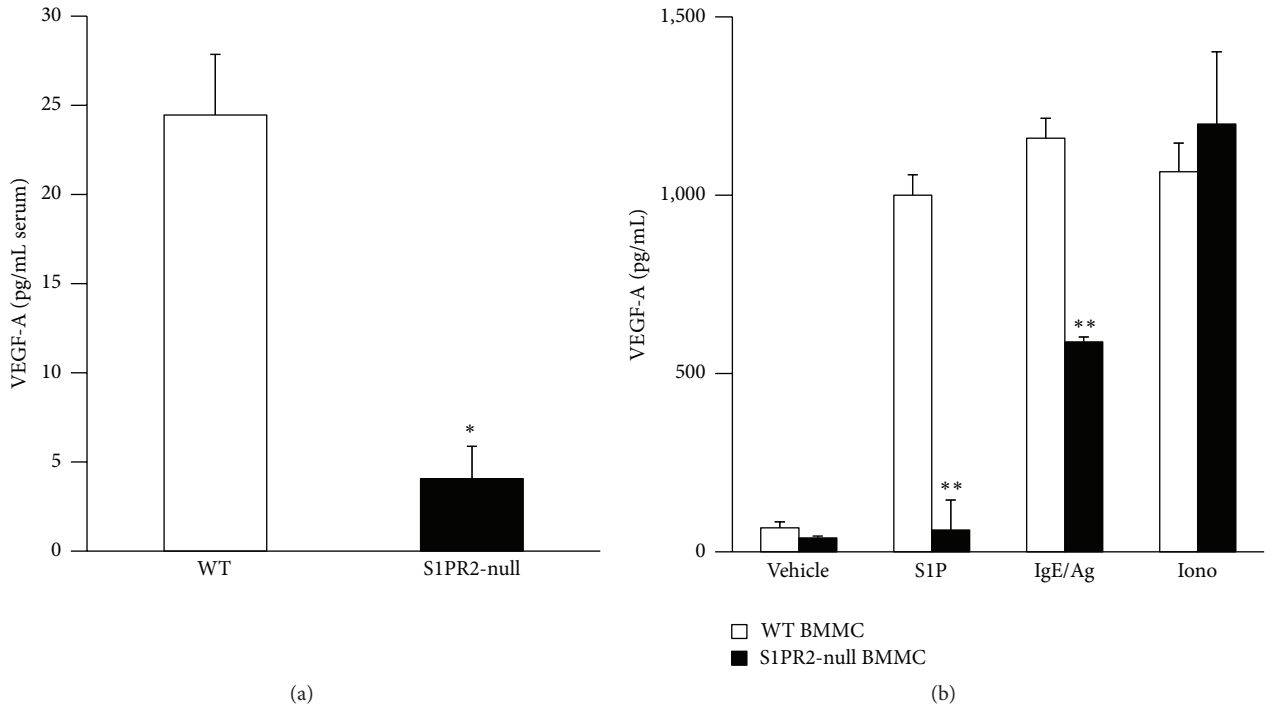

Figure 1: Role of S1P/S1PR2 signaling in VEGF-A secretion. (a) Blood was collected from allergenically challenged WT (open bar, n = 5-6 mice) and S1PR2-null (black bar, n = 5-6 mice) mice, euthanized 2 hours after Ag challenge, and serum VEGF-A levels were measured in duplicate determination for each animal. (b) Bone marrow-derived mast cells (BMMC, three independent populations) from both genotypes were stimulated for 24 hours with vehicle (DMSO/PBS/4 mg/mL fatty acid-free BSA), S1P (100 nM), IgE/Ag, or ionomycin (Iono) and VEGF-A was measured in MC supernatants, in duplicate determinations. Error bars show standard error of means. $^{*}p < 0.05$, $^{**}p < 0.005$.

consisted of 10 s at 95°C, followed by 20 s annealing/extension at 59°C and extension at 72°C. All reactions were performed in duplicate. Data were analyzed with CFX Manager software (Bio-Rad) and are normalized expression directly proportional to the amount of mRNA of the target genes VEGF-A and MMP-2 relative to the amount of mRNA of the reference gene, GAPDH. Primers were synthesized and purchased from Thermo Fisher Scientific, Inc. (Waltham, MA), with melting temperatures ranging from 59.9 to 64.5°C.

2.7. Statistical Analysis. Data are expressed as means ± SEM and were analyzed by using unpaired two-tailed Student's t-test for comparison of two groups (Prism 6; GraphPad Software, La Jolla, CA). Significance for statistical tests is shown in figures. Experiments were repeated at least three times in duplicate or triplicate with consistent results. *In vivo* experiments were repeated twice (n = 5-6 mice per experimental group).

3. Results

3.1. MC- and IgE-Dependent Acute Allergic Reactions Trigger Systemic VEGF-A Detection in WT Mice That Is Significantly Mitigated in S1pr2-Null Mice. We previously reported that substantial levels of circulating chemokines were detected in the serum of WT mice 2 hours after eliciting a MC-dependent allergenic challenge [4]. We also showed that chemokine production was significantly decreased in the absence of extracellular S1P [4] or in mice genetically ablated for *S1pr2* or pretreated with a selective S1PR2 antagonist [3]. Similarly, circulating levels of VEGF-A were measured in the serum of WT and *S1pr2*-null mice in the same preclinical model. Figure 1(a) shows that as early as 2 hours after Ag administration significant levels of VEGF-A were detected in the serum of sensitized WT while the absence of *S1pr2* severely mitigated serum VEGF-A levels in sensitized *S1pr2*-deficient mice. Of note, VEGF-A was undetectable in the serum of either genotype prior to allergenic challenge (data not shown). We further substantiated the relevance of *S1pr2* in VEGF-A secretion by stimulating *in vitro* mouse BMMC from both genotypes and measuring VEGF-A in activated BMMC supernatants. Figure 1(b) shows that S1P (100 nM) and IgE/Ag stimulation trigger significant release of VEGF-A in the supernatants of activated WT BMMC collected after 24 hours that was significantly decreased in *S1pr2*-deficient BMMC, further emphasizing the importance of S1PR2 signaling on MC for VEGF-A secretion. Of note, ionomycin, a calcium ionophore and receptor-independent MC stimulus, also triggered significant VEGF-A release from BMMC that was unaffected by the absence of S1PR2.

3.2. In Vitro S1P- or IgE/Ag-Mediated Stimulation of Human Primary Mast Cells Results in the Release of Angiogenic Factors VEGF-A and MMP-2. Next, to validate the physiological relevance of these findings, primary human mast cells were assessed for their ability to release angiogenic factors. As

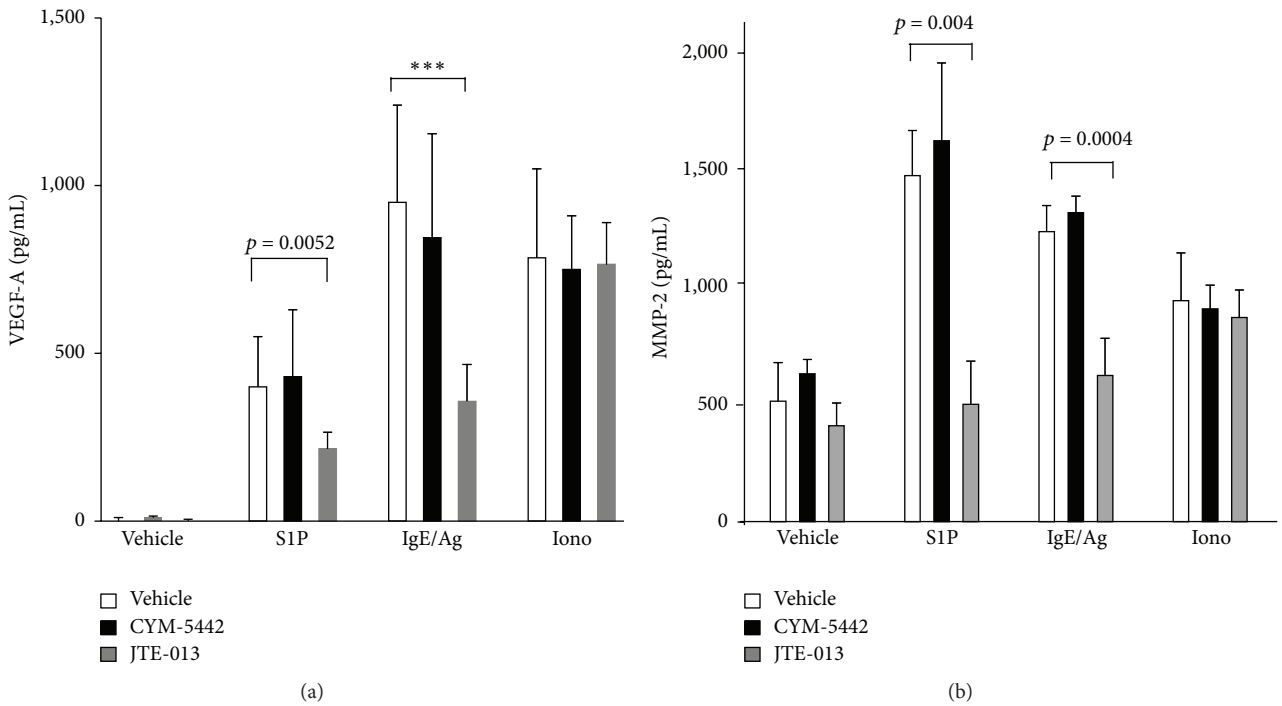

Figure 2: Human MC secrete proangiogenic factors upon exposure to S1P in a S1PR2-dependent manner. (a) VEGF-A levels were measured in the supernatants of activated human MC in the absence (open bars, vehicle (DMSO/PBS/4 mg/mL fatty acid-free BSA)) or presence of a 30-minute pretreatment with CYM-5442 (filled bars, S1PR1 agonist, 1 μM) or with JTE-013 (gray bars, S1PR2 antagonist, 1 μM), followed by the indicated stimuli similar to Figure 1(b). (b) Matrix metalloproteinase- (MMP-) 2 levels were measured in supernatants of MC activated exactly as described in Figure 1(b). All supernatants were collected 24 hours after stimulation. Activation experiments were conducted using five independent human skin MC populations generated from five donors. Activation was conducted in triplicate determinations and measurements were conducted in duplicate determinations for each individual determination. When reaching significance, statistics are indicated in each figure. Error bars show standard error of means. $^{***}p < 0.0001$.

shown in Figure 2(a), addition of exogenous S1P or Ag to sensitized human skin MC stimulates VEGF-A secretion that occurs at very low levels spontaneously. Ionomycin, a calcium ionophore and receptor-independent MC stimulus, also triggers VEGF-A release from human primary skin MC. The pretreatment of cells for 30 minutes in the presence of CYM-5442, a selective S1PR1 agonist, did not induce any VEGF-A secretion from MC on its own. Furthermore, exposure to CYM-5442 (1 μM) prior to S1P or IgE/Ag did not alter the levels of VEGF-A released by either of these two stimuli. However, pretreating MC with JTE-013, a potent and selective antagonist for S1PR2, significantly inhibited both S1P- and IgE/Ag-mediated VEGF-A release from MC. Figure 2(b) demonstrates that S1P, IgE/Ag, and ionomycin stimuli also induce the secretion of large amounts of MMP-2 from human skin MC. Similar to Figure 2(a), pretreatment with CYM-5442 prior to stimulant exposure did not prevent MMP-2 secretion from human MC (Figure 2(b)). By contrast, preexposure to JTE-013, a pharmacological antagonist for S1PR2, significantly mitigated S1P- and IgE/Ag-dependent release of MMP-2 from human MC (Figure 2(b)), establishing that MC-derived VEGF-A and MMP-2 secretion requires functional MC-expressed S1PR2. It is noteworthy that S1P, itself potentially proangiogenic, promoted the release of VEGF-A and MMP-2 from human skin MC.

3.3. S1P Stimulation Transcriptionally Upregulates VEGF-A and MMP-2 mRNA Expression in Human but Not in Mouse Primary Mast Cells. Since *in vitro* stimulation of primary MC is conducted for 24 hours, we next sought to determine whether S1P could also activate VEGF-A and MMP-2 mRNA production during this time. To this end, primary human skin MC were stimulated for different periods of time in the presence of S1P (100 nM) and kinetics of VEGF-A (Figure 3(a)) and MMP-2 (Figure 3(b)) mRNA expression was investigated. Figures 3(a) and 3(b) show that stimulation of human skin MC for 3 hours triggered a significant increase in both VEGF-A and MMP-2 mRNA expression that decreased with increased incubation time but remained higher than in the absence of stimulation. Results were consistent for human skin MC derived from 5 different donors. In contrast, similar stimulation of BMMC did not result in upregulation of transcription for either of these two genes (Figures 3(c) and 3(d)). These results were replicated using three different BMMC populations.

4. Discussion

Inflammation first manifests itself with vascular alterations, including increased vascular permeability and edema [28]. MC are tissue-dwelling cells located around blood vessels and

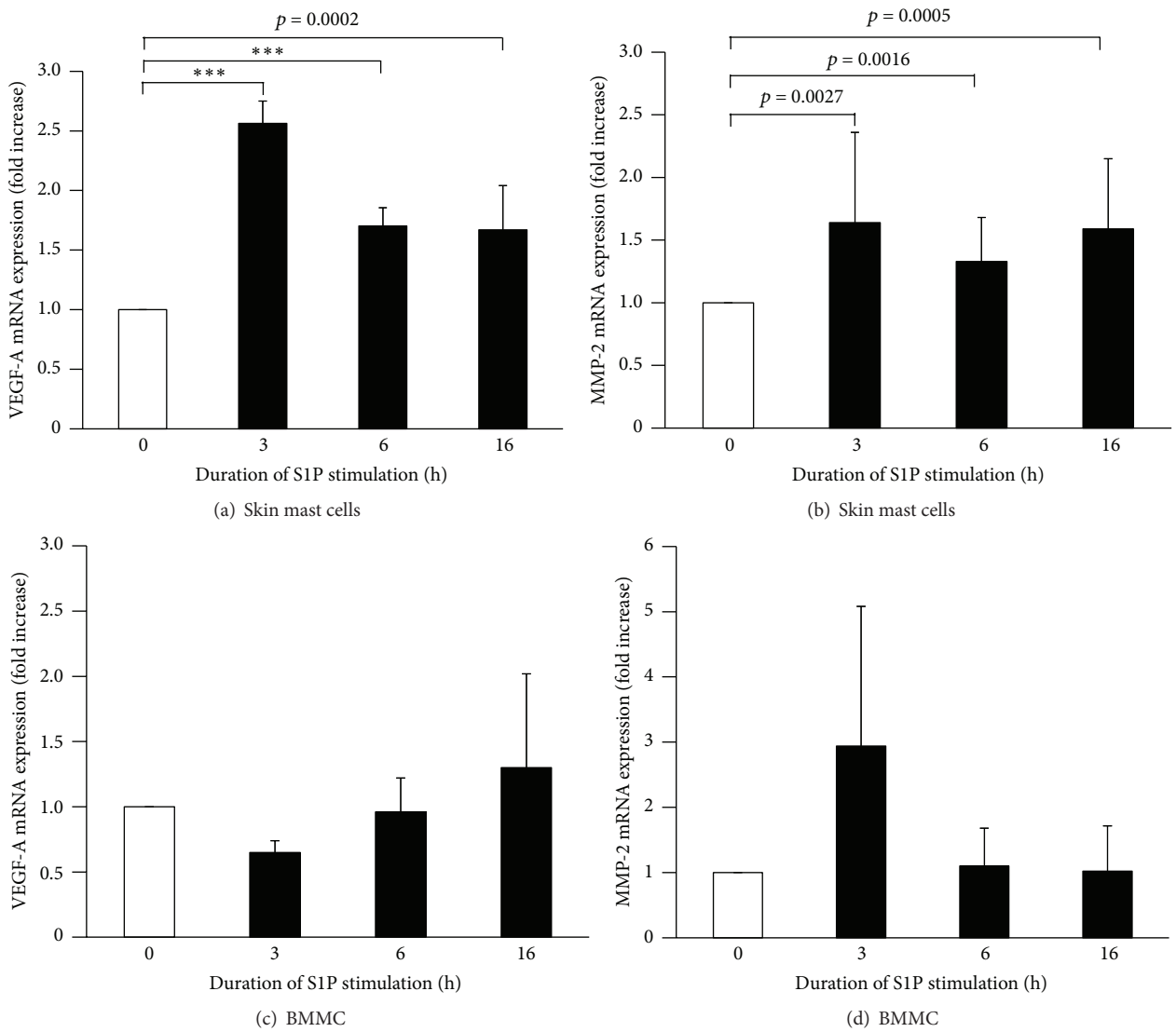

Figure 3: Effect of S1P on VEGF-A and MMP-2 mRNA expression. Human (a, b) and mouse (c, d, BMMC) MC were stimulated in the presence of S1P (100 nM) for different periods of time at which VEGF-A (a, c) and MMP-2 (b, d) mRNA levels were measured by QPCR. mRNA was prepared from 3 independent BMMC and 5 independent human MC populations, in duplicate. Kinetics of S1P stimulation was repeated 3 to 4 times; each QPCR experiment was performed in duplicate determination for each individual sample. When reaching significance, statistics are indicated in each figure. Error bars show standard error of means. $^{***}p < 0.0001$.

can release a number of vasoactive mediators that act directly on the vasculature, promoting vasodilation, increased endothelial permeability, and subsequent extravascular leakage of proteins and fluid [5]. Among the panoply of MC-derived bioactive mediators affecting the endothelium, histamine is released within minutes after activation [28]. We and others have shown that, upon allergenic activation of MC, concomitant sphingosine kinase activation occurs, leading to phosphorylation of sphingosine to rapidly produce S1P [6, 29–31]. We have also reported that S1P could be exported out of and act on the MC that produced it in an autocrine/paracrine manner [32] by ligation to S1PR1 or S1PR2, the two S1PR subtypes expressed on the MC surface [31]. This creates an inflammatory amplification loop that we have shown is essential to the onset of acute allergic inflammation [3, 4, 31, 33]. Importantly, S1P is also constitutively released from human MC in the absence of stimulus [32]. This is an important point, as MC could be a source of local S1P at homeostasis in tissues, further prominent in inflamed sites [33].

Angiogenesis is an important feature of development and perpetuation of allergic inflammation [34]. The levels of VEGF-A are increased in the airways of asthmatic patients and MC constitute the majority of VEGF-A positive cells in bronchial biopsies from asthmatics [35]. We report for the first time that S1P stimulation of mouse and human primary MC is a potent inducer of VEGF-A release. Many studies have reported MC as a major source of VEGF [35–39]. We had previously shown that *S1pr2* deficiency in mice or in MC drastically reduced acute allergic responses and

early T-cell recruitment [3, 4]. Remarkably, our data indicate that acute allergen challenge of sensitized mice triggered immunodetectable levels of circulating VEGF-A as early as two hours after Ag challenge. In comparison, sensitized *S1pr2*-null mice display impaired systemic VEGF-A at the same time point after Ag challenge, suggesting an important function of S1PR2 signaling in VEGF-A secretion. Interestingly, similar findings have been reported in neuroblastoma cells, which also exhibited enhanced formation of S1P that ligated S1PR2 to induce VEGF expression [40]. In agreement with our study, the effect of S1P on VEGF mRNA expression also occurred at the transcriptional level [40] in human but not in mouse MC, suggesting contrasted and species-specific mechanisms regulating transcription. Supporting our findings, S1PR2 has been shown to be essential for normal and pathological angiogenesis [41, 42]. Our results are important because they show for the first time that MC/S1P/S1PR2 axis could promote proangiogenic VEGF-A secretion from MC.

The cross talk between S1P and VEGF signaling has been identified more than a decade ago but was typically assigned to S1PR1 [22, 43]. Whether they directly affected each other remained elusive. Our current study establishes that S1P can indeed stimulate VEGF-A secretion from primary MC through ligation to S1PR2, as CYM-5442, a selective pharmacological agonist of S1PR1 [24], did not induce VEGF-A secretion nor did it alter S1P- or IgE-Ag-mediated VEGF-A release. By contrast, JTE-013, a potent and selective antagonist for S1PR2, significantly impaired S1P and IgE/Ag-mediated VEGF-A secretion from primary MC. Of note and although S1PR2 functions in cancer are still obscure [40, 44, 45], our data suggest that S1P/S1PR2 signaling in MC may promote local VEGF production and therefore angiogenesis, potentially linking inflammation to cancer.

Moreover, S1P and IgE/Ag we show can stimulate the secretion of MMP-2 from human primary MC possibly extending their contribution as connectors of inflammation to metastatic cancer [46, 47]. Interestingly, active forms of MMP have been associated with the development of vasogenic edema and disruption of blood vessel integrity in stroke reperfusion therapy and S1PR2 has recently been shown to be critical in MMP-9 activation [48]. MC have long been known to produce MMP-9 [33, 49, 50] and MMP-2 [33, 51]. In agreement, we found that S1PR2 ligation by S1P resulted in MMP activation in primary MC.

It is important to mention that MC harbor nonconventional yet proangiogenic granule-associated mediators, including tryptase and chymase [46], particularly because they can activate latent forms of MMP [52]. It is tempting to speculate that S1P can directly activate MC to release MMP-2. Alternatively, because (1) we and others have previously reported that S1P contributes to MC activation and degranulation and (2) tryptase and chymase are granule-associated and as such released from MC upon activation, we can hypothesize an indirect influence of S1P on active MMP-2 release from MC through tryptase and/or chymase exocytosis, which, in turn, could activate MMP-2. These equally intriguing hypotheses are not mutually exclusive and highlight potentially new mechanistic insights warranting further investigations relevant to inflammation and cancer.

In sum, our findings demonstrate that S1P produced by MC in inflammatory settings can induce VEGF-A release from MC by ligation to S1PR2, further emphasizing a critical role of MC as pivotal cells influencing the conversion of acute inflammation to chronicity and perhaps carcinogenesis. Moreover, based on our previous reports demonstrating a downregulatory effect of *S1pr2* deficiency or functional antagonism on MC responsiveness, this report further suggests the importance of fine-tuning S1P signaling through MC S1PR2 as a relevant anti-inflammatory strategy and, potentially, a novel approach to chemoprevention of inflammation.

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

Fostering Inflammatory Bowel Disease: Sphingolipid Strategies to Join Forces

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Complex sphingolipids are essential structural components of intestinal membranes, providing protection and integrity to the intestinal mucosa and regulating intestinal absorption processes. The role of sphingolipid signaling has been established in numerous cellular events, including intestinal cell survival, growth, differentiation, and apoptosis. A significant body of knowledge demonstrates that intestinal sphingolipids play a crucial role, as such and through their signaling pathways, in immunity and inflammatory disorders. In this review, we report on and discuss the current knowledge on the metabolism, signaling, and functional implications of sphingolipids in inflammatory bowel disease (IBD), focusing on the different aspects of sphingolipid actions on inflammatory responses and on the potential of sphingolipid-targeted molecules as anti-IBD therapeutic agents.

1. Introduction

Complex sphingolipids, including sphingomyelin (SM) and glycosphingolipids (GSLs), are essential components of intestinal membranes, providing protection and integrity to the mucosa and regulating intestinal digestion and absorption processes. As in other organs, in the intestine, simple sphingolipids/sphingoids, which are intermediates of sphingolipid metabolism, are involved in the control of key cellular events such as survival, proliferation, differentiation, and apoptosis. Indeed, the metabolism of complex sphingolipid includes enzymes involved in different signaling pathways, which lead to the formation of bioactive molecules, including ceramide (Cer) and sphingosine (Sph), as well as their 1-phosphorylated derivatives ceramide-1-phosphate (C1P) and sphingosine-1-phosphate (S1P).

The role and impact of sphingolipids and sphingolipid-mediated signaling emerged in their relevance in intestinal disorders, when aberrations in their metabolism lead to an altered sphingolipid homeostasis. Herein, we review our current knowledge on the impact of sphingolipid disequilibrium on intestinal inflammation, focusing on inflammatory bowel disease (IBD).

2. Inflammatory Bowel Disease

The term IBD encompasses a group of common chronic inflammatory disorders affecting the gastrointestinal tract [1]. The major types of IBD are Crohn's disease (CD) and ulcerative colitis (UC). Despite some overlapping clinical features, these diseases are characterized by distinct inflammatory profiles, gut microbiota composition, and symptomatology [2, 3]. CD potentially affects any portion of the alimentary tract and is characterized by a discontinuous and ulcerous transmural inflammation, associated with complications (e.g., intestinal granulomas, obstructions, abscesses, strictures, and fistulas) [3]. In UC, a continuous inflammation involves only the superficial layers of the intestinal mucosa and is localized to regions of the gut most highly colonized by bacteria, specifically at the rectum and moving proximally along the large bowel [4].

The pathogenesis of IBD is complex (Figure 1) and for many aspects remains unclear. The general hypothesis is that IBD develops as a result of a persistent alteration of intestinal homeostasis, leading to a perturbation of the balance between the intestinal mucosa and the gut microbiome [1]. Diverse factors, such as genetic, environmental, and immunologic

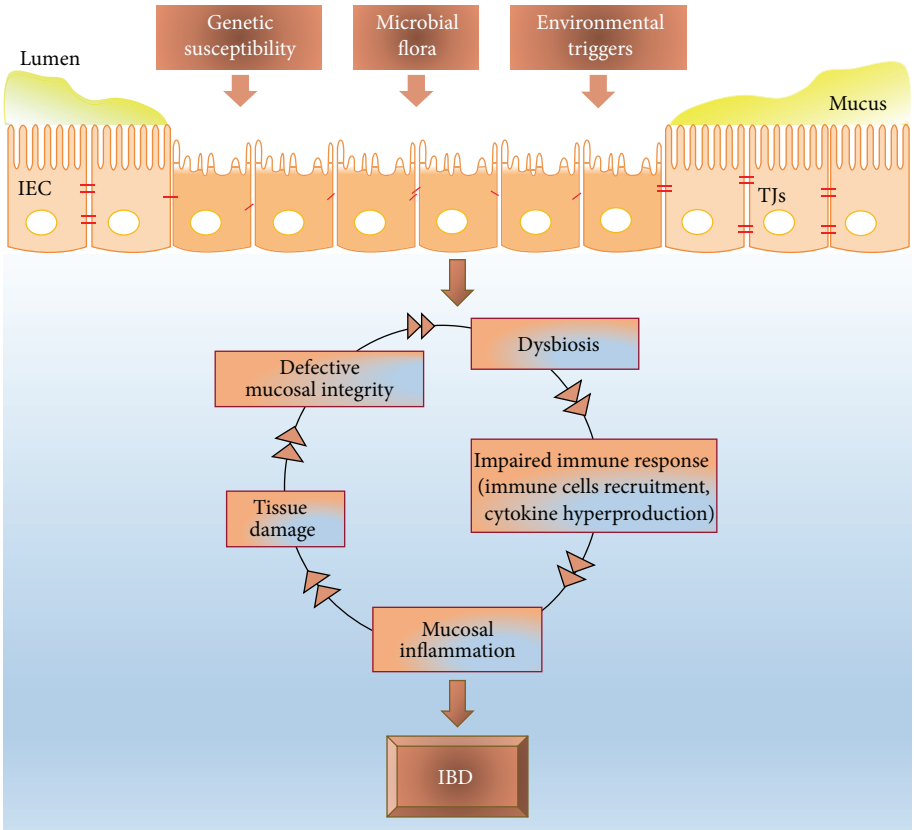


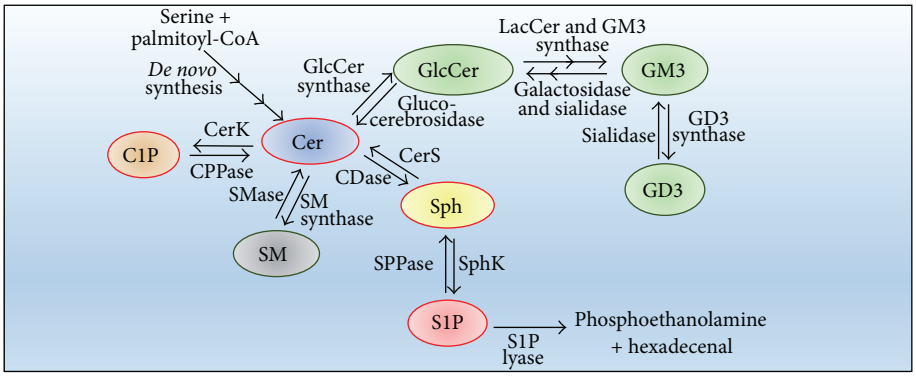
Figure 1: The pathogenesis of IBD. Genetic, microbial, and environmental factors participate to disrupt the intestinal barrier. The defective mucosal integrity starts a complex vicious cycle that leads to, enhances, and perpetuates IBD.

variations, participate to and influence the onset and reactivation of this disease [4, 5]. There is compelling evidence that an inherited/acquired genetic predisposition that leads to barrier disruption and overreaction of the mucosal immune responses to enteric/environmental antigens are major factors contributing to the pathogenesis of IBD [6–8]. The dysregulated reaction of the mucosal immunity to normal intestinal microflora may be induced by defects in the epithelial barrier (increased intestinal permeability), adherence of bacteria, or expression of the "defensins" proteins.

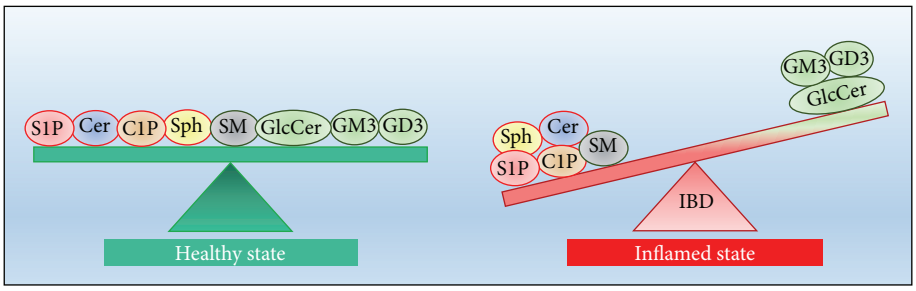
The interaction among intestinal epithelial cells (IECs), intestinal microbes, and local immune cells plays a crucial role in the maintenance of the intestinal homeostasis and is disrupted in IBD, leading to overreaction of the mucosal immune response to normal intestinal microflora. Indeed, a common histopathological feature of IBD is an excessive immune activation, characterized by an exaggerated infiltration of mast cells, monocytes/macrophages, and polymorphonuclear leukocytes into the intestinal epithelium. This overabundance of immune cells is accompanied by continuous and dramatic production of proinflammatory stimuli, including cytokines, growth factors, and adhesion molecules, as well as of inflammatory mediators (especially those of the eicosanoid family) and reactive oxygen species (ROS) [9, 10]. All this results in the development of a severe and pervasive inflammation that promotes and exacerbates IBD.

3. Intestinal Sphingolipid Equilibrium

The small intestine is lined by a single layer of self-renewing IECs, which cover the surface of fingers-like projections called villi, and that of flask-like structures around the base of villi called crypts. The large intestine does not contain villi. Complex sphingolipids are present throughout the intestinal tract, with preferential localization in the apical membrane of polarized IECs, endowing its architecture with enhanced stability and digestive/absorptive capacity. Enterocytes of the small intestine are characterized by the selective abundance of SM and glucosylceramide (GlcCer), whose levels account for more than twofold that of the colonic mucosa and about 40% of total lipids [11]. The high content of sphingolipids in the small intestine is associated with selective enrichment and localization of several species in the apical membrane of the absorptive villous cells, which parallels the continuous process of mucosal cell differentiation throughout the crypt-villus axis [12]. Indeed, individual sphingolipids are differently distributed in villus and crypt cells, higher amounts of Sph, GlcCer, and GM3 being present in villi and Cer, trihexosyl-Cer, and GD3 ganglioside in crypts [13].



(a)



(b)

Figure 2: Metabolism and balance of intestinal sphingolipids in physiological conditions and IBD. (a) Interconnected pathways of intestinal sphingolipid metabolism. (b) In the healthy state, intestinal sphingolipids are in a "functional equilibrium" (left); in the inflamed state of IBD, an unbalance of this equilibrium occurs, favoring the inflammatory disease (right).

Sphingolipids have rapid turnover, and their levels are controlled by the balance between synthesis and degradation. As in most cells, the overall metabolism of intestinal sphingolipids is complex and intricate (Figure 2(a)) and involves multiple enzymes, also present in different isoforms and subcellular districts. Sphingolipids of the intestinal tract are synthesized via either "*de novo* pathway" (initiated by serine condensation with palmitoyl-CoA through serine palmitoyltransferase) or the "salvage pathway" (the recycling of free Sph derived from sphingolipid catabolism). In both pathways, the action of ceramide synthase (CerS) is required to produce Cer, and five of the six known isoforms of CerS are expressed in the intestinal mucosal cells [11]. The newly formed Cer in the endoplasmic reticulum is then transferred to the Golgi apparatus through either the protein (CERT) mediated transport (for SM biosynthesis) or a vesicle-mediated route (for both SM and GSLs formation) [14]. Two types of SM synthases (SMS) (SMS1 and SMS2) have been identified and cloned in the intestinal cells. SMS1 resides in the Golgi whereas SMS2 mainly presents at the plasma membrane. GlcCer synthase is highly expressed in the small and large intestine, rendering GlcCer the major intestinal GSL. GlcCer is also the substrate of galactosyltransferase and then of sialyltransferase abundant in villous cells, to provide lactosyl-Cer and GM3 [11].

Concerning sphingolipid catabolism, the intestine is characterized by the ability to hydrolyze both endogenous and exogenous (dietary-derived) sphingolipids. Intestinal SM degradation, catalyzed by sphingomyelinase (SMase) and ceramidase (CDase), has been the object of different studies. SMase and CDase can act both inside mucosal cells and as ectoenzymes, being present either in the intracellular environment or on the outer surface of the cell membrane or in the intestinal lumen. So far, three isoforms of SMase, acid (A-SMase), neutral (N-SMase), and alkaline (Alk-SMase), are figured out in the intestine with different compartmentalization. A-SMase (and in minor amounts N-SMase) is mainly localized in highly proliferating crypt cells, particularly of the proximal intestine, and appears to be mainly involved in the hydrolysis of SM internalized by endocytosis [11]. Alk-SMase is found preferentially in the middle part of the intestine, primarily at the brush border, and is recognized as the major enzyme for dietary SM digestion, even if it is also able to hydrolyze the plasma membrane SM of mucosal cells [15].

Besides SMase, also CDase is present in the intestine as three isoforms, including the neutral (N-CDase), the alkaline, and the acid one, the former exhibiting the highest catalytic activity in the presence of bile salts [16]. Although direct absorption of SM and Cer in humans cannot be excluded [17], there is ample evidence that free Sph, produced through Alk-SMase and N-CDase digestion, is the major absorbed product of dietary SM. Once internalized into the enterocytes, Sph is rapidly metabolized, mainly to S1P by sphingosine kinase (SphK). In the small intestine of mice, the total SphK activity was shown to be about twofold that of colon, and SphK1 contributed approximately 40% to the total kinase activity in

small intestine, whereas it was the prevalent isoform in colon [18]. Interestingly, an unidentified SphK with high activity toward phytosphingosine was reported to be the prominent SphK in the small intestine [18]. S1P lyase that degrades S1P to phosphoethanolamine and palmitaldehyde is expressed at high levels in the intestinal mucosa [19], most probably to reduce the level of S1P (and Sph) derived from the digestion of dietary sphingolipids.

This intricate network of metabolic reactions leads to the physiological presence of multiple sphingolipid molecules, which play fundamental roles in the digestive, absorptive, protective, and defense properties of the intestine.

4. Aberrant Sphingolipid Metabolism Leads to a "Proinflammatory" Sphingolipid Pattern in IBD

There is compelling evidence that dysregulated production of several sphingolipid molecules occurs along with IBD and contributes as a major factor to the pathogenesis and maintenance of this disorder. Indeed, sphingolipid metabolism and the cellular level/distribution of different sphingolipids exhibit significant changes in IBD. These variations not only alter the healthy sphingolipid equilibrium essential for intestinal functions, but also implicate cell-signaling responses that precipitate the pathology.

Accumulating pieces of evidence demonstrate that a significant increase in some sphingolipid species, including SM, Cer, and the sphingoids mediators C1P and S1P, is associated with IBD and is counterbalanced by the decrease of other molecules, especially GlcCer and GM3 (Figure 2(b)). Indeed, SM and Cer are significantly increased in a colitis mouse model [20], as well as in feces of animals with dextran sulfate sodium- (DSS-) induced colitis (an experimental model of IBD) [21]. In agreement, and of relevance, high levels of SM and Cer were reported in the ileum from CD patients [22], providing evidence that SM and Cer generation accompanies and possibly aggravates chronic intestinal inflammation. Different enzymatic alterations appear to contribute to the increased SM in IBD. First, a decrease in Alk-SMase activity was demonstrated in human chronic colitis [23], possibly acting as a key mechanism to induce SM accumulation. In addition, colon samples from mice with a DSS-induced IBD exhibit downregulation of N-SMase, and, of relevance, concomitant upregulation of SMS2 [20].

Both the increase of glucocerebrosidase (responsible for GlcCer hydrolysis to Cer) and CerS activation were found in IBD and were suggested to be responsible for Cer elevation [24]. Moreover, in colon cancer cells, tumor necrosis factor α (TNF-α), a proinflammatory cytokine that plays a pivotal role in IBD, was shown to upregulate the *de novo* pathway of sphingolipid biosynthesis [25], and it is conceivable that this effect could contribute to Cer and SM accumulation in IBD. A further study on mice revealed that also Sph level is elevated in colon inflammation [26], most likely as a consequence of the increased expression and activity of N-CDase. Indeed, it was shown that N-CDase expression and activity increase in colon epithelium of mice during DSS-induced IBD [26]. On the contrary, it was shown that the ganglioside content of inflamed intestinal mucosa is significantly decreased [27]. Whether enhanced catabolism of gangliosides and/or decreased biosynthesis from Cer are responsible for ganglioside depletion in IBD remains unknown. In a rat model of intestinal inflammation, the 1-phosphorylated forms of Cer (C1P) and Sph (S1P) were shown to increase in a time-dependent fashion with inflammation [28]. The increase of S1P levels is of particular relevance in IBD colon and occurs not only in the intestine but also in blood, as a consequence of the enhanced expression of SphK1 [29]. On the contrary, the deficiency of SphK2 was shown to significantly reduce IBD severity [30].

It is unknown whether alterations of sphingolipid metabolism precede the onset of the chronic inflamed state of IBD, or the inflammatory condition induces them. It appears plausible that both conditions are operative, sphingolipid aberrations favoring and on their turn being favored by the IBD condition.

5. Sphingolipid Unbalance: A Multiarmed Force of IBD

IBD has many tiers of initiation, progression, and evolvement to reach the summit of intestinal damage. The multiple and significant alterations of sphingolipids and bioactive sphingoids level associated with IBD result in a variety of effects on these tightly interrelated tiers, including the epithelial barrier integrity, immune cell targeting and signaling, and innate/adaptive immune responses (Figure 3).

5.1. Sphingolipid Alterations Favor the Disruption of the Intestinal Barrier in IBD. A critical function of the intestinal epithelium is to form a selective permeable barrier that allows the appropriate absorption of nutrients but limits the permeation of noxious agents, such as pathogens, toxins, and antigens, from the luminal environment [31]. This specialized permeable barrier is achieved by different structures, including mucus and intercellular tight junctions (TJs), which control luminal molecules-intestinal cells interactions and paracellular permeability [32]. It is recognized that the disruption of the intestinal TJ barrier, followed by the permeation of luminal noxious agents, and perturbation of the mucosal immune system and inflammation, acts as a key trigger of IBD development [33]. IBD patients exhibit increased intestinal permeability, which favors bacterial/viral infections and thus onset/relapse of IBD. Observations on IBD patients pointed out the role of bacteria in this inflammation, and antibiotics were found to be effective in some patients with IBD [34]. In agreement, most mouse models of colitis require intestinal bacteria to occur [35].

It is noteworthy that the intestinal permeability is also influenced by the content and pattern of IEC sphingolipids (Figure 3). Both Sph and its N-acylated derivative Cer are elevated in IBD and emerged as antibarrier effectors (Figure 3(a)). By using fumonisin B1 (a fungal inhibitor of CerS that leads to Sph accumulation and depletion of complex sphingolipids), it was found that the toxin induces a primary

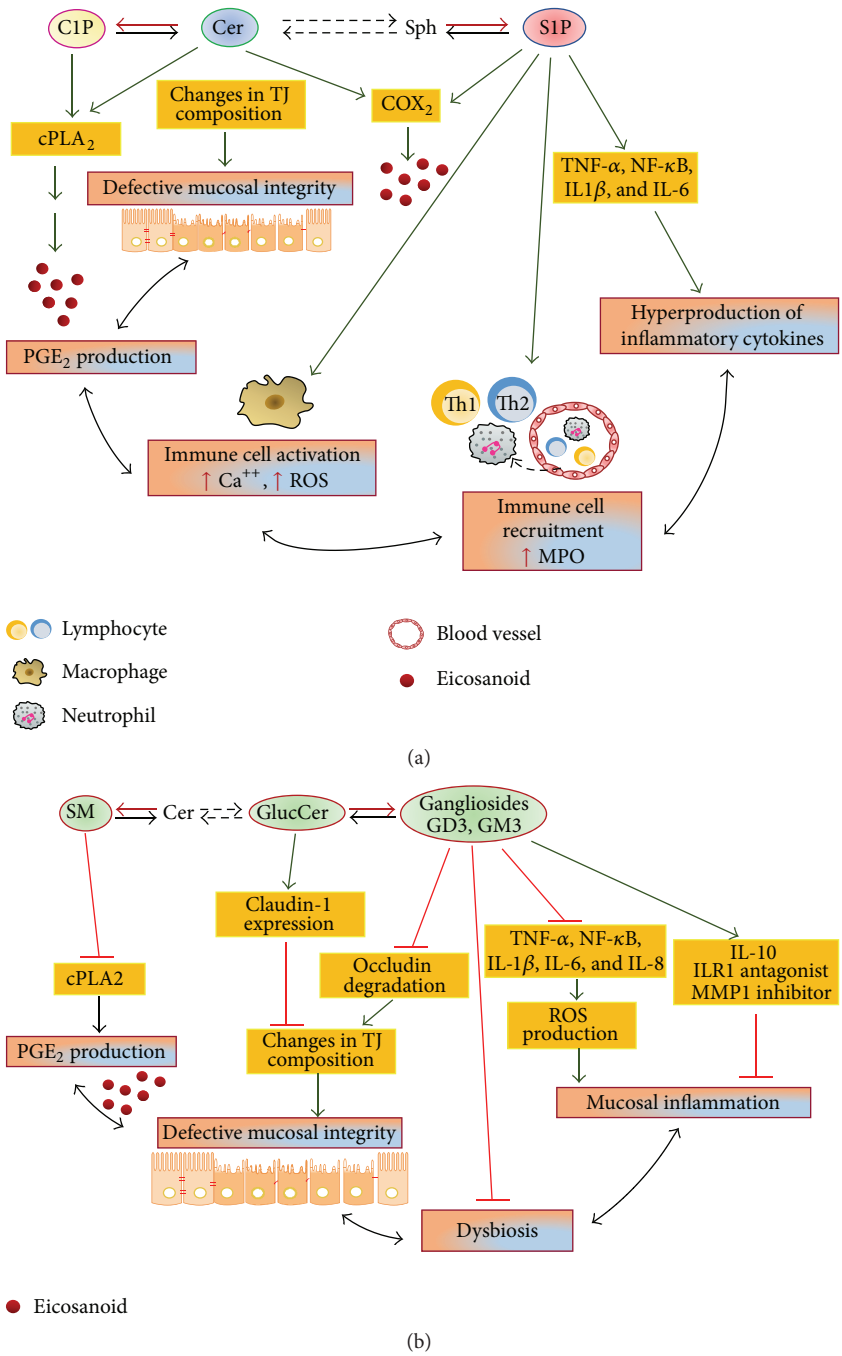


Figure 3: Inflammatory roles of sphingolipids in IBD. (a) Simple sphingolipids (C1P, Cer, and S1P) act as potent proinflammatory messengers, favoring and exacerbating the IBD condition. (b) Complex sphingolipids (SM and GSLs) exert several anti-inflammatory effects.

defect in barrier function, increasing intestinal epithelial permeability [36]. In addition, fumonisin B1 treatment in pigs resulted in a significant increase of intestinal colonization by pathogenic *E. coli* [37]. As far as Cer is concerned, it is recognized that it selectively accumulates in cholesterol- and sphingolipid-enriched membrane microdomains, which also include TJs [33]. The packing of Cer in these microdomains alters the lipid rafts composition, contributing to a disturbed barrier function [38]. In agreement, the neutralization of cell surface Cer prevented the loss of barrier function induced by platelet-activating factor (PAF) [38].

Opposite to the negative effects of Sph and Cer at the intestinal barrier, GSLs (gangliosides and GlcCer), enriched on the luminal membrane of enterocytes, protect intestinal mucosa from injuries induced by bile salts, gastric juice acidity, and toxins/pathogens entry (Figure 3(b)). In particular, through their negative charge, gangliosides such as GM1 and GM3 are able to bind, and then inactivate, the toxins of

Vibrio cholerae, *E. coli*, and *Shigella* [39–41]. Of interest, these antibacterial effects can be improved by dietary gangliosides. A recent *in vivo* study on lipopolysaccharide- (LPS-) induced inflammation revealed that dietary gangliosides inhibit the degradation of gut occludin, a major protein of intestinal TJs [42], implicating them in the intestinal barrier properties. In addition, dietary GlcCer was shown to induce the expression of claudin-1, thus improving TJ properties [43].

Overall, it emerges that the significant reduction of GSLs, especially GM3 and GlcCer, associated with the concomitant elevation of Sph and Cer, markedly concurs to alter the permeability properties of the intestinal epithelium in IBD.

5.2. Sphingolipids and Cytokine Networks in IBD. A well-recognized feature of IBD is the overproduction of proinflammatory cytokines, mainly represented by TNF-α, interleukin- (IL-) 6, and IFN-γ [44]. In the IBD intestine, excess of macrophages and subpopulations of macrophages not normally present in the lamina propria of the intestine is present, indicating ongoing recruitment to the inflamed bowel. The abundance and activation of these innate and adaptive immune cells in the intestinal mucosa result in increased local levels of proinflammatory mediators [44]. In active IBD, an elevated expression of TNF-α occurs, consequent to the high density of TNF-α producing cells, especially macrophages within the lamina propria [45]. The high level of TNF-α contributes to the inflammatory tissue damage, by impairing the integrity of epithelial and endothelial membranes and increasing the recruitment of inflammatory cells [46].

Different studies implicate a strong network connection between cytokines and different sphingolipids as critical in the regulation of inflammatory pathways and immune reactions in IBD. In this connection, SphK1 emerged as key actor, being activated by several cytokines, among which TNF-α plays a prominent role [47]. SphK1 acts as important mediator of various cellular processes in IBD, most likely downstream of TNF-α and upstream of cyclooxygenase- (COX-) 2. Indeed, in a DSS-induced colitis model, Snider et al. [29] reported that SphK1 depletion failed to induce colonic COX-2 and the consequent prostaglandin (PG)E2 production, which contributes to the exacerbation of the inflammatory colon damage. In the same study, it was reported that S1P produced by SphK1 is able to promote neutrophil infiltration into the crypts and lamina propria of the colon [29]. Thus, S1P emerged as a chemoattractant for granulocytes in the inflamed intestine either directly or through the production of other local chemoattractants, and in both cases the SphK1/S1P pathway promoted the tissue inflammatory reaction. In a follow-up study, the same group provided further insights into the distinct roles of hematopoietic (bone marrow-derived) and extrahematopoietic (intestinal) SphK1/S1P pathway in the pathogenesis of IBD [48]. Hematopoietic-derived SphK1 was found as major contributor to circulating S1P, which, on its turn, was implicated in lymphocyte egress from the spleen, and circulating neutrophil increase [48]. This led to a high neutrophil/lymphocyte ratio, a steady indicator of systemic inflammation in colitis [49]. In addition, both hematopoietic and extrahematopoietic S1P were involved in the increased expression of IL-1β and IL-6, as well as in the phosphorylation of STAT3, a tightly regulated transcription factor implicated in chronic colitis associated cancer [48, 50]. As far as the extrahematopoietic SphK1/S1P is concerned, this was shown to be essential for the autocrine induction of COX-2 in the colon tissue [48].

Although the absence of SphK1 was protective against DSS-induced colitis [51], SphK2 depletion resulted in enhanced proliferation and proinflammatory cytokine production, and thus IBD progression [30, 51]. Indeed, knockout mice for SphK2 were shown to develop more severely damaged and necrotic colonic mucosa, with pronounced loss of crypt structures and extensive infiltration of inflammatory cells, eosinophils, and neutrophils [30]. In SphK2(−/−) mice, mesenteric lymph node cells overproduced proautoimmunity cytokines, and T-cells induced more rapid and robust IBD in scid recipients which enhanced the pathological phenotypes of colitis severity, suggesting that SphK2 negatively modulates inflammatory responses and significantly reduces IBD severity [30]. These findings support the hypothesis that therapeutic enhancement of SphK2 activity and/or depletion of SphK1 may be effective treatments for the exuberating inflammation of IBD.

Different reports demonstrate that Cer generated by SMase is able to exert powerful effects, even opposite, on IBD cytokine production and signaling. In the inflammatory processes of the gastrointestinal tract, the infiltration of inflammatory cells into the intestinal mucosa leads to a marked increase of IL-1β and then to NF-κB activation [44]. Both IL-1β and NF-κB exert detrimental effects, by inducing the expression of proinflammatory mediators that orchestrate and sustain the inflammatory response, finally determining tissue damage. A study by Homaidan et al. [52] on murine IECs demonstrated that IL-1β activates N-SMase through NF-κB, thus inducing significant accumulation of Cer. Cer was found to mimic IL-1β in enhancing PGE2 generation through COX-2 induction and by promoting the rise of chloride and water secretion, a typical alteration of IBD patients [52, 53]. Cer generated by N-SMase was shown to induce apoptosis in human colon HT-29 cells, through NF-κB activation and IL-8 expression [54]. In addition, also the A-SMase-derived Cer was apoptotic, but by activating NF-κB on distinct κB complexes [54]. Besides IL-1β and TNF-α, also LPS induces the release of inflammatory cytokines from monocyte/macrophages via A-SMase activation, and Cer generation leads to the expression of a variety of genes and results in inflammatory responses [55].

It is recognized that Cer can exert opposite effects depending on its enzymatic origin and/or site of production [56]. Indeed, opposite to the proinflammatory effects of Cer produced by N- and A-SMase in the intestine, Cer generated by Alk-SMase was shown to promote intestinal anti-inflammatory pathways. In fact, the conversion of the apical membrane SM to Cer by intestinal Alk-SMase was found to inhibit cholera toxin endocytosis and host cells intoxication [57]. Further support for the anti-inflammatory action of intestinal Alk-SMase was provided by a report showing that, in rats with DSS-induced colitis, the intrarectal instillation of Alk-SMase induced TNF-α inhibition, significantly reduced

the inflammation score, and protected the colonic epithelium from inflammatory destruction [58]. These studies, together with the finding that Alk-SMase is downregulated in IBD [23], support the notion that the decrease of this anti-inflammatory enzyme may be of relevance in IBD pathogenesis and progression.

The excessive cytokine production in IBD appears to be favored also by the decrease of gangliosides occurring in this disease. It is recognized that gangliosides in the apical intestinal surface can influence numerous processes, including microbial attachment, toxin production, and infectivity of several intestinal pathogens (Figure 3(b)), and increased ganglioside catabolism occurs in IBD and results in increased proinflammatory signaling [59]. Intriguingly, dietary gangliosides emerged as effective elements in reducing proinflammatory mediators of IBD. Indeed, an induced ganglioside increase was shown to inhibit both TNF-α and IL-1β proinflammatory signals in rats [27]. Moreover, in a cultured infant bowel exposed to LPS in hypoxic conditions, gangliosides were able to reduce both IL-6 and IL-8 production [60].

5.3. Cross-Communication of Sphingolipid Messengers with Eicosanoid and Glycerolipid Signaling. Aberrant production of bioactive lipids of the eicosanoid family, especially PGs, was identified to drive chronic inflammation by dysregulating signaling pathways and/or cellular events and leading to abnormal immune functions [61]. Eicosanoids, including PGs, are generated from arachidonic acid, released from cell membrane phospholipids mainly by phospholipase-A2 (PLA2). Arachidonic acid is then converted to PGs via the COX pathway. Of the two known COX isoforms (COX-1 and COX-2), it is COX-2 that is induced during acute or chronic inflammation [62].

Increased PG production occurs within the gastrointestinal mucosa of patients with IBD, and in experimental models of IBD [63]. As a consequence, PGs and especially PGE2 act as proinflammatory factors and tool in IBD. Different studies progressively revealed a key role of sphingolipids in the modulation of eicosanoid production in inflammatory processes [64] (Figure 3(a)). Initial studies showed that Cer and Sph were able to induce PGE2 production [65]. Cer was shown to enhance cytosolic PLA2 (cPLA2) activity and PG formation through direct binding to the enzyme [66]. Subsequent studies demonstrated that both C1P and S1P are involved in the regulation of PGE2 production [67–69], by acting through different mechanisms. C1P was shown to activate cPLA2 either directly or by promoting its translocation to intracellular membranes [70–72]. After binding to different subtypes of its receptors, S1P acts downstream of Cer, by causing strong and prolonged expression of COX-2 [73], through inducing transcription and mRNA stabilization [74, 75].

The proinflammatory cytokines IL-1β and TNF-α were shown to regulate both cPLA2α and COX-2 to promote SM metabolism and to produce C1P and S1P formation [76]. This signaling pathway starts with A-SMase activation, which results in the reduction of the SM-mediated inhibition of cPLA2α. Moreover, the two cytokines activate SphK1 and ceramide kinase 1, the Cer pool generated by A-SMase finally promoting both C1P and S1P biosynthesis. In response to proinflammatory cytokines, C1P and S1P exhibit a significant synergistic effect on the activation of cPLA2α and COX-2, and thus on the production of the key inflammatory mediators PGE2 [67].

Further support to IBD maintenance derives from the different catalytic activities exhibited by the Alk-SMase. This enzyme is able to hydrolyze not only SM, but also other choline phospholipids, including lysophosphatidylcholine, with reduction of lysophosphatidic acid, and PAF [15, 77]. Thus, the Alk-SMase reduction of IBD appears to be functional to signaling alterations that potentiate IBD, by reducing the anti-inflammatory actions exerted by SM and PAF and by promoting the proinflammatory lysophosphatidic acid.

Although much remains to be understood about these multiple signaling interactions, the actual knowledge linking sphingolipids with eicosanoids and glycerolipid messengers provides a new conceptual view on how alterations of sphingolipid signaling in IBD act not only as such but also through regulation of other signaling molecules. This leads to increased efficacy in promoting and maintaining the chronic state of IBD.

6. Sphingolipids: Potential Therapeutic Benefits/Targets in IBD

The global incidence of IBD is increasing with time [78]. Actually, a step-up strategy is used in the management of IBD, therapeutic treatments including 5-aminosalicylic acid, glucocorticoids, immunomodulators, and anti-TNF agents [79]. Despite advancements in therapy with the introduction of anti-TNF agents [80, 81], IBD gradually causes serious effects in terms of morbidity, work disability, and quality of life [82]. The finding that multiple sphingolipid alterations are associated with IBD has prompted investigations on the targeting of sphingolipids as a potential field of therapy for managing progression and morbidity of this disease.

6.1. Dietary Bioactive Sphingolipids: A Gift from Nourishment. 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 compounds and, among them, dietary lipids have emerged as major determinants of the intestinal immune responses, mainly after conversion into lipid mediators [83]. Indeed, as described above, sphingolipid metabolites act as multifaceted bioactive molecules in IBD, and different studies demonstrated that dietary complex sphingolipids, including gangliosides and SM, possess the potential of effective compounds.

Initial studies on dietary gangliosides demonstrated that the sialylated GSLs of human milk exert beneficial effects in infant bowel infections, through enterotoxin-inhibiting activity and maintenance of the intestinal barrier integrity [84]. Thereafter, it was shown that milk gangliosides were able to inhibit adhesion and suppress the growth of *E. coli*. In particular, an infant formula, specifically enriched with GD3 and GM1 gangliosides, lowered *E. coli* content and concomitantly

increased that of bifidobacteria, suggesting a ganglioside role in the development of intestinal immunity [85]. Furthermore, Schnabl et al. [60] demonstrated that ganglioside preexposure to LPS treatment reduces bowel necrosis and endothelin-1 production in response to LPS, by suppressing infant bowel production of different proinflammatory mediators, such as nitric oxide, leukotriene B4, PGE2, hydrogen peroxide, IL-1β, IL-6, and IL-8. Moreover, very recently, it was reported that gangliosides, especially GD3, ameliorate intestinal injury not only by suppressing proinflammatory mediators, but also by upregulating anti-inflammatory molecules, such as chemokines and the cytokine IL-10 [86] (Figure 3(b)).

Besides gangliosides, also dietary SM was shown to exert biomodulatory activities in intestinal inflammation, though with contrasting results. In the mouse colon, it was observed that the simultaneous administration of SM and DSS prevents the increase of myeloperoxidase (MPO) activity, a marker of neutrophil influx into the inflamed colon [87]. Moreover, Mazzei et al. [88] recently reported that dietary SM decreased colonic inflammatory lesions and disease progression in mice with DSS-induced colitis. On the contrary, two recent papers by Hausmann's group showed that, after metabolism to Cer, dietary SM triggers apoptosis in murine IECs and aggravates intestinal inflammation in acute DSS-induced colitis and in IL-10 knockout mice [21, 89]. The reasons of these opposite findings on dietary SM in intestinal inflammation remain to be clarified. Although it cannot be excluded that the use of different mouse strains may be responsible for the anti- and proinflammatory effects of dietary SM, it appears likely that the different composition and intestinal hydrolysis of the administered SM (of milk and egg origin, resp.) play a major role.

Taken together, these findings suggest that the dietary assumption of specific types of complex sphingolipids might be beneficial as preventive/therapeutic strategy against IBD.

6.2. Regulation of Sphingolipid Metabolites by Dietary Materials. In addition to dietary sphingolipids, the assumption of dietary compounds able to modify the intestinal sphingolipid metabolism has emerged as an intriguing possibility to influence the IBD status.

It has been demonstrated that a diet enriched with fibers and fats is able to influence the expression of both intestinal SMases and CDases. Psyllium, a water soluble fiber partly digested by bacterial flora, increased the activity of Alk-SMase and decreased that of A-SMase and N-CDase alleviating inflammation, whereas a high-fat diet exerted opposite effects [90]. Moreover, in IL-10 KO mice with colitis, it was demonstrated that the VSL#3 probiotic upregulates Alk-SMase activity, suggesting its potential as anti-inflammatory agent [91].

Luteolin, a tetrahydroxyflavone present in a variety of vegetables, fruits, and medicinal herbs, has been shown to function as an antioxidant, anti-inflammatory, and anti-cancer agent [92]. Very recently, we reported that dietary luteolin is able to unbalance the sphingolipid rheostat by inhibiting both S1P biosynthesis and Cer traffic in colon cancer cells [93], suggesting its dietary introduction/supplementation as a potential strategy to improve inflammation in colorectal cancer. These findings prompt further investigations on dietary molecules able to modulate sphingolipid metabolism as anti-IBD foods.

6.3. Targeting Sphingolipids: A Potential Anti-IBD Strategy. The discovery of alterations of sphingolipid metabolism and signaling in IBD paved the way for the discovery of pharmacological approaches to prevent/treat this disease. Up to now, several compounds have been discovered to manipulate the SM cycle and S1P metabolism/receptors, and these were evaluated for their potential therapeutic benefit generally in inflammation, and especially in IBD.

So far, the most high-profile drug that improves inflammation via S1P receptor (S1PR) modulation and is assessed *in vivo* is the fungal metabolite fingolimod (FTY720). Differently from typical immunosuppressive drugs, its mode of action is not via inhibition of T-cell function, but via downregulation of S1P1 on lymphocytes, leading to the inhibition of their egress from the lymph nodes [94]. As a consequence, FTY720 reduces the number of T-cells circulating between lymph nodes and the peripheral site of tissue inflammation. In agreement with this action, Mizushima et al. [95] demonstrated the efficacy of FTY720 in decreasing the severity of spontaneous colitis in the IL-10-deficient mouse model. FTY720 induced accelerated homing and sequestration of T-cells into Peyer's patches and mesenteric lymph nodes, resulting in a reduction of CD4+ T lymphocytes into the lamina propria [95]. An additional study provided new evidence on the immunosuppressive properties of FTY720. In particular, in a chemically induced mouse model of colitis, FTY720 treatment markedly reduced the severity of intestinal inflammation [96]. This therapeutic effect of FTY720 was associated with downregulation of different proinflammatory cytokines, paralleled by a functional activity of CD4+CD25+ regulatory T-cells and prominent upregulation of anti-inflammatory mediators [96].

The finding that S1P1 modulation was the major mechanism of FTY720's efficacy prompted the search for second-generation compounds, with high selectivity to S1P1, and lower clinical adverse events (linked to its agonistic activity to S1P3), such as symptomatic bradycardia [97]. Among new drugs, KRP-203, S1P1/4/5 agonist prodrug with a molecular structure resembling FTY720 with high selectivity to S1P1, was developed for immunomodulation in autoimmune diseases and organ transplantation [98]. In the IL-10-deficient mouse, a validated model of human IBD, KRP-203 regulated not only T-cell response but also B-cell one [99]. Its treatment resulted in a significant reduction in the severity of colitis and in the number of CD4(+) T-cell and B220(+) B-cell subpopulations and T-helper 1 (Th1) cytokine production in colonic mucosa, a histopathological hallmark of IBD [99]. Thus, the use of KRP-203 emerged as an option to be tapped into effective immunointervention in IBD.

W-061, a novel and potent S1P1 agonist, structurally different from Sph and active *in vivo* without undergoing phosphorylation, showed effectiveness for alleviating multiple aspects of chronic intestinal inflammation in DSS-induced colitis, such as preventing mucosal thickness and mucin depletion induced by DSS. The novel effect attributed

exclusively to W-0671 as S1PR agonist is the suppression of Th17 and Th1 increase in the lamina propria, by the sequestration of these cells into secondary lymphoid tissues [100]. It has been recently demonstrated that SEW 2871, another selective S1P1 agonist, ameliorates colitis in IL-10-deficient mice, by reducing proinflammatory cytokines production and promoting the expression of TJ typical proteins [101]. A further, highly specific S1P1 agonist, named RPC 1063, has been successfully tested in animal models of IBD and is currently being used as an oral therapeutic in phase II clinical trials of UC [102].

Besides S1PR antagonists, other drugs exhibiting a promising therapeutic benefit in IBD are those targeting the S1P axis and acting as SphK inhibitors. Different studies demonstrated that the orally available inhibitors of SphKs, ABC747080 and ABC294640, are effective in inhibiting the severity of colitis [29, 103, 104]. This protection is likely due to the significant decrease, induced by the inhibitors, of a broad spectrum of inflammatory mediators, such as TNF-α, COX-2, IL-1β, IFN-γ, MPO, and IL-6, including S1P. Moreover, SphK inhibitors were able to attenuate the effects of TNF-α-induced increase in the expression of the adhesion proteins physiologically involved in leukocyte recruitment.

Of notice, in both acute and chronic models of UC, it has been reported that SphK inhibitors and Dipentum, an FDA-approved anticolitis drug, exhibit similar effects in reducing inflammation extent and severity, with the best results in a combinatory delivery [103]. These results are encouraging and shed light on a possible combination of SphK inhibitors and anti-IBD drugs for further clinical IBD improvement. Overall, it emerged that SphK is an excellent target for the development of new anti-IBD drugs.

Additional studies investigated the SM cycle as therapeutic target in IBD. By using SMA-7, an inhibitor of A-SMase and N-SMase, it was found that, besides suppressing Cer production, it was effective in inhibiting NF-κB activation and inflammatory cytokine release from macrophages [55]. In murine models of colitis, the oral administration of SMA-7 induced a significant decrease in the NF-κB-dependent cytokine levels and reduced alterations and damage in the colonic mucosal layer [55]. Whether N- and A-SMases play a different role in the regulation the IBD inflammation and the contribution of Cer inhibition and/or S1P reduction as key effectors of the SMA-7 effect remain to be clarified.

Finally, also glycosylated sphingolipids recently emerged as compounds of potential benefit in IBD. In particular, cerebroside D, a glycoceramide compound from fungal culture, was presented for its immunosuppressive activity and efficiency in improving DSS-induced colitis in mice [105]. Its anti-inflammatory mechanism involves multiple effects against activated T-cells in colon, such as regulation of the cytokine profile and apoptosis induction in activated T-cell effectors. Thus, by interfering with naïve T-cell activation, cerebroside D appears to be of relevance for IBD management.

7. Closing Remarks

Taken together, a huge and increasing volume of experimental evidence points to the importance of sphingolipids/sphingoids molecules, and their enzymes, in IBD development and fate. However, at present, several gaps in our knowledge and many questions need to be understood better. Among them, more knowledge is required on the mechanisms underlying the *in vivo* digestion/absorption of sphingolipids in the gastrointestinal system, as well as on the effective concentrations shifting them from food components to dietary bioactive nutrients. The influence of specific lipids, and their modifying enzymes, on intestinal lipid-barrier control is an area of great promise that needs to be investigated further to develop novel treatment strategies that strengthen the intestinal barrier and possibly halt, or at least slow down, the initial alterations leading to IBD. It will be also of relevance to better understand the multifaceted role of sphingolipid molecules in IBD and their subcellular localization, as well as cross talk with other intestinal factors involved in this disease. The potential of diet components in changing the sphingolipid amount and pattern in the gut remains an open question. In the "future food" field, it is a hard challenge to find out new compounds targeting sphingolipid metabolism and able to self-actualize, or to combine with other drugs, for effective IBD treatment with minimal side effects. Based on the preclinical findings of the anti-IBD potential of sphingolipid, hard work remains to establish their efficacy and to upgrade and improve treatments to clinical endpoints. Deeper understanding of the proper role of sphingolipids and their enzymes in controlling the intestinal properties and in promoting the pathogenesis and progression of IBD will generate new perspectives in the development of "sphingolipid-centered" therapeutic strategies that control the onset and perpetuation of this disabling inflammation.

Abbreviations

IBD:	Inflammatory bowel disease
SM:	Sphingomyelin
GSLs:	Glycosphingolipids
Cer:	Ceramide
Sph:	Sphingosine
C1P:	Ceramide-1-phosphate
S1P:	Sphingosine-1-phosphate
CD:	Crohn's disease
UC:	Ulcerative colitis
IECs:	Intestinal epithelial cells
ROS:	Reactive oxygen species
GlcCer:	Glucosylceramide
CerS:	Ceramide synthase
SMS:	SM synthases
SMase:	Sphingomyelinase
CDase:	Ceramidase
Alk-SMase:	Alkaline sphingomyelinase
N-SMase:	Neutral sphingomyelinase
A-SMase:	Acid sphingomyelinase
N-CDase:	Neutral ceramidase
SphK:	Sphingosine kinase
DSS:	Dextran sulfate sodium
TNF-α:	Tumor necrosis factor alpha
IFN-γ:	Interferon-γ
TJ:	Tight junction
LPS:	Lipopolysaccharide

PAF: Platelet-activating factor
IL: Interleukin
COX: Cyclooxygenase
NF-κB: Nuclear factor kappa B
PG: Prostaglandin
cPLA2: Cytosolic phospholipase-A2α
S1PR: S1P receptor.

Conflict of Interests

The authors declare that no financial conflict of interests exists.

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Review Article

Role of Sphingolipids in the Pathobiology of Lung Inflammation

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Sphingolipid bioactivities in the respiratory airways and the roles of the proteins that handle them have been extensively investigated. Gas or inhaled particles or microorganisms come into contact with mucus components, epithelial cells, blood barrier, and immune surveillance within the airways. Lung structure and functionality rely on a complex interplay of polar and hydrophobic structures forming the surfactant layer and governing external-internal exchanges, such as glycerol-phospholipids sphingolipids and proteins. Sphingolipids act as important signaling mediators involved in the control of cell survival and stress response, as well as secreted molecules endowed with inflammation-regulatory activities. Most successful respiratory infection and injuries evolve in the alveolar compartment, the critical lung functional unit involved in gas exchange. Sphingolipid altered metabolism in this compartment is closely related to inflammatory reaction and ceramide increase, in particular, favors the switch to pathological hyperinflammation. This short review explores a few mechanisms underlying sphingolipid involvement in the healthy lung (surfactant production and endothelial barrier maintenance) and in a selection of lung pathologies in which the impact of sphingolipid synthesis and metabolism is most apparent, such as acute lung injury, or chronic pathologies such as cystic fibrosis and chronic obstructive pulmonary disease.

1. A Brief Overview on Sphingolipids within the Lung Environment

The interest in sphingolipid presence and bioactivities in the respiratory airways has produced a steady number of reports since the 1970s. However, a host of publications in the last few years have provided an increasingly detailed picture of the role played in the lungs by this class of lipids and by the proteins that handle them. As vital respiratory organs that mediate air-blood gas exchanges, lungs must undergo delicate and tightly controlled developmental transitions. Antenatally, a 20-week human fetus displays lungs that have branched to generate all airways, but it is not before ~28 weeks of gestation that alveolarization begins from primordial saccular structures and type I alveolar cells differentiate from the cuboidal epithelium. Concomitantly, at this stage endothelial cells shape the alveolar capillary bed and type II alveolar cells appear, to demarcate alveolar septal junctions. Type II cells start producing surfactant, which accumulates to increasing concentrations by term. The initiation of autonomous ventilation at birth represents a dramatic switch in postnatal lung function. While throughout gestation a chloride-ion driven liquid secretion creates a positive pressure that distends the lungs and stimulates growth, a sudden reversal from net secretion to net adsorption takes place at birth under the effect of O_2 and hormones (epinephrine, glucocorticoids, and thyroid hormones), enabling the rapid elimination of lung liquid. From this moment on, lung lumen will maintain a low-level chloride-ion based liquid secretion to generate a surface liquid layer, known as surfactant and formed by specific secreted lipids and proteins, and a robust absorptive capacity will prevent alveolar flooding and edema.

Equally important, being permanently exposed to inhaled particles and microorganisms from birth, pulmonary immunity must be tuned to effectively dispose of them, while minimizing immunopathology to preserve appropriate gas exchange. Thus, the first-line lung defenses, prior to immunity, are based on mechanical weapons including cilia, mucus, and the cough reflex, which concur to prevent pathogen access to the lower airways and in so doing avoid an overt inflammatory response. This is one of the major reasons why lungs are particularly sensitive to the sphingolipid (and

other lipids) metabolism equilibrium: pulmonary physiology relies on lipids for important extracellular activity ensured by surfactant and consisting of a sphingolipid/glycerolipid network. Indeed, most successful respiratory pathogens have evolved the ability to gain access to the lower airways in the alveolar compartment, the critical lung functional unit involved in gas exchange. Inherited conditions such as cystic fibrosis are prone to lung infection, partly as a consequence of a compromised mechanical clearance. Upon infection or in sterile inflammatory conditions, adjoining epithelial and endothelial layers in the alveoli, which with their fused basal lamina form the next leakproof barrier against microbes, must become temporarily permeable to allow leukocyte migration. This way, bone marrow derived macrophages, neutrophils, and dendritic cells can enter the extra-epithelial space where they meet a resident population of yolk sac-derived macrophages. A complex cross talk between specific cell populations (immune cells, epithelial cells, endothelial cells, stromal cells, and platelets), mediators, and coagulation and complement cascades will orchestrate the immune response and inflammation from start to resolution. However, disruption of alveolar integrity may occur in aging and in a number of lung diseases.

How do sphingolipids fit into the above sketch? As in most other tissues and organs, in the lung too, sphingolipids play a crucial role as signaling molecules as well as components of membranes and extracellular fluids, in both normal development/functioning and pathological settings involving inflammation. A lung-specific action is the one involving sphingolipids as both minor structural components of lipid-protein surfactant and regulators of its synthesis and release. This short review explores a few mechanisms underlying sphingolipid involvement in the healthy lung (surfactant production and endothelial barrier maintenance) and in a selection of lung pathologies where the impact of sphingolipid metabolites is most apparent. Drawing upon recent evidence from studies in humans and in animal models and *in vitro*, including those in our laboratory, we attempt to highlight in particular the reciprocal controls intertwining sphingolipid metabolism with that of other bioactive lipids (i.e., glycerophospholipids) and the vicious circle linking sustained cellular stress and the imbalance of *de novo* sphingolipid synthesis, as a significant mechanism acting in favor of tissue injury and against inflammation resolution in inflammatory lung diseases. Further, current therapeutic perspectives in using compounds that target the sphingolipid pathway for countering development of lung injury will be discussed. For a more extensive coverage of additional lung pathologies and the role of sphingolipids therein, the reader is referred to other recent reviews [1–4]. In addition, here we shall take for granted the general notions about sphingolipid biosynthesis, transport, and breakdown, which have been extensively treated in many reviews, and shall just recall those that are relevant to our narrative.

2. Sphingolipid Synthesis during Pulmonary Functional Maturation

About five decades ago, we learned of the presence of a layer of surface lipids, with structural and active signaling roles, consisting of lecithin, dipalmitoyl-lecithin, sphingomyelin, phosphatidyl ethanolamine, phosphatidylinositol, phosphatidylglycerol, cholesterol, and small amounts of other lipids, along with proteins of 18 and 36 kDa (termed surfactant proteins), beginning to form at 28 days of gestation [5, 6]. This notion led to the use of a lecithin/sphingomyelin (L/S) ratio, from amniotic fluids, in the clinical diagnosis of fetal lung maturity [7] and in the prediction of neonatal respiratory distress syndrome RDS caused by an insufficient amount of pulmonary surfactant [8, 9]. An L/S ratio less than 2.0 indicates a potential risk of RDS. The risk is nearly 75–80% when the L/S ratio is 1.5. Experiments conducted in rats demonstrated that the prenatal development of this surface lipid activity sees sequentially ordered changes in components, including glycerophospholipids (phosphatidylserine and phosphatidylinositol) [10] together with the sphingolipid sphingomyelin [11]. Similarly, sequential changes in the tissue expression of these lipid-related enzymes were shown, identified in the microsomal fraction of respiratory epithelia and in the alveolar lavage. During the third trimester, the fetal lung synthesizes primarily sphingomyelin, and the majority of stored glycogen is converted to fatty acids and then to surfactant lipids. The enzymes responsible for the biosynthesis of phosphatidyl glycerolipids (CTP: Phosphatidate Cytidylyl Transferase and CDP-Diacylglycerol: Glycerolphosphate Phosphatidyl Transferase and Phosphatidyl Glycerol Phosphate Phosphatase) demonstrated a coordinate increase in activity in fetal rat lung at term when the demand for pulmonary surfactant increases. A fall in one phospholipid is accompanied by an increase in another. The same phenomenon is observed among phospholipids in human amniotic fluid, suggesting a similar development and further confirming the continuity between fetal lung secretions and the amniotic fluid. At birth, the expression of the major enzymes involved in phosphatidyl choline synthesis (CTP and CDP) as well as the expression of Serine Palmitoyl Transferase and Sphingomyelin Synthase further increases, respectively, in type I [10] and type II alveolar cells [11]. Ceramide Synthase 5 is the predominant isoform detected in lung epithelia and its expression is also upregulated. Concomitantly synthesized at ER [12], phosphatidylcholine and sphingomyelin are both secreted in the surfactant, whereas lung tissue, other than surfactant, contains only small amounts of these phospholipids. It is noteworthy that sphingolipid and glycerolipid metabolisms overlap at the enzymatic step where ceramide competes with diacylglycerol for the phosphocholine (deriving from CDP-choline or phosphatidylcholine) to give rise to sphingomyelin (Figure 1). In adult life, enzyme activity reaches a plateau, and the ratio between the key enzymes of the two pathways, Serine Palmitoyl Transferase over Glycerol 3-phosphate Acyl Transferase, is significantly higher in microsomal lung (and pancreas) than in most other adult rat tissues; accordingly, the percentage of sphingomyelin is higher in the total phospholipid content in these fractions [13].

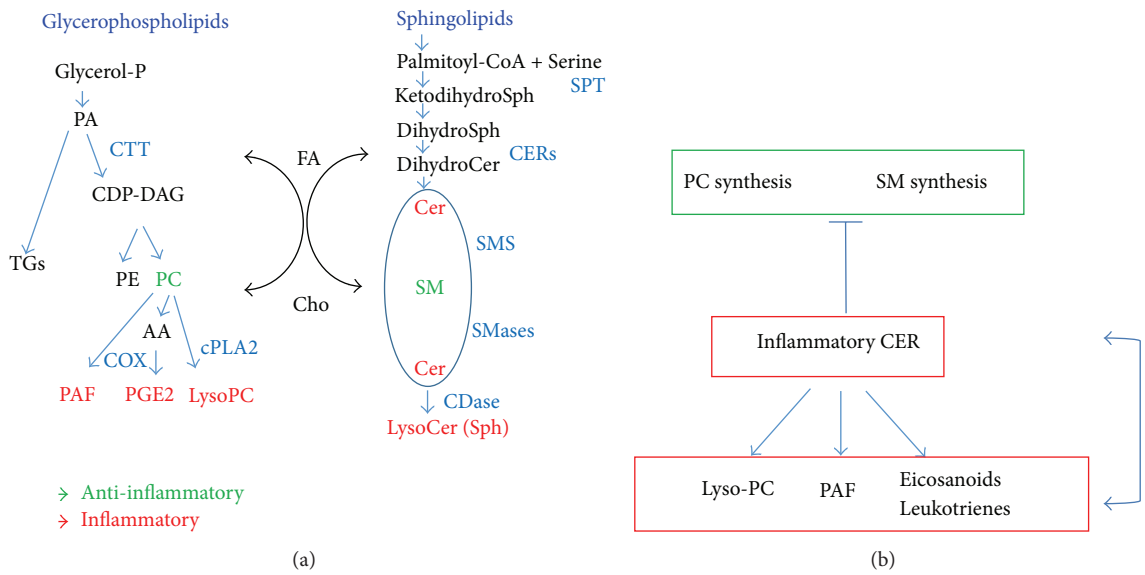

Figure 1: Sphingolipids metabolites form a network with other inflammatory lipids in the lung. (a) Intracellular and secretory lipids regulating inflammation in mucus and mucosa. Red: inflammatory lipids and major controlled enzymatic activities. Green: anti-inflammatory lipids and major controlled enzymatic activities. P, phosphate; FA, fatty acids, Cho, choline; CDP, citidyl diphosphosphate; DAG, diacylglycerol; TGs, triglycerides; PE, phosphatidyl ethanol ammine; PC, phosphatidyl choline; PAF, platelet activating factor; AA, arachidonic acid; PGE2, prostaglandins E2; Sph, sphingosine; Cer, ceramide, SM, sphingomyelin; CTT, CTP: Phosphocholine Cytidyl-Transferase; SMase, Sphingomyelinase, SMS, Sphingomyelin Synthase, and CERS, Ceramide Synthases; SPT, Serine Palmitoyl Transferase; cPLA2, Cytosolic Phospholipase 2. (b) Ceramide accumulation controls other inflammatory and anti-inflammatory lipids. Red: inflammatory lipids and major controlled enzymatic activities. Green: anti-inflammatory lipids and major controlled enzymatic activities.

Fetal lung maturation allows protection from maternal infection, but this maturation process can be altered by pathogens [14]. Surfactant components generally decrease harmful inflammatory responses [15]. Preterm labor-inducing inflammatory ligands (interleukin-1 or lipopolysaccharide) cause a robust induction of the surfactant complex in order to lower the risk of respiratory distress syndrome (RDS). Data from clinical studies suggest that surfactant can be used successfully in neonates with congenital pneumonia, due to a comprehensive contribution by surfactant to host defense from pathogens [16, 17]. However, such trials obtained mixed results and much evidence in animal studies demonstrates that surfactant therapy may also enhance inflammation and reduce the ability of macrophages to clear pathogens [18–20], raising the hypothesis that surfactant physiologic activity relies on a complex equilibrium, whose alteration drives a pathological setting. Thus, an unbalanced sphingolipid metabolism impact on surfactant composition and pulmonary function as well as pharmacological intervention aimed at regulating sphingolipids mediators can promote or block surfactant production. A more extensive comprehension of sphingolipid moiety of surfactant would help to identify therapies or adjuvants for most lung diseases.

3. A First Glance at the Small Picture: Sphingolipid Molecules Take Part in Airway Cell Signaling in Inflammatory Responses

Before dealing with pulmonary disease, we should review better the evidence collected for sphingolipid signaling in lung-derived cell cultures under pathologic stress. The very first evidence of sphingolipid involvement in lung inflammation concerns the production of autoantibodies against glycosphingolipids (aGM1 and GM1b) in response to pulmonary infection with *Mycoplasma pneumonia* [21]. Damaged or regenerating respiratory epithelial cells, typical of cystic fibrosis or lungs suffering from emphysema, exhibit increased expression of receptors for sialylated glycosphingolipids [22], which are recognized by Gram-positive and Gram-negative organisms. Upon engagement, these receptors activate the inflammatory signaling cascades of the acute innate phase [23]. Moreover, alveolar macrophages undergo maturation steps, showing surface expression of aGM1 [24, 25].

In addition to infection, it is possible to reproduce lung inflammation, in an *in vitro* setting, by stressing cells with oxidative agents (e.g., hydrogen peroxide), which represent the by-products of inflammatory damage. Alternatively, cells can be stressed directly with inflammatory mediators or by blocking trophic and prosurvival stimuli, which are dramatically reduced in pulmonary low oxygen pathological conditions (i.e., by inhibiting Vascular Endothelium Growth Factor (VEGF) receptor signaling). Most of the *in vitro* evidence suggests that ceramide can be pharmacologically targeted to reduce reactive oxygen and nitrogen species and inflammatory damage in airway cells. In 2000, Chan and coauthors demonstrated, at a molecular level in the human airway epithelial cell line (HAE), the dependency of hydrogen peroxide-induced apoptosis on ceramide generation following the activation of the glutathione-sensitive neutral Sphingomyelinase [26, 27]. A few years later, Goldkorn and

his group demonstrated that hydrogen peroxide upregulates neutral Sphingomyelinase 2 and consequently increases ceramide levels in human airway epithelial cells [28, 29]. IL-8 plays a pivotal role in lung injury, serving as a recruiter for neutrophils. Neutrophil invasion causes a massive release of oxygen radicals, proteases, and other toxic moieties, responsible for the subsequent tissue destruction and loss of barrier function, leading to pulmonary edema, intrapulmonary shunt, and hypoxemia hallmarks. IL-8 neutralization can be envisaged as a therapeutic approach [30]. Glutathione inhibition of neutral Sphingomyelinase counteracts part of the oxidative stress-induced signaling (p38 activation, inhibition/degradation of the bound phosphatase calcineurin) that sustains IL-8 transcriptional activation [31]. Apart from reactive oxygen species, such as hydrogen peroxide, reactive nitrogen species (RNS) are involved in the pathophysiology of inflammatory lung diseases. NO exposure (via NO donors) was able to induce ceramide accumulation but not apoptosis in airway epithelial cells, by stimulating Ceramide Synthases. This accumulation was inhibited by fumonisin B1 (inhibitor of Ceramide Synthases) [32]. According to the notion that different pools of ceramide can be raised by different stress inducers and take part in specific responses [33], not only neutral Sphingomyelinase but also the activity of Ceramide Synthases is therefore modulated in airway epithelia inflammation. The combined presence of high levels of NO and superoxides generated peroxynitrite (ONOO(−)), which is responsible not only for the ceramide increase but also for apoptosis induction. Such apoptosis was prevented by silencing acid Sphingomyelinase [34].

Taken together, these findings support the hypothesis that the inflammatory stress in airway epithelia, driven by oxygen and nitrogen oxidative species, modulates different pools of ceramide, possibly involving its *de novo* synthesis until the stress is tolerated and evolving to Sphingomyelinases activation to induce apoptosis.

Ceramide can be deacylated by Ceramidases to give rise to the toxic sphingosine, which can be immediately phosphorylated to sphingosine-1-phosphate. Among all the sphingolipids, sphingosine-1-phosphate is a minor species in terms of intracellular concentration but is endowed with potent proliferative and prosurvival activity. The high level of plasma sphingosine-1-phosphate, bound either to albumen or to lipoproteins, has important homeostatic functions in the maintenance of vascular integrity [35] and its gradient is crucial for immune cell trafficking during inflammatory reactions [36]. The role of sphingosine-1-phosphate in inflammation is partially controversial. Although *in vivo* it exerts mainly an anti-inflammatory role (discussed in the following sections), a few reports demonstrate proinflammatory activities of this lipid mediator. H441 lung epithelial cell treatment with exogenous sphingosine-1-phosphate induced an increase in IL-8 mRNA and its secretion. TNFα can also activate Sphingosine Kinase [37, 38], and sphingosine-1-phosphate leads to IL-8 gene expression *via* ERK and p38 MAPK activation and increased AP-1 inflammatory transcriptional activity in alveolar macrophages [39]. Thus sphingosine-1-phosphate recapitulates the action previously ascribed to ceramide and possibly exerted by sphingosine-1-phosphate. A putative explanation may be that sphingosine-1-phosphate, either supportive or alternative to ceramide, could exert different actions not only on cell culture treatment versus *in vivo* but also in physiological and protective inflammatory responses [26], as opposed to pathological inflammation.

Human pulmonary artery endothelial cells effectively utilize exogenous sphingosine-1-phosphate as a prosurvival and permeability regulator, via extracellular conversion to sphingosine by Lipid Phosphate Phosphatase-1 and uptake of sphingosine followed by intracellular phosphorylation by Sphingosine Kinase-1 [40, 41]. VEGF receptor is engaged by a trophic factor required for the survival of endothelial cells and abundantly expressed in the lung [42]. The inhibition of VEGF receptor initiates apoptosis and alveolar destruction, morphologically resembling emphysema, and it is concomitant to an increased ceramide synthesis in the alveolus [43, 44]. FTY720, a sphingosine analogue which can be phosphorylated [45], acts as a downregulator of sphingosine-1-phosphate receptor 1 by inducing its ubiquitination, internalization, and degradation [46, 47], but it was also proved to directly inhibit Ceramide Synthases activity in human pulmonary artery endothelial cells [48]. Consequently FTY720 may at the same time target ceramide synthesis and sphingosine-1-phosphate signaling, which is downstream of ceramide catabolism. A contrasting but interesting report supports the hypothesis of a complex and fine regulation of ceramide signaling pools in which TNFα-induced ceramide in lung epithelial cells is necessary to downregulate IL-8 synthesis. In line with previously reported ceramide activation of the Protein Phosphatase 2A (PP2A) [49] and ceramide impairment of the binding of noncompetitive biological inhibitors of PP2A [50], Cornell et al. reported that TNFα-induced ceramide, in respiratory epithelial cells, activates PP2A, which is responsible for dephosphorylation/inactivation of MAPK pathways (JNK, p38, and ERK) and consequently for the inhibition of their downstream NF-kB promoter activity. This would explain the downmodulation of the IL-8 transcription. The authors showed that both desipramine (inhibitor of acid Sphingomyelinase) and fumonisin B1 pretreatments (for a few hours) block the initial ceramide increase upon TNFα receptor engagement, indicating a double pathway leading to ceramide accumulation from *de novo* synthesis and from the sphingomyelin cycle. This initial ceramide wave is, in their hypothesis, important in a feedback regulation of inflammatory signaling and its absence enhances IL-8 release [51]. These apparently contrasting data, suggesting a ceramide role of "inflammation homeostasis keeper," can be explained if we consider an overall picture of a physiological inflammatory setting versus chronic inflammatory pathology. Acute stimulation of early inflammatory inducers, such as TNFα, may trigger a controlled remodeling of sphingolipid mediators, during which ceramide can be driven into metabolic transformation such as the sphingomyelin cycle or the phosphorylation to ceramide-1-phosphate. The latter, involved in inflammation with contrasting evidence [52], was shown to mediate TNFα-induced IL-10 production, thus representing the missing link that

relates proinflammatory to anti-inflammatory/physiological inflammation resolution response [52, 53].

In this line of thinking, an initial ceramide-driven reaction to inflammation could be aimed at inflammation resolution, but it is conceivable that deregulation of ceramide metabolism enzymes, due to excessive stimuli and stress, can become an effector arm that easily pushes the whole inflammatory machine to collapse into pathological chronic inflammatory signaling.

4. Sphingolipid Metabolites Form a Network with Other Inflammatory Lipids in the Lung

PAF is a potent lipid mediator which is involved in asthma, sepsis, and acute lung injury, responsible for vasoconstriction, bronchoconstriction, vascular permeability, and pulmonary and extra-pulmonary edema formation. PAF-induced increase in vascular permeability is mediated by PGE2 and ceramide, derived from acid Sphingomyelinase activation, in endothelial cells [54, 55]. On the other side, PAF-induced activation/maturation of macrophages during inflammation was related to *de novo* ceramide synthesis induction [56]. Moreover, the Sphingomyelinase activation and ceramide accumulation promote Cyclooxygenase 2 expression and its release of PGE2 [57].

Being a major surfactant component, phosphatidyl choline is largely used in clinical therapy as a potent anti-inflammatory agent for intestinal mucosa protection [58, 59]. In airway epithelia cells, the interrelation of sphingolipids and glycerolipids metabolism was assessed by demonstrating that cytokine-induced catabolism of sphingomyelin, related to the inflammatory ceramide release, and the inhibition of the anti-inflammatory phosphatidyl choline synthesis are directly dependent on one another, suggesting that a complex program of lipid adjustment is targeted to initiate inflammatory response. TNFα-derived ceramide and sphingosine inhibit Phosphatidyl-Choline: Ceramide Phosphocholine Transferase (Sphingomyelin Synthase) [60, 61], blocking the "consumption" of phosphatidyl-choline to form sphingomyelin and diacylglycerol. At the same time, in H441 cells, TNFα-induced ceramide reduces phosphatidyl choline synthesis, probably because of its inhibition of CTP: Phosphocholine Cytidylyl-Transferase (CTT), which is the rate-limiting enzymatic step in *de novo* phosphatidyl choline synthesis [60, 61]. In this view, the inflammatory stimulus TNFα raises ceramide release, *via* Sphingomyelinase activation. Ceramide activates cytosolic PLA2, thus increasing lysophosphatidyl choline, which on the contrary is a proinflammatory mediator [62] and inhibits the phosphatidyl choline synthesis (CTT activity). The arachidonic acid, released by cPLA2 upon lysophosphatidyl choline formation, stimulates the synthesis of leukotrienes, which in turn raise intracellular Ca^{2+} levels and complete the activation of $cPLA_2$ [60], as well as lysosomal phospholipases [39] (Figure 1). Interestingly, the overexpression of Ceramide Synthase 5, the predominant Ceramide Synthases isoform detected in lung epithelia, also reduced phosphatidyl choline synthesis, but maximal inhibition was achieved when Ceramide Synthase 5 was coexpressed with a plasmid encoding a neutral Sphingomyelinase, enhancing sphingomyelin hydrolysis [63]. Thus, the modulation of sphingolipid metabolism drives the formation of glycerolipids inflammatory molecules as well as the eicosanoids family of inflammatory lipids. Altered surfactant is undoubtedly related to inflammatory stress within the lungs. Inflammatory cytokines regulate the alveolar pool of sphingomyelin. Sphingomyelin hydrolysis, induced upon inflammation, causes a twofold increase in the amount of surfactant-associated ceramide, tending to decrease the sphingomyelin mass, thus impairing the biophysical properties of the alveolar surfactant film [64]. Moreover, ceramide can interfere with surfactant production and release. All these lipids and surfactant components are secreted by type II pneumocytes by regulated exocytosis of secretory vesicles, termed lamellar bodies. The fusion of lamellar bodies with the plasma membrane is inhibited by treatment with the ceramide analogue C2-ceramide, which inhibits phospholipase D activity [65]. Moreover, in H441 airway epithelial cells, ceramide decreased SP-B surfactant production. This was shown to occur by a unique ability of ceramide to bind to a region located within the −233/−80 bp region of human SP-B promoter. Ceramide binding was shown to reduce the transactivation capability of thyroid transcription factor 1 (TTF-1/Nkx2.1), a key factor for SP-B promoter activity [66]. An overall view of sphingolipid interaction with other lipids and of their inflammatory and anti-inflammatory activities is provided in Figures 1(a) and 1(b).

5. Sphingolipid in Pulmonary Inflammatory Pathologies: Acute and Chronic Inflammation Are Sustained by Sphingolipid Mediators

Pathological inflammation in the lung involves surfactant and mucus production, epithelial cell reaction, endothelial permeability, immune response, parenchyma, and matrix damage. The major outputs are leukocyte infiltrate, releasing damaging molecules such as radicals and proteases; tissue edemas and fibrosis remodeling; small terminal airway damage (bronchiolar loss) with low oxygen-hypoxia induction; capillary damage and hypertension; necrosis and emphysema. In the following paragraphs we will review the involvement of sphingolipid mediators and the adjustments of sphingolipid metabolism in the setting of pulmonary inflammation, focusing only on acute lung injury and on two major chronic diseases, namely, cystic fibrosis and chronic and obstructive pulmonary disease.

5.1. Pulmonary Inflammation and Acute Respiratory Distress Syndrome (ARDS). Pulmonary inflammation, generally known as Adult Respiratory Distress Syndrome (ARDS), occurs in individuals who sustain systemic or localized insults (sepsis, aspiration of toxins, emboli, circulatory collapse, and metabolic neurological hematological disorders) that cause diffuse lung injury. Acute lung injury (ALI) is the most severe form. The major event is fluid leaking into the lungs from damaged capillaries (edema). Disappointing results from therapeutic approaches, targeting known involved mediators

such as TNFα, PAF, or PGEs, suggest that several parallel and interacting mechanisms are involved. Sphingolipid altered metabolism was shown to take part in this pathological process.

As early as 1985, Merrill and his collaborators noticed a significant decrease in sphingomyelin in the lung microsomal fraction of rats maintained in elevated versus normal oxygen levels [67]; later, alteration in oxygen supply (hyperoxia) was shown to be a potent cause of ceramide accumulation and of lung injury and inflammation [68–70]. This early evidence shed light on the hypothesis, largely validated later on, that sphingolipids are modulated during inflammatory processes relating to airway oxygenation and may actively take part in inflammation responses.

Besides surfactant phospholipids, bronchoalveolar lavage from ARDS patients contains a significant amount of ceramides and glycosphingolipids (lactosyl ceramides and paraglobosides), appearing during lung injury, that are present just in traces in healthy people [71]. The glycosphingolipids take part in damage because they are able to inhibit the surfactant system *in vitro* by increasing surfactant tension obtainable at minimum bubble size [71]. Ceramide derivatives are markedly elevated in bronchoalveolar lavage fluid of patients with ARDS [71] and plasma ceramide levels correlate with mortality [72].

Göggell and coworkers extensively analyzed the role of ceramide in lung edema, demonstrating that ceramide accumulation in lung tissue and fluids, upon acid Sphingomyelinase increased activity, is an important cause of edema formation [73]. Mice treated with PAF developed pulmonary edema which was reduced by ~50% in acid Sphingomyelinase deficient animals. A further decrease was obtained if deficient animals were treated with acetylsalicylic acid, indicating a common involvement of acid Sphingomyelinase and cyclooxygenase in the pathogenesis of pulmonary edema. Moreover, the perfusion of rat lungs with TNFα or PAF rapidly induced an increased ceramide concentration in the alveolar fluid, suggesting increased activity of extracellular acid Sphingomyelinases. Antisera against ceramide as well as the pharmacological inhibitors of the membrane cycle sphingomyelin/ceramide (xanthogenate D609 for Sphingomyelin Synthase and imipramine for acid Sphingomyelinase) prevented PAF-triggered pulmonary edema. Nonetheless, inhibition of ceramide release from the plasma membrane had no effect on other PAF actions, such as reduction in pulmonary vasoconstriction and bronchoconstriction. The authors exclude that ceramide may derive from a biosynthesis pathway because fumonisin B1, an inhibitor of Ceramide Synthase, had no effect on pulmonary edema. In a neonatal piglet model of airway inflammation, induced upon repeated lavages, surfactant plus desipramine administration prevented edema, inflammatory marker upregulation, leukocyte alveolar influx, and increase in ceramide content. Similar results were obtained in acid Sphingomyelinase deficient mice treated with surfactant, which were protected from inflammation unlike Sphingomyelinase-expressing control animals [74].

Acute pulmonary injury can be modeled by bleomycin-induced inflammation and fibrosis in mice. Such pathological consequences correlated with the rapid activation of acid Sphingomyelinase, and injury was markedly attenuated in the absence of the enzyme (knockout mice). Along with the elevated acid Sphingomyelinase activity, there was an increase in acid ceramidase activity, which was sustained for up to 14 days after bleomycin treatment, suggesting a possible bioactivity of the induced accumulation of sphingoid back-bone mediators such as sphingosine and sphingosine-1-phosphate. Consistently with this hypothesis, bleomycin treatment induced acid Sphingomyelinase and acid Ceramidase increased activity and accumulation of sphingosine-1-phosphate in NIH3T3 fibroblasts [75]. Nonetheless, the formation of sphingosine-1-phosphate was able to counteract the inflammation-induced endothelial permeability [76, 77] and pharmacological use of its analogues (FTY720, s-FTY720-phosphonate, and SEW2871) was proposed as a therapeutic approach in ALI [78].

Although these data suggest a key role for acid Sphingomyelinase activation and ceramide formation to cause edema, studies in human airway epithelial cells have shown that neutral Sphingomyelinase, but not acid Sphingomyelinase, is activated to induce cell death by inflammatory stress, such as cigarette smoke [28]. Hyperinflammation, such as LPS treatment, is associated with induced suppression of spontaneous neutrophil apoptosis, whose peculiarity is to provide for their own suppression, being short-lived differentiated cells. A massive and uncontrolled presence of neutrophils in the lung contributes to inflammation, thus entering a pathological vicious cycle. Lin and colleagues demonstrated significantly higher ceramide levels in alveolar neutrophils from ARDS patients than in those from healthy subjects, indicating an association between inflammatory granulocytes and increased ceramide level, specifically in the alveolar areas [79]. These authors showed that Sph-24 (neutral Sphingomyelinase inhibitor) and SKI-II (Sphingosine Kinase I inhibitor, triggering the enzyme lysosomal degradation [80]) antagonized the antiapoptotic effect of LPS [79]. Interestingly the acid Sphingomyelinase inhibitor CHL (inducing the enzymes lysosomal degradation [81]) had no effect on the regulation of neutrophil apoptosis in response to LPS stimulation.

Thus ceramide is one of the required triggers of inflammation, endothelial leaking, and edema in pulmonary injury; the acute inflammatory stress would cause a burst in sphingolipid catabolism, both at the acidic lysosomal compartment and at the plasma membrane neutral compartment, thus involving multiple inflammatory signaling. Sphingosine and sphingosine-1-phosphate accumulation may derive from lysosomal-related ceramide formation and possibly contribute to inflammation with diverse effects depending on the cell type and sphingosine-1-phosphate increase may impair neutrophils spontaneous apoptosis. The underlying idea, stemming from these reports and suggested by von Bismarck and colleagues, is that it may be possible to create a "fortified surfactant preparation," enriched in anti-inflammatory lipids and/or enzyme inhibitors (i.e., Sphingomyelinases inhibitors), with therapeutic activity against newborn lung inflammation.

5.2. Cystic Fibrosis. Cystic fibrosis (CF) is the most common life-threatening recessive genetic disease in the Caucasian population, affecting approximately 70,000 individuals worldwide, with median predicted life expectancy around the age of 40. This genetic disorder, caused by a mutation of the CF transmembrane conductance regulator (CFTR) gene, is mostly characterized by recurrent lower respiratory infections, exocrine pancreatic insufficiency (85% of patients), and increased electrolyte concentration in sweat. The CFTR gene encodes a member of the ATP-binding cassette transporter superfamily, involved in multidrug resistance. The encoded protein functions as a chloride/carbonate exchange channel, driving anions through different lipid-encased cellular compartments [82].

CFTR resides in many endosomal membranes, trafficking to the epithelial surface and back again; it is localized within lipid rafts and alters membrane lipid composition and in particular ceramide-driven membrane lipid rafts [83, 84]. CFTR mutation can be considered *per se* as a real cause of inflammatory disease and, even in sterile condition, CF fetuses exhibit atrophy or metaplasia and absence of villi. Moreover, the tracheal epithelium in infants and young children exhibits dilated airways with thicker epithelial walls [85, 86]. Human CF fetus allografts of lung small airways into mice with severe combined immunodeficiency (missing lymphocytes and NK cells) stimulate an immediate neutrophil over response and tissue damage, suggesting that innate immunity is hyperactivated even in the absence of infection [87].

It was reported that wild type CFTR, possibly acting as a scavenger, uptakes sphingosine-1-phosphate and the structurally related lipids dihydrosphingosine-1-phosphate and lysophosphatidic acid, thus modulating cell responses to these lipids. In the presence of CFTR, sphingosine-1-phosphate intake increases, thus leaving less ligand available for interaction with sphingosine-1-phosphate receptor and its signaling towards proliferation, migration, and angiogenesis. This would explain the abnormal angiogenesis, responsible for fibrosis, in CF disease, due to a higher availability of sphingosine-1-phosphate and to its stimulation of excessive angiogenesis in response to inflammation [88, 89].

CFTR inhibitors GlyH-101 and CFTRinh172 caused a dose-dependent increase in permeability of the pulmonary or bronchial endothelial monolayer. Increased endothelial sphingosine-1-phosphate, either by exogenous treatment or by endogenous inhibition of its degradation, significantly improved the barrier function in CFTR-inhibited monolayers [90].

In addition to the role of sphingosine-1-phosphate in CF, the most significant aspect of CFTR dysfunction, according to the published papers overall, is an imbalance of sphingolipid homeostasis, due to ceramide release from sphingomyelin within the membranes [91] and to a disease-related increase in ceramide synthesis [83, 92].

A considerable amount of evidence indicates that ceramide may be a pharmacological target in CF, since its accumulation significantly contributes to sustaining hyperinflammation and inability to fight lung infection. The first evidence of lipid and sphingolipid imbalance in CF was reported in the late seventies with the analysis of various lipids, including glycosylated ceramides, in the bronchial lavage and sputum of CF patients [93, 94]. Ceramides, mainly bearing C16:0, C18:0, and C20:0 acyl chains, were shown to accumulate progressively in the lower airway, as the disease advanced, in cystic fibrosis patients, compared with pulmonary hypertension and emphysema patients and healthy donors. Ceramide accumulation correlated with infiltrate presence (neutrophils) [95]. UIrich and coauthors demonstrated extensive inflammation and tissue remodeling in the alveolar tissues from CF patients with advanced lung disease, showing increased myofibroblasts, intercellular adhesion of molecule-1 and collagen expression, and decreased elastin fibers. In these patients alveolar type II cells were markedly stained with anti-ceramide antibodies, demonstrating a close association between inflammation ceramide accumulation and damage within the lower airways [96].

This evidence contrasts with Becker and coworkers' reported data, showing a decrease in ceramides and docosahexaenoic acid (DHA) and an increase in arachidonic acid (AA) content in CF plasma compared with healthy controls. The reduced levels of circulating ceramides, showed by the authors, may be ascribed to CF patients' low plasma HDL, LDL, and total cholesterol levels, due to malabsorption and altered liver function [97]. In these authors' hands, the administration of fenretinide to patients corrected ceramide and other lipids levels [98]. Fenretinide is a known chemotherapeutic agent that modulates sphingolipids by inhibiting a dehydrogenation reaction that forms ceramide, thus accumulating dihydroceramide. Data supporting a mechanism which could explain the plasma ceramide upregulation upon fenretinide treatment are missing. However, fenretinide has different intracellular targets leading to apoptosis that are not related to the inhibition of dihydroceramide desaturation and that may in turn cause ceramide release from apoptosis-related Sphingomyelinases activation [99].

A more thorough knowledge of sphingolipid signaling in CF is obtained from studies in animal models. It is worth noting that both circulating and pulmonary lipid analyses must be obtained only in normal diet fed CF animal models. These can be either low CFTR expressing or CFTR knockout but corrected for wild type CFTR expression in the gut only [83, 100]. In these models, most reports confirm the hypothesis that accumulation of ceramide in the lung promotes apoptosis, DNA deposition, and granulocyte hyperactivity, thus facilitating infection [91].

The alveolar epithelium comprises two main cell types: alveolar type I and alveolar type II cell. Type I cell is a complex branched cell with multiple cytoplasmic plates that are greatly attenuated and relatively devoid of organelles; these plates represent the gas exchange surface in the alveolus. On the other hand, type II cell responds to damage of vulnerable type I cell by dividing and acting as a progenitor cell for both type I and type II cells. In addition, it synthesizes, stores, and releases pulmonary surfactant into the alveolar hypophase, where it acts to optimize conditions for gas exchange [101]. Alveolar spaces are primarily involved in inflammatory

responses, being endowed with extended capillary distribution and resident phagocytes. Moreover, alveolar epithelial cells cooperate with alveolar macrophages in immune response and pathogen clearance, by expressing TLRs upon infection [102]. The greatest accumulation of ceramide in CF animals is in the lower airways and, specifically, in alveolar type II cells [96], although a marked accumulation of ceramide was also seen in tracheal and intestinal epithelial cells of low CFTR expressing (solid diet fed) mice [103]. The increased ceramide concentration in alveolar epithelia may significantly contribute to inflammatory signaling and impaired pathogen clearance exerted by these particular cells, addressing the lower airways as the specific therapeutic targets in chronic lung inflammation and infection (Caretti A. and Signorelli P. unpublished data).

Teichgräber et al.'s lab demonstrated an accumulation of ceramide located in intracellular vesicles (not restricted to lysosomes) in CFTR deficient respiratory epithelial cells. According to these authors, CFTR deficiency increases the pH of this vesicular compartment (around pH 6), causing an imbalance between the enzymatic activities of acid Sphingomyelinase and Ceramidase. While acid Sphingomyelinase activity was partially compromised by the pH variation, acid ceramidase activity was dramatically reduced by 90%. Such alteration of activities ensures a release of ceramide from sphingomyelin and then overcomes its clearance by degradation [91]. The same group, based on previously reported data [104, 105], claimed that CFTR deficiency increases the pH of secretory vesicles produced by alveolar macrophages upon *P. aeruginosa* infection, correlating with a reduced production of ROS and reduced bactericidal activity and with ceramide intracellular accumulation and altered lipid raft formation on the plasma membrane [106].

In support of the ion imbalance hypothesis in CFTR deficiency, Noe et al. suggested that increased pH and ceramide are responsible for aberrant angiogenesis, leading to fibrosis in CF disease. Impairment of physiological apoptotic response to stress in the endothelium may lead to abnormal angiogenesis and chronic inflammation. Ceramide is a key regulator of survival and apoptosis in endothelial cells. Oxidative stress, as from hydrogen peroxide, induces ceramide increase mainly via *de novo* sphingolipid synthesis in endothelial cells. Inhibition by CFTR(inh)-172 of endothelial cell channel activity prevented the increases in the ceramide: sphingosine-1-phosphate ratio induced by hydrogen peroxide, impairing caspase activation and apoptosis [107], thus promoting aberrant proliferation under stress and pathological angiogenesis. This evidence would suggest that CFTR deficiency modulates ceramide by altering the pH of intracellular compartments, leading to diverse outcomes according to the cell type.

In line with the hypothesis of an increase in ceramide due to a pH dependent inactivation of acid ceramidase; sphingosine presence on the surface of nasal epithelial cells from CF patients was shown to be almost undetectable (by means of an anti-sphingosine antibody), whereas it is abundantly expressed on the luminal surface of human nasal epithelial cells obtained from healthy individuals. Similar results were obtained in bronchial cells from CF mice. With the aim of demonstrating that sphingosine deficiency may favor bacterial colonization, the inhalation of purified acid ceramidase or sphingosine or its analogue FTY720 was obtained in pulmonary infected CF mice. The treatment not only corrected the anomalous absence of mucosal sphingosine in CF murine airways but also significantly reduced increased ceramide levels and allowed an effective response against lung colonization by *P. aeruginosa*. These results were obtained by treating either prior to or after infection, indicating this therapy as preventive as well as curative against bacterial infection [108]. Although the idea of a reservoir of mucosal sphingosine as a toxic compound, to be spent against pathogens invasion, is attractive, the hypotheses put forward by these authors will certainly require further studies for validation. A major issue that needs to be cleared up, in our opinion, is as follows: given that sphingosine is a toxic compound for eukaryotic cells, how is it possible to obtain beneficial effects in terms of killing bacteria by exogenous administration, avoiding damage to the host?

Although this "pH theory" would fit the above reported published data, by means of CFTR overexpression systems and loss-of-function studies, other investigators reported that neither the secretory (Golgi and TGN) nor the endocytic organelles (endosomes, lysosomes, and phagosomes) display a CFTR-dependent acidification defect (revised in [109]), questioning the technical methods previously used to measure intracellular pH [110]. These latter studies clearly show that CFTR-independent and overall counter-ion permeability was remarkably higher than the passive proton permeability of endosomes, lysosomes, and phagolysosomes of respiratory epithelia and primary or immortalized mouse macrophages. Therefore, CFTR activation cannot interfere with the endosomal pH regulation [109] and ceramide modulation should derive from mechanisms other than pH deregulation of Ceramidase.

In line with the observations arising from their patients' studies, Teichgräber and his coworkers investigated the hypothesis of a hyperactivation of acid Sphingomyelinase in CF animal models. Although the baseline activities of acid Sphingomyelinase and acid Ceramidase in lung homogenates from CFTR deficient animals are equivalent to those in control mice [91], they achieved partial inhibition of the activity of acid Sphingomyelinase either by intraperitoneal injection of amitriptyline or by heterozygous deficiency for acid Sphingomyelinase and observed reduced airway inflammation, phagocyte recruitment, and susceptibility to infection by *Pseudomonas aeruginosa* [91, 111].

Other authors confirm the long term efficacy of amitriptyline to inhibit the robust burst of inflammatory response in CFTR deficient (gut corrected) mice subjected to *Pa*-LPS-induced acute lung injury. In such a model, the authors also demonstrated an early anti-inflammatory activity of fumonisin B1, which is lost at a longer time. Although not explained, the early anti-inflammatory effect of this last inhibitor can be related to its ability to activate Sphingosine Kinase-1 [43, 112, 113] and, therefore, to the upregulation of sphingosine-1-phosphate levels. Moreover we believe that fumonisin B1 can also reduce a burst of ceramide arising from *de novo* synthesis activation in

inflammation [92], which in turn sustains the activation of Sphingomyelinase(s) [44]. Nonetheless, the inhibition of Ceramide Synthase by fumonisin B1 is associated with the inevitable mounting, over time, of toxic sphingoid bases. The bioactivity of these metabolites may later on overcome the initial anti-inflammatory action of the inhibitor [114].

CFTR deficiency in mice results in the upregulation of CD95, crucially involved in aseptic inflammation, bronchial cell death rate, and susceptibility to infection. CD95 is activated upon increase of membrane ceramide concentration; CD95 also contributes to stimulating further ceramide release, possibly concurring in the inflammatory pathology of CF [97].

Whereas CF mice exhibit upregulation of inflammasome components and an altered presence of tight junction proteins in lung epithelia, knocking out (heterozygous gene deletion) acid Sphingomyelinase in the same CF mice model resulted in animals similar to the healthy control mice [115]. In addition, CF mice exhibit increased rates of cell death, increased cytokines concentration, and ceramide levels in the trachea and intestine. The inhibition of acid Sphingomyelinase activity ensured the concomitant normalization of cell death, inflammatory cytokines, and ceramide concentration in these body districts [103]. These findings suggest that ceramide plays a crucial role in inflammation and in increased rates of cell death in several organs of cystic fibrosis mice.

The "take home message" derived from the above reported findings is that ceramide and/or its metabolite sphingosine play a crucial role in inflammation and increased rates of cell death in several organs of cystic fibrosis mice [103]. The encouraging results led to the initiation of clinical trials with amitriptyline: the drug was administered to patients 25–50 mg/d twice daily for 28 days. Increased FEV1 and reduced ceramide in nasal and lung cells were observed, with no evident sign of toxicity for the patients [116, 117].

Although ceramide accumulation in CF is clearly established, in our opinion further studies are required in order to consider amitriptyline as a pharmacological tool in CF. The mechanism-based hypothesis of pH induced Ceramidase inactivation and acid Sphingomyelinase hyperactivity is questionable (as above explained) and, to the best of our knowledge, there are no data demonstrating either a transcriptional or a posttranscriptional control of this enzyme *in vivo* in CF or CF models. Data obtained from acid Sphingomyelinase deficient mice are never accompanied by an overview on the sphingolipid metabolism rearrangement in this model nor are they characterized for any possible alteration in their immune responses. The reported quantitations of ceramide increase in CF mice versus wild type are also puzzling, with an approximately 8-fold increase when measured by DAG Kinase total ceramide phosphorylation and Thin Layer Chromatography separation [111, 115], compared with mass spectrometry analytical methods (around 20–30% increase) [92, 100]. Prolonged treatment with amitriptyline (6 months) was performed on CF mice, monitoring the reduced level of ceramide, but there are no data on the response to infection and on the overall immune response of the treated animals [111]. Finally, the requirement for acid Sphingomyelinase activation in macrophages and immune cells (i.e., ROS production and bactericidal activity [106]), to give rise to proper signaling during infection, leaves an open question as to the efficacy of this treatment in patients with chronic infections.

An alternative explanation of sphingolipid metabolite imbalance in CF is derived from studies conducted by Wargall's group and our own. It relies on the hypothesis that CFTR deficiency induces intracellular stress leading to an upregulation of the rate-limiting step of sphingolipid *de novo* synthesis, with consequent accumulation of ceramide. Since an intracellular increase of ceramide stimulates Sphingomyelinases (as effectively as TNFα), thereby amplifying the Sphingomyelinase activation [44], this mechanism may also explain the effect of amitriptyline administration in CF models. By means of radioactive precursor treatment (^{3}H serine and ^{3}H sphinganine), elegant metabolism studies revealed that sphingolipid synthesis is significantly enhanced in IB3 CF epithelial cells compared to normal C38 cells: increased rate of radioactivity incorporation was assessed, for *de novo* as well as the recycle path of ceramide synthesis and for sphingomyelin synthesis. Moreover, a markedly enhanced expression of Serine Palmitoyl Transferase 1 subunit was found in CF cells, and its expression inversely correlates with CFTR expression in airway epithelial cells. The mass of C16-dihydroceramide and C22- and C24-ceramide species increased compared with controls, whereas C18-ceramide and C18:1-ceramides mass decreased [83]. Similar results were obtained by our group comparing the same cell lines under inflammatory stimulation: we concluded that TNFα was able to significantly enhance Serine Palmitoyl Transferase 1 transcript in IB3 cells and that inflammatory cytokines transcription and release, induced by TNFα, were impaired by treating cells with myriocin [92], the inhibitor of Serine Palmitoyl Transferase (the rate-limiting enzyme in the sphingolipid *de novo* synthesis pathway [13]). Next, we demonstrated that the response against *P. aeruginosa* acute lung infection in CF mice is ameliorated by intratracheal treatment with myriocin and that both ceramide and inflammatory mediators pulmonary levels of these mice were corrected to those of wild type mice [92].

Although enhanced sphingolipid synthesis in CF was shown to induce ceramide and sphingomyelin mass increase, the CFTR silenced human airway epithelial cell line exhibited ~60% lower GM1 ganglioside than control cells and was unable to migrate, showing impaired activation of β1-integrin, phosphorylation of focal adhesion kinase, and Crk-associated substrate. Exogenously added GM1 partially restored migration of CFTR silenced cells [118]. This deficiency in gangliosides may be related to dysregulated intracellular trafficking of neosynthesized ceramide and may account for reduced antibacterial responses, since gangliosides are involved in bacteria interaction (as previously mentioned). It should be noted that the loss of CFTR function leads to altered cholesterol trafficking, resulting in increased cholesterol synthesis. Excessive cholesterol causes pathological conditions such as atherosclerosis. It is recognized that sterols can modulate the levels of other lipids to attain lipid homeostasis;

thus, excess free cholesterol may play a role in modulating compensatory sphingolipid pathways [119] and it is suggested that this perturbation in cholesterol regulation contributes to the inflammatory response present in CF [120].

The overall data suggest that decreased CFTR expression would reflect a state in which augmented membrane lipid synthesis, including sphingolipids, is necessary to compensate the dysfunction and maximally increase membrane stability. This hyperanabolism state either associates with or possibly promotes hyperinflammation, with ceramide being a major signaling mediator. This concept is further sustained in the following paragraph, dealing with sphingolipids involvement in COPD pulmonary inflammation [83].

The evidence discussed above is summarized in Figure 2.

5.3. Chronic Obstructive Pulmonary Disease. Chronic obstructive pulmonary disease (COPD) is an inflammatory respiratory disease, estimated to become the third leading cause of death worldwide by 2020, after ischemic heart disease and cerebrovascular disease [121, 122]. The Global Initiative for Chronic Obstructive Lung Disease (GOLD) defines COPD as follows: COPD, a common preventable and treatable disease, is characterized by airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases. Exacerbations and comorbidities contribute to the overall severity in individual patients (global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: revised 2014; Global Initiative for Chronic Obstructive Lung Disease (GOLD), available online: http://www.goldcopd.org/). Salvi and Barnes, from population-based studies, proposed that cigarette smoke is a risk factor only for approximately half of COPD patients, and alternative genetic and epigenetic environmental risk factors are clearly implicated in the disease's etiology. The triggering causes of COPD include indoor and outdoor air pollution (i.e., biomass fuel, dust, and fumes [123]). A central role in the pathophysiology of COPD has been shown to be played by chronic inflammation of the airways [124]. Although cause related, the inflammation process characterizing COPD persists long after cessation of stress (i.e., quit smoking) and relies on cells and mediators ranging from innate to adaptive immunity, ROS overproduction and induced damage, imbalance of local proteolysis/antiproteolysis, fibrosis, and altered angiogenesis. The outcome is a thickened (and also dysfunctional) epithelial layer, increased thickness of the smooth muscle layer of the airway, disruption of the alveolar walls with varying levels of fibrosis, hypoxic vasoconstriction [125], the narrowing and progressive loss of terminal bronchioles and increased peripheral airway resistance, and arterial hypertension [126]: all these features precede emphysematous demise of the COPD lung structure [127]. Depending on the patients (age, susceptibility to infection, etc.), hyperinflammation is conducive to an increase in the number and size of mucus-secreting glands. Such condition, named mucus metaplasia, causes mucus hypersecretion, increased mucin stores in the epithelium according to airflow limitations, and increased luminal mucus obstructing the airways and evolves into chronic bronchitis [128]. Thus chronic bronchitis and emphysema often overlap in COPD patients, with the burden of one or the other prevailing. Susceptibility to exacerbations is defined by background inflammation in the lung tissue, microbiota equilibrium, and comorbidities. Individual immune responsive ability and autoimmunity have been indicated as possible initiators of the pathologic inflammation of COPD [129]. Up to the 1960s, the imbalance between proteases and antiproteases induced during inflammation was ascribed as the major mechanism initiating emphysema and COPD pathogenesis. In the last decade, however, the collapsing of the alveolar structure was explained in terms of apoptosis of epithelial and endothelial cells and of excessive inflammatory stress. Interestingly, autophagy was demonstrated to be significantly impaired, with marked accumulation of p62-enriched vesicles in the epithelia of COPD patients, accounting for accumulation of damaged material and unbalanced homeostasis between degradation and resynthesis [130, 131].

In line with this evidence, metabolic changes responsible for proinflammatory metabolites accumulation can be considered as one of the triggering causes concurring in COPD etiology: specifically, sphingolipid mediators contribute to the inflammatory process, driving the onset and progression of the pathology.

In 2005, Petrache and colleagues induced emphysema in mouse lung by subcutaneous treatment with the VEGF receptor 1 and receptor 2 inhibitor, SU5416. They demonstrated that apoptosis was mediated by ceramide, whose accumulation was localized at the alveolar septal cells and not in bronchi, colocalized with caspase-3 activation and most importantly preceded (by about twenty days) alveolar enlarging and damage. The accumulation of ceramide depended on its *de novo* synthesis and the authors were able to prevent VEGFRs inhibitor-induced damage by systemic administration of fumonisin B1 or myriocin. Indeed, a later activation of acid Sphingomyelinase was found in its secreted form, released as a feed-forward mechanism in response to enhanced sphingolipids synthesis, thus increasing the pool of paracellular ceramide and amplifying lung inflammation and injury. Anti-ceramide antibody i.p. administration was partially able to neutralize extracellular ceramides and attenuated lung apoptosis, induced by the VEGFR inhibitor. To better prove that the altered sphingolipid metabolism severely affects lung physiology, they administered fumonisin B1 to untreated normal mice and observed alveolar enlargement and damage, which was inhibited by replenishment of sphingoid bases (sphingosine-1-phosphate and FTY720 combinatorial treatment, with the aim of reintroducing sphingoid bases but simultaneously downregulating the sphingosine-1-phosphate receptor 1 signaling). FTY720 and exogenous sphingosine exert a protective effect on airspace enlargement, concomitant with attenuation of VEGFR inhibitor-induced lung apoptosis, possibly by decreasing the ceramide/sphingosine-1-phosphate ratio (by FTY720 inhibition of Ceramide Synthesis and induction of Sphingosine Kinase, resp.) [43]. Moreover, intratracheal instillation of synthetic ceramide analogue C12 was able to induce septal apoptosis and emphysema, by triggering the

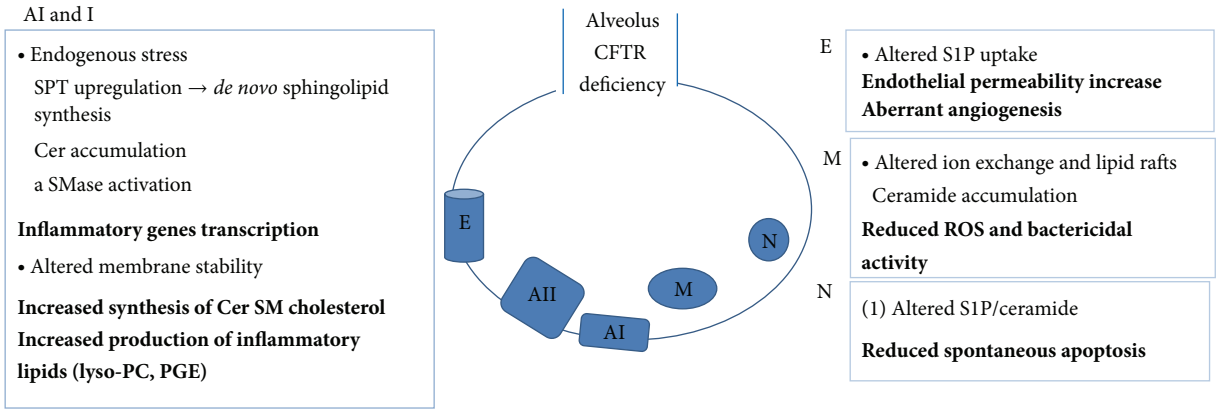

Figure 2: Sphingolipids modulation and bioactivity in cystic fibrosis: prevalent sphingolipids alteration in the alveolar lung compartment and induced responses in endothelial cells (E), alveolar epithelial cells (alveolar type 1 AI, alveolar type 2, AII), macrophages (M), and neutrophils (N). SPT, Serine Palmitoyl Transferase; Cer, ceramide; a SMase, acid Sphingomyelinase; SM, sphingomyelin; lyso-PC, lysophosphatidyl choline; PGE, prostaglandin; S1P, sphingosine-1-phosphate; ROS, reactive oxygen species.

synthesis of long-chain endogenous ceramide, which accumulated twofold [44]. As alveolar cell apoptosis and oxidative stress mutually interact to mediate alveolar destruction [39], the same research group demonstrated, shortly afterwards, that intratracheal ceramide instillation decreased cytosolic SOD activity and increased superoxide production in the lungs. They also demonstrated that overexpressing human Cu/Zn SOD in mice significantly protected from intratracheal ceramide-induced superoxide production, apoptosis, and air space enlargement. It is to be noted that the lung activation of acid Sphingomyelinase, in response to ceramide treatment, was abolished when overexpressing SOD. Such evidence demonstrates that exogenous ceramide treatment and the consequently stimulated endogenous neosynthesized ceramide merge in ROS formation and acid Sphingomyelinase activation. This latter activity amplifies injury through redox-dependent mechanism. Both ROS accumulation and acid Sphingomyelinase derived ceramide are the major effectors of the damage, leading to the conclusion that enhanced neosynthesis of lung ceramides is upstream of redox-dependent inflammatory damages [132]. Knowing that cigarette smoke induces oxidative stress and is one of the causes of emphysema, mass spectrometry analysis proved that cigarette smoke is able to induce ceramide accumulation in the murine lung [132]. Accordingly, lung ceramide levels were markedly higher in patients with emphysema due to chronic cigarette smoking compared with patients without emphysema, as measured by Diacylglycerol Kinase assay, followed by Thin Layer Chromatography and ceramide quantitation. Immunohistochemistry revealed that ceramide was almost exclusively localized to alveolar septal cells and alveolar macrophages. Human emphysematous lungs exhibit a significant increase in long-chain ceramides. Even smoking alone (without a pathological diagnosis of emphysema) changed the lung ceramide expression profile, suggesting that not only absolute levels but also patterns of ceramide species expression may be upregulated and contribute to emphysema induction. These studies highlight the concept that ceramide accumulates in specific lung compartment and there induces damage in epithelial cells, which in turn supports inflammation in a vicious loop. Ceramide accumulation results from deregulation of sphingolipid metabolism. The accumulation of newly synthesized sphingolipids (bearing long acyl chains) leads to the extracellular release of ceramide(s). In addition, an increased synthesis of catabolism enzymes, such as Sphingomyelinases, is required in response to the sphingolipids accumulation, and eventually the extracellular release of these enzymes occurs too. These events are linked to prolonged extracellular inflammatory/damaging signaling [44]. Levy and colleagues substantiated the hypothesis that cigarette smoke induces the activation of neutral Sphingomyelinase 2 and apoptosis in human respiratory epithelial cells and glutathione administration was able to inhibit apoptosis [133]. Mice exposed to cigarette smoke exhibit a twofold increase in lung ceramide (measured by Diacylglycerol Kinase assay) as well as a significant increase in neutral Sphingomyelinase expression (evaluated by immune histochemical staining of the protein with noncommercial antibodies), both in the bronchial epithelium and in alveolar septal cells. Diet supplementation with N-acetyl-cysteine (glutathione precursor), or intranasal installation of biotin-labeled neutral Sphingomyelinase 2 siRNA, showed a significant reduction in this enzyme staining and of lung apoptosis, measured both by TUNEL assay and by evaluation of cleaved caspase-3 levels. Immunostaining for neutral Sphingomyelinase 2 on lung biopsies from emphysematous smoker patients versus healthy people showed an enhanced expression of the enzyme [134]. Finally, the oncogene product Src Kinase was shown to activate the serine kinase p38, responsible for phosphorylation and activation of neutral Sphingomyelinase 2, upon oxidative stress [135].

The catabolism of ceramide gives rise not only to sphingosine but also to the opposing bioactive sphingosine-1-phosphate, known to promote proliferation and survival, even though in very low intra- or extracellular concentration [43]. Systemic administration of sphingosine, via daily

intraperitoneal injection, increased sphingosine-1-phosphate levels in a dose-dependent manner. In the presence of VEGFR blockade, stimulation of sphingosine-1-phosphate activity, by either administering its precursor D-erythro-sphingosine or the agonist of its receptor 1, SEW2871, led to a decrease in the ceramide to sphingosine-1-phosphate ratio and markedly reduced apoptosis and lung injury [43].

Sphingosine-1-phosphate receptors 1–3 (S1PRs) are involved in the control of pulmonary vascular function by altering endothelial and epithelial barriers as well as smooth muscle cell function [48, 136, 137]. In mice, S1PR3 receptor activation induced pulmonary edema by opening narrow junctions between alveolar type I and type II cells [137], and S1PR2 deficient mice and mice with reduced S1P3 receptor expression were protected against LPS-induced disruption of the alveolar barrier [136, 137]. A recent report, analyzing the expression of the genes related to sphingosine-1-phosphate synthesis/degradation and of its receptors in human lung, showed that relative mRNA expression of S1PR5 was significantly reduced in COPD patients compared with control [138]. A possible scenario in which different S1PRs are differentially modulated in the airways to promote inflammation can be envisaged from these reports.

Environmental stresses, including cigarette smoke and hypoxia, and chronic inflammation have recently been shown to reduce CFTR function, and this suggests that common mechanisms contribute to the progression of both CF and COPD [114]. Immune histochemical analysis of tissue sections reveals that CFTR expression is inversely correlated with the severity of emphysema and with ceramide accumulation in COPD subjects, compared with control subjects [84]. Bodas and colleagues also demonstrated that acute exposure to cigarette smoke induces a significant downregulation of CFTR in lung and in particular in membrane rafts. From antibody staining, they claim that the absence of CFTR in rafts induces the accumulation of ceramides within the membrane, possibly as a compensatory mechanism for the altered stability of the membrane [84]. Although it is risky to draw conclusions on ceramide levels from quantitation obtained by staining with anti-ceramide antibodies, these data are in line with other published results, which suggest that deficiency of CFTR switches on an "alarm" mechanism that leads the cell to modulate the synthesis of membrane components [83]. In this view, an upregulation of sphingolipid synthesis not only fulfills the requirement for supporting raft anomalies but also triggers an alarm signal that evolves toward a chronic inflammatory state.

Treatment with CFTR inhibitors GlyH-101 and CFTR(inh)-172 caused a dose-dependent increase in human and rat pulmonary endothelial monolayer permeability, redistribution of the junctional protein β-catenin, and scattered actin stress fiber formation. Endothelial cells treated with exogenous sphingosine-1-phosphate or with endogenous sphingosine-1-phosphate lyase inhibitor exhibited a significantly smaller decrease in permeability in response to CFTR inhibition. In this model, exposure to cigarette smoke markedly enhanced the loss of endothelial barrier function [90].

To the degree that the catabolism of ceramide gives rise to sphingoid bases and these become phosphorylated to produce sphingosine-1-phosphate, the metabolism of ceramide via direct phosphorylation forms ceramide-1-phosphate, another metabolite opposing ceramide and signaling towards proliferation and survival. In a recent publication, ceramide-1-phosphate (bearing either the short chain analogous C8 acyl chain or the C16 natural acyl chain) was administrated intratrachea. The administration occurred after acute cigarette smoke inflammation or it lasted for the last three months, overlapping with the last part of a seven-month cigarette smoke treatment (long treatment to model chronic inflammation). Ceramide-1-phosphate significantly reduced inflammation (cytokines lung expression and BAL infiltrate) as well as alleviating lung emphysema occurring after chronic stress [52]. Ceramide-1-phosphate effects were recapitulated *in vitro*, blocking cigarette smoke induced hyperinflammation in human airway epithelial cells and neutrophils from COPD patients [52].

It is intriguing to note that the above reported data suggest that ceramide-1-phosphate and sphingosine-1-phosphate, known to modulate inflammation with different mechanisms and outcomes, may be applied in pulmonary chronic inflammatory disease with a mere anti-inflammatory action. Our hypothesis is that, in stress conditions such as chronic inflammation, the ER major and ubiquitous cellular sensors stimulate *de novo* synthesis of ceramides as an intrinsic response to the stress, affecting the whole network of the sphingolipid metabolism, leading to membrane reorganization and signaling modulation. In order to balance the increased signaling of ceramide, its opposite metabolites ceramide-1-phosphate and sphingosine-1-phosphate, even if present at lower concentrations than the ceramide, may be able to counteract ceramide-induced outcomes. Thus the bioactivity of ceramide should rather be considered not *per se* but as a ceramide/ceramide-1-phosphate or ceramide/sphingosine-1-phosphate ratio. It is also necessary to take into account the possibility of ceramide-forming sphingosine, which can be considered as proinflammatory "lyso-ceramides," in opposition to forming sphingomyelin and glycosphingolipids. This complex picture might be envisaged for therapeutic approaches, although requiring much more thorough translational studies.

Lung chronic disease is to be considered as a systemic inflammatory disease and medical research focuses on the identification of early new markers in blood and body fluids [139, 140]. A massive presence of neutrophilic infiltrate along with increased inflammatory mediators signaling (i.e., IL-6) has been detected in the sputum of patients with COPD [141] as well as in bronchoalveolar lavage fluid (BALF) [142]. Bahr and colleagues studied the peripheral blood mononuclear cells expression profile in 136 subjects of the COPD gene cohort. Among others they found an overexpression of acid ceramidase in COPD and emphysema patients but not in those with chronic bronchitis. Looking for metabolites, to validate the gene expression results, they found an accumulation of lactosyl ceramide. The authors did not clearly explain the link between the enzyme and the metabolite [143]; anyhow these evidences confirm the spread

of inflammation. One year later, the analysis of sputum samples demonstrated that there was an increase in different sphingolipids species (ceramides, sphingomyelin, and gangliosides) in smoking compared with nonsmoking COPD patients. Specifically, ceramide concentration is inversely related to the severity of the disease. A part of these sphingolipids was significantly reduced upon cessation of smoking [144]. A hypothesis for these apparently contrasting data was launched in a recent large-scale study of more than 250 subjects from the COPD gene cohort, demonstrating that plasma sphingomyelin, ceramide, ganglioside GM3, monohexosyl ceramide, and sphingosine-1-phosphate levels inversely correlated with COPD worsening phenotype (low FEV1) and emphysema, whereas trihexosylceramides correlated directly with exacerbations. In agreement with previous data from peripheral blood mononuclear cells, no correlation with chronic bronchitis phenotype was traced. Plasma sphingolipids may derive from shed cellular plasma membrane, apoptotic bodies, microvesicles, or exosomes. The decreased levels of sphingomyelin in plasma were ascribed to increased catabolism activities by enhanced/activated plasma secreted acid Sphingomyelinase and neutral Ceramidase [74, 145]. These data suggest that, in severe COPD and its exacerbations, plasma accumulation of sphingolipid catabolites and related secreted enzymes possibly contributes to systemic inflammation [146].

An impairment of apoptotic cell phagocytosis in emphysema lungs, leading to secondary necrosis and promoting inflammation, has been reported [147, 148]. Being a regulator of autophagy, ceramide lung enrichment may interfere with autophagosomes clearance. Intratracheal instillation of ceramide (PEG-C16-ceramide) after intrapulmonary introduction of apoptotic (PI-targeted) human thymocytes significantly decreased the phagocytic uptake of the exogenous cells by alveolar macrophages, recovered from BAL [149]. The authors conclude that, upon occurrence of inflammatory events, such as the exogenous ceramide instillation, endogenous ceramide accumulates and gives rise to sphingoid bases that are primarily responsible for phagocytosis inactivation: alveolar macrophages recovered their phagocytic ability, impaired by cigarette smoke extracts, when pretreated with myriocin (to inhibit *de novo* synthesis of sphingolipids) or MAPP or siRNA to inhibit acid ceramidase but not with fumonisin B1 (to inhibit sphingosine acylation) nor Sphingomyelinase inhibitors [149]. Moreover, the sphingosine intracellular increase caused by exposure to cigarette smoke was responsible for an altered lipid raft assembling, impairing the presence in the membrane of Rac1 GTPases, which promotes membrane ruffling and phagosome closure [149].

Emphysema is caused by alveolar structure demise. This destructive process particularly targets lung microvascular endothelial cells and alveolar epithelial cells. Besides apoptosis, aberrant lung endothelial cell responses may contribute to pulmonary vascular remodeling, frequently observed in COPD in response to the low oxygen level caused by terminal bronchiolar loss, favoring endothelia permeability and inflammation [150, 151]. Petrache's group conducted an interesting study using primary human lung microvascular endothelial cells derived from smokers or nonsmoking healthy subjects. Following the hypothesis that ceramide synthesis is increased upon exposure to smoke and that this ceramide is released and acts paracellularly, they treated endothelial cells with palmitoyl ceramide (Cer16). While cells from nonsmokers responded with apoptosis, the smoker-derived cells were found to be resistant to exogenous ceramide and exhibited marked proliferation and increased autophagy. This involved a baseline-increased phosphorylation of Akt (a known prosurvival kinase, inhibited by ceramide and possibly enhanced as a feed-forward mechanism in an environment with baseline-increased ceramide) and increased levels of high-mobility group box 1 (an inflammatory protein, elevated in sputum and plasma of COPD patients and directly involved in vasculature remodeling in emphysema [150]). The authors hypothesize that deregulated sphingolipid metabolism and increased endogenous sphingosine, arising from increased ceramide production, may contribute to inflammation by promoting the expression and the activity of high-mobility group box 1 and aberrant vasculogenesis.

A correlation between lactosyl ceramide accumulation and increasing severity of emphysema was found in lung tissues from COPD subjects and in cigarette smoke treated mice lungs. The increase in this sphingolipid was impaired by pharmacological inhibition of lactosyl Ceramide Synthase. Moreover, in bronchial-epithelial cells (BEAS2B) and macrophages (Raw264.7), lactosyl Ceramide Synthase inhibitors impaired aberrant-autophagy (p62-accumulation) and apoptosis induced by cigarette smoke extracts [152].

Overall, the evidence cited above (summarized in Figure 3) traces an important role for ceramide synthesis and its derived metabolites in sustaining chronic lung inflammation.

6. Conclusions

Sphingolipid molecules play an active part in the extracellular equilibrium of proinflammatory and anti-inflammatory lipids which finely regulate mucosal activities and immunity in the lungs. In addition to being structural membrane components and endogenous mediators, sphingolipids exert an additional paracrine signaling role which is required for physiological immune response, defense from pathogens, and inflammation resolution. Deregulation of sphingolipid mediator homeostasis alters the development of the fetal lung and impairs its maturation and functionality. In adult life, deregulation of sphingolipid mediators is associated with a derangement of the inflammatory cascade. As outlined above, a major distinction can be traced between the roles of sphingolipids in acute and chronic lung inflammation. Acute inflammation is triggered by a robust and sudden stress, which sets off the rapid formation of alarm signals. The hydrolysis of membrane sphingomyelin is primarily involved in this external incident-intracellular response setting. Local ceramide increase produced by Sphingomyelinases activates different pathways cooperating in the activation of immune defense, cell stress response, and eventually apoptosis in case of cell failure. Chronic inflammation, on the other hand, is *per se* the cause of a prolonged hidden and borderline stress,

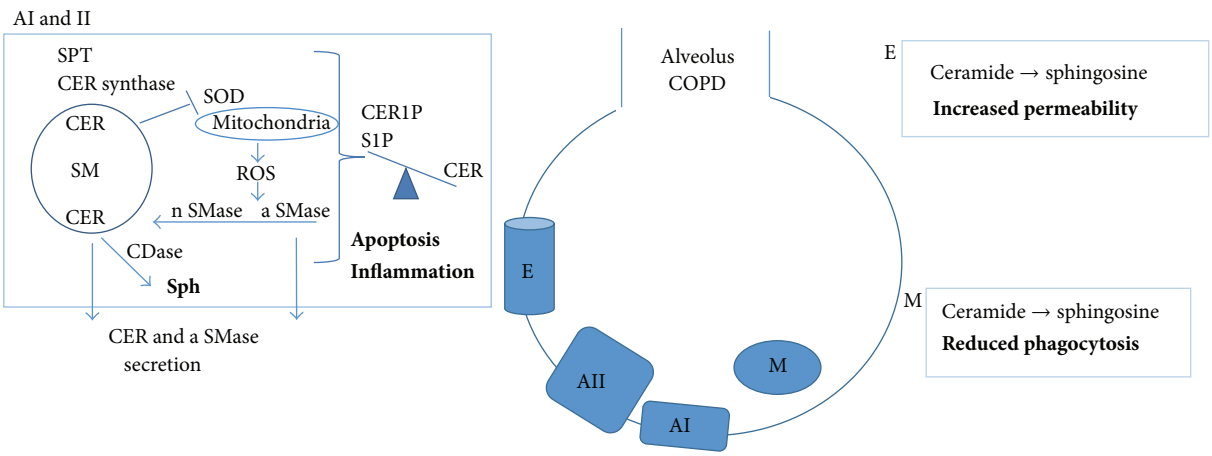

Figure 3: Sphingolipids modulation and bioactivity in COPD: prevalent sphingolipids alteration in the alveolar lung compartment and induced responses in endothelial cells (E), alveolar epithelial cells (alveolar type 1 AI, alveolar type 2, AII), and macrophages (M). SPT, Serine Palmitoyl Transferase; Cer, ceramide; SM, sphingomyelin; Sph, sphingosine; a SMase, acid Sphingomyelinase; n SMase, neutral Sphingomyelinase; CDase, Ceramidase; S1P, sphingosine-1-phosphate; Cer1P, ceramide-1-phosphate; ROS, reactive oxygen species.

which alters cell features and eventually tissue physiology and function. In this scenario, as a consequence of either a genetic defect or environmental toxic factors such as pollutants, the whole sphingolipid metabolic network undergoes a stable shift requiring transcriptional regulation. An overall increase in sphingolipid synthesis, coordinated with that of other lipids devoted to forming surfactant, reflects an attempt to enhance mucosal protection. This is a fragile equilibrium that may be compromised if an additional external insult, such as an infection, supervenes; the equilibrium can then collapse into a self-sustaining hyperinflammatory reaction, and the upregulation of the metabolic rate itself turns out to be a pathogenetic rather than protective adjustment. Insofar as this picture is correct, the goal of future translational research must be to obtain a preventive, disease-personalized medicine, targeting the alteration of metabolism induced by the disease. Within the lung, sphingolipid metabolism may constitute a key target because sphingolipid synthesis and ceramide levels seem to control the synthesis of other lipid mediators involved in inflammation, and ceramide modulatory agents have a profound biological effect in processes such as epithelial apoptosis, endothelial permeability and immune cells mobilization, mucus production, and pathogen clearance.

Abbreviations

PG: Prostaglandin
PAF: Platelet activating factor
TNF: Tumor necrosis factor
ROS: Reactive oxygen species
NO: Nitric oxide.

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

Sphingolipids as Regulators of the Phagocytic Response to Fungal Infections

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Fungal infections pose a significant risk for the increasing population of individuals who are immunocompromised. Phagocytes play an important role in immune defense against fungal pathogens, but the interactions between host and fungi are still not well understood. Sphingolipids have been shown to play an important role in many cell functions, including the function of phagocytes. In this review, we discuss major findings that relate to the importance of sphingolipids in macrophage and neutrophil function and the role of macrophages and neutrophils in the most common types of fungal infections, as well as studies that have linked these three concepts to show the importance of sphingolipid signaling in immune response to fungal infections.

1. Introduction

Beginning in the 20th century, fungi have emerged as important human pathogens. Increases in the population of immunocompromised individuals, due to AIDS or medical interventions, have allowed for invasive fungal infections to take hold in the human population worldwide [1]. Although much work remains to be done in understanding interactions between host and invasive fungi, it is well established that phagocytes serve a central role in the immune response to fungal pathogens [2]. Phagocytes, such as macrophages and neutrophils, are essential effector cells of the innate immune system and are responsible for recognition and killing of fungal pathogens [2–6]. Recent published work has revealed a role for a class of bioactive signaling lipids, known as sphingolipids, in regulating the antimicrobial activity of host phagocytic cells [7–11]. This review will center on the involvement of host sphingolipids in macrophage and neutrophil function during fungal infection. For general reviews on innate antifungal immunity, the reader is referred to [2, 5, 6]. For reviews on microbial sphingolipids in pathogenesis, the reader is referred to [12, 13].

2. Invasive Fungal Infections

Unlike bacteria and viruses, systemic fungal diseases were not described until the late 19th century and were considered to be extremely rare. Today, fungal infections are on the rise and there is a pressing need for research focused on immune responses to these relatively "new" human pathogens [14]. It is estimated that there are nearly 1.5 million fungal species; of those species, only a small subset (approximately 300) has been reported to be pathogenic to humans [15]. Although superficial fungal infections, which affect the outer layers of the skin, nails, and hair, are the most common fungal infections in humans, invasive infections pose a more serious threat to human health. Despite the availability of several antifungal drugs, mortality associated with invasive fungal infections remains unacceptably high and is estimated to be over 50% for most mycoses. As a group, fungal infections cause over a million deaths annually worldwide [16]. The most common global opportunistic invasive fungi are *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans*, but there are many other fungal species that infect humans including endemic fungi such as *Blastomyces*

dermatitidis, *Coccidioides immitis*, and *Histoplasma capsulatum* [16, 17].

2.1. Candidiasis. Candidiasis is caused by commensal *Candida* species, which live in the human gastrointestinal tract and vagina. The most commonly found species is *C. albicans* [5]. In a healthy host, phagocytic cells of the innate immune system are able to recognize and eliminate any invading *Candida* [18]. Under immunosuppressed conditions, *Candida* is able to breach the integrity of mucosal barriers and cause systemic infection. Infection may also occur in patients with a central venous catheter in which *Candida* on the skin is able to bypass cutaneous barriers and a significant amount of fungi enters the circulation [18, 19]. *Candida* has the unique ability to switch between yeast and hyphal forms [18, 20]. The ability to reversibly convert from isotropic (yeast) growth to apical (hyphal and pseudohyphal) growth has been theorized to contribute to virulence [21]. Virulence is attenuated in both yeast and hyphal locked mutants and infection sites are populated by both morphological forms, which points to a role for both forms in the pathogenesis of candidiasis [21].

2.2. Aspergillosis. Aspergillus is ubiquitously found in the environment. The most common pathogenic *Aspergillus* is *A. fumigatus* [22, 23]. Infection occurs via inhalation of conidia into the lungs. Healthy human hosts are typically able to clear invading conidia [21] and prevent germination and spread into the lung [24]. Invasive *Aspergillus* infection occurs primarily when neutrophils are somehow impaired (i.e., chronic granulomatous disease, or neutropenia) and thus unable to contain and clear invasive hyphal growth in the lungs [22–24].

2.3. Cryptococcosis. Cryptococcosis is a systemic fungal infection in immune compromised hosts that results in deadly meningitis once the fungus has disseminated to the central nervous system (CNS) [25–27]. *C. neoformans*, the most common cause of cryptococcosis, is a yeast commonly found in the environment, and thus exposure is fairly prevalent but rarely progresses to disease in healthy individuals [28–30]. Immunocompetent individuals are able to combat and contain *Cryptococcus* in the lung after inhalation of spores to prevent spread to the CNS. A successful immune response results in killing of *Cryptococcus* by phagocytes and granuloma formation that is thought to prevent *Cryptococcus* from accessing the vasculature and causing infection of the CNS. In the case of an immunocompromised host, *Cryptococcus* is not successfully cleared by phagocytes and spreads through the vasculature and across the blood brain barrier causing life threatening meningitis [26, 30].

2.4. Challenges in Development of Therapies against Invasive Fungal Infections. Together, these infections present a unique set of challenges for treatment. Most systemic fungal infections occur in immunocompromised individuals who may be suffering from AIDS, cancer, or organ failure, adding another layer of complexity to the disease [17]. Additionally, there are only a limited number of therapeutic interventions widely available. None of the available classes of drugs are wide spectrum and there is high toxicity associated with the most effective therapies [31]. This relative scarcity of available compounds is owing to the relatedness of fungi to humans compared to viruses and bacteria. Many essential pathways are conserved between fungi and humans, which forces researchers to search for structures and pathways unique to fungi [31]. As an alternative, phagocytes can be exploited as a cell-based therapy in conditions of immune suppression [32, 33]. Therefore, understanding the intracellular pathways that contribute to the killing mechanisms of these immune cells (such as sphingolipid signaling) may provide new means for the development of novel therapeutic strategies against fungal infections.

3. Role of Macrophages in Fungal Infections

The name macrophage comes from Greek and means "big eater." Macrophages are professional phagocytic cells capable of detecting a multitude of signals to bind and consume opsonized pathogens, as well as dying cells and cell debris [34]. Macrophages derive from the myeloid lineage and develop from both monocytic precursors and embryonic progenitors during embryonic development [35]. Tissue macrophages are responsible for immune surveillance and upon recognition of pathogen-associated molecular patterns (PAMPS) will drive inflammation by recruiting other leukocytes including monocytes and neutrophils [36]. They secrete a variety of cytokines including tumor necrosis factor-alpha (TNF-α), interleukin 1 (IL1), and nitric oxide (NO), which contribute to activation of antimicrobial defense, and interleukin 12 (IL12) and interleukin 23 (IL23), which direct differentiation of inflammatory T helper cells [37]. They have also been shown to be capable of releasing antimicrobial extracellular traps (ETs) that may play a role in clearance of infections [38].

3.1. Candida. Macrophages are one of the most important lines of defense against *C. albicans* in tissues and the blood stream [39]. Evidence for the role of macrophages in *Candida* infection has been demonstrated in different mouse models. For instance, depletion of mouse splenic macrophages (but not neutrophils) with liposome-entrapped clodronate was shown to increase susceptibility of both BALB/cByJ and nude mice strains to disseminated candidiasis [40]. In addition, inactivation of macrophages with intraperitoneal injection of carrageenan was shown to increase susceptibility in an oropharyngeal candidiasis model in BALB/c and CBA/CaH mice [41]. Finally, depletion of alveolar macrophages by 2-chloroadenosine resulted in delayed mortality of BALB/c in a lung injury model but reduced *Candida* clearance and neutrophil recruitment in the lung [42]. From these results, it is thought that macrophages are important for recognition, killing, and recruitment of other cell types but must be activated by T helper 1 cytokines for efficient killing [41]. Macrophages recognize *Candida* through Toll-like receptors

2 and 4, Dectin-1, mannose receptor, and Dectin-2 [18, 43–46]. Mannan has been shown to be one of the most important pattern-associated molecular patterns for recognition of *Candida* by macrophages [18]. Under immune sufficient conditions, the yeast form of *Candida* is effectively controlled by phagocytic action of macrophages, but under certain conditions, *Candida* is able to overgrow and may switch to filamentous hyphal growth that is more of a challenge for the immune system. Once infection is able to take hold (such as under the condition of immune suppression), yeasts that are able to transition to the hyphal form are able to escape macrophages by physically destroying cells due to their size or by inducing pyroptosis [20, 47].

3.2. Aspergillus. There is a growing body of evidence for the role of alveolar macrophages in the initial defense against *Aspergillus* conidia that are inhaled into the lung [48]. Alveolar macrophages efficiently uptake *Aspergillus* conidia in a Dectin-1 dependent manner and have the capacity to kill conidia intracellularly [49]. Additionally, invasive aspergillosis has been reported in a patient with inflammatory defective macrophages [50]. Despite this evidence, in 2009, it was reported that macrophages were dispensable in a C57BL/6 mouse model in which clodronate liposomes were used to deplete alveolar macrophages [24]. It was shown that, in the absence of macrophages, neutrophils were capable of mounting a sufficient response to prevent hyphal tissue invasion [24]. Another group has designed transgenic monocyte depleting mice, which utilizes diphtheria toxin induced cell ablation directed toward CCR2 expressing cells. That group reported no difference in lung burden in their depleted mice but showed that they were essential to the priming and expansion of CD4+ T cells [51]. Counter to these results, in 2011, another group has published that depletion of macrophages with clodronate in BALB/c mice results in increased fungal burden in the lung and that the elevated levels of neutrophils failed to control the infection [49]. Altogether, the evidence points to an important supportive role for macrophages in *Aspergillus* infection.

3.3. Cryptococcus. In cryptococcal infections, macrophages have been shown to play a critical role in normal host defense but may also have a role in development of disease in immunocompromised individuals. Depletion of macrophages using transgenic diphtheria toxin induced cell ablation directed toward CD11c expressing cells showed increased susceptibility in the mouse model [52]. In an experiment that compared two model hosts, one susceptible (mouse) and one resistant (rat), it was found that clodronate liposome depletion in each species had very different results [53]. While macrophage depletion in mice leads to decreased fungal burden, depletion in rats leads to increased fungal burden and dissemination [53]. Additionally, depletion of alveolar macrophages proved to be protective to immunodeficient mice infected with a glucosylceramide deficient mutant of *Cryptococcus* (Δ*gcs1*) but showed no effect when these same mice were infected with wild type *C. neoformans* H99 [54]. Importantly, the results with the *C. neoformans* Δ*gcs1* strain are of particular clinical relevance since this strain mimics the infection pattern of human cryptococcosis in that it is avirulent in immunosufficient mice and it becomes virulent in T and NK cell deficient mice [54]. Thus, altogether, these findings demonstrate the paradoxical role that macrophages play in cryptococcosis: good cop in case of immunocompetency when macrophages are able to kill the fungus, and bad cop in case of immunosuppression, when they are unable to kill the fungus and rather provide a safe environment for *C. neoformans* to replicate and be transported elsewhere (favoring dissemination). Indeed, in immunocompetent subjects, clearance of internalized *Cryptococcus* is thought to depend on T helper 1 mediated response which results in formation of a granuloma and production of TNF-α and Interferon gamma (IFNγ) [55]. These cytokines cause macrophages to become classically activated and upregulate NADPH oxidase to allow for production of nitric oxide which kills internalized *Cryptococcus* [56]. On the other hand, in an immunocompromised host, *Cryptococcus* is able to survive and proliferate within macrophages leading to eventual dissemination into the blood stream and central nervous system [26, 57]. There is further evidence for this transcellular passage theory, also known as "Trojan horse" model. An experiment which inoculated mice with macrophages already containing *Cryptococcus* showed increased fungal burden in the lung and spleen and also the brain at later stages of infection as compared to mice inoculated with the same number of free yeasts [58]. It was also shown that late stage depletion of macrophages (72 hours after intravenous infection) resulted in decreased disease severity and fungal burden [58]. As another way to subvert macrophage processes and disseminate, *Cryptococcus* has also been shown to extrude itself from macrophages, leaving both macrophage and yeast intact [59]. Altogether, this evidence supports a protective role for macrophages in an immunocompetent host but strongly supports the subversion of macrophages in the condition of immunosuppression resulting in increased dissemination. Generally, an efficient uptake of *Cryptococcus* by macrophages requires the opsonization by complement or specific antibodies [60, 61] while the presence of a large capsule on *Cryptococcus* prevents phagocytosis *in vitro*.

4. Role of Neutrophils in Fungal Infections

Neutrophils are considered to be the most important cell type for fungal killing. They sense pathogens with an array of pattern recognition receptors (PRRs), which include Toll-like receptors, C-type lectin receptors, glycosphingolipids (GSLs), and cytoplasmic sensors for ribonucleic acids [62, 63]. PRRs, along with signals from other immune cells (such as macrophages), work together to help neutrophils sense their environment, undergo chemotaxis, and initiate inflammatory responses [62, 64, 65]. Neutrophils are equipped with an arsenal of granule proteins that have various enzymatic activities designed to neutralize pathogens, including defensins, myeloperoxidase, proteases, lactoferrin, and gelatinase [65, 66]. Once activated, neutrophils carry

out effector functions, which include phagocytosis, mobilization of granules, production of reactive oxygen species (ROS), release of neutrophil extracellular traps (NETs), and secretion of lytic enzymes, antimicrobial peptides, and neutrophil derived cytokines. These activities ultimately lead to pathogen destruction by both intracellular and extracellular killing and recruitment of additional immune cells [64–66].

4.1. Candida. Neutrophils are thought to be critical for controlling systemic candidiasis. Patients suffering from induced neutropenia or genetic neutrophil defects are at high risk for invasive *Candida* infection [67, 68]. In the mouse, ablation of neutrophils using RB6-8C5 (anti-Gr-1, anti-Ly6G/Ly6C) antibody causes increased susceptibility to systemic, vaginal [69], and oropharyngeal challenge with *Candida* [41]. Three mechanisms have been described by which neutrophils kill *Candida* in healthy individuals. The first is killing of unopsonized *Candida* and it depends on complement receptor 3 (CR3) and caspase recruitment domain-containing protein 9 (CARD9). A second mechanism of killing targets opsonized *Candida* in an Fcγ receptor (FcγR), protein kinase c (PKC), and NADPH oxidase dependent manner [67]. Finally, a third mechanism involves a newly discovered function of neutrophils in the generation of neutrophil extracellular traps (NETs). NETs are weblike structures extruded by neutrophils composed of decondensed chromatin and over 30 different neutrophil proteins [70]. NETs are generated in response to *Candida* hyphae [71] and contain the antifungal protein calprotectin [72]. It is thought that while intact neutrophils are able to clear yeast forms of *Candida*, NETs may have evolved as a way to defend against hyphae that evade phagocytosis due to their size [71].

4.2. Aspergillus. Neutrophils are essential to defend the host against *Aspergillus* infection. Like *Candida* infection, neutropenia and neutrophil defects (such as chronic granulomatous disease) are major risk factors for invasive aspergillosis [73]. It has been confirmed that depletion of neutrophils via monoclonal antibody RB6-8C5 (anti-Gr-1, anti-Ly6G/Ly6C) during the earliest phase of infection is associated with high mortality which shows that neutrophils provide essential defense during inhalation and germination of *Aspergillus* [24]. It is still unclear how neutrophils control *Aspergillus* in healthy individuals. One theory is that neutrophils spread and degranulate onto the surface of hyphae [74]. New research suggests that NETs may also play a role. NETs are formed in response to *Aspergillus* hyphae [71] and restoration of NET formation using gene therapy to add the gp91(phox) gene (encoding a subunit of NADPH oxidase) in a patient with chronic granulomatous disease was shown to rapidly cure aspergillosis [73].

4.3. Cryptococcus. Although macrophages are considered the first line of defense against *C. neoformans*, the role of neutrophils is equally important because, once recruited, they are extremely efficient in killing *C. neoformans* and other fungal cells [75, 76]. Studies on the role of neutrophils during *C. neoformans* infection have not been pursued much, mainly because primary neutropenia is not a risk factor for cryptococcosis. However, this does not mean that neutrophils are not important for protection against cryptococcosis, and it only suggests that the decrease of neutrophils is not sufficient to render the host susceptible to *C. neoformans*. On the other hand, neutrophils might play an important role for protection once the infection has occurred. This is exemplified by many observations. First, patients in which neutrophil killing activity is decreased may actually develop cryptococcosis [77, 78]. Second, in late stages of human immune deficiency virus (HIV) infection, with low number of CD4+ T cells and when cryptococcosis occurs, the defensive mechanisms of macrophages and neutrophils are depressed [79]. Thus, it is largely accepted that most, if not all, opportunistic infections in acquired immune deficiency syndrome (AIDS) patients (including cryptococcosis) also develop because neutrophils and macrophages are not fully activated [79, 80]. Third, macrophage-mediated chemotaxis, phagocytosis, production of cytokines, superoxide, extracellular traps, and antimicrobial peptides and their killing activity are not optimal in the late stages of AIDS [80–83]. Fourth, although it is reported that cryptococcosis is not usually associated with human neutropenia or defective neutrophil function, neutropenia is often present in HIV positive patients, especially when patients have been diagnosed with AIDS [80, 81, 84]. Fifth, there are also reports showing that apparent immunocompetent individuals with pulmonary cryptococcosis have impaired killing activity of neutrophils and monocytes due to deficient production of TNF-α, IL-1β, and nitric oxide [77]. These studies clearly highlight that neutrophils are important to control *Cryptococcus* infection in humans.

Studies in mice are controversial mainly because murine neutrophils are notoriously weak compared to humans as they do not produce (and secrete) fully activated defensins [85]. Consequently, the role of neutrophils in *C. neoformans* infection is still unresolved: only a very limited amount of published work has addressed this issue using animal models and depending on the model used (mouse and/or *C. neoformans* strains and/or size and route of the inoculum) the results seem to differ [52, 86–88]. For instance, Casadevall's group found that depletion of neutrophils in BALB/c mice infected with the weak *C. neoformans* strain D52 (a mouse model in which mice succumb to the infection) enhanced resistance of the host [86] whereas other *in vivo* studies have implied a protective role for neutrophils when a mouse strain (SJL/J) relatively resistant to cryptococcosis was employed [88]. In the first model of infection (BALB/c mice with D52 *C. neoformans*), depletion of neutrophils before intratracheal *Cryptococcus* instillation resulted in protection of mice [86]. In this study, however, only a single depletion of neutrophils (effective for approximately 3 days) was performed; indeed, at day 7 of infection, neutrophil numbers were up again to the level of control mice. This early and short window of intervention points to a damaging role of neutrophils during the initial phase of the infection [52, 86] and it does not allow for formulating an overall conclusion regarding the role of neutrophils in the final outcome of *Cryptococcus* infection.

Considering that neutrophils continue to accumulate considerably also in the later phases of infection [86, 88], the question remains as to whether neutrophils exert different roles in different stages of the disease especially before an effective T cell mediated response is mounted (2-3 weeks). To definitively assess the role of neutrophils during cryptococcosis, we depleted neutrophils throughout the infection, in two different mouse strains (CBA/J or SJL/J) infected with a clinical isolate and highly virulent *C. neoformans* (H99) (Figure 1). Neutrophils were depleted by injecting 300 μg of RB6-8C5 monoclonal antibody intraperitoneally, as indicated (Figure 1). Confirmation of neutropenia, defined here as a decrease of at least 70% of neutrophils, was confirmed before *Cryptococcus* challenge and throughout the survival experiment by blood neutrophil count. In our hand, 300 μg of RB6-8C5 was the minimum dose required to ensure the 70% decrease of neutrophils. As a negative control, 300 μg of LTF-2 isogenic mAb was administered using a similar dose regime and neutrophils were also routinely counted in these mice and no depletion was found. Mice were then challenged with *C. neoformans* H99 strain intranasally and survival was monitored and recorded. The average survival of CBA/J and SJL/J neutropenic mice was 15.4 ± 7 and 16.8 ± 7.8, respectively, whereas the average survival of nonneutropenic mice was 32.2 ± 9.7 and 35 ± 8.5, respectively ($P < 0.05$) (Figure 1). These results clearly indicate that neutrophils are important to control *Cryptococcus* infection in mice. In line with our novel observations, other mouse models also supported a protective role for neutrophils [88, 89]. In one model, which employed the rather resistant mouse strain SJL/J (similar to CBA/J) infected with the *C. neoformans* strain D52, the T helper 1 response was preceded by accumulation of neutrophils in the lung as early as 3 hours after infection together with increased macrophage inflammatory protein-1α (MIP-1α), monocyte chemotactic protein 1 (MCP 1/CCL2), and keratinocyte chemoattractant (KC), which are neutrophil and macrophage chemoattractants. The number of neutrophils in the lung progressively and greatly increased in the following days and weeks while the fungal burden decreased [88]. In another study, *in vivo* imaging was used to show neutrophils directly removing *C. neoformans* from the brain vasculature [89]. Additionally, it was shown that depletion of neutrophils enhanced fungal burden in the brain [89]. Thus, from these studies, it is obvious that the apparent conflicting results in the literature are likely due to the use of different mouse models, *Cryptococcus* strains, and most importantly the time frame of the induced neutropenia. Altogether, these studies and our new results (Figure 1) strongly point to the fact that neutrophils are important to control *Cryptococcus* infection, especially when the infection has already developed.

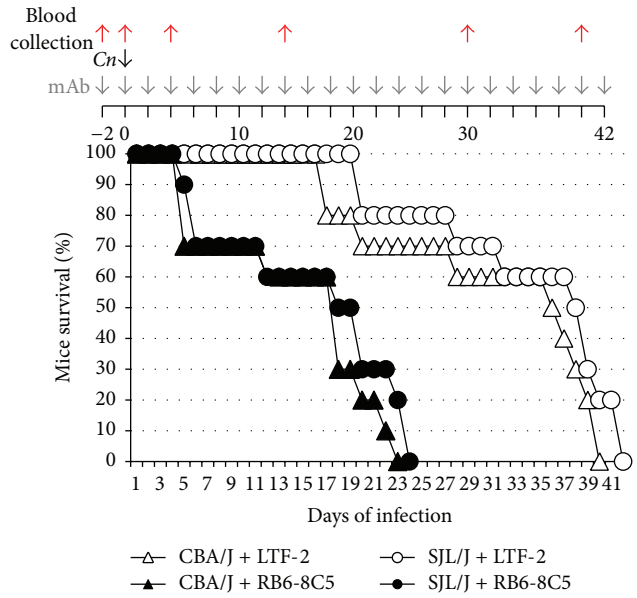

Figure 1: Neutrophils are important to control cryptococcosis in mice. Four six-week-old mice (CBA/J or SJL/J model) were treated intraperitoneally every other day with 300 μg of Rb6-8C5 monoclonal antibody (mAb, gray arrows) directed against neutrophils. After 2 days from the first dose (day 0), mice were infected intranasally with a lethal dose of *C. neoformans* cells (5×10^5) (black arrow). As controls, mice were treated with LTF-2 mAB (an IgG2 isotype for Rb6-8C5). Before mAb treatment and *C. neoformans* challenge, and during infection, blood was collected for neutrophil count (red arrows).

5. The Role of Sphingolipids in the Immune Responses

Sphingolipids are a family of lipids defined by a backbone mostly composed of an eighteen-carbon amino alcohol, referred to as the sphingoid backbone. The simplest sphingolipids are sphingosine, phytosphingosine, and dihydrosphingosine, which can be modified to produce an array of more complex sphingolipids, some of which have regulatory functions in important cell processes. For general reviews on sphingolipid metabolism and signaling, the reader is referred to [90–94].

Among the bioactive sphingolipids that have been implicated in the regulation of the immune response against fungal infections are sphingosine-1-phosphate (S1P), sphingomyelin (SM), and glycosphingolipids (GSLs) (Figure 2) [94].

5.1. Sphingosine-1-Phosphate. S1P is produced by the phosphorylation of sphingosine by one of two sphingosine kinases (SK1 and SK2) [94]. Once phosphorylated, S1P is recognized by a family of G-protein coupled receptors (S1PR1-5) that activate downstream effectors such as small GTPases (Rho, Rac, and Ras), adenylate cyclases, PI-3-kinase, phospholipase C, protein kinase C, or intracellular calcium [91]. The distribution of the receptors on different cell types and the coupling of receptors to different G-proteins allow S1P to differentially exert its influence in many different pathways, including inflammation [95]. S1P may also signal independently of S1PRs as an intracellular second messenger [96].

5.2. Sphingomyelin. SM is produced by the addition of a phosphocholine moiety from phosphatidylcholine (PC) onto

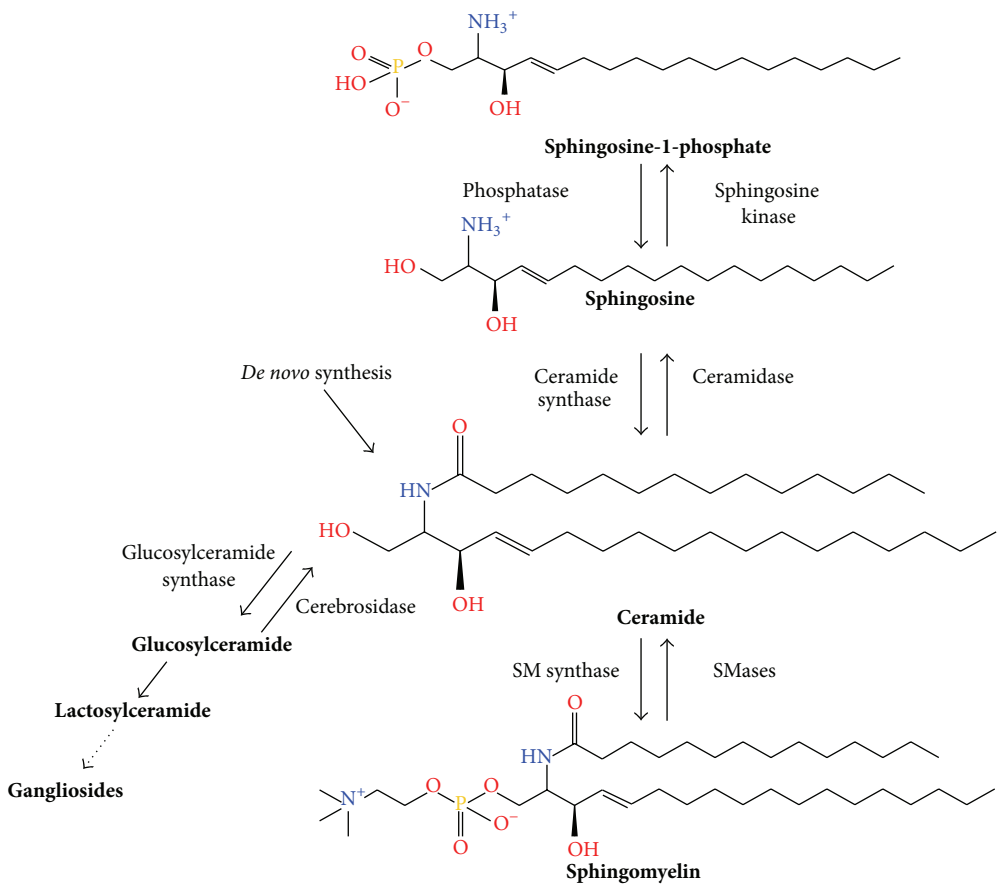

Figure 2: Overview of sphingolipid synthesis. Sphingolipids contain a sphingosine backbone that is modified to produce an array of metabolites. Ceramide serves a central role and can be synthesized by breakdown of sphingomyelin, addition of fatty acid by ceramide synthase, or *de novo* synthesis from serine palmitoyltransferase. Ceramide and sphingosine can be phosphorylated by their respective kinases to form bioactive metabolites. More complex sphingolipids are formed from ceramide, including sphingomyelin and glycosphingolipids.

ceramide by a family of enzymes known as sphingomyelin synthases. In mammals, there are two sphingomyelin synthases, SMS1 and SMS2. SM is an abundant component of cell membranes and is important for the formation of ordered membrane domains known as lipid rafts in model membranes [97, 98]. It is thought that lipid rafts play important roles in many processes such as GPI-anchored protein sorting, receptor clustering [99], endocytosis, exocytosis, vesicle formation, and budding [100, 101]. Thus, the ability of SM to contribute to lipid raft homeostasis may have important implication in the functions of phagocytes whose activities rely on receptor activation, endocytosis, and secretion. So far, it has been shown that SMS2 deficiency prevents TNF-α stimulated lipid raft recruitment of TNF receptor 1 and prevents NFκB activation in macrophages [102]. Additionally, SM can also be broken down by the sphingomyelinase (SMase) enzymes to produce ceramide and phosphocholine, thus serving as a major source of the bioactive sphingolipid, ceramide [103, 104]. During synthesis of SM, SMSs also produce the bioactive product diacylglycerol (DAG) [105–107] which can activate DAG-binding targets, such as protein kinase D (PKD). Indeed, PKD is a key regulator of protein trafficking and secretion, and it has been shown to control neutrophil secretion of antifungal factors [8, 105].

5.3. Glycosphingolipids. GSLs are composed of a sugar moiety attached to ceramide. More than 400 types of GSLs have been identified based on the attached sugar structure, but the ceramide chain lengths are also highly variable [108, 109]. Glycosphingolipid biosynthesis occurs via the action of specific glycosyl transferases, which add galactose or glucose moieties to ceramide [94]. These can be further modified to produce an array of carbohydrate structures [110]. Major relevant GSL species in phagocytes include lactosylceramide and gangliosides [10, 111, 112]. GSLs are another major component of lipid rafts and have also been found to have direct interaction with both cytosolic and membrane proteins; they play roles in cell adhesion, motility, growth, and neutrophil function [111, 113–115]. Importantly, GSLs have been shown to be able to directly bind to pathogens which is a crucial step in initiating phagocytosis [111, 116, 117]. For example, *Chlamydia pneumoniae* and *Chlamydia trachomatis* have been shown to bind both Asialo-GM2 and GM1 [118], while influenza virus

binds poly$_{(\rightarrow 50)}$ glucosylceramides and other GSLs [119]. For a thorough discussion on the topic, please refer to [116].

6. The Role of Host Sphingolipids in Fungal Infections

6.1. Candida. There is evidence for the role of host sphingolipids in the regulation of the immune response to *Candida*. It has been shown that inhibition of sphingosine synthesis with myriocin in *Galleria mellonella*, a commonly used insect model for studying fungal infections [120], increases mortality during *Candida* infection [121]. In the mouse model, sphingolipid synthesis inhibition with myriocin or fumonisin B1 treatment impairs phagocytosis of *C. albicans* by macrophages in culture [122]. Fumonisin B1 treatment of mice increased susceptibility to tail vein injected *C. albicans* [122]. Additionally, the importance of the GSL lactosylceramide (LacCer) in neutrophil function has been studied and it was reported that LacCer is expressed on the plasma membrane of neutrophils [10, 115]. It is important for superoxide generation and the formation of domains with the Src family kinase Lyn [114, 115]. These observations are important in light of the evidence supporting the role of neutrophils in *Candida* infection. Furthermore, LacCer can bind *Candida* directly [123] and it also acts as a pattern recognition receptor to promote chemotaxis of neutrophils in response to *Candida* soluble beta-D-glucan [63]. Additionally, GSLs and specifically gangliosides have been shown to play essential roles in adhesion and motility, both important processes for phagocytes to serve their function [113].

More recently, sphingolipids have been implicated in the production of NETs. Neumann et al. demonstrated that treatment of primary blood-derived human neutrophils with bacterial sphingomyelinase, which hydrolyzes SM into ceramide and phosphocholine, causes spontaneous generation of NETs [124]. Although the mechanism for this observation is unknown, the breakdown of SM could alter signaling complexes that localize to rafts and lead to spontaneous NET generation. This observation points to a role for rafts in controlling the generation of NETs and suggests that SM and GSL pathways could contribute to clearance of *Candida* by NETs. Since the importance of neutrophils and macrophages for fighting *Candida* infections is well established, these insights into sphingolipid involvement in phagocyte function could aid in developing alternative therapeutic strategies against this fungus.

6.2. Cryptococcus. Host sphingolipids have been shown to play an important role in controlling *Cryptococcus* infections. In particular, S1P plays a role on multiple levels. In an obligate intracellular murine model of *Cryptococcus* infection (Δ*gcs1*), which forms granulomas, SK1, the enzyme responsible for production of S1P, was found to be essential to granuloma formation. In fact, knockout of SK1 prevented formation of granulomas by reducing the amount of S1P in the bronchoalveolar lavage fluid which resulted in lowered levels of MCP-1 and TNF-α [9, 25]. Additionally, S1P was found to directly affect phagocytic cells. While addition of S1P to macrophages increased their ability to uptake *Cryptococcus* via the action of S1P receptor 2 [125], addition of S1P to neutrophils increased their ability to kill *Cryptococcus* extracellularly [9]. Sphingomyelin may also play a role in regulating the response of phagocytic cells to *C. neoformans*. In fact, some work has hinted at a role for lipid rafts in phagocytosis of *Cryptococcus* as disruption of lipid rafts with methyl-β-cyclodextrin results in decreased uptake of *Cryptococcus* by macrophages *in vitro* [126]. Since SM and glycosphingolipids are key constituents of lipid rafts, these studies warrant further investigation on the requirements also for these complex sphingolipids in the recognition and phagocytosis of *C. neoformans* by macrophages [97]. Finally, inhibition of SMS, the enzyme responsible for SM biosynthesis, impairs the killing ability of neutrophils by preventing the release of antifungal factors through a DAG-PKD dependent mechanism [8, 105].

6.3. Aspergillus. There is a dearth of information concerning host sphingolipid involvement in *Aspergillus* infection. It is known that neutrophils and NETs play an important role in clearance of infection. As discussed in the previous sections, sphingolipids are important for many neutrophil antifungal activities, including secretion of antifungal factors, and possibly regulating NET formation. This warrants further study to extend work that has been done in other fungi to include *Aspergillus* and other emerging fungi.

6.4. Other Fungal Infections. There is an increasing amount of evidence that lipid rafts play a role in the interaction between phagocytes and fungi. Both complement receptor 3 and Dectin-1 are major fungal pattern recognition receptors and they have been shown to colocalize in lipid raft microdomains in response to *Histoplasma capsulatum* [127]. This finding shows the importance of these sphingolipid rich domains especially during fungal infections, many of which are recognized through these receptors.

7. Conclusions and Future Directions

Sphingolipids have been shown to play an important role in many cellular processes, including the function of phagocytic cells, which play critical roles in invasive fungal infections. Signaling lipids such as S1P are able to directly bind proteins to affect cellular pathways, while SM and GSLs may affect cellular processes by altering domain formation on the plasma membrane or serving as pattern recognition receptors themselves (LacCer). Findings that highlight the roles of sphingolipids in phagocytes are particularly useful in light of the critical role that these cells play in controlling fungal infections and may serve as a key to overcome the challenges associated with treating these types of infections. In the future, it is important to apply what we learned about phagocytes into understanding how sphingolipids affect the interactions between phagocytes and fungi. Much work that has been done concerning this has not yet been validated for other species. Another unexplored pathway is the possible

connection between sphingolipids and formation of extracellular traps and whether this could be another avenue to fight off hyphal growth. In the future, understanding of host pathways in phagocytes could lead to cell-based therapies that exploit the strengths of phagocytes to combat fungal infections in the context of an immunocompromised system.

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

Sphingolipids in High Fat Diet and Obesity-Related Diseases

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Nutrient oversupply associated with a high fat diet (HFD) significantly alters cellular metabolism, and specifically including sphingolipid metabolism. Sphingolipids are emerging as bioactive lipids that play key roles in regulating functions, in addition to their traditional roles as membrane structure. HFD enhances *de novo* sphingolipid synthesis and turnover of sphingolipids via the salvage pathway, resulting in the generation of ceramide, and more specifically long chain ceramide species. Additionally, HFD elevates sphingomyelin and sphingosine-1 phosphate (S1P) levels in several tissues including liver, skeletal muscle, adipose tissue, and cardiovascular tissues. HFD-stimulated sphingolipid generation contributes to systemic insulin resistance, dysregulated lipid accumulation, and cytokine expression and secretion from skeletal muscle and adipose tissues, exacerbating obesity-related conditions. Furthermore, altered sphingolipid levels, particularly ceramide and sphingomyelin, are involved in obesity-induced endothelial dysfunction and atherosclerosis. In this review, HFD-mediated sphingolipid metabolism and its impact on HFD-induced biology and pathobiology will be discussed.

1. Introduction

Obesity is emerging as a significant public health concern in developed countries. Obesity contributes significantly to increasing medical costs by exacerbating other chronic diseases, such as cardiovascular disease, type 2 diabetes, and certain types of cancers. In the United States, more than one-third (34.9%) of adults and 17% of the youth are obese [1]. A western diet comprised of a high fat content is a primary contributing factor for obesity which has led to extensive studies using models of high fat diet (HFD) to delve into the pathobiologies of obesity-related diseases.

Sphingolipids are bioactive lipids which are involved in cellular signaling and regulatory functions [2]. These lipid species have been implicated as potent mediators in the regulation of several diseases such as cancer and inflammatory diseases [2, 3]. Based on these studies, interest for the role of sphingolipids in obesity-induced pathobiology is emerging. This is of particular interest as HFD significantly changes energy metabolism, including sphingolipid levels, suggesting the possibility that HFD-induced dysregulation of sphingolipid metabolism contributes to HFD and obesity-related pathologies. This review will discuss sphingolipid metabolism altered by HFD and its impact on HFD-induced biology and pathobiology.

2. Sphingolipid Metabolism

Sphingolipid metabolism is a complex network composed of numerous metabolizing enzymes that function to generate sphingolipids through either *de novo* synthesis or the salvage pathway. Sphingolipid levels are tightly regulated by these enzymes, allowing them to function as bioactive mediators. *De novo* sphingolipid synthesis begins with the enzyme serine palmitoyltransferase (SPT). SPT functions as a heterodimer with subunits SPTLC1 and SPTLC2, or SPTLC3, primarily using palmitoyl-CoA as a substrate. SPTLC3 has recently been shown to utilize myristoyl-CoA as well [4]. Condensation of serine and palmitoyl-CoA (or myristoyl-CoA) which forms 3-ketosphinganine is then reduced to form dihydrosphingosine by NADH-dependent reductase. Ceramide synthases (CerS), of which there are six, catalyze

the acylation of dihydrosphingosine to dihydroceramide. Subsequent desaturation yields the generation of ceramide from dihydroceramide. Ceramide can also be generated by sphingomyelinases (SMase) and glucosylceramidase (GCase) from various membrane glycolipids and sphingolipids in the salvage pathway. Once formed, ceramide acts as a central hub in the sphingolipid network. Ceramide can be phosphorylated by ceramide kinase to form ceramide 1-phosphate (C1P). Sphingomyelin synthase (SMS) and glucosyl- or galactosylceramide synthases (GCS) incorporate ceramide into sphingomyelin and glycosyl or galactosylceramide, respectively. Finally, one of five ceramidases (CDase) facilitates the deacylation of ceramide to produce sphingosine, followed by conversion to sphingosine-1 phosphate (S1P) by sphingosine kinases (SKs) or reacylation to ceramide by CerS. S1P can be degraded by one of two enzymes, sphingosine phosphate phosphatase (SPP) or S1P lyase. SPP dephosphorylates S1P to sphingosine, allowing for the reformation of ceramide, whereas S1P lyase irreversibly breaks down S1P to ethanolamine phosphate and hexadecanal, resulting in exit from sphingolipid metabolism.

3. The Effects of HFD on Sphingolipid Metabolism

3.1. Ceramide. Dysregulation of ceramide in response to nutrient oversupply, specifically saturated fatty acids, has been known to be a key factor in the impairment of cellular homeostasis and function [5]. During *de novo* synthesis, one of the six isoforms of CerS generates ceramide species with specific fatty acid chain lengths [6]. The long chain fatty acid palmitate (palmitoyl-CoA) is the main source of fatty acid in *de novo* sphingolipid synthesis. HFD administration and/or palmitate treatment have been shown to increase ceramide content independent of tissue or cell type (Table 1). Several tissues including liver, adipose, skeletal muscle, and heart demonstrate elevated total ceramide and long chain ceramide levels upon HFD administration. Diabetic models using HFD have also shown elevations in circulating ceramides [7]. Additionally, several studies have demonstrated C16:0 and C18:0 ceramides are consistently elevated by HFD/palmitate [8–13], compared to ceramides with the other chain lengths. Interestingly, very long chain ceramides, C24:0 and C24:1, have been shown to significantly increase upon HFD/palmitate treatment in some studies [8, 14–16] but are decreased in others [9, 11, 17] (Table 1). Additionally, ceramide induction in response to HFD has also been demonstrated as an aging process with some studies indicating augmented accumulation of ceramide in muscle in aging individuals [18–21]. The mechanism for this age-related increase in ceramide is still unclear, but the membrane protein CD36/FAT that facilitates the uptake of fatty acid has been suggested as a potential mechanism in HFD administration [20].

HFD-induced ceramide generation is primarily due to increased *de novo* synthesis, whereby excessive fatty acid supply supplies a continuous substrate supply for this pathway. Radioactive palmitate treatment in HepG2 cells (human hepatocarcinoma cell line) and C2C12 cells (murine myotubes) increased incorporation of palmitate into ceramide, demonstrating that fatty acids from exogenous sources can be utilized for sphingolipid synthesis [14, 22]. In addition to increasing substrate, HFD/palmitate alters expression and activity of the enzymes involved in *de novo* synthesis. HFD increased transcription of SPT subunits, SPTLC1, SPTLC2, and SPTLC3 [9, 18, 23–25], as well as activity of SPT [26]. CerS have also been shown to be upregulated by HFD [9, 17, 24, 25], specifically CerS1 and CerS6, which are key in the formation of long chain ceramide species. This upregulation resulted in the generation of C16:0 ceramide in response to HFD [6, 17]. In addition to CerS1 and CerS6, CerS2 and CerS4 expression has been reported to increase in response to HFD/palmitate [23, 24].

Upstream regulation of *de novo* sphingolipid synthesis by HFD has not been well studied but there are suggestions in the literature. TLR4, suggested as a receptor for saturated fatty acids [27], has also been shown to mediate several HFD-induced cell signaling events including HFD-induced *de novo* sphingolipid synthesis [28]. TLR4 deletion in skeletal muscle inhibited increases in ceramide levels upon HFD administration. Additionally, silencing the downstream signaling mechanism NF-κB suppressed induction of mRNA expression in *de novo* sphingolipid enzymes, such as SPTLC1, SPTLC2, CerS1, CerS2, CerS5, and CerS6. Cannabinoid-1 receptor (CB_1R) is another potential regulatory mechanism for *de novo* sphingolipid synthesis, as hepatic CB_1R deletion or blockade suppressed HFD-induced SPTLC3, CerS1, and CerS6 upregulation and subsequent ceramide increases [9]. Additionally, the energy sensing molecule, AMP-activated protein (AMPK), has been shown to regulate *de novo* sphingolipid synthesis. The AMPK activator, AICAR, suppressed HFD-induced transcription of SPTLC2 in skeletal muscle, suggesting AMPK as an upstream regulator of SPTLC2 [23]. Adipocyte-derived plasminogen activator inhibitor-1 (PAI-1) is important in the regulation of ceramide synthesis in adipose tissue as well [16]. PAI-1 deficient mice exhibited decreased expression of SPTLC2, SPTLC3, and CerS1 upon HFD feeding, resulting in reduced adipose ceramide level [16].

Enzymes of the salvage pathway have also been implicated in dietary manipulations of ceramide levels. Radio-labeled palmitate has been used to demonstrate the involvement of CerS and not *de novo* sphingolipid synthesis, as labeled palmitate was found in the acyl chain of ceramide, and not in the sphingoid backbone [14]. In addition to CerS, HFD administration enhanced mRNA expression and activity of acid SMase (aSMase) and neutral SMase (nSMase) in liver [24, 29] and adipose tissue [16]. Pharmacological inhibition of aSMase, using amitriptyline, inhibited ceramide induction by HFD in plasma and adipose tissue [30]. However, compared to studies examining *de novo* sphingolipid synthesis, regulation of the salvage pathway has not been well-studied.

Taken altogether, the above studies demonstrate that HFD and palmitate regulate global sphingolipid metabolism through both *de novo* sphingolipid synthesis and the salvage pathway. These diet-induced alterations in ceramide demonstrate that nutrition has the ability to alter sphingolipid metabolism and in turn downstream signaling pathways to

Table 1: Ceramide regulation by HFD or fatty acids.

Tissue	Experimental model	Ceramide species	Alteration	Reference
Liver	60% HFD for 5 weeks in Wistar rats	Total ceramide	Increased	[40]
	60% HFD for 16 weeks in C57BL/6 mice	C14:0, C16:0, C18:0, C20:0, and C24:0	Increased Decreased	[9]
	30% HFD with 40% fructose for 2 weeks in Syrian Golden hamsters	C14:0, C18:0, C18:1, and C20:0	Increased	[39]
	34% HFD for 3 weeks in Wistar rats	C14:0, C16:0, C18:0, C18:1, and C24:1 C14:0, C16:0, C18:0,	Increased (nuclei) Increased (total)	[29]
	42% HFD for 16 weeks in C57BL/6 mice	C18:0, and C20:0 C24:0, C24:1	Increased Decreased	[11]
	42% HFD for 3 weeks in C57BL/6 mice	C20:0, C22:0 C24:0, C24:1	Increased Decreased	[11]
	42% HFD for 6 weeks in C57BL/6 mice	C18:0, C20:0, C22:0, C24:1, C24:0, and total ceramide	Increased	[8]
	60% HFD for 4 weeks in C57BL/6 mice	C16:0, C22:0, and total ceramide	Increased	[12]
	0.3 mM palmitate for ≤24 h in primary rat hepatocytes	C16:0	Increased	[43]
Skeletal muscle	42% HFD for 16 weeks in C57BL/6 mice	C16:0, C18:0	Increased	[11]
	42% HFD for 3 weeks in C57BL/6 mice	C18:0	Increased	[11]
	42% HFD for 6 weeks in C57BL/6 mice	C16:0, C18:0, C20:0, C22:0, C24:1, C24:0, and total ceramide	Increased	[8]
	10-week HFD in 25-month-old Wistar rat	Total ceramide	Increased	[21]
	300 μM palmitate for 3 days during differentiation in human myotubes	Total ceramide, primarily C16:0, but except for C24:1	Increased	[18]
	Obese patient samples	C16:0, C18:0, C20:0, C22:0, and total ceramide	Increased	[10]
	60% HFD for 6 weeks in C57BL/6 mice	C16:0, C18:0, C18:1, and total ceramide	Increased	[41]
	60% HFD for 10 weeks in C57BL/6 mice	C16:0, C18:0, C18:1, and total ceramide	Increased	[13]
	42% HFD for 6 weeks in C57BL/6 mice	C16:0, C18:0, and total ceramide C24:1	Increased Decreased	[68]
	0.75 mM palmitate for 16 h in C2C12 myotubes	C16:0, C22:0, and total ceramide	Increased	[12]
	60% HFD for 4 weeks in C57BL/6 mice	C16:0, C18:0, and total ceramide	Increased	[12]
	0.75 mM palmitate for 16 h in C2C12 myotubes	Total ceramide, total glucosylceramide	Increased	[65]
	0.75 mM palmitate for 16 h in C2C12 myotubes	Total ceramide	Increased	[23]
	1.25 mM palmitate for 14 h in C2C12 myotubes	C16:0, C18:0, C24:0, C24:1, and total ceramide	Increased	[14]
Adipose tissue	42% HFD for 16 weeks in C57BL/6 mice	C16:0, C18:0, C20:0, and C22:0	Increased	[11]
	Obese patient samples	C14:0, C16:0, C18:0, C18:1, and C22:1	Increased	[17]
	55% HFD for 18 weeks in C57BL/6 mice	C16:0, C18:0 C22:0, C24:0	Increased Decreased	[17]
	60% HFD for 16 weeks in C57BL/6 mice	C16:0, C18:0, and C18:1 C16:0, C18:0, and C18:1	Increased Increased	[16]
Plasma/serum	60% HFD for 16 weeks in C57BL/6 mice	C16:0, C18:0, C20:0, and C24:1	Increased	[16]
	31% HFD in diabetic Rhesus Macaque monkeys	C14:0, C16:0, C18:0, C20:0, C22:0, and C24:0	Increased	[7]
	60% HFD for 13 weeks in C57BL/6 mice	C20:0, C22:0, C24:0, and total ceramide	Increased	[30]
	60% HFD for 8 weeks in Long Evans rats	Total ceramide	Increased	[24]

Table 1: Continued.

Tissue	Experimental model	Ceramide species	Alteration	Reference
Aorta	100 μM palmitate for 8 h in primary bovine aortic endothelial cells	C16:0	Increased	[89]
	60% HFD for 2 weeks in C57BL/6 mice	C16:0, C18:0, and C22:0	Increased	[89]
Heart	45% HFD for 16 weeks in 18-month-old C57BL/6 mice	Total ceramide	Increased	[19]
	41% palmitate-enriched HFD for 12 weeks in C57BL/6	C16:0, C20:0, and C20:1	Increased	[98]
	60% HFD for 12 weeks in 40–44-week-old C57BL/6 mice	C18:0	Increased	[20]
	60% milk-fat based HFD for 8 weeks in C57BL/6 mice	d16:1-base ceramide	Increased	[25]
	60% HFD for 16 weeks in C57BL/6 mice	C16:0, C18:0, C18:1, C20:0, and C24:1	Increased	[15]

Table 2: Sphingomyelin regulation by HFD or fatty acids.

Tissue	Experimental model	Sphingomyelin species	Alteration	Reference
Liver	60% HFD for 5 weeks in Wistar rats	NS	Increased	[40]
	34% HFD for 3 weeks in Wistar rats	SM16:0, SM18:0, and SM18:1	Increased (nuclei)	[29]
		SM16:0, SM18:0	Increased (total)	
	58% HFD for 16 weeks in C57BL/6 mice	NS	Increased	[54]
Skeletal muscle	60% HFD for 6 weeks in C57BL/6 mice	SM18:1	Increased	[41]
		SM18:0, total SM	Decreased	
Adipose tissue	42% HFD for 16 weeks in C57BL/6 mice	SM14:0, SM16:0, SM16:1 SM18:0, and SM18:1	Increased	[11]
Plasma/serum	45% HFD for 14 weeks in C57BL/6 mice	SM16:0, SM18:0, SM16:1, SM18:1, and SM22:1	Increased	[33]
	58% HFD for 16 weeks in C57BL/6 mice	NS	Increased	[54]
Heart	60% HFD for 16 weeks in C57BL/6 mice	SM 16:0, SM18:0, and SM18:1	Increased	[15]
	41% palmitate-enriched HFD for 12 weeks in C57BL/6	SM16:0, SM20:0, SM24:0, and SM24:1	Increased	[98]

NS = not specified.

induce obesity-related conditions, such as insulin resistance and ectopic lipid accumulation.

3.2. Sphingomyelin. Sphingomyelin, one of the most abundant sphingolipid species, plays key roles in membranes and has been suggested to function in lipid rafts [2, 31]. Concentrations of sphingomyelin are higher than ceramide, suggesting that sphingomyelin may act as a pool for the rapid generation of ceramide. It has been demonstrated that high concentrations of serum sphingomyelin correlate with coronary artery disease in obese individuals [32]; and several studies demonstrated that HFD or palmitate administration increased sphingomyelin levels in cells and tissues, including serum (Table 2) (although the magnitude of sphingomyelin increase was smaller than that of ceramide). Similar to alterations in ceramide species, long chain sphingomyelin species, mostly C16:0 and C18:0, have been reported to increase by HFD or palmitate treatment in liver, adipose tissue, and plasma [11, 29, 33] (Table 2). Macrophages stimulated with palmitate demonstrated increases in specific species of sphingomyelin, specifically 18:0, 20:0, 22:0, and 22:1 [34]. HFD-induced changes in sphingomyelin have been attributed to SMS2 activity and expression. Mice overexpressing liver-specific SMS2 exhibited elevated HFD-induced sphingomyelin in plasma. Additionally, this study demonstrated loss of SMS2 *in vivo* repressed HFD-induced increases in sphingomyelin [35].

The role for sphingomyelin in HFD-induced biologies has been less studied than ceramide; however, sphingomyelin levels are elevated in diverse tissues, including liver, skeletal muscle, adipose, and cardiac, as well as plasma. These studies suggest that sphingomyelin may directly regulate cell functions and/or serve as a reservoir for the generation of ceramide.

3.3. Sphingosine/S1P. Sphingosine, the breakdown product of ceramide, is known to regulate apoptosis and cell-cycle arrest [36–38]. There are few studies examining the role of sphingosine in HFD-mediated processes. It is possible that sphingosine is rapidly converted to S1P (or reacylated to ceramide) in HFD-mediated sphingolipid metabolism. However, the exact role of sphingosine in HFD-mediated effects has yet to be determined. S1P, a potent bioactive sphingolipid, is present in low nanomolar concentrations in the cell and has high affinity to S1PRs functioning as a signaling molecule. Despite its potent capacity as a bioactive lipid, studies for S1P (and its precursor, sphingosine) upon HFD administration are limited (compared to those examining ceramide). However, S1P (and sphingosine) levels exhibited significant increases in liver, skeletal muscle, and plasma in response to several experimental models of HFD. Conversely, there are also a few opposing studies that have demonstrated decreases in the levels of these lipids (Table 3). While S1P in liver and skeletal muscle was unchanged by HFD or palmitate in some studies [39–41], S1P in plasma was consistently elevated, which suggests that HFD-enhanced S1P levels in circulation may mediate some of the systemic effects associated with HFD and obesity. Furthermore, elevated plasma S1P positively correlated with body fat percentage, body mass index, waist circumference, and fasting plasma insulin in obese humans [42]. In cell culture palmitate stimulation of mouse derived pancreatic beta cells, MIN6, and primary rat hepatocytes resulted in increased S1P secretion [43, 44].

HFD-induced S1P seems to be due to augmented expression and activity of SK. Two isoforms for mammalian SK have been identified, SK1 and SK2. These two isoforms have suggested differences in subcellular localization, cytosol for SK1 and nucleus for SK2, as well as different physiological functions [45–47]. With respect to HFD, both enzymes have been shown to mediate HFD-induced S1P; however,

Table 3: Sphingosine and S1P regulation by HFD or fatty acids.

Tissue	Experimental model	Species	Alteration	Reference
Liver	0.3 mM palmitate for ≤24 h in primary rat hepatocyte	S1P	Increased	[43]
	30% HFD with 40% fructose for 2 weeks in Syrian Golden hamsters	Sphingosine	Increased	[39]
		S1P	No Change	
	60% HFD for 5 weeks in Wistar rats	Sphingosine, S1P	No Change	[40]
	58% HFD for 16 weeks in C57BL/6 mice	Sphingosine	Increased	[54]
Skeletal Muscle	60% HFD for 6 weeks in C57BL/6 mice	Sphingosine, S1P	No Change	[41]
	0.75 mM palmitate for 16 h in C2C12 cells	S1P	Increased	[65]
	1.25 mM palmitate for 14 h in C2C12 cells	S1P	Increased	[14]
		Sphingosine	No Change	
	0.75 mM palmitate for 16 h in primary mouse myotube	S1P	Increased	[48]
Adipose tissue	58% HFD for 16 weeks in C57BL/6 mice	Sphingosine	Increased	[54]
Plasma	42% HFD for 6 weeks in C57BL/6 mice	S1P	Increased	[42]
	42% HFD for 6 weeks in C57BL/6 mice	S1P	Increased	[8]
	58% HFD for 16 weeks in C57BL/6 mice	S1P	Increased	[54]
		Sphingosine	Increased	

regulation of these enzymes is suggested to be different. Skeletal muscle from mice fed a HFD for 16 weeks exhibited increases in only SK1 mRNA expression, and not SK2 [48]. In contrast, liver from rats fed a HFD for two weeks exhibited increased expression and activity of SK2, rather than SK1 [49].

The studies examining the effects of HFD on sphingosine and S1P levels suggest that S1P levels are elevated in several tissues. Moreover, S1P levels in plasma were elevated in response to HFD and have been suggested to be secreted from tissues including the liver and pancreas. This S1P has been shown to be generated by both isoforms of SK which are altered by HFD; however, they seem to be independently regulated each by independent mechanisms that have yet to be fully elucidated.

4. Sphingolipids in HFD-Induced Pathobiology

Obesity is well-known to induce several pathologic conditions, including insulin resistance. Insulin is responsible for clearance of redundant nutrients in circulation by facilitating their uptake and storage in liver, skeletal muscle, and adipose tissue. Under normal physiologic conditions, increased blood glucose from the diet stimulates insulin secretion and subsequent reduction of blood glucose, working as a negative feedback mechanism. However, when insulin fails to clear glucose in bloodstream this results in "insulin resistance." At the molecular level, insulin triggers signaling through the insulin receptor and subsequent phosphorylation of the insulin receptor substrate (IRS) [50]. Phosphorylated IRS recruits phosphatidylinositol 3-kinase (PI3K), which results in phosphorylation of Akt and its various substrates, including glycogen synthase kinase 3β (GSK3β) regulating glucose metabolism and glycogen, lipid, and protein synthesis [50, 51]. In insulin resistance, this signaling pathway is disrupted and ceramide has been reported to play a key role in the processes involved in insulin resistance [5].

Disruption of various enzymes in sphingolipid metabolism including SPT, CerS6, aSMase, and SMS2 suppressed weight gain by HFD [17, 30, 52, 53]. Specifically, myriocin, an inhibitor of *de novo* sphingolipid synthesis, enhanced energy expenditure and improved leptin resistance associated with HFD inhibiting obesity [52]. Additionally, for decades the role for sphingolipids in postobesity events such as ectopic lipid accumulation and insulin resistance has been established. Induction of ceramide by palmitate has been shown to disturb insulin signaling via inhibition of Akt signaling mediated by TLR4-NF-κB in myotubes [28]. Many studies demonstrated that inhibition of *de novo* synthesis of sphingolipids suppressed blood glucose and insulin induction [17, 40, 52, 54]. In addition to these systemic effects, aberrant sphingolipid regulation plays a significant role in HFD-induced dysregulation of various cellular signaling pathways. Understanding the specific effects in specific tissues will begin to lend insight into sphingolipid metabolism as a therapeutic target for HFD-induced pathologic conditions.

4.1. Liver. The liver is the central organ for the regulation of metabolism by action of insulin. Obesity-induced hepatic insulin resistance results in expansion of peripheral insulin resistance due to elevated fasting blood glucose and subsequent blood insulin levels. Sphingolipids play a significant role in energy metabolism; therefore, there has been an effort to elucidate the role for sphingolipids, particularly, ceramide and S1P, in hepatic insulin resistance.

Increased hepatic ceramide has been suggested to be a major mechanism in the regulation of insulin resistance, and suppression of ceramide induction has been demonstrated to improve insulin signaling. Direct treatment of Hu7 hepatoma cells with short chain C6:0 ceramide decreased

phosphorylation of Akt and GSK3β [24]. The CB_1R agonist anandamide increased endogenous C16:0 ceramide in hepatocytes via *de novo* synthesis, resulting in inhibition of insulin receptor substrate 1 (IRS1) phosphorylation, increased Akt phosphatase Phlpp1 expression, and suppression of Akt signaling in response to insulin [9]. Moreover, inhibition of ceramide synthesis through pharmacological inhibitors and gene deletion improved insulin signaling. Myriocin treatment and CerS6 deletion restored insulin-induced Akt signaling previously disrupted by ceramide [9, 17]. In contrast, CerS2 haploinsufficiency reduced very long chain ceramide and inhibited insulin sensitivity. This contradictory result was suggested to be due to CerS6 upregulation and subsequent C16:0 ceramide synthesis upon HFD [55]. These studies suggest that the roles for ceramide in energy metabolism regulation may be species-dependent and that many enzymes may generate ceramide in the liver during insulin resistance.

Hepatic S1P and its receptors are also suggested to function as key molecules in the regulation of insulin signaling. Palmitate-induced production and secretion of S1P from hepatocytes led to not only abrogation of insulin-induced Akt activation, but also reduced glucokinase expression and consequent glycogen synthesis [43]. These signaling pathways are reversed by SKII and JTE-013 treatment, an SK1 inhibitor, and an S1P receptor 2 (S1PR2) antagonist, respectively [43]. SK2 overexpression enhanced insulin sensitivity in liver from HFD-fed mice [56], implicating localization of S1P may be important for regulation of hepatic insulin resistance.

In addition to ceramide and S1P, sphingomyelin also may be involved in the regulation of insulin sensitivity. SPTLC2 or SMS2 deletion and subsequent decreases in sphingomyelin levels in the plasma membrane restored insulin signaling disrupted by HFD. Also exogenous sphingomyelin treatment impaired insulin-stimulated Akt phosphorylation [54].

Nonalcoholic steatohepatitis (NASH) is a major obesity-related disease that involves elevated proinflammatory cytokine activation, oxidative stress with mitochondrial dysfunction, leading to fibrogenesis, and finally liver cirrhosis [57–59]. The progress of these diseases is closely associated with dysregulated intracellular lipid accumulation and metabolism [57], and recently the involvement of sphingolipids in this process has been demonstrated. Inhibition of *de novo* sphingolipid synthesis with myriocin reduced triglyceride (TG) accumulation in liver [40]. Similarly, CerS6 deletion reduced CD36/FAT expression and increased palmitate β-oxidation, resulting in suppressed lipid accumulation [17]. Increased ceramide breakdown by aCDase induction prevented TG accumulation in liver from HFD feeding [60]. It has also been well documented that obesity induces ER stress [61] and important mechanism in the activation of lipogenesis [62]. Indeed, inhibition of the salvage pathway via aSMase deletion ameliorated HFD-induced hepatic steatosis via protection from ER stress [63]. However, acute ER stress augmented SK2 expression, inhibiting HFD-induced lipid accumulation in the liver [56], potentially functioning as an initial defense mechanism against HFD-induced lipotoxicity. In addition to the study demonstrating overexpression of SK2 suppressed lipid accumulation [56], deletion of SK2 significantly intensified HFD-induced hepatic steatosis [49]. The latter study demonstrated nuclear S1P was critical for histone acetylation and followed global gene transcription in lipid metabolism [49]. These studies highlight the importance of subcellular localization of sphingolipid metabolizing enzymes and their lipid products suggesting that specific pools of sphingolipids function to differentially regulate hepatic insulin resistance.

These studies demonstrate that HFD-induced alteration in sphingolipid metabolism disrupts normal physiology in liver. Specifically, increases in ceramides have been demonstrated to play a key role in insulin resistance, while future studies will be needed to determine the specific roles of sphingomyelin, sphingosine, and S1P.

4.2. Skeletal Muscle. Skeletal muscle is a primary site for glucose utilization and is significantly affected by HFD. Similar to the liver, HFD induces insulin resistance in skeletal muscle, inhibiting glucose uptake [64]. In myotubes palmitate-induced ceramide generation and exogenous C2-ceramide treatment impaired insulin-stimulated Akt activation [65]. Additionally, ceramide-induced PKCζ activation triggered the inhibitory phosphorylation of Akt [66]. Inhibition of nSMase, which should decrease ceramide generation, abolished palmitate-induced JNK and NF-κB activation and ER stress, conferring improvement in insulin resistance [67]. Furthermore, ceramide has also been implicated in the regulation of ectopic lipid accumulation in skeletal muscle and aggravation of insulin resistance [21, 68].

Recently, skeletal muscle has emerged as secretory tissue for cytokines, termed myokines [69]. Interleukin-6 (IL-6), the first described myokine, was found to be released from contracting skeletal muscle [70]. In relation to HFD, in C2C12 and mouse primary myotubes, palmitate stimulated IL-6 expression and secretion in an SK1- and S1PR3-dependent manner [48]. These data suggest that SK1, S1P, and S1PRs may play a role in skeletal muscle myokine generation. However, the definitive role for IL-6 from skeletal muscle is controversial in HFD-induced metabolic disorders [64].

4.3. Adipose Tissue. The traditional perception of adipose tissue is that of long-term energy storage. However, since the discovery of increased of tumor necrosis factor alpha (TNFα) in adipose tissue from obese individuals [71], this tissue has become the focus of numerous studies on the secretion of proinflammatory cytokines and consequent pathobiologies including insulin resistance [72, 73]. After identification of TNFα, various cytokines including leptin, PAI-1, and adiponectin have been defined as adipokines [74–76]. Adipose tissue consists primarily of adipocytes with minor population of preadipocytes and immune cells, such as lymphocytes and macrophages. Infiltration of macrophages occurs via recruitment by chemokines and cytokines secreted by adipose tissue resulting in exacerbation of obesity-induced proinflammatory responses [73]. HFD-induced ceramide levels have also been implicated in adipokine induction and secretion. CerS6 deletion, and subsequent decreased ceramide levels, inhibited expression of proinflammatory cytokines including IL-6 in response to HFD [17]. In addition, *de novo* synthesis of ceramide contributed to HFD

stimulation of monocyte chemoattractant protein-1 (MCP-1), suggesting that ceramide may play a role in recruitment of immune cells in adipose tissue [52]. Furthermore, this may suggest a role for adipose-derived ceramide in chronic and systemic inflammation via adipokine regulation in response to HFD.

In addition to adipokine regulation, ceramide has been shown to function in the regulation of adipose tissue. Inhibition of dihydroceramide desaturase reduced ceramide formation and increased dihydroceramide accumulation in 3T3-L1 mouse derived adipocytes and white adipose tissues in mice, leading to impaired differentiation and lipid accumulation [77]. This study implicates ceramide and dihydroceramide regulation of adipose tissue homeostasis.

C1P, generated by ceramide kinase, has not been well studied in response to HFD. The study that has been performed demonstrated that deletion of ceramide kinase abrogated increased mRNA expression of adipokines IL-6, TNFα, and MCP-1 and decreased macrophage recruitment to adipose tissue, in response to HFD [78]. This study suggests that ceramide kinase, and perhaps C1P, may function similarly to ceramide in the generation of adipokines.

S1PR modulators are being extensively utilized to define S1PR-mediated signaling events and pathobiologies [2]. Upon phosphorylation by SK2, FTY720 is able to bind S1PR1 inducing internalization and degradation of the receptor and sequestration of lymphocytes in secondary lymphoid tissues [79, 80]. In response to HFD, FTY720 prevented HFD-induced lymphocyte and macrophage infiltration in adipose tissue (although the mechanism for macrophages is unclear), resulting in reduced local inflammation and improved insulin resistance [81]. In addition to immune cell regulation, FTY720 has been shown to directly mediate HFD-induced effects on adipocytes. Phospho-FTY720 inhibited preadipocyte differentiation into mature adipocytes and stimulated lipolysis, reducing fat mass in HFD-fed mice [82]. In addition to receptor mediated effects on adipocytes, S1P itself has been implicated downstream of SK1 in lipid accumulation. Pharmacological or genetic inhibition of SK1 attenuated lipid accumulation in differentiating 3T3-L1 adipocytes [83]. These studies suggest involvement of S1P-S1PR signaling in HFD-induced adipose tissue dysregulation.

Sphingolipids in adipose tissue are potent mediators for adipokine expression and secretion in response to HFD. Moreover, sphingolipid enzymes and their product lipids may also regulate subsequent immune cell infiltration and proinflammatory signaling in adipose tissues in HFD and obesity.

4.4. Cardiovascular System. Systemic insulin resistance and excessive cytokine release due to obesity significantly alter the cardiovascular environment including blood pressure, coagulation, and fibrinolysis, ultimately leading to endothelial dysfunction and atherosclerosis [84]. Endothelial dysfunction is the failed balance between endothelium-dependent vasodilation and contraction. In addition to this imbalance, endothelial activation confers susceptibility to atherosclerosis [85]. Sphingolipids are significantly altered by HFD and palmitate treatment in the cardiovascular system, and these altered sphingolipids, specifically ceramide, seem to be essential in the regulation of obesity-induced cardiovascular diseases [86–89].

Endothelial dysfunction is mainly caused by dysregulation of endothelial nitric oxide synthase (eNOS), the enzyme that generates the potent vasodilator nitric oxide (NO) [85]. Palmitate- and HFD-induced generation of ceramide resulted in endothelial dysfunction, abolishing vascular endothelial growth factor (VEGF) or insulin-stimulated eNOS activation and subsequent NO production from endothelial cells [88, 89]. Inhibition of *de novo* synthesis with myriocin and SPTLC2 haploinsufficiency restored HFD-induced eNOS phosphorylation and achieved the normal range of vasodilation [88]. In addition, palmitate impaired angiogenesis in human umbilical vein endothelial cells (HUVECs) upon VEGF treatment, resulting in reduced tube formation in matrigel. This reduction in angiogenesis was rescued by myriocin, demonstrating the involvement of *de novo* generated sphingolipids in VEGF function [89].

Plasma sphingomyelin levels are reported to correlate with the increased risk of coronary heart disease [32]. In addition, high levels of sphingomyelin in low density lipoprotein (LDL) contributed to aggregation and retention of LDL in arterial wall. High levels of LDL-sphingomyelin supplied sufficient substrate for arterial SMase to increase LDL-ceramide, conferring aggravated atherosclerotic damage [90]. Consistently, LDL from SMS2 transgenic mice exhibit proatherogenic properties with increased *in vitro* aggregation upon SMase treatment [35]. Also, SMS2 overexpression in mice with adenovirus exaggerated atherosclerotic inflammation with elevations in cyclooxygenase-2 (COX-2) and matrix metalloproteinase-2 (MMP-2) in aorta [91]. Myriocin treatment decreased sphingomyelin and ceramide levels in plasma and consequently resulted in less atherosclerotic lesions in aorta from HFD-fed Apo-E deficient mice [87, 92, 93]. Moreover, bone marrow-derived SPTLC2 haploinsufficient macrophages also reduced atherosclerotic lesions in mice suggesting the importance of immune cell-derived sphingolipids in this disease (similar to adipose tissue) [86]. Meanwhile, the effect of glycosphingolipids and their metabolizing enzymes in atherosclerosis has been controversial [94–97]. The studies that have been conducted suggest that glycosphingolipids are necessary in atherosclerosis progress, but not a sufficient therapeutic target for this disease.

Altered sphingolipid metabolism has also been implicated in cardiomyopathy. A single study suggested that palmitate-induced ceramide and sphingomyelin accumulation in cardiac tissue resulted in Ca^{++} dysregulation and consequent systolic contractile dysfunction [98]. In a recent study by Russo et al., cardiac tissue was found to be enriched in SPTLC3, which has been shown to utilize the C14:0 saturated fatty acid myristate. This results in the *de novo* generation of d16:0/d16:1-base sphingolipids. Elevation of these d16:0/d16:1 sphingolipids resulted in increased apoptosis [25]. This study suggested the involvement of novel sphingolipid species in obesity-induced cardiomyopathy.

HFD-mediated dysregulation of sphingolipids contributes to obesity-related cardiovascular disease. In addition, ceramide and sphingomyelin contribute to endothelial dysfunction,

atherosclerosis, and cardiomyopathy in cardiovascular tissues. Together these studies suggest that dietary fat overload and obesity alter cardiovascular function are at least partially due to altered sphingolipid metabolism.

5. Conclusion

Sphingolipid metabolism is significantly affected by dietary nutrient oversupply, including HFD. Due to the complexity of the sphingolipid metabolism network, it is hard to define the regulation and role of a single sphingolipid species in HFD-involved biologies and pathobiologies. However, HFD significantly alters numerous sphingolipids species, impacting the downstream sphingolipid-mediated cellular signaling pathways. These changes in cellular signaling often contribute to HFD-induced toxicity in affected tissues: liver, muscle, adipose, and cardiovascular. Therefore, dissecting HFD-mediated sphingolipid metabolism and understanding the mechanisms by which HFD regulates these changes may lend insight into potential therapeutic and nutritional targets in obesity-related diseases.

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

Role of Ceramide from Glycosphingolipids and Its Metabolites in Immunological and Inflammatory Responses in Humans

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Glycosphingolipids (GSLs) are composed of hydrophobic ceramide and hydrophilic sugar chains. GSLs cluster to form membrane microdomains (lipid rafts) on plasma membranes, along with several kinds of transducer molecules, including Src family kinases and small G proteins. However, GSL-mediated biological functions remain unclear. Lactosylceramide (LacCer, CDw17) is highly expressed on the plasma membranes of human phagocytes and mediates several immunological and inflammatory reactions, including phagocytosis, chemotaxis, and superoxide generation. LacCer forms membrane microdomains with the Src family tyrosine kinase Lyn and the Gαi subunit of heterotrimeric G proteins. The very long fatty acids C24:0 and C24:1 are the main ceramide components of LacCer in neutrophil plasma membranes and are directly connected with the fatty acids of Lyn and Gαi. These observations suggest that the very long fatty acid chains of ceramide are critical for GSL-mediated outside-in signaling. Sphingosine is another component of ceramide, with the hydrolysis of ceramide by ceramidase producing sphingosine and fatty acids. Sphingosine is phosphorylated by sphingosine kinase to sphingosine-1-phosphate, which is involved in a wide range of cellular functions, including growth, differentiation, survival, chemotaxis, angiogenesis, and embryogenesis, in various types of cells. This review describes the role of ceramide moiety of GSLs and its metabolites in immunological and inflammatory reactions in human.

1. Introduction

Biological membranes are mainly composed of phospholipids, sphingolipids, cholesterol, and membrane-associated proteins. These molecules are nonhomogeneously distributed in membranes and can rearrange, leading to the formation of membrane "domains" with highly differentiated molecular compositions and supramolecular architectures, which are stabilized by lateral interactions among the membrane components. Although glycosphingolipids (GSLs) were originally thought to be structural components of plasma membranes [1], several experiments suggested that GSLs are involved in the regulation of numerous cellular functions [2]. The membrane lipid bilayer is a stable structure, constituting a physical boundary between intra- and extracellular environments. GSLs are expressed on the surface of cellular membranes. Based on their physicochemical properties, especially their many hydroxyl and acetamide groups, which can act as hydrogen bond donors and acceptors, GSLs form clusters through cis interactions [2]. There is a general consensus on the roles played by the ceramide moiety of GSLs in promoting the formation and stabilization of membrane lipid domains. In addition, ceramide was also shown to be involved in GSL-mediated functions and several biological activities [3, 4]. Ceramide is composed of sphingosine and fatty acid chains. We recently showed that very long fatty acid chains of ceramide, such as C24:0 and C24:1, are responsible for the direct connection between lactosylceramide (LacCer, CDw17) and palmitoylated signal transducer molecules [5]. Moreover, the phosphorylated product of sphingosine,

sphingosine-1-phosphate (S1P), was shown to be important in immunological, especially inflammatory reactions [4, 6].

This review describes the role of the fatty acid chains of ceramide in GSL-mediated outside-in signaling in promoting GSL-enriched domain-mediated cellular functions, as well as the activities of S1P in inflammatory reactions of keratinocytes in human.

2. Organization of GSL-Enriched Lipid Microdomains

GSLs on biological membranes tend to form specific domains with several types of molecules. The most studied GSL-enriched domains are membrane lipid microdomains, called lipid rafts, defined by their GSL- and cholesterol-rich nature, enrichment in GPI-anchored proteins and membrane-anchored signaling molecules, and cytoskeletal association [7, 8]. As shown in artificial membrane models, GSLs tend to form clusters [9], with this cluster formation confirmed in intact cells by immunoelectron microscopy [10–12]. The GSL-enriched microdomains on plasma membranes have a diameter of 50–100 nm and include signal transducer molecules, such as Src family kinases [11, 12]. GSLs that contain saturated fatty acid chains with higher transition temperatures [13] show ordered, less fluid, liquid phase. Cholesterol is composed of a highly hydrophobic sterol-ring system and 3-hydroxy moiety, the only hydrophilic part of the molecule. The small cholesterol sterol-ring system and the ceramide moiety of sphingolipids are thought to interact via hydrogen bonds and hydrophobic van der Waal's interactions [14]. In addition, hydrophilic interactions between sugar moieties of GSLs promote the lateral association of GSLs and cholesterol. In contrast, phospholipids have low acyl chain melting temperatures and unsaturated acyl chains. Phospholipids tend to be loosely packaged in bilayers, resulting in the formation of liquid-disordered membranes that allow rapid lateral and rotational movement of lipids [15]. These interactions result in the separation of GSL- and cholesterol-enriched lipid microdomains from other phospholipids in the cell membrane and the formation of distinct domains.

Electron microscopy using labeled anti-GSL antibodies has revealed GSL clusters on the surface of glycosphingolipid/phosphatidylcholine (PC) liposomes, even in the absence of sphingomyelin (SM) and cholesterol [2]. LacCer forms clusters, consisting of LacCer-enriched microdomains, on plasma membranes [11]. The anti-LacCer mAbs T5A7 and Huly-m13 recognized LacCer on human neutrophils, but only T5A7 recognized LacCer on mouse neutrophils. Interestingly, Huly-m13 but not T5A7 can be used for immunoprecipitation [16], suggesting a difference in binding and/or cluster formation of Huly-m13 and T5A7 to LacCer-enriched microdomains. Indeed, stimulated emission depletion (STED) superresolution microscopy showed that T5A7 and Huly-m13 bind to different regions of the same LacCer/dioleoylphosphatidylcholine (DOPC) liposomes (Figure 1) [17]. LacCer-enriched microdomains are composed of LacCer, SM, phospholipids, and cholesterol. Surface plasmon resonance analysis showed that reduction of the LacCer content in the DOPC/cholesterol/LacCer/SM lipid layer markedly decreased Huly-m13 but not T5A7 binding to LacCer [17], suggesting that the content of LacCer in LacCer-enriched microdomains affects the binding avidity of Huly-m13 to LacCer. In contrast, the molecular species of PC, including DOPC, dipalmitoylphosphatidylcholine (DPPC), and palmitoyl-oleoyl-phosphatidylcholine (POPC), did not affect the binding avidity of Huly-m13 to LacCer-coated plastic wells. Lactose inhibited the binding of Huly-m13 to LacCer/DOPC liposome-coated and DOPC/LacCer mixture-coated plastic wells, suggesting that Huly-m13 binds only to LacCer clusters in LacCer-enriched microdomains. In contrast, the binding avidity of T5A7 to LacCer-coated plastic wells was much weaker than its binding avidity to DOPC/LacCer-, POPC/LacCer-, and DPPC/LacCer mixture-coated wells, suggesting that the binding of T5A7 to LacCer is affected by PC. The ability of lactose to inhibit the binding of T5A7 to DOPC/LacCer liposome-coated plastic wells was similar to its ability to inhibit the binding of Huly-m13 [17]. In contrast, lactose inhibition of T5A7 binding to DOPC/LacCer mixture-coated plastic wells was significantly lower than its inhibition of Huly-m13 binding, suggesting that T5A7 recognizes the PC-enhanced three-dimensional structure of LacCer clusters. Thus, Huly-m13 may bind to the core region of lactose clusters in LacCer-enriched domains, while T5A7 binds to "dispersed" LacCer clusters in the phase boundary regions of these microdomains. These findings suggest that the specificities of these antibodies against the same GSLs are dependent on the organizations of the GSLs and molecules surrounding the GSL-enriched domains.

3. GSL Metabolism Diseases

Disorders of the degradation of GSLs sometimes cause human diseases [18, 19]. For degradation, GSLs are endocytosed and reach endosomes and other organelles. Then, those molecules are constitutively degraded by their suitable catabolic enzymes. When the activities of lysosomal enzymes are impaired, degradation is not able to proceed normally and undegraded molecules accumulate in the organelle and intracellular membranes, causing several metabolism diseases. For instance, genetic disorder of glucocerebrosidase (GBA) (EC 3.2.1.45; [20]), Gaucher disease, results in accumulation of GlcCer and its deacetylated form glucosylsphingosine is caused by abnormality of GBA. Gaucher disease is a multisystem disorder whose features include peripheral blood cytopenias, hepatosplenomegaly, bone disease, and neurological manifestations in some cases [21]. The form of intravenous enzyme replacement therapy in the 1990s has been developed and resulted in dramatic improvements in haematological and visceral disease [22]. Recognition of complications, including multiple myeloma and Parkinson disease, has challenged the traditional macrophage-centric view of the pathophysiology of this disorder. However, the pathways by which enzyme deficiency results in the clinical manifestations of this disorder remain obscure. In spinal

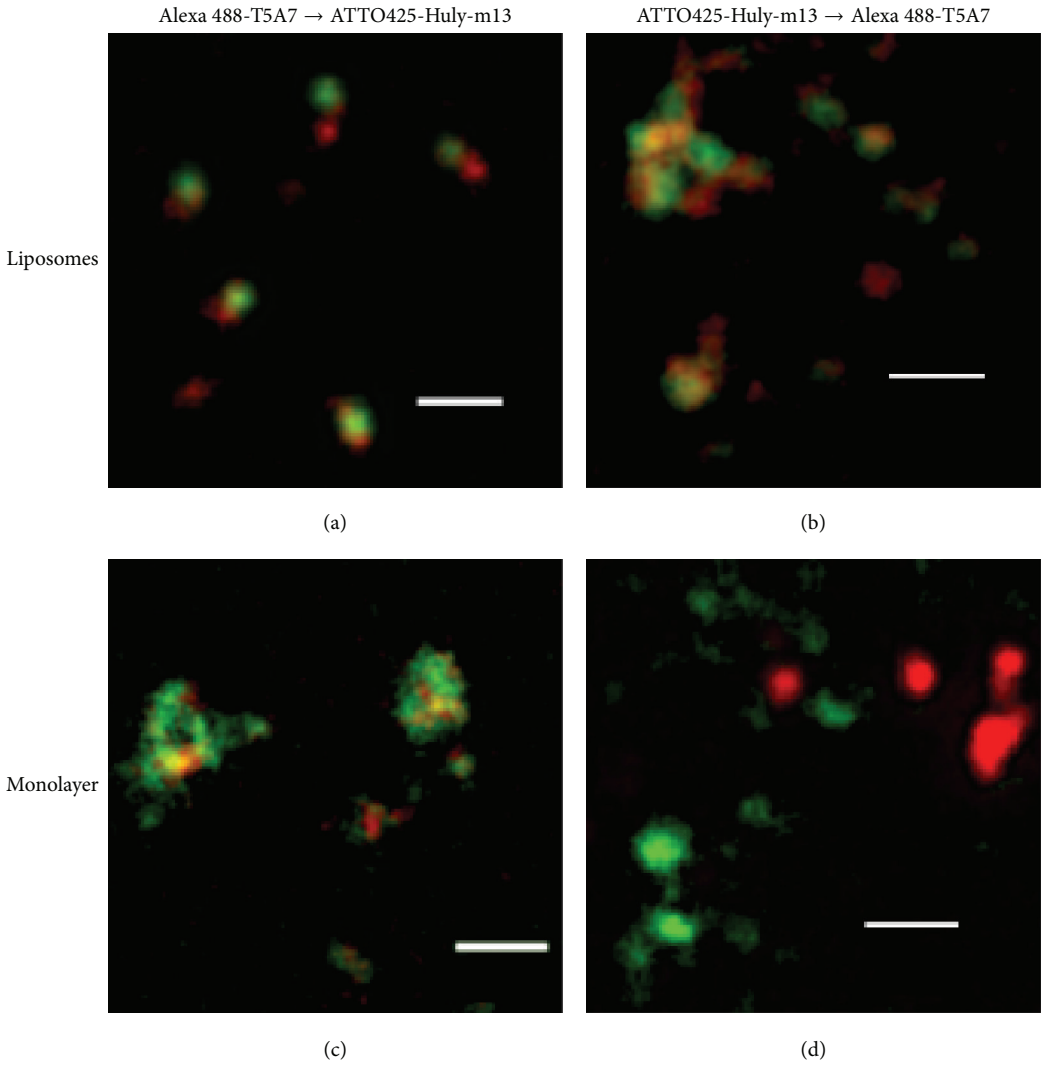

Figure 1: Stimulated emission depletion (STED) microscopic observation. The LacCer/DOPC liposomes (a, b) and LacCer and DOPC in ethanol (c, d) were coated onto the back surfaces of 96-well NUNC Immunoplates, followed by overnight incubation at room temperature with gentle shaking. The chemical condition of the backside surface of the plate was the same as the surfaces of the wells. The coated plates were blocked with BSA, sequentially stained with Alexa 488-T5A7 (green) ATTO425-Huly-m13 (red) (a, c) or ATTO425-conjugated Huly-m13 (red) → Alexa 488-conjugated T5A7 (green) (b, d), and viewed under a TCS STED CW superresolution microscope (Leica), with signals detected using a GaAsP hybrid detection system (Leica). Deconvolution was performed using Huygens STED deconvolution software (Leica). The panels on the right show enlargements of those on the left. White bars depict 1 μm.

cords of amyotrophic lateral sclerosis (ALS) patients, levels of GM1, GM3, LacCer, GlcCer, GalCer, and ceramide were significantly elevated [23]. Furthermore, glucocerebrosidase-1, glucocerebrosidase-2, hexosaminidase, galactosylceramidase, α-galactosidase, and β-galactosidase activities were also elevated in those patients. Inhibition of glucosylceramide synthesis accelerated disease course in ALS model mice, whereas infusion of exogenous GM3 significantly slowed the onset of paralysis and increased survival. These observations suggest that GSLs and their metabolism are likely important participants in pathogenesis of ALS. Further studies about GSL metabolism pathways in GSL-related disease will serve to advance our understanding of other associated disorders.

4. GSL- and Ceramide-Enriched Membrane Microdomains Are Binding Targets for Pathogenic Microorganisms

Over the last 30 years, many studies have indicated that GSLs expressed on the cell surface may act as binding sites for microorganisms. The binding avidities of microorganisms to several types of GSL [24–27] suggest that GSLs are involved in host-pathogen interactions. Indeed, microorganisms have been shown to recognize and enter host cells *via* GSL-enriched membrane microdomains on the cells [28]. Among GSLs, LacCer has been well described to bind to several kinds of microorganisms, including viruses and

fungi [27]. For instance, *Candida albicans* specifically bind to LacCer though the binding of β-1,6-long glucosyl side-chain-branched β-1,3-glucan to LacCer-enriched domains [26, 29]. It is also well known that microorganisms-derived toxins, such as Shiga toxin, specifically bind to GSLs [30–32]. Furthermore, a sphingolipid metabolite, ceramide, has been demonstrated to play a crucial role in pulmonary infection and inflammation [33]. Ceramide, which is degraded product of GSLs and sphingomyelin, has been reported to form ceramide-rich membrane platforms and involve uptake of several microorganisms including *Pseudomonas aeruginosa*. Abnormal amounts of enzymes involved in the synthesis of ceramide have been demonstrated in emphysematic smokers and in patients with severe sepsis [34]. Therefore, GSLs and their metabolites play important roles in infection and inflammation.

5. Fatty Acid Chains of Ceramide Are Indispensable for GSL-Mediated Signaling

GSLs have been reported to interact with membrane proteins and modulate the properties of these proteins [2, 25]. In addition, certain proteins, including glycosylphosphatidylinositol- (GPI-) anchored and palmitoylated proteins, tend to enter GSL-enriched membrane microdomains [13]. These observations suggested that GSLs may be involved in transferring information across membranes. However, the mechanism by which GSLs interact with proteins and mediate outside-in signaling is unclear. The ceramide moiety consists of a long chain base linked to a fatty acid chain. Sphingosine containing C18 carbons [(2*S*,3*R*,4*E*)-2-amino-1,3-dihydroxy-octadecene] is generally the main structure in mammals, but a structure containing 20 carbons is relatively abundant in neurons. However, the fatty acid content of GSL ceramide is highly heterogeneous [35]. Ceramide is synthesized by ceramide synthases (CerS) 1–6, each of which uses a restricted subset of fatty acyl-CoAs for N-acylation of the sphingoid long chain base [36]. The expression levels of genes encoding CerS are tissue specific, suggesting that the molecular varieties and expression patterns of GSLs are associated with the functions of these cells [37].

Although GSL-enriched microdomains have been implicated in a number of important membrane events [2, 38, 39], the molecular mechanisms responsible for GSL-mediated cell functions are still unclear. One of the main issues centers around the association of GSLs with signal transducer molecules localized on the cytosolic side. However, we recently analyzed LacCer-enriched microdomains in human neutrophilic lineage cells [38]. LacCer, along with the Src family kinase Lyn, forms lipid microdomains on the plasma membranes of human neutrophils and is involved in several cellular functions, including chemotaxis, phagocytosis, and superoxide generation, highly dependent on Lyn [16, 29, 38]. HL-60 cells differentiated into neutrophilic lineage cells by DMSO (D-HL-60) were found to acquire superoxide generating activity, but not through LacCer, despite their expression of LacCer on plasma membranes [38]. Most LacCer and Lyn were recovered in the microdomain fractions of neutrophils and D-HL-60 cells. Lipidomics analysis revealed that LacCer in the neutrophil plasma membrane was mainly composed of molecular species containing C16:0, C24:1, and C24:0 fatty acid chains, whereas over 70% of LacCer in the plasma membranes of D-HL-60 cells contained C16:0 fatty acid chains, but only about 14% were C24:1 and C24:0 [11]. Lyn was immunoprecipitated by anti-LacCer antibody in neutrophils but not D-HL-60 cells. Importantly, Lyn was coimmunoprecipitated by anti-LacCer antibody from the detergent resistant membrane (DRM) fraction of plasma membranes from C24:0 and C24:1, but not C16:0 or C22:0, LacCer-loaded D-HL-60 cells. Anti-LacCer antibody induced superoxide generation from D-HL-60 cells loaded with C24:0-LacCer, but not C16:0-LacCer. Lyn colocalized with LacCer-enriched domains of D-HL-60 cells loaded with C24:0-LacCer, but not C16:0-LacCer. These results suggested that the C24 fatty acid chain of LacCer is indispensable for connecting Lyn with LacCer-enriched microdomains. Knockdown of Lyn molecules by human Lyn-specific short interfering RNA (siRNA) in D-HL-60 cells completely abolished the effects of C24:1-LacCer loading function [11], suggesting that Lyn is crucial for C24-LacCer-mediated neutrophil function. Experiments using azide-photoactivatable tritium-labeled C24- and C16-LacCer revealed that C24- but not C16-LacCer directly associated with Lyn and a heterotrimeric G protein subunit Gαi. These results confirm a specific direct interaction between C24-LacCer and the signal transduction molecules Lyn and Gαi, which are associated with the cytoplasmic layer via palmitic acid chains (Figure 2). LacCer species with very long fatty acids are indispensable for Lyn-coupled LacCer-enriched membrane microdomain-mediated neutrophil functions.

GPI-anchored proteins are composed of glycerol phospholipids, which do not have C24 fatty acid chains, suggesting that GPI-anchored proteins are not able to form large clusters by themselves and cannot directly connect with signal transduction molecules through fatty acid chains. To mediate cell functions, GPI-anchored proteins require signal transduction molecule-coupled transmembrane proteins or GSL-enriched domains, such as LacCer-enriched domains [40]. Further studies are required to determine the organization and signaling mechanisms of membrane microdomains.

6. Role of Ceramide Metabolite Sphingosine-1-phosphate in Immunological Reactions of Human Keratinocytes

The epidermis consists of a single layer of proliferating undifferentiated keratinocytes, the stratum basale, and several superficial layers of the stratum spinosum and stratum granulosum (SG), which form the stratum corneum (SC). The SC acts as an air-liquid interface barrier to avoid drying of tissues in contact with air. Ceramide is the main component of SC and is important for the water retention and permeability barrier functions of SC. Ceramides account for 30–40% of SC lipids [41, 42]. All ceramide molecules in the SC are derived from GlcCer and SM [43]. CDase hydrolyzed ceramide to yield sphingosine and fatty acids. Sphingosine can be phosphorylated by sphingosine kinase

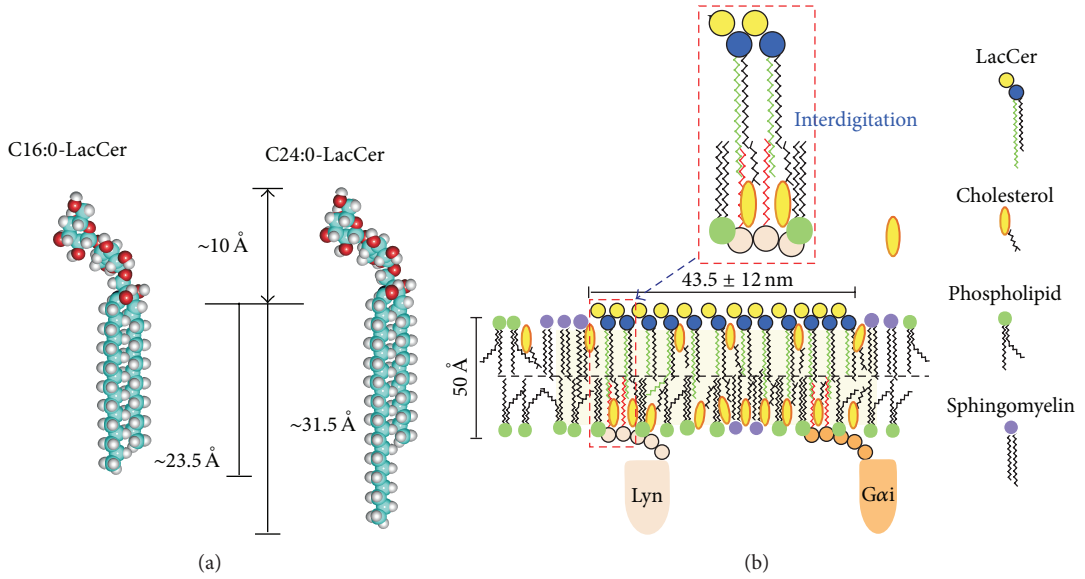

Figure 2: LacCer-enriched microdomains. (a) Size of LacCer microdomains containing C16:0 and C24:0 fatty acid chain. (b) LacCer forms lipid microdomains on plasma membrane of human neutrophils and acts as a signal transduction platform. The C24 fatty acid chains of LacCer interdigitate into inner leaflet of plasma membranes and directly interact with Lyn and Gαi. These molecules associate with LacCer to mediate signaling from outside to inside, resulting in neutrophil chemotaxis, migration, and phagocytosis.

to form S1P, a molecule involved in a wide range of cellular functions, including growth, differentiation, survival, chemotaxis, angiogenesis, and embryogenesis, in various types of cells [44, 45]. S1P was shown to inhibit keratinocyte proliferation, to promote corneocyte differentiation [46], and to chemoattract keratinocytes. Roles of S1P in skin immunological functions have been demonstrated in mouse models [45, 47–51]. Mice are the good experimental tool of choice for the majority of immunologists, and the study of immune responses in mice has provided considerable insight into human immune system function. However, there are significant differences in immunological reactions between mice and human [52]. Little is known, however, about the role of ceramide metabolites in the immunological functions of differentiating keratinocytes.

A neutral CDase from *Pseudomonas aeruginosa* AN17 (PaCDase) isolated from a patient with atopic dermatitis (AD) was shown to require detergents to hydrolyze ceramide [53]. *Staphylococcus aureus*-derived lipids, which consist primarily of cardiolipin and phosphatidylglycerol, enhanced the PaCDase hydrolysis of normal ceramide and of human skin-specific omega-hydroxyacyl ceramide in the absence of detergents [11]. A three-dimensionally cultured human primary keratinocyte (3D keratinocyte) culture system has been utilized to simulate epidermal differentiation at its air-liquid interface, resulting in the generation of basal, spinous, and granular layers and an SC, with the latter displaying permeability barrier functions [54]. Treatment of 3D keratinocytes with PaCDase and water-soluble stimulants of keratinocytes, including trypsin, *Dermatophagoides pteronyssinus* class 1 allergen (Der p1), and *Dermatophagoides farinae* allergen (Der f1) had no effect on the expression of any of the genes in our DNA microarray analysis [55], indicating that the SC of the 3D keratinocyte culture acts as a permeability barrier. Triton X-100 is a detergent that reduces permeability barrier functions, thereby moderately increasing transepidermal water loss and the production of erythema on human skin [56]. In the presence of 0.1% Triton X-100, PaCDase markedly enhanced TNF-α mRNA expression in 3D keratinocytes, an increase not observed in cells treated with Triton X-100 alone [55]. TNF-α mRNA expression was not enhanced by heat-inactivated or mutant PaCDase, suggesting that ceramide metabolites induce TNF-α mRNA expression in keratinocytes. TNF-α, a critical cytokine in several dermatological diseases [57], is secreted by keratinocytes [58] and shown to be involved in the progression of atopic dermatitis (AD) [59]. Among the metabolites of ceramide, only sphingosine and S1P enhanced TNF-α mRNA levels in 3D keratinocytes. S1P is synthesized from sphingosine by sphingosine kinase (SphK) and stimulates 3D keratinocytes through specific receptors [60]. Both the specific SphK inhibitor CAS 1177741-83-1 and the S1P receptor antagonist VPC 23019 suppressed the PaCDase-induced expression of TNF-α mRNA in 3D keratinocytes. S1P is generally considered to stimulate cells through plasma membrane G protein-coupled receptors, for example, S1P1–S1P5 [61]. S1P was recently shown to activate NF-κB [62] independently of S1P receptors [63]. However, VPC2301, a competitive antagonist for S1P1 and S1P3 receptors, inhibited the PaCDase-enhanced gene expression not only of TNF-α but also of endothelin-1 and IL-8 [55]. Thus, the S1P-induced production of these inflammatory mediators is mediated by S1P receptors in human primary keratinocytes in a 3D culture system. cDNA microarray analysis showed that S1P strongly upregulated the expression of endothelin-1, CXCL1, TNF-α, β-defensin 5, IL-8, CXCL2, interferon

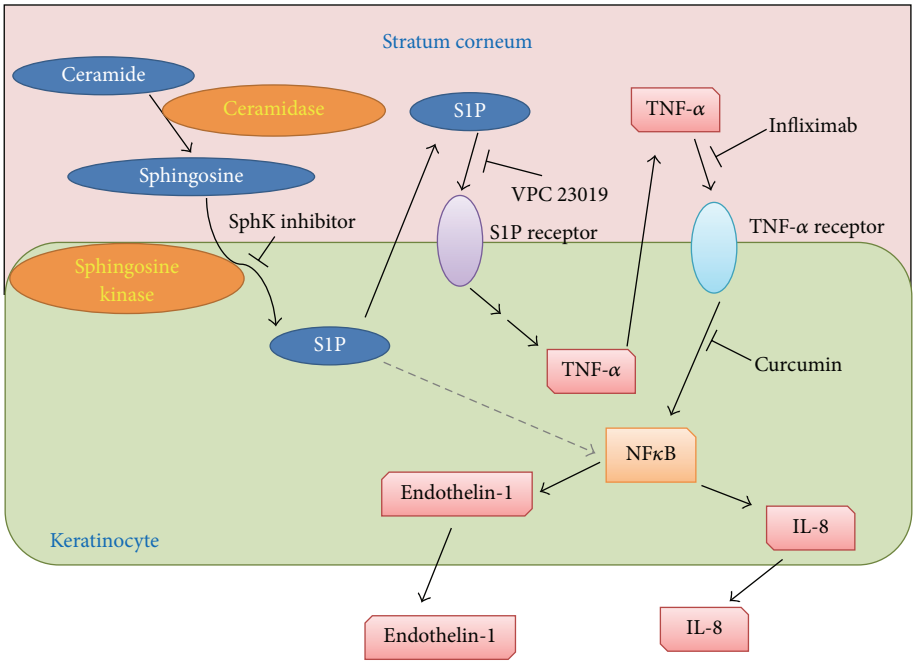

Figure 3: Schematic mechanism of the production of inflammatory mediators by ceramide metabolites in human keratinocytes. PaCDase degrades ceramide into sphingosine in the stratum corneum, and sphingosine is converted to S1P by SphK of keratinocytes. S1P is then released extracellularly and binds to S1P receptors, resulting in the production and release of TNF-α. The released TNF-α binds to TNF-α receptors, activating NF-κB and inducing the production of IL-8 and endothelin-1.

regulatory factor 1, GADD45 gamma, and IL-23α subunit mRNAs [55]. IL-8, CXCL1, and CXCL2 have been reported to be upregulated in the lesional skin of patients with AD and psoriasis [64]. S1P also enhanced the expression of claudin-4 mRNA, which has been observed in more layers of psoriatic than normal epidermis [65]. TNF-α can induce the production of endothelin-1 and IL-8 by human keratinocytes [66, 67]. Epidermal keratinocytes produce and respond to TNF-α via TNFR1 [68]. Infliximab, a chimeric IgG1κ monoclonal antibody against human TNF-α, inhibits the TNF-α-mediated production of IL-8 by keratinocytes [69]. PaCDase-induced phosphorylation of NF-κB p65 was markedly suppressed by infliximab [55]. The NF-κB inhibitor curcumin inhibited PaCDase-induced expression of IL-8 and endothelin-1 mRNAs but not of TNF-α mRNA. TNF-α induces IL-8 production via NF-κB [70]. Therefore, it is likely that S1P induces TNF-α production and release from 3D keratinocytes via S1P receptors, resulting in TNF-α induction of cytokine production through NF-κB-mediated signal transduction (Figure 3). TNF-α is a critical cytokine in psoriatic immunopathology, and the development of an effective strategy is required to counteract its effects [57]. Infliximab, which is used to treat patients with plaque psoriasis, psoriatic arthritis, pustular psoriasis (excluding localized type), and psoriatic erythroderma [71, 72], downregulates antiapoptotic proteins in regressing psoriatic skin [72]. The effects of infliximab have also been evaluated in other inflammatory dermatoses and in systemic diseases involving the skin, pityriasis rubra pilaris, pyoderma gangrenosum, and cutaneous sarcoidosis [73]. AD is characterized by a marked reduction in ceramides in the SC of lesional and nonlesional forearms [74, 75] and by increased activities of the enzymes ceramidase (CDase). The metabolic conversion of ceramide to S1P has been found to protect keratinocytes against UVB-induced, ceramide-mediated apoptosis [76]. These observations suggest that ceramide metabolites, especially S1P, are involved in AD. AD is a common pruritic, inflammatory skin disorder [77]. Chronic, localized, or even generalized pruritus is the diagnostic hallmark of AD. Histamine H1-receptor blockers are used to treat all types of itch resulting from serious skin diseases, such as AD, as well as from renal and liver diseases. However, they often lack efficacy in chronic itch, a profound clinical problem that decreases quality of life [78]. Nerve density in the epidermis is partly involved in itch sensitization in pruritic skin diseases, such as AD [79]. Endothelin-1 has been shown to elicit itch in humans [80–82]. The molecular pathways that contribute to the transduction of itch responses to endothelin-1 do not require either PLCβ_3 or TRPV1 of neurons, which mediate histamine- and serotonin-induced itch responses, respectively [83]. Thus, keratinocyte-produced S1P may be involved in endothelin-1-mediated pruritus in AD. Therefore, atopic dermatitis may be exacerbated by treatment with S1P analogue FTY720.

7. Conclusion

Several reports have described the roles of ceramide metabolites in immunological and inflammatory diseases [84–88]. However, the physiological roles of GSL-enriched

microdomains are largely undetermined, although much is known about the organization and functions of LacCer-enriched microdomains [24, 89, 90]. The analogous patterns of GSLs and motifs of PAMPs result in the generation of autoantibodies against these GSLs, inducing severe autoimmune inflammatory diseases [91]. Antibodies against neuronal tissues are involved in immune-mediated neurological disorders, with expression of several of these antibodies found to correlate with the pathophysiology of these diseases [92, 93]. Therefore, elucidation of their organization and structural specificities, based on interactions between GSLs and surrounding molecules, are important for understanding the physiological functions of GLS-enriched microdomains and their related diseases.

Abbreviations

GSL:	Glycosphingolipid
PaCDase:	*Pseudomonas aeruginosa*-derived neutral ceramidase
S1P:	Sphingosine-1-phosphate
3D keratinocytes:	Three-dimensionally cultured human primary keratinocytes
SphK:	Sphingosine kinase.

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

Sphingosine-1-Phosphate and Its Receptors: A Mutual Link between Blood Coagulation and Inflammation

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Sphingosine-1-phosphate (S1P) is a versatile lipid signaling molecule and key regulator in vascular inflammation. S1P is secreted by platelets, monocytes, and vascular endothelial and smooth muscle cells. It binds specifically to a family of G-protein-coupled receptors, S1P receptors 1 to 5, resulting in downstream signaling and numerous cellular effects. S1P modulates cell proliferation and migration, and mediates proinflammatory responses and apoptosis. In the vascular barrier, S1P regulates permeability and endothelial reactions and recruitment of monocytes and may modulate atherosclerosis. Only recently has S1P emerged as a critical mediator which directly links the coagulation factor system to vascular inflammation. The multifunctional proteases thrombin and FXa regulate local S1P availability and interact with S1P signaling at multiple levels in various vascular cell types. Differential expression patterns and intracellular signaling pathways of each receptor enable S1P to exert its widespread functions. Although a vast amount of information is available about the functions of S1P and its receptors in the regulation of physiological and pathophysiological conditions, S1P-mediated mechanisms in the vasculature remain to be elucidated. This review summarizes recent findings regarding the role of S1P and its receptors in vascular wall and blood cells, which link the coagulation system to inflammatory responses in the vasculature.

1. Introduction

Sphingosine-1-phosphate (S1P), a highly active lipid mediator, exhibits a broad range of cellular activities including proliferation, survival, adhesion, and migration [1, 2]. S1P is critical for mammalian cardiac development and for maturation of the systemic circulatory system [3]. These biological actions are carried out by predominantly intracellularly produced S1P via sphingosine kinase (SphK), of which two isoforms SphK1 and SphK2 exist [4, 5]. Moreover, S1P has emerged as an intracellular second messenger involved in regulation of cell proliferation and in mobilization of internal calcium stores by a protein kinase C independent pathway [6]. Further reports suggest that S1P found within the extracellular space is not merely derived from intracellular generation but biosynthetic enzymes of the S1P metabolism appear to subsist in the extracellular space [7]. Indeed, the majority of studies have focused on the functions of extracellular S1P. This "outside the cell" S1P acts in an autocrine or paracrine manner as an agonist for a unique family of G-protein-coupled receptors which to date comprises the five S1P receptors (S1PRs) S1PR1–S1PR5 [8, 9]. Extracellular S1P regulates proliferation and migration of vascular endothelial cells (ECs) [10] and smooth muscle cells (VSMCs) [11] and critically determines lymphocyte egress and angiogenesis [12]. Both S1P and S1PRs regulate vascular tone either by directly modulating the smooth muscle layer or by stimulating ECs to release bioactive molecules which regulate VSMCs responses in a paracrine manner [13].

The levels of S1P in plasma and tissues are tightly regulated by the balance between its synthesis by sphingosine

kinases and degradation [2, 14]. The role of S1P and the processes involved in its biosynthesis, that is, regulation of the metabolizing enzymes, for controlling vascular integrity has been studied thoroughly *in vitro* and *in vivo* [15]. Vascular proliferative disorders such as atherosclerosis and persistent proinflammatory challenges of the vessel wall [16] are characterized by the activation of the coagulation cascade and platelet activation, both processes which elevate local S1P concentrations [17]. This may play an important role in directing immune cells to sites of local injury and directly links the coagulation system to S1P-mediated inflammatory responses *in vivo*. After vascular injury, the coagulation cascade is initiated by activating the clotting factors X (FXa) and ultimately thrombin, which are both key regulators of subsequent tissue repair and remodeling [18, 19]. FXa-mediated thrombin generation initiates and is itself amplified by subsequent platelet activation, finally leading to cleavage of fibrinogen and eventually the formation of the mural thrombus [20, 21]. In addition to their physiological function in hemostasis, the clotting proteases thrombin and FXa are also accountable for clinically relevant pathological responses such as postphlebitic inflammatory and tissue repair reactions [16, 22]. The biological effects of FXa and thrombin are mediated via a family of G-protein-coupled receptors, protease-activated receptors 1, 2, 3, and 4 (PAR-1–PAR-4) [23, 24]. Thrombin initiates signaling through PAR-1, PAR-3, and PAR-4, while FXa acts via PAR-1 and PAR-2. Previous reports, including studies from our group, have reported that PARs stimulate VSMCs proliferation and migration, modify the composition of the extracellular matrix of blood vessels, and mediate proinflammatory responses in the vessel wall [25–28]. Because proliferation and migration of VSMCs are considered key events in the development of atherosclerosis and vascular remodeling, these cellular effects of thrombin and FXa may directly contribute to the pathogenesis of vascular diseases such as progression of atherosclerosis and restenosis after vascular injury. In addition, recent studies highlight numerous interactions between blood coagulation and the S1P signaling system [17, 29].

This review discusses the recent findings concerning the role of S1P and its receptors in vascular and blood cells which are interlinked with the coagulation system. Particularly, hemostasis-related mechanisms which increase local S1P availability and the regulation of PAR receptor expression by S1P are highlighted. Elucidating the complex interactions between blood coagulation and the S1P signaling network further may bear the potential to discover and develop novel targets for the therapy of inflammation-prone vascular diseases.

2. Biosynthesis, Degradation, and Functions of S1P in the Vascular System

S1P biosynthesis is tightly interlinked with the metabolism of ceramide. Ceramide is formed either *de novo* from serine, palmitoyl CoA, and fatty acid or from breakdown of membrane-resident sphingomyelin [30, 31]. Ceramide is further converted to sphingosine by enzymatic action of ceramidase. Finally, the bioactive lysophospholipid S1P is produced by phosphorylation of sphingosine. This reaction is catalyzed by the two sphingosine kinase isoenzymes SphK1 and SphK2. Maintaining a balance between S1P generation and degradation is critical for regulation of cell growth and plays a key role in pathological processes such as carcinogenesis [32]. S1P degradation is achieved via reversible dephosphorylation by two S1P-specific phosphatases (SPP1 and SPP2) or irreversible hydrolysis by S1P lyase. S1P exerts actions either by binding to its intracellular targets or through its specific receptor in autocrine, paracrine, and/or endocrine manner [31].

S1P is secreted, stored, and exported by the cells of the vessel wall, VSMCs, and ECs, respectively. Recent observations highlight the critical role of the putative S1P transporter spinster homolog 2 (Spns2) in endothelial S1P release and in lymphocyte trafficking [33, 34]. In other cell types, that is, breast cancer and mast cell, the ABC (ATP-binding cassette) transporter family members ABCC1 and ABCG2, known regulators of inflammatory processes, facilitate export of S1P across the cell membrane [35, 36]. S1P regulates a diverse range of cellular processes that are important in immunity, inflammation, and inflammatory disorders [37, 38]. Once secreted, most of the S1P binds to albumin or serum lipoproteins [39]. Whether this carrier-bound serum S1P or rather locally produced S1P is important for the diverse cellular functions is a matter of current debate [40]. The metabolism and distinct vascular functions of S1P are highlighted in Figure 1.

3. Interactions of S1P Receptors and Thrombin Receptors Affect Endothelial Function

Endothelial cells synthesize and secrete large amounts of S1P and contribute substantially to generating the high S1P level present in the blood [40, 41]. Of the five S1PRs, endothelial cells express S1PR1, S1PR2, and S1PR3 [29]. S1P modulates diverse endothelial activities including proliferation [42], survival [43], migration [44], and regulation of proinflammatory responses [45] and controls the endothelial barrier function [46–49]. S1PR1 is highly expressed in endothelial cells [50] and regulates cytoskeletal structure, migration, and vessel maturation [51, 52]. In S1PR1 receptor deficient embryos, blood vessels were incompletely covered by VSMCs, indicating that S1PR1 also regulates vascular maturation [53]. Thus, S1PR1 appears to mediate predominantly physiological functions while particularly S1PR2 regulates inflammatory endothelial responses and is upregulated during inflammatory conditions such as atherosclerosis [45, 54]. These assumptions are in agreement with recent observations of varying S1P concentrations resulting in differential receptor activation [55] and the differential regulation of S1PR1 and S1PR2 expression during conditions of hyperglycemia-induced endothelial cell dysfunction [56].

A key regulator of endothelial function is the coagulation system with factors such as thrombin known to affect its permeability [57] as well as endothelial inflammation [58]. Thrombin causes induction of endothelial cell contraction

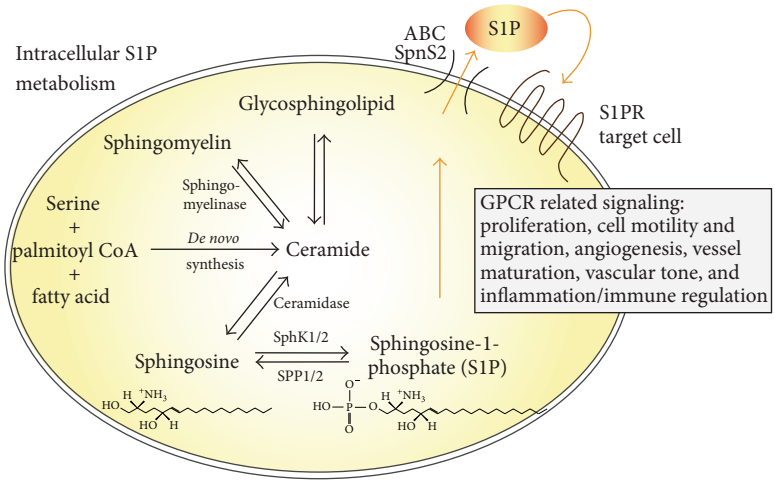

Figure 1: Synthesis of S1P and functions in the vascular system. *De novo* ceramide synthesis in general originates from condensation of serine, palmitoyl CoA, and fatty acid, a multistep enzyme catalysed process. Ceramide can be converted reversibly into sphingomyelin by sphingomyelinase or to glycosphingolipids. It is further metabolised by ceramidase to sphingosine, which can then be phosphorylated into S1P by sphingosine kinase isoforms 1 and 2 (SphK1/2). This phosphorylation can be reverted by the S1P phosphatases SPP1 and SPP2 or irreversible degradation by S1P lyase can occur. S1P may act intracellularly or is exported out of the cell via ABC transporters or Spns2, dependent on the cell type, and may bind to one of its receptors (S1PR1–S1PR5) to initiate G-protein mediated signaling. S1P modulates key processes of vascular pathogenesis which involve but are not restricted to modulation of cell proliferation and migration and regulation of vascular tone and immune functions.

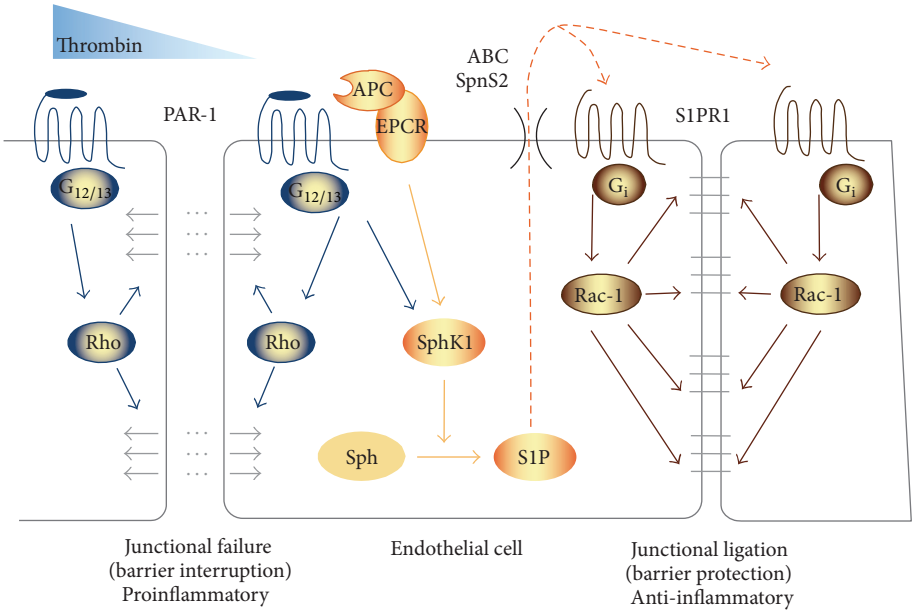

Figure 2: Thrombin effects in endothelial cells involve S1P signaling. Activation of the classical thrombin receptor PAR-1 interrupts endothelial barrier integrity by induction of endothelial contraction through stimulation of $G_{12/13}$ subunit and Rho signalling pathway (left). Conversely, thrombin also induces expression of SphK1 and increases S1P generation. This involves PAR-1-induced signaling via activated protein C (APC) and the endothelial protein C receptor (EPCR). S1P in turn transactivates S1PR1 leading to G_i-dependent activation of the Rac-1 signaling pathway. This effect improves endothelial integrity to counteract and limit thrombin-induced endothelial damage and vascular leakage (right).

and disruption of endothelial barrier integrity via the PAR-1 receptor [57]. This involves signaling through the endothelial protein C receptor and includes cross talk with the S1PR system [59]. S1P/S1PR signaling can counteract this detrimental effect of thrombin and appears to protect from vascular leakage and tissue damage such as edema formation [60]. Thus, thrombin may enhance endothelial S1P generation and signaling within the endothelium to limit its own actions of inducing vascular leakage via mutual PAR-1 mediated S1P/S1PR1 actions (Figure 2).

Table 1: Mechanistic studies which directly link S1P and its receptors to the thrombin or FXa receptors PAR-1 to PAR-4, their (patho)physiological actions, and associated signaling pathways.

Receptor(s)/stimuli	G-protein binding	Signaling pathway	Physiological action(s)	Reference
S1P/thrombin	Not described	NF-κB, EGR-1/ERK1/2	Enhanced tissue factor expression in endothelial cells	[59]
S1P/thrombin	GIT1 and GIT2	Focal adhesion kinase (FAK)/Src	Regulation of endothelial barrier function	[46]
S1PR3/PAR-1 (via SphK1)	$G_{12/13}$	IL-1B	Induces tissue factor production, inflammation, and coagulation	[37]
S1PR1 and S1PR3/PAR-1	G_i; $G_{12/13}$	Rac-1/Rho	Inflammatory responses	[76]
S1P/PAR-1 (via SphK1)	$G_{12/13}$	NF-κB	Regulation of endothelial function	[75]
SphK1/FXa via PAR-1 and PAR-2	Gq	Rho-kinase, PKC	Mitogenesis and migration of VSMCs	[79]
S1P/FXa	Gα; $G_{12/13}$	Rho-A/GTPases	Proliferation/survival	[80]
S1PR3/PAR-4	G_i	Akt, p38 MAPK	Migration, chemotaxis	[107]

In certain systemic diseases such as sepsis, signaling through PAR-1 exerts multiple and partly opposing functions. This has been attributed to either promoting dendritic cell-dependent coagulation and inflammation or reducing sepsis lethality due to protein C activation and involves regulation of the balance between differential vascular S1PR (S1PR1/S1PR3) signaling pathways [59]. Thus, not only PAR signaling but also S1P actions in endothelial cells appear to involve opposing mechanisms and cellular effects. On the one hand, S1P enhances barrier integrity to counteract thrombin-mediated disturbance of permeability to restore vascular homeostasis after injury; on the other hand, it synergizes with thrombin in upregulating the expression of TF in endothelial cells [61]. Thereby, S1P may enhance generation of thrombin under proinflammatory conditions such as atherosclerosis. In this context, a recent study from Campos et al. is of interest, which showed that the functional S1P receptor antagonist fingolimod [62] reduces infarct size and enhances blood-brain barrier integrity in rodent models of stroke [63]. To determine whether this observation, besides an effect on barrier function, involves direct thrombotic or antithrombotic mechanisms of S1P signaling requires further investigations. The mechanistic studies which directly link S1P and its receptors to the thrombin or FXa receptors, their (patho)physiological actions, and associated signaling pathways are summarized in Table 1.

4. Role of Coagulation Factor-S1P Interactions in the Vessel Wall

Proliferation and migration of vascular VSMCs are fundamental features in physiological processes such as maturation of blood vessel [64] as well as during vascular lesion formation [65]. Numerous growth factors and inflammatory molecules like cytokines regulate VSMCs proliferation and migration. Early studies also suggested a function of S1P for DNA synthesis and migration in VSMCs [66]. Since then, S1P has rapidly been gaining attention as a key regulator of VSMCs functions and vascular development as well as a critical factor for vascular damage. Like endothelial cells, VSMCs obtained from different vascular beds express S1PR1, S1PR2, and S1PR3 receptors [67–71]. Kluk and Hla reported that S1P via activation of S1PR1 significantly stimulates both proliferative and migratory responses for VSMCs [70]. This is in agreement with the observation that S1P induces VSMCs migration through a Gαi-linked, Ras- and PI3-K-coupled, ERK1/2-dependent process [71]. A further role of S1PR2 receptor in vascular physiology and pathology has been established through regulation of intracellular signaling pathways, such as Rho GTPase, the phosphatase PTEN, and VE-cadherin pathways [72]. Nodai et al. found high mRNA levels of the receptors S1PR2 and S1PR3 in rat VSMCs [73]. They suggest that predominantly S1PR3 stimulates expression of COX-2 through mechanisms involving calcium-dependent PKC and Src-family tyrosine kinase [73].

The relevance of S1P in the regulation of vascular permeability, lymphocyte trafficking, and vascular development is well documented *in vivo* [41]. S1PR1 deficiency resulted in impaired vascular maturation [74] whereas SphK1 and SphK2 null mice have shown disturbed angiogenesis resulting in embryonic lethality [75]. Furthermore, Kono et al. reported that S1PR1, S1PR2, and S1RP3 function coordinately during embryonic angiogenesis [76]. Taken together, these studies suggest S1P governs physiological vascular homeostasis and is also an important mediator during pathophysiological conditions such as inflammation.

The coagulation system has been well recognized as a key regulator of inflammation. An interaction between thrombin-induced PAR-1 signaling and the S1P system via enhanced expression of SphK1 and elevated S1P synthesis has first been observed in epithelial and in endothelial cells [77]. In addition, the S1P system has been suggested as a downstream component of thrombin signaling also in other cell types. Work from Niessen et al. revealed a critical role of cross talk between PAR-1 and the S1PR3 receptor in dendritic cells in the amplification of inflammation during sepsis [78]. Further studies indicate direct involvement of thrombin in regulating key processes of cellular proinflammatory responses

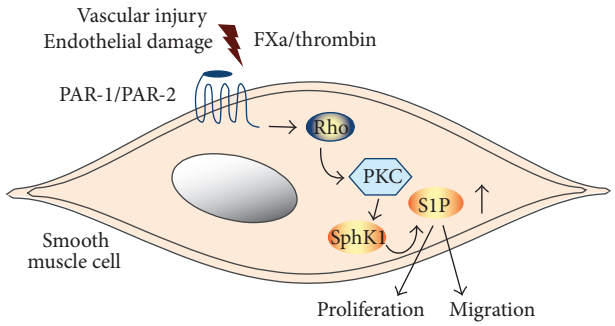

Figure 3: The coagulation proteases thrombin and activated factor X (FXa) enhance S1P synthesis and release from vascular smooth muscle cells (VSCMs). During vascular injury, local generation of thrombin and FXa enhances the synthesis and the release of S1P via activation of PAR-1 and PAR-2, respectively. This signaling pathway involves activation of the small GTPase Rho and PKC signaling leading to transcriptional upregulation of SphK-1. The resulting S1P activities regulate proliferation and migration of VSMCs.

in VSMCs. This involves activation of classical inflammatory transcription factors such as NF-κB [79], but also immune regulators that have more recently become of interest, that is, the forkhead-box-O transcription factor family [80].

In addition to thrombin, FXa can independently activate PAR-1 and PAR-2. Recent work from our laboratory has shown that FXa regulates transcription of SphK1 and elevates S1P biosynthesis in human vascular smooth muscle cells (Figure 3) [81]. This stimulatory effect, observed in cultured cells, was seen at FXa concentrations (3 to 30 nM) which have been shown to occur during thrombus formation *ex vivo* [26]. Expression of SphK1 by FXa was attenuated by inhibitors of the Rho-associated kinase and of classical PKC isoforms. In addition, FXa caused activation of the small GTPase RhoA in human smooth muscle cells. This is particularly interesting, because small GTPases are known to play key roles in mediating signaling responses of the S1P receptor [82], suggesting a mutual interaction of S1P receptor-initiated signaling and regulation of S1P synthesis. Interestingly, FX/FXa appears to be already present within human carotid artery plaques (plaque material is well known to be highly thrombotic) and colocalizes with SphK1 expression [81]. The presence of active coagulation factors in atherosclerotic tissue has also been shown by others [83]. This observation suggests a close relation between coagulation factor signaling and progression of the atherothrombotic disease. Whether possible antiproliferative or antiatherogenic actions of the novel oral coagulation inhibitors involve affecting SphK1 expression and possibly modification of S1P-mediated signaling in patients requires further investigations.

5. Release Mechanisms of S1P from Activated Platelets

The biological effects of S1P released from activated platelets in the vasculature include inhibition of platelet aggregation [84], angiogenesis, vascular development, and thrombosis-related vascular diseases such as the acute coronary syndrome [47, 85, 86]. Platelets were originally suggested to be the prime source of plasma S1P, because they exhibit high SphK activity. In human platelets, SphK2 is the predominant isoform [87]. Surprisingly, however, although platelets do express S1P receptors [88] during *in vitro* platelet function testing such as light transmission aggregometry, S1P does not appear to function as a potent direct platelet agonist [89].

Due to lack of S1P lyase activity in platelets [90], S1P abundantly accumulates intracellularly. However, S1P plasma levels in thrombocytopenic mice were found to remain largely unchanged [91], suggesting that resting platelets may not substantially contribute to circulating S1P concentrations in plasma. Platelets release huge amounts of S1P during blood clotting or upon direct activation with agonists of PKC signaling like thrombin [92, 93]. Work from our laboratory suggests a critical role of thromboxane in regulating the release of S1P from human platelets [89]. Secretion of S1P was induced after activation of platelets with potent agonists such as thrombin or selective PAR-activating peptides (PAR-APs) or with a high concentration of collagen. This effect was largely prevented after inhibition of thromboxane formation by classical inhibitors of cyclooxygenase-1 (COX-1), such as aspirin, diclofenac, or ibuprofen (Figure 4 and [89]). Thus, one pathway mediating release of platelet-derived S1P after platelet activation depends on COX-1-derived thromboxane.

Since S1P represents an amphiphilic anion, its translocation across the plasma membrane supposedly does require active transport proteins. As mentioned above, several studies in various cell types point to the involvement of a transporter of the ABC family [94, 95]. However, the biological functions of these proteins are by far not completely understood. In activated platelets, S1P secretion was affected by several compounds that are known to inhibit members of the multidrug resistance protein (MRP/ABCC) subfamily of ABC transporters [89]. A variety of transporters including MRPs are expressed in platelets that exert important functions for translocation and storage of signaling molecules [96–98]. Further studies are required to identify the proteins involved in the export of S1P out of the cells. The elucidation of the mechanisms of S1P release would also provide the opportunity of pharmacological modulation of the transport process.

6. Interactions of the S1P-PAR System for Inflammatory Monocyte Responses

During vascular inflammation, monocytes secrete several proinflammatory cytokines and adhesion molecules, a response mechanism which facilitates recruitment and adherence to the inflamed and activated endothelium [99]. Thrombin is one of the key factors controlling the migratory and secretory behavior of monocytes [100]. Human peripheral blood monocytes predominantly express PAR-1 and PAR-3 [101]. Interestingly, during differentiation into macrophages, that is, by colony-stimulating factors, the expression levels of PAR-1, PAR-2, and PAR-3 are highly elevated indicating dynamic adaptation mechanisms

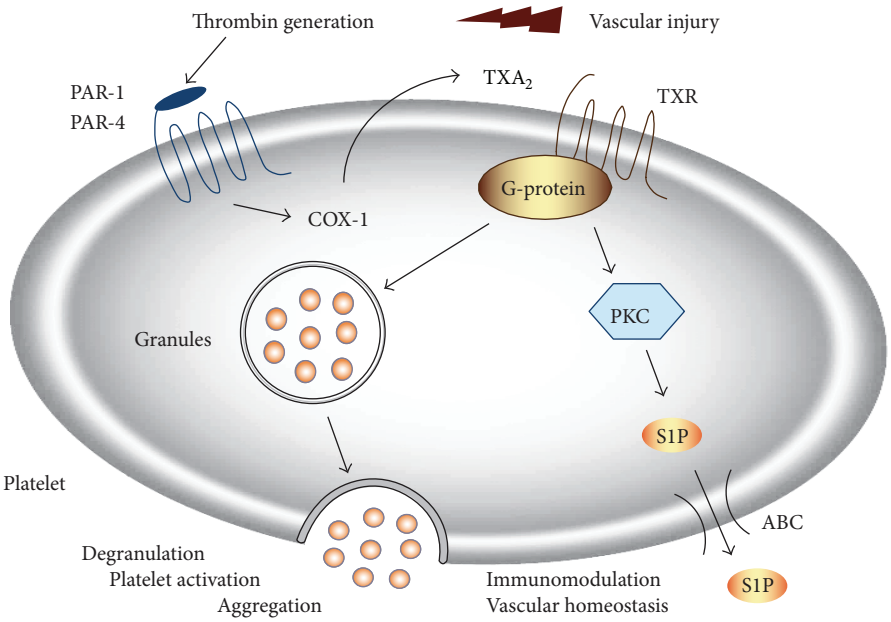

Figure 4: Thrombin-stimulated S1P secretion in human platelets. Thrombin triggers COX-1-mediated synthesis of thromboxane A_2 (TXA_2) via activation of platelet PAR-1. Consecutive TX release and activation of thromboxane A_2 receptor (TXR) enhances platelet degranulation and aggregation. In parallel, TXA_2-TXR signaling stimulates platelet S1P secretion. This pathway involves activation protein kinase C (PKC) and appears to be mediated by an ATP-dependent transport mechanism (ABC, ATP-binding cassette transporter). In a paracrine manner, platelet-derived S1P may modulate endothelial and immune cell responses at sites of injury.

of the system [101]. A recent report indicates that monocytes from patients with antiphospholipid syndrome expressed PAR-1 to PAR-3 but not PAR-4 [102]. Other authors have described a role for PAR-4 in the release of inflammatory markers from monocytes, such as IL-6 [103]. Thus, different PARs may be differentially regulated in response to various stimuli during vascular pathogenesis.

S1P is a recently recognized novel regulator also of monocyte functions [104, 105]. Human monocytes express all five S1PRs at the mRNA and protein levels [106], possibly mediating the regulation of monocyte apoptosis and chemotaxis [107]. In leukocytes, S1P contributes to P-selectin-dependent rolling through endothelial S1PR3 [108]. In dendritic and endothelial cells, involvement of S1P in the signaling pathways of the prototypic thrombin receptor PAR-1 has been suggested [80]. However, little information is to date available about a possible cross talk between S1P and PARs in monocytes. Recent data from our laboratory provide evidence that (i) S1P directly enhances expression of the thrombin receptors PAR-1 and PAR-4 in human monocytes and that (ii) this results in enhanced PAR-4-mediated chemotaxis and elevated generation of COX-2 in response to thrombin [109].

S1P induced PAR-1 and PAR-4 mRNA and total protein expression in human monocytes and U937 cells in a concentration- and time-dependent manner, respectively. However, only PAR-4 cell-surface expression was increased significantly by S1P, whereas cell-surface PAR-1 remained unaffected. This response was associated with activation of the Akt, ERK1/2, and p38 pathway and induction of COX-2 but not COX-1. PAR-4-mediated induction of COX-2 was prevented by pharmacological inhibition of the PI3 kinase pathway and incubation of human monocytes with S1P resulted in an enhanced PAR-4-dependent chemotaxis response to thrombin. Thus, S1P enhances monocyte responses to thrombin via upregulation of PAR-4 protein and cell-surface expression, which promotes migration and COX-2 abundance. These studies establish a direct link between S1P receptor activation and regulation of thrombin receptor expression in human monocyte and the subsequent cellular responses to thrombin. This mechanism may facilitate monocyte recruitment to sites of vessel injury and inflammation (Figure 5).

7. Summary and Perspective

Taken together, complex (patho)physiological interactions between blood coagulation factors and S1P and their respective signaling receptors are being increasingly recognized (see Table 1). This involves regulation of endothelial, smooth muscle, and immune cell functions. Of particular interest for the clinic is the use of new selective modulators of the S1P-S1PR signaling system such as fingolimod as therapeutic agents. In the cardiovascular system, the role of S1P as therapeutic target or as a potential biomarker in cardiovascular diseases is still unclear. For example, the role of S1P levels and release, that is, from thrombin-activated platelets during myocardial infarction, is not finally defined to date. Recent studies indicate that S1P levels substantially vary during cardiovascular disease entities [110, 111]. An important future issue is the definition of circulating S1P levels in defined study populations as well as in clinical cohorts such as patients

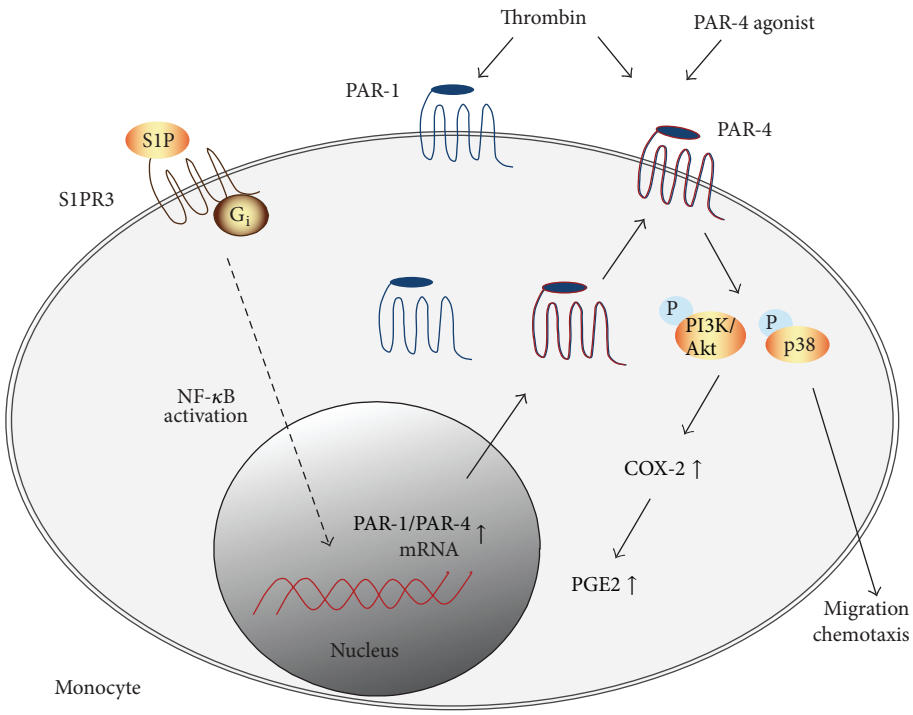

Figure 5: Interactions of S1PRs and PARs in monocyte function. In human monocytes, S1P significantly enhances expression of the thrombin receptors PAR-1 and PAR-4 at the mRNA and protein level. Elevation of PAR-1/PAR-4 abundance appears to be mediated via activation of S1PR3 and results in increased migration of the monocyte toward thrombin. These responses are associated with PI3K/Akt-mediated expression of COX-2. At sites of vascular injury, increased levels of S1P, for example, released from aggregating platelets, may enhance the inflammatory response of local monocytes, thereby modulating tissue-targeted events such as thrombosis and vessel injury.

with acute coronary syndrome. The clinical relevance and therapeutic potential of altering S1P levels or receptor activity in atherothrombosis associated diseases is to date unclear and warrants future studies.

Abbreviations

ABC:	Adenosine triphosphate-binding cassette transporter
APC:	Activated protein C
COX-1:	Cyclooxygenase-1
EGR-1:	Early growth response protein 1
EPCR:	Endothelial protein C receptor
FAK:	Focal adhesion kinase
FXa:	Activated coagulation factor X
NF-κB:	Nuclear factor-κB
PAR-1/PAR-2/PAR-4:	Protease-activated receptor 1/2/4
p38 MAPK:	p38 mitogen-activated protein kinases
PI3K:	Phosphatidylinositol-3-kinases
PAR-4AP:	PAR-4 activating peptide
PGE2:	Prostaglandin E2
PKC:	Protein kinase C
Rac-1:	Ras-related C3 botulinum toxin substrate 1
Rho:	Ras-homologue GTPase family member
S1P:	Sphingosine-1-phosphate
S1P1–S1P3:	S1P receptors 1 to 3
Sph:	Sphingosine
SphK-1:	Sphingosine kinase-1
Spns2:	Spinster homolog 2
TXA_2:	Thromboxane A_2
W146:	S1PR1 antagonist
CAY:	S1P3 antagonist
LY:	An inhibitor of PI3K upstream of Akt
SB:	p38 MAPK inhibitor.

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

Exogenous S1P Exposure Potentiates Ischemic Stroke Damage That Is Reduced Possibly by Inhibiting S1P Receptor Signaling

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Initial and recurrent stroke produces central nervous system (CNS) damage, involving neuroinflammation. Receptor-mediated S1P signaling can influence neuroinflammation and has been implicated in cerebral ischemia through effects on the immune system. However, S1P-mediated events also occur within the brain itself where its roles during stroke have been less well studied. Here we investigated the involvement of S1P signaling in initial and recurrent stroke by using a transient middle cerebral artery occlusion/reperfusion (M/R) model combined with analyses of S1P signaling. Gene expression for S1P receptors and involved enzymes was altered during M/R, supporting changes in S1P signaling. Direct S1P microinjection into the normal CNS induced neuroglial activation, implicating S1P-initiated neuroinflammatory responses that resembled CNS changes seen during initial M/R challenge. Moreover, S1P microinjection combined with M/R potentiated brain damage, approximating a model for recurrent stroke dependent on S1P and suggesting that reduction in S1P signaling could ameliorate stroke damage. Delivery of FTY720 that removes S1P signaling with chronic exposure reduced damage in both initial and S1P-potentiated M/R-challenged brain, while reducing stroke markers like TNF-α. These results implicate direct S1P CNS signaling in the etiology of initial and recurrent stroke that can be therapeutically accessed by S1P modulators acting within the brain.

1. Introduction

Cerebral ischemia produced during stroke is triggered by sudden lack of blood flow and subsequent reperfusion of the ischemic area. Within a few minutes of onset, neurons in the ischemic core are irreversibly injured, which in part determines the fate of brain tissue in the penumbra areas after stroke [1]. Brain damage results from a cascade of cellular and molecular events, including energy failure, excitotoxicity, oxidative stress, and neuroinflammation [2], the latter of which is characterized by CNS infiltration of immune cells and activation of neuroglia such as microglia and astrocytes; neuroinflammation also results in the production of a variety of neurotoxic molecules, including proinflammatory cytokines, all of which produce brain damage [1, 3, 4].

Recurrent stroke, which is a common sequel to an initial stroke, leads to worsened patient outcomes and is thought to be a major cause of morbidity and mortality among initial stroke survivors. Neuroinflammation has been associated with an increased risk of recurrent stroke following transient ischemic attack and may contribute to more severe damage [5–10]. Several proinflammatory factors have been reported to be active in recurrent stroke, including IL-6, TNF-α, lipoprotein-associated phospholipase A_2, C-reactive protein, and fibrinogen [9, 11–14].

Another molecule implicated in neuroinflammation is the lysophospholipid known as sphingosine 1-phosphate (S1P), produced by the phosphorylation of sphingosine by two kinases, sphingosine kinases 1 and 2 (SPHK1 and SPHK2) [15], which acts predominantly as an extracellular signaling

molecule through 5, cognate G protein-coupled receptors [16]. This lipid signaling system has been extensively studied in neuroinflammatory processes associated with multiple sclerosis (MS) [17–20] through actions on both immune and CNS cells, where reductions in signaling promote therapeutic efficacy [21–26]. In addition to MS, S1P signaling has also been implicated in other CNS pathologies including Sandhoff disease and demyelination [21, 26–30]. Prior reports implicated S1P signaling in cerebral ischemia where it was presumed to act through effects on immune cells, including elevated S1P levels [31] and that the nonselective S1P receptor modulator, FTY720 (fingolimod), a current therapy for MS [17–20], reduces brain damage in cerebral ischemia [32–36]. Intriguingly, fingolimod improved outcomes in a proof-of-concept clinical trial of 23 patients with intracerebral hemorrhage at both acute (days) and chronic (months) time points [37], consistent with S1P signaling effects in human stroke.

In this study, we have assessed the possibility of direct CNS S1P receptor signaling in M/R models of stroke and focusing on changes occurring within the brain. We report that local increases in S1P within the brain potentiate damage produced by transient focal cerebral ischemia (M/R), which may represent a new model for recurrent stroke, particularly in view of the effects on markers like TNF-α. Importantly, we report that S1P receptor modulation using FTY720 can reduce stroke damage in both primary and recurrent stroke models.

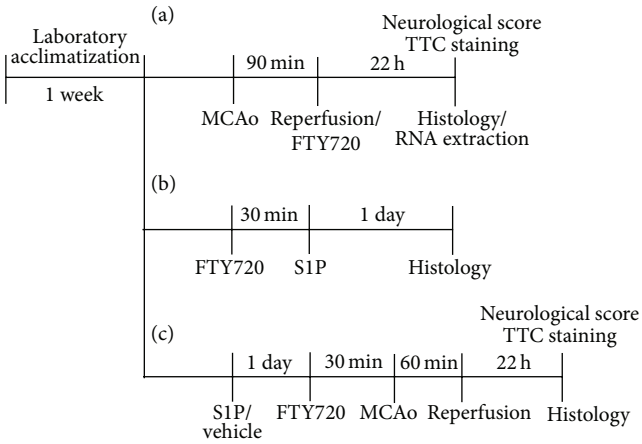

Figure 1: Schematic representation of experimental protocols. (a) Scheme for Figures 2 and 4 (an initial stroke model). Mice were challenged by 90 min occlusion followed by 22 h reperfusion. FTY720 (3 mg/kg, i.p.) was administered to mice immediately after reperfusion. (b) Scheme for Figure 3. S1P was microinjected into the corpus callosum (CC) and brain samples were prepared 1 day after microinjection. FTY720 was administered 30 min prior to S1P microinjection. (c) Scheme for Figures 5 to 7 (a recurrent stroke-mimicking model). S1P was at first microinjected into the CC. One day later, mice were challenged by 60 min occlusion followed by 22 h reperfusion. FTY720 (3 mg/kg, i.p.) was administered to mice 30 min prior to MCAO.

2. Materials and Methods

2.1. Animals. All animal experiments were conducted in accordance with the Center of Animal Care and Use (CACU) guidelines of Lee Gil Ya Cancer and Diabetes Institute (LCDI) at Gachon University (numbers of approved animal protocols: LCDI-2012-0075 and LCDI-2014-0016). Adult male ICR mice (28–32 g, 7 weeks old) were purchased from the Orient Co., Ltd. (Korea), and were housed under controlled temperature (22 ± 2°C), constant humidity, and a 12 h light/dark cycle (light on 07:00–19:00), with food and water made available *ad libitum*. After S1P microinjection or middle cerebral artery occlusion (MCAO) and reperfusion (M/R) surgery (Figure 1, experimental scheme), mice were housed 4 per cage with moist food and soft bedding materials to reduce suffering until they were sacrificed by CO_2 inhalation or used for sampling.

2.2. Materials. S1P [D-erythro-sphingosine-1-phosphate] was purchased from Avanti Polar Lipid (Alabaster, AL). FTY720 [2-amino-2-[2-(octyl-phenyl) ethyl]-1,3-propanediol hydrochloride] was kindly provided by Novartis AG (Basel). 2,3,5-Triphenyltetrazolium (TTC), 3,3′-diaminobenzidine tetrahydrochloride (DAB), fatty-acid-free BSA (FAF-BSA), mouse monoclonal anti-glial fibrillary acidic protein (GFAP) antibody, anti-β-actin antibody, cresyl violet acetate, and protease inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO). Silicon (Variotime) and Zoletil 50 were obtained from Heraeus Kulzer GmbH (Germany) and Virbac (Carros, France), respectively. Goat polyclonal anti-Iba1 and rabbit polyclonal anti-TNF-α antibodies were purchased from Abcam (Cambridge, UK). Avidin-biotin-peroxidase complex (ABC) kit and Vectashield were purchased from Vector Laboratories, Inc. (Burlingame, CA). Fluoro-Jade B was purchased from Chemicon (Temecula, CA).

2.3. Microinjection of S1P at Corpus Callosum (CC). S1P was dissolved in DMSO with 1 N HCL (95 : 5 v/v, 20 mM) and diluted in 10% FAF-BSA to make a stock (2 mM; 1 nmole/0.5 μL). S1P (1 nmol/0.5 μL dissolved in 10% FAF-BSA) was injected at 0.1 μL/min into the right CC of mice anesthetized with the mixture of Zoletil 50 (10 mg/kg, i.m.) and Rompun (3 mg/kg, i.m.). Stereotaxic coordinates were as follows: AP (anteroposterior) = +0.9 mm anterior to bregma, ML (mediolateral) = ±1.0 mm, and DV (dorsoventral) = −2.15 mm. For control mice, 10% FAF-BSA solution containing the same amount of DMSO and HCl was used as vehicle instead of S1P. These mice were used for additional experiments 24 h after microinjection, including M/R challenge (60 min of MCAO) and histological analysis.

2.4. Induction of Transient Focal Cerebral Ischemia. M/R-induced focal ischemia was produced by an intraluminal suture method as reported [38, 39]. Briefly, mice were anesthetized with 3% isoflurane in N_2O and O_2 (70 : 30) and maintained on 1.5% isoflurane. MCAO was induced by inserting a 9 mm long 5-0 nylon monofilament coated with silicon from the bifurcation to the MCA. In general,

blood flow was restored 90 or 60 min after MCAO by carefully withdrawing the monofilament to allow complete reperfusion of the ischemic area under anesthesia. The latter condition (60 min of MCAO) was used to determine damage in recurrent stroke-mimicking situations. Sham-operated animals underwent the same surgical procedure without insertion of nylon monofilament. During the surgery, body temperature was maintained at 37 ± 0.5°C using a heating pad (Biomed S.L., Spain).

2.5. FTY720 Administration. FTY720 was dissolved in saline (0.15 M NaCl) and intraperitoneally (i.p.) administered into mice at 3 mg/kg 30 min before S1P microinjection or MCAO surgery to determine its effect on neuroinflammation via S1P exposure or on brain damage by S1P + M/R challenge. Alternatively, FTY720 was administered to mice immediately after reperfusion to determine its therapeutic effect in 90 min M/R-challenged mice. For the control group, an equal volume of saline was administered.

2.6. Measurement of Functional Neurological Deficit Score and Infarct Volume. Twenty-two hours after reperfusion, the neurological functions of mice were assessed, including motor function, sensory function, reflex, and balance, using a well-known modified neurological severity score (mNSS), as described previously [39, 40].

Brains obtained 22 h after reperfusion were used to measure infarct volume by staining brain sections (2 mm thickness) with 2% TTC in saline for 30 min. TTC-stained sections were photographed and analyzed using an image J software (National Institute of Mental Health, Bethesda, MD). The infarct volume (%) was calculated for each mouse brain by dividing the lesion volume with the total volume.

2.7. Histology. Mice were anesthetized with the mixture of Zoletil 50 (10 mg/kg, i.m.) and Rompun (3 mg/kg, i.m.) and perfused transcardially with ice-cold 50 mM phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde. The brains were removed, postfixed in 4% paraformaldehyde containing 30% sucrose solution (in 50 mM PBS), and frozen with OCT solution. Cryostat sections (20 μm) were used for staining or immunohistochemistry.

For the determination of cell survival or death, cryostat sections were processed for Nissl or Fluoro-Jade B staining as in our previous report [39].

Tissue sections were also used for immunohistochemistry as follows. Tissue sections were treated with 1% hydrogen peroxide in PBS for 15 min, blocked with 5% normal serum containing 0.3% Triton-100, and labeled with primary antibodies, such as goat anti-Iba1 (1 : 500), mouse anti-GFAP (1 : 500), or rabbit anti-TNF-α (1 : 100) antibody. The sections were labeled with appropriate biotinylated antibodies (1 : 200) followed by incubation with ABC solution (1 : 100) and then developed with a solution containing 0.02% DAB and 0.01% H_2O_2.

Images were taken from each section using a bright-field or fluorescent microscope equipped with a DP72 camera (Olympus Co., Tokyo, Japan). For quantification of immunopositive cells, brain sections of 3~5 mice were analyzed: the number for a mouse brain section was taken after calculating a mean value from 3 images (20x) of each section.

2.8. Quantitative Real-Time PCR (qRT-PCR) and Semiquantitative RT-PCR. Total RNA was extracted using RNAiso Plus (Takara) from mouse brain hemisphere subjected to surgical procedure after perfusion with ice-cold PBS and cDNA was synthesized according to the manufacturer's protocols (AffinityScript reverse transcription). qRT-PCR was performed using a Stratagene Mx3005p (Agilent Technologies, Inc., USA) and SYBR Green PCR master mix (Agilent Technologies). Gene expression was quantified using the comparative threshold method and data were calculated as fold changes relative to each gene of sham group after normalization to a reference gene, β-actin. Alternatively, 2x master mix (Takara, Japan) was used to conduct semiquantitative RT-PCR. The sequences of all primer sets are listed in Table 1.

2.9. Statistical Analysis. All data are presented as mean ± SEM and statistical analysis was carried out using GraphPad Prism software (GraphPad Software Inc., San Diego, CA) as specified. Differences among the groups were analyzed by one-way ANOVA followed by Newman-Keuls test for multiple comparisons. Comparisons between the two groups were performed using paired Student's t-test. The statistical significance was set at $P < 0.05$.

3. Results

3.1. Expression Levels of S1P Signaling-Related Genes Are Altered in M/R-Challenged Mouse Brain. We examined whether transient cerebral ischemia influences gene expression levels of S1P receptors ($S1P_{1-5}$) and S1P-producing enzymes (sphingosine kinase 1/2, SPHK1/2) within the brain. Temporal changes in S1P receptors and SPHK1/2 gene expression were assessed by qRT-PCR or semiquantitative RT-PCR, 22 h after M/R reperfusion, as compared to β-actin controls. In the normal mouse brain, 4 of 5 S1P receptors were expressed, including $S1P_1$, $S1P_2$, $S1P_3$, and $S1P_5$, with particularly high expression of $S1P_1$ (Figure 2(a)). In M/R-challenged brains, mRNA expression of $S1P_3$ and SPHK1 was significantly upregulated compared with sham-operated brains, with differences 3- to 4-fold higher (Figure 2(b)). In contrast, $S1P_1$ was downregulated in the M/R group (Figure 2(b)). When semiquantitative RT-PCR analysis was employed, $S1P_1$ downregulation was confirmed as observed in data from qRT-PCR analysis. Interestingly, the lowered expression level of $S1P_1$ was still higher than the upregulated $S1P_3$ in M/R-challenged brains (Figure 2(c)). These results indicate that S1P receptor expression is altered by cerebral ischemia.

3.2. Local S1P Microinjection Activates Microglia and Astrocytes. Local injection of S1P into the brain induces astrocyte activation [23], which may have relevance to cerebral

Table 1: Primers used for PCR analysis.

Gene	Direction	Sequence	Gene accession number
β-actin	Forward	5′-AGCCTTCCTTCTTGGGTATG-3′	NM_007393
	Reverse	5′-CTTCTGCATCCTGTCAGCAA-3′	
S1pr1	Forward	5′-AGGGAACTTTGCGAGTGAG-3′	NM_007901
	Reverse	5′-GTTACAGCAAAGCCAGGTCAG-3′	
S1pr2	Forward	5′-ATAGACCGAGCACAGCCAAC-3′	NM_010333
	Reverse	5′-GTGTTCCAGAACCTTCTCAGG-3′	
S1pr3	Forward	5′-TTGCAGAACGAGAGCCTATT-3′	NM_010101
	Reverse	5′-TTCCCGGAGAGTGTCATTTC-3′	
S1pr4	Forward	5′-ACCTTCAGTCTGCTCTTCACG-3′	NM_010102
	Reverse	5′-AAGAGCACATAGCCCTTGGAG-3′	
S1pr5	Forward	5′-AGATTTCCAATAGCCGCTCTC-3′	NM_053190
	Reverse	5′-AGCTTGCCGGTGTAGTTGTAG-3′	
Sphk1	Forward	5′-AGTCATGTCCGGTGATGGTC-3′	NM_011451
	Reverse	5′-CCAGTTGGCCTTGGTAGATG-3′	
Sphk2	Forward	5′-ATCTCTGAAGCTGGGCTGTC-3′	NM_203280
	Reverse	5′-GAAGAAGCGAGCAGTTGAGC-3′	

ischemia in view of changes to S1P signaling molecules. To determine whether direct activation of S1P receptors induces changes in activation of microglia and astrocytes, immunohistochemistry was used to assess the microglia/macrophage-specific marker Iba1 or the astrocyte-specific marker GFAP. S1P microinjection was used to localize S1P at the level of the corpus callosum via defined stereotaxic coordinates (see Section 2) to produce uniform and reproducible exposure. Immunohistochemistry of normal, injected brains revealed increased Iba1-immunopositive cell numbers as compared with vehicle-injected controls (18.50 ± 11.36 to 67.80 ± 11.41: 370%) (Figure 3(a)). In addition, S1P microinjection induced an increase in GFAP-immunopositive cells (116.4 ± 14.91 to 244.4 ± 59.45: 210%) (Figure 3(b)). These neuroinflammatory outcomes were reduced by pretreatment of FTY720 (3 mg/kg, i.p.; Figure 3), a nonselective S1P receptor modulator that acts as a functional antagonist of, at least, $S1P_1$ [17, 36]. These results indicate that activation of S1P receptors induces neuroinflammatory changes for neuroglia that can be prevented by pharmacological modulation of S1P receptor activities.

3.3. FTY720 Reduces M/R-Induced Brain Infarction and Neuroglial Activation. To determine the role of S1P receptor signaling in the pathogenesis of cerebral ischemia, mice were challenged by M/R (90 min of occlusion) and compared to the same challenge except that animals were exposed to FTY720 (3 mg/kg, i.p.) immediately after reperfusion. Brain damage as a percentage of total brain was then assessed by TTC staining of sampled serial sections from the entire brain taken 22 h later (Figures 4(a) and 4(b)). M/R induced brain infarction by 28.08 ± 2.347%, which was reduced by FTY720 administration to 22.00 ± 1.586% (Figure 4(b)). Brain damage was also determined 22 h after reperfusion based on neurological score, showing that FTY720 exposure recovered damaged neurological functions in cerebral ischemia (Figure 4(c)). Neuroglial activation was assessed in M/R groups treated with saline (M/R + sal) or FTY720 (M/R + FTY720) (Figures 4(d) and 4(e)). M/R-challenged mice displayed microglial activation (Iba1-immunopositive cells, Figure 4(d)) and astrogliosis (GFAP-immunopositive cells, Figure 4(e)), which were both markedly decreased in M/R + FTY720 group compared with saline-treated M/R group controls. These data demonstrate that FTY720 significantly decreases brain damage in M/R-challenged mice that is associated with reduced astrocyte and microglial activation, supporting S1P receptor signaling in the brain as a pathological mediator of cerebral ischemia that can be altered to reduce neuroinflammatory changes and brain damage produced by M/R.

3.4. Brain Damage Is Augmented by S1P Microinjection. Neuroinflammation during initial cerebral ischemia is strongly correlated with recurrent cerebral ischemia, in which more severe brain damage occurs [7–10]. Based on findings that S1P receptors are involved in neuroglial activation and M/R-induced damage, S1P microinjection was used to activate local neuroglia followed by M/R challenge followed by assessments of brain damage. To determine the augmentation clearly, mice were challenged by a shorter M/R (60 min of occlusion and reperfusion) 24 hours after S1P microinjection. S1P microinjection followed by M/R (S1P + M/R) significantly increased damage compared to M/R after vehicle injection (veh + M/R) (Figures 5(a) and 5(b)). Cerebral infarct volume in veh + M/R group was 18.40 ± 3.638% whereas the S1P + M/R group was 29.57 ± 4.867% (Figure 5(b)). This secondary, augmented brain damage produced by initial S1P microinjection was reduced by FTY720 administration prior to M/R challenge (Figures 5(a) and 5(b)). Infarct volume in the S1P + FTY + M/R group was 16.73 ± 2.493 (Figure 5(b)). These results were confirmed by assessments of neurological deficit (Figure 5(c)) and neural cell death using Fluoro-Jade B staining (Figure 5(d)). These data showed

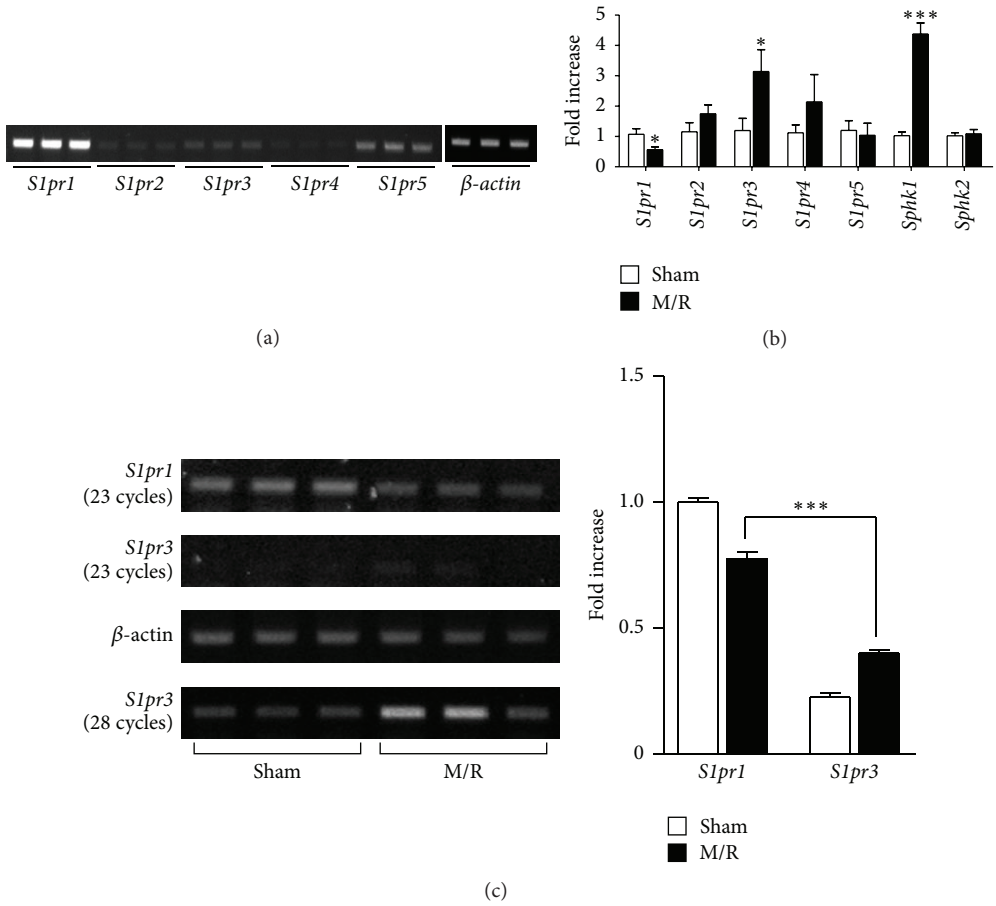

Figure 2: mRNA expression levels of S1P receptors and S1P-producing enzymes are altered in an M/R-challenged brain. (a) Normal brains were used to determine mRNA expression levels of S1P receptors (*S1pr1*, *S1pr2*, *S1pr3*, *S1pr4*, and *S1pr5*) and SPHKs (*Sphk1* and *Sphk2*) based on semiquantitative RT-PCR analysis (28 cycles for all targets). (b, c) Mice were challenged by 90 min occlusion followed by 22 h reperfusion. (b) Brain samples were used to determine changes of S1P receptors and SPHKs based on qRT-PCR analysis. $^{*}P < 0.05$ and $^{***}P < 0.001$, compared with the sham group (t-test), $n = 5$ per group. (c) Brains from sham and M/R-challenged mice were used to determine mRNA expression levels of $S1P_1$ and $S1P_3$ by semiquantitative RT-PCR analysis. Band intensity (bar graph) was calculated as fold increase relative to *S1pr1* level of sham groups after normalization to β-actin. $^{***}P < 0.001$, compared with *S1pr1* level of M/R group (Newman-Keuls test), $n = 3$ per group.

that the increased brain damage in S1P + M/R group was blocked by FTY720 administration and indicated that S1P receptor signaling that activates neuroglia—astrocytes and microglia—exacerbates M/R-induced brain damage, possibly representing a model for increased damage observed in recurrent cerebral ischemia.

3.5. FTY720 Reduces Neuroglial Activation Occurring in S1P-Primed M/R Challenge. Local S1P microinjection augmented M/R damage, indicating that CNS S1P receptor signaling potentiates damage produced by ischemic insult. To determine whether local neuroglial activation was also occurring in S1P-primed damage, activated microglia and astrocytes were examined using immunohistochemical markers from animals challenged under various M/R conditions as compared to sham controls. Immunohistochemically observed microglial activation (Iba1-immunopositive cells, Figure 6(a)) and astrogliosis (GFAP-immunopositive cells, Figure 6(b)) were both increased in S1P + M/R group compared to the M/R only group (veh + M/R) and sham controls. The activation was then assessed in animals that had received FTY720 administration prior to M/R challenge: this reduced activation of microglia and astrocytes (S1P + FTY + M/R) (Figure 6).

3.6. S1P Microinjection Induces TNF-α Expression. The priming of M/R damage by S1P microinjection into the brain was suggestive of changes seen in recurrent stroke, raising the question of whether markers for recurrent stroke might be expressed in the S1P-primed model. Increased TNF-α expression is associated with a risk of recurrent stroke [13]. TNF-α immunolabeling in the cortex identified significant increases in the number of cells expressing TNF-α after S1P microinjection alone (S1P) or M/R alone (veh + M/R) (Figure 7). Notably, the number of TNF-α-positive cells was

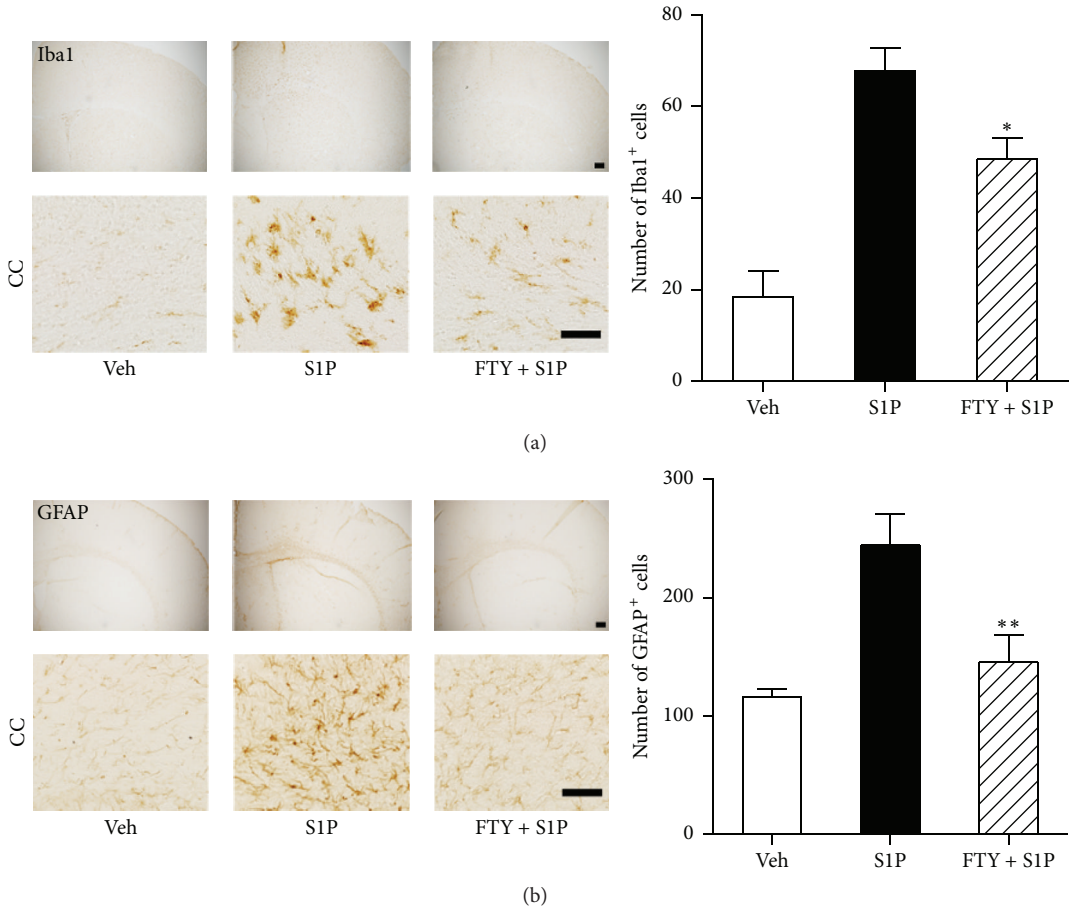

Figure 3: Microglia and astrocytes are activated in the brain following S1P microinjection into the corpus callosum. S1P was microinjected into the corpus callosum (CC), and activation of microglia or astrocytes was assessed 1 day after microinjection. FTY720 (FTY) was administered 30 min prior to S1P microinjection. Representative microphotographs of brain sections immunolabeled against Iba1 (a) or GFAP (b) and their quantitative analysis in groups of vehicle (veh), S1P, and FTY + S1P. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with the S1P-injected group (S1P) (Newman-Keuls test). $n = 5$ per group. Scale bar, 200 (upper panel) or 50 μm (lower panel).

highest in brains challenged by M/R after initial S1P microinjection (S1P + M/R) (Figure 7). All conditions showed a reduction in TNF-α expression following FTY720 exposure (FTY + S1P and S1P + FTY + M/R) (Figure 7). These data indicate that S1P receptor-mediated changes can produce pathological changes consistent with recurrent stroke, which can be reduced by modulation of S1P signaling by FTY720 exposure.

4. Discussion

The present study has identified activation of S1P receptor signaling within the brain as a factor in transient focal cerebral ischemic (M/R) brain damage, particularly involving activation of astrocytes and microglia. In particular, local brain delivery of S1P—which is independent of M/R, produced astrocyte, and microglial activation—was found to potentiate ischemic brain damage, supporting direct CNS activities of S1P signaling in stroke. FTY720 reduced neuroglial activation and ischemic brain damage and this neuroprotective effect was associated with neuroinflammation [41, 42] wherein neuroglia, such as astrocytes and microglia, are activated by immune cells within the CNS. These data implicate modulation of S1P receptors in forms of stroke, including recurrent stroke, which can be therapeutically accessed by S1P receptor modulation.

Receptor-mediated S1P signaling has previously been suggested to play a role in cerebral ischemia based upon protective effects of the S1P receptor modulator, FTY720. In rodent models of cerebral ischemia, FTY720 reduced ischemic brain damage [32–36], with consistent results observed in a proof-of-concept clinical trial that reported improved neurological endpoints with FTY720 (fingolimod) treatment of brain hemorrhagic stroke patients [37]. The proposed mechanism of FTY720 efficacy in stroke models [36] was similar to that initially proposed for multiple sclerosis (MS) wherein a reduction of pathogenic lymphocytes entering the brain occurs, produced by S1P-dependent alterations of lymphocyte trafficking [18]. This effect is consistent with reported lymphocyte involvement in cerebral ischemia [35].

In addition, evidence for nonimmunological S1P signaling mechanisms occurring within the brain itself has

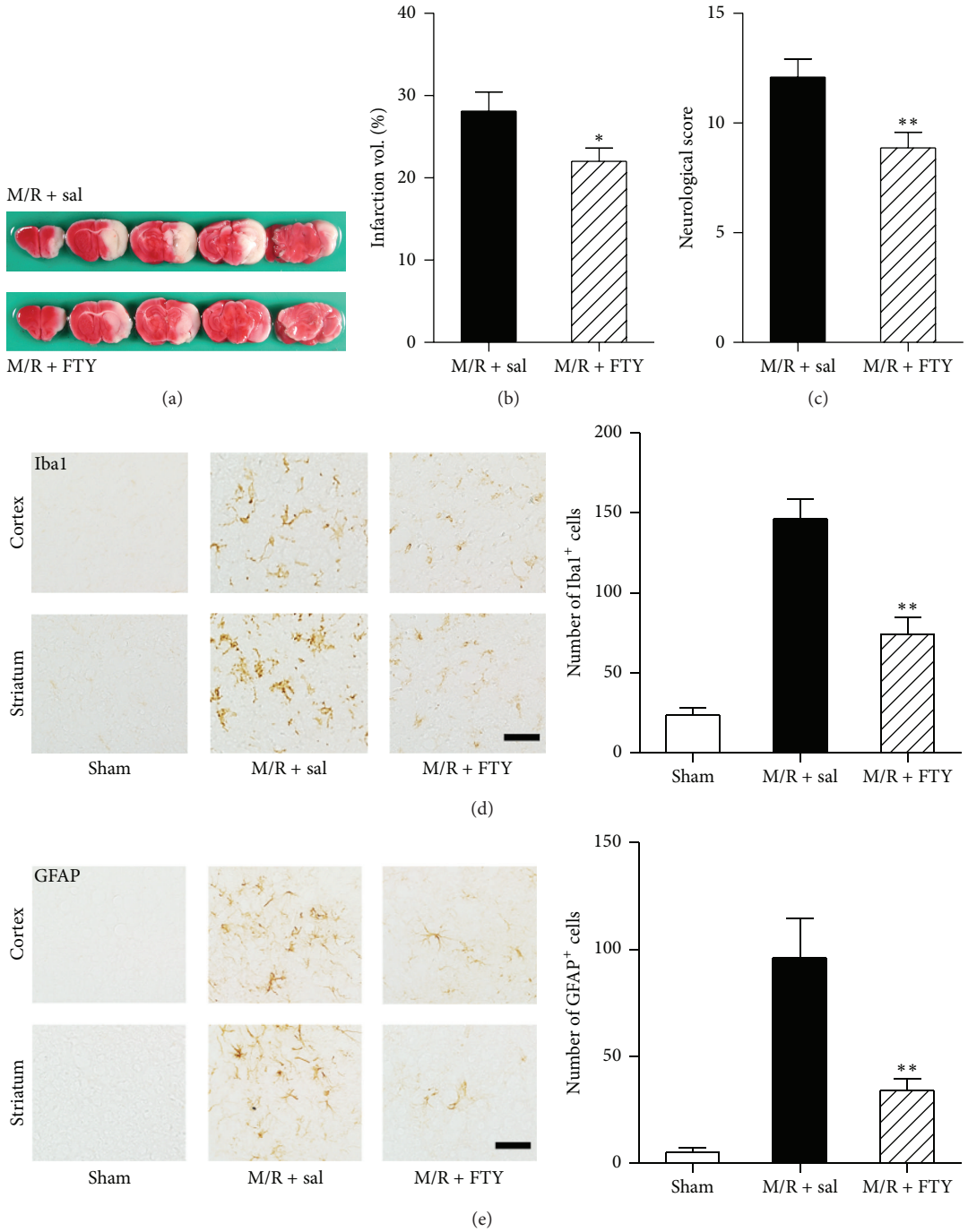

Figure 4: FTY720 reduces brain damage and neuroinflammation in M/R-challenged mice. Mice were challenged by 90 min occlusion and brain infarction or neuroinflammation was assessed 22 h after reperfusion. FTY720 (FTY, 3 mg/kg, i.p.) was administered to mice immediately after reperfusion. (a) Representative TTC-stained brain slices of M/R + saline (sal) and M/R + FTY. Photographs are coronal brain sections stained with TTC showing infarct area (white) and intact area (red). (b) Percentage of infarct volumes calculated from the TTC-stained brain slices. Infarct volume was measured using Image J software, and the percentage of infarction was assessed. (c) Neurological score demonstrating neurological functions. $^{*}P < 0.05$ (t-test), compared with the saline-treated group (M/R + sal) (t-test). $n = 12\sim15$ per group. (d, e) Representative microphotographs of cortex and striatum regions immunolabeled against Iba1 (d) or GFAP (e) and their quantitative analysis in groups of sham, M/R + sal, and M/R + FTY. $^{**}P < 0.01$, compared with the saline-treated group (M/R + sal) of each set (Newman-Keuls test). $n = 3$ per group. Scale bar, 50 μm.

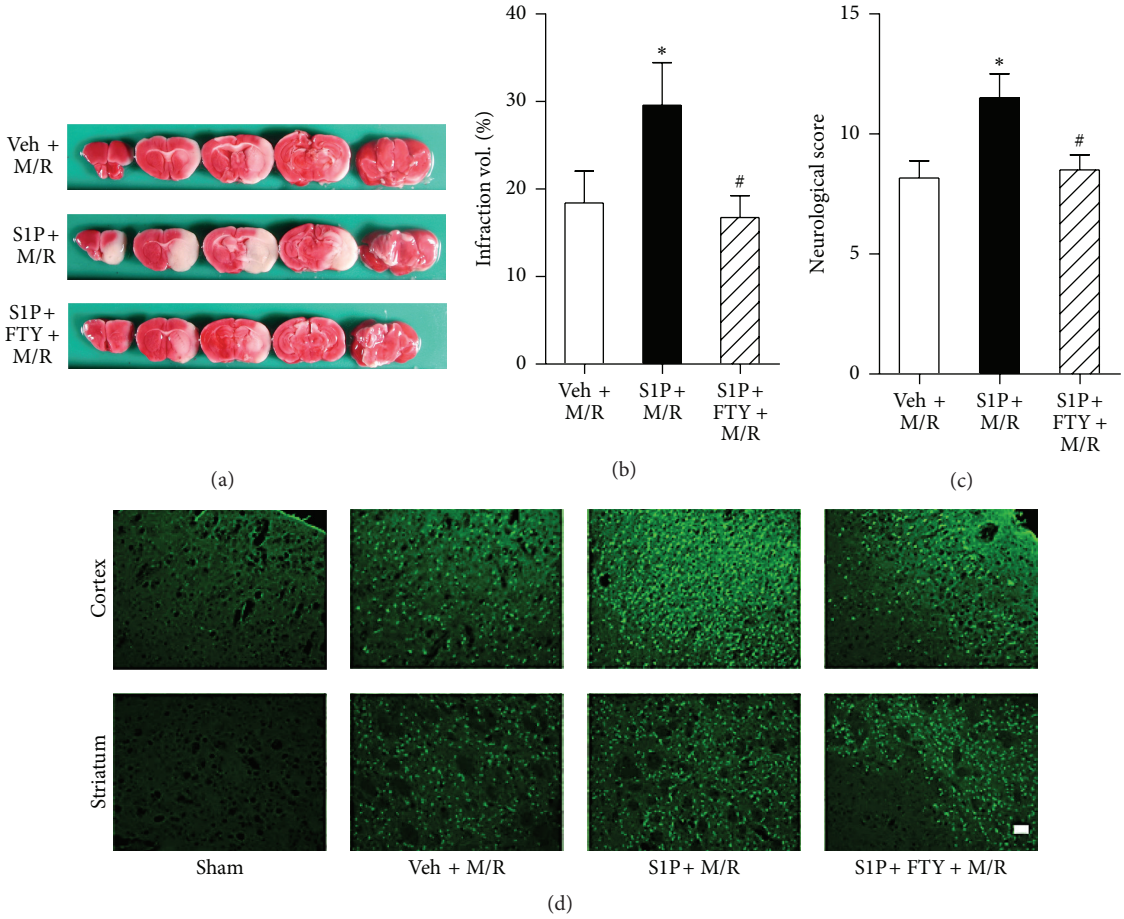

Figure 5: Activation of S1P signaling induces augmented brain damage following M/R injury. S1P or vehicle (veh) was microinjected into the corpus callosum 24 h prior to M/R challenge (60 min occlusion followed by 22 h reperfusion). FTY720 (FTY) was administered into mice 30 min prior to 60 min occlusion. Brain infarction or neuroinflammation was assessed 22 h after reperfusion. (a) Representative TTC-stained brain slices of veh + M/R, S1P + M/R, and S1P + FTY + M/R. Photographs are coronal brain sections stained with TTC showing infarct area (white) and intact area (red). (b) Percentage of infarct volumes calculated from the TTC-stained brain slices. Infarct volume was measured using Image J software and the percentage of infarction was assessed. (c) Neurological score demonstrating neurological functions. $^{*}P < 0.05$ and $^{\#}P < 0.05$, compared with the M/R group (veh + M/R) and S1P + M/R group, respectively (Newman-Keuls test). $n = 6\sim8$ per group. (d) Representative microphotographs of cortex and striatum regions stained with Fluoro-Jade B. Scale bar, 50 μm.

emerged as an explanation for FTY720 efficacy in MS [21, 26–30], which might also be relevant to stroke. Notably, selective removal of the S1P receptor subtype $S1P_1$ from astrocytes was found to reduce astrogliosis, disease severity, and FTY720 efficacy in EAE (experimental autoimmune encephalomyelitis), an animal model of MS [26], despite the maintenance of $S1P_1$ in the immune system. Reductions in astrogliosis observed here during FTY720 exposure support the operation of a similar protective mechanism involving astrocyte reductions in $S1P_1$ signaling. These observations support a direct effect of S1P receptor signaling on the severity of damage produced during stroke, which may involve nonimmunological mechanisms relevant to neuroprotection or repair. Consistent with this possibility, a proof-of-concept clinical trial examining FTY720 effects on hemorrhagic stroke patients reported not only short-term effects that might be immunologically driven, but also longer-term neurological improvement (3 months after event) [37]. Future identification of specific S1P receptor subtypes beyond $S1P_1$ and the involved CNS cell types, such as microglia, will assist in elucidating the precise mechanisms of FTY720 efficacy in cerebral ischemia models, which also appears to be relevant to the recurrent stroke model accessed by S1P pretreatment within the brain in view of both damage potentiation by S1P and the increased presence of recurrent stroke markers like TNF-α.

Additional support for the importance of S1P signaling mechanisms relevant to stroke may come from studies on S1P itself and its biosynthetic enzymes, SPHK1 and SPHK2. In cerebral ischemia, S1P levels are increased [31] and S1P-producing enzymes may be upregulated in lesion sites [43, 44] even with a contrasting report for the latter [34]. In the normal brain, SPHK2 is more abundantly expressed than SPHK1 [45] and is thought to play a protective role [46]. SPHK2 upregulation in the ischemic or hypoxic brain has been reported [43, 44] but requires further study in view of

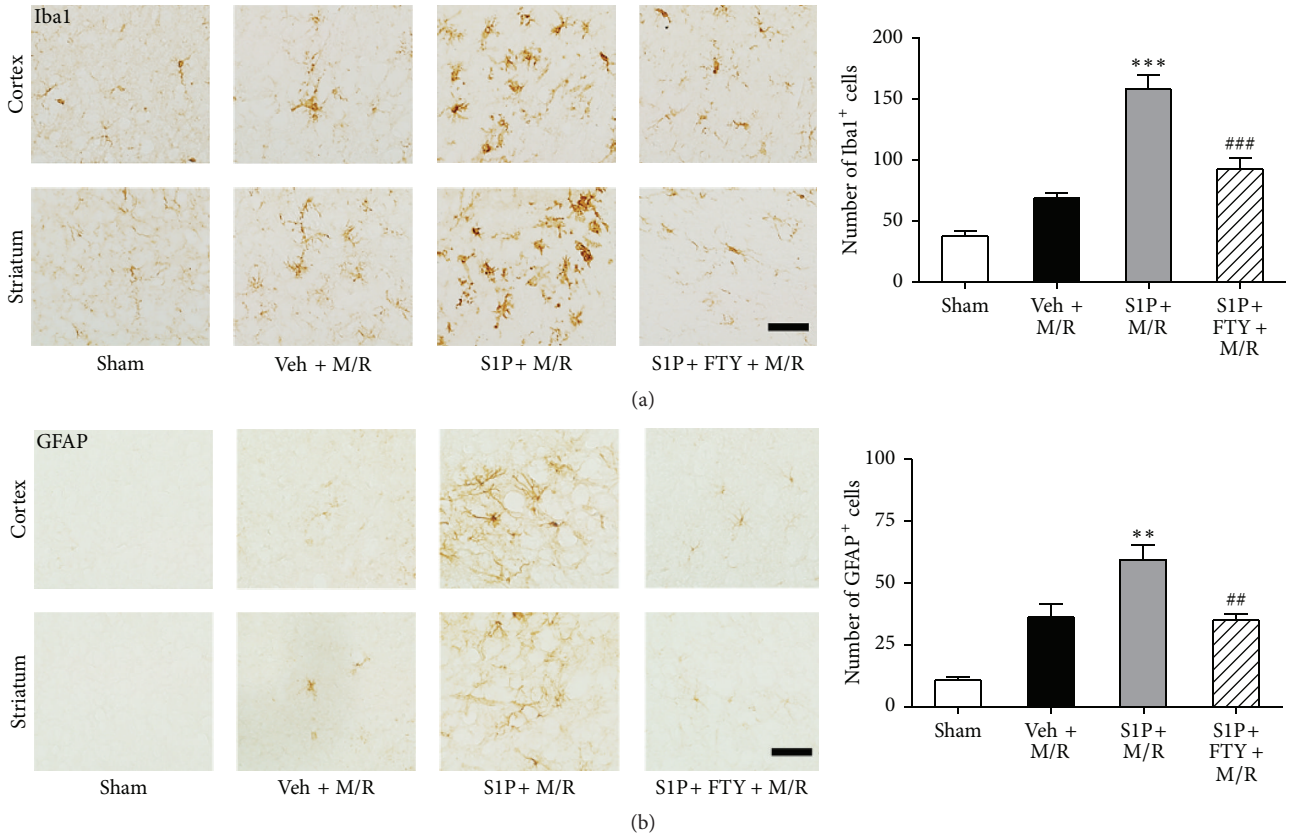

Figure 6: Activation of S1P signaling induces augmented microglial activation and astrogliosis following M/R injury. S1P or vehicle (veh) was injected into the corpus callosum 24 h prior to M/R challenge (60 min occlusion followed by 22 h reperfusion). FTY720 (FTY) was administered into mice 30 min prior to 60 min occlusion. Activation of microglia or astrocytes was assessed 22 h after reperfusion. Representative microphotographs of brain sections immunolabeled against Iba1 (a) or GFAP (b) and their quantitative analysis in groups of sham, veh + M/R, S1P + M/R, and S1P + FTY + M/R. $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with M/R group (veh + M/R) (Newman-Keuls test). $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$, compared with the S1P + M/R group (Newman-Keuls test). $n = 3$ per group. Scale bar, 50 μm.

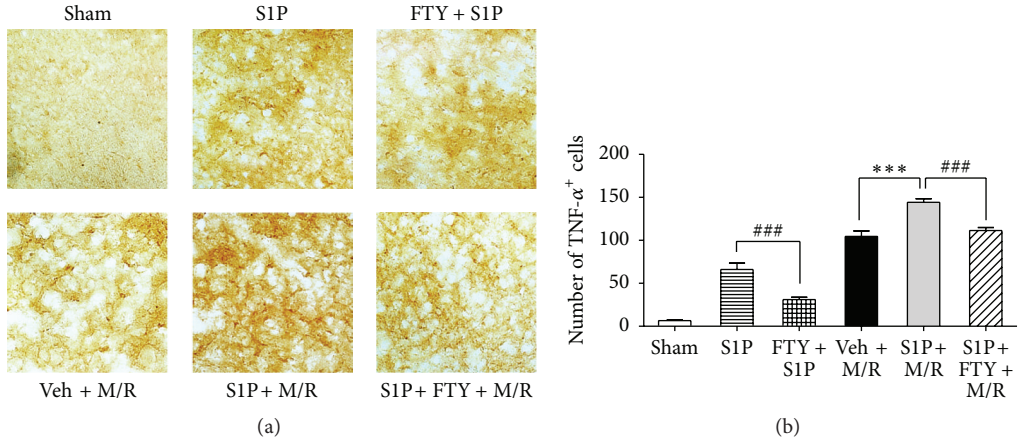

Figure 7: Activation of S1P signaling induces augmented TNF-α expression following M/R injury. S1P or vehicle (veh) was injected into the corpus callosum 24 h prior to M/R challenge (60 min occlusion followed by 22 h reperfusion). FTY720 (FTY) was administered into mice 30 min prior to S1P microinjection (FTY + S1P) or 60 min occlusion (S1P + FTY + M/R). Cells expressing TNF-α were assessed 1 day or 22 h after S1P microinjection or reperfusion. Representative microphotographs of brain sections immunolabeled against TNF-α (a) and their quantitative analysis (b) in groups of sham, S1P, FTY + S1P, veh + M/R, S1P + M/R, and S1P + FTY + M/R. Significance was presented only for the main groups as indicated. $^{***}P < 0.001$, compared with M/R group (veh + M/R) (Newman-Keuls test). $^{\#\#\#}P < 0.001$, compared with the S1P or S1P + M/R group (Newman-Keuls test). $n = 3$ per group. Scale bar, 50 μm.

a contrasting report that SPHK2 was not upregulated [34] and our finding that SPHK1 rather than SPHK2 was upregulated in the ischemic brain. Although this study suggests the importance of SPHK1 in cerebral ischemia based on its mRNA upregulation, a functional role of SPHK1 needs to be clarified. In addition to upregulation of ligand-producing enzyme, our data indicate altered mRNA expression levels of S1P receptors, including $S1P_3$ upregulation and $S1P_1$ downregulation. It is of note that basal mRNA expression of $S1P_1$ is much higher than $S1P_3$ both in normal and M/R stroke conditions and that the reduced expression level of $S1P_1$ mRNA is still higher than the upregulated level of $S1P_3$ in M/R. In fact, there is a report that $S1P_1$ is downregulated in the infarcted area 24 h following M/R challenge (2 h MCAo followed by 24 h of reperfusion) in rats possibly through the decreased cerebral blood flow along with ATP depletion in the ipsilateral hemisphere (dead cell regions) [47]. Another study reported the downregulation of $S1P_1$ in the kidney 24 h after ischemia/reperfusion [48]. These two independent reports are consistent with our observation that $S1P_1$ is downregulated by ischemic challenge. However, the exact mechanism regarding the $S1P_1$ downregulation following M/R is still unclear and could be pursued as another study. In addition, it is unclear why FTY720 that is supposed to downregulate $S1P_1$ has a protective effect in cerebral ischemia where $S1P_1$ is downregulated. Of note, $S1P_1$ was also reported to be downregulated at the gene level in rat spinal cords of EAE [49] consistent with overactivation by increased S1P levels, while EAE symptoms were reduced by genetic deletion of $S1P_1$ or its functional antagonism by FTY720, consistent with a critical role for the receptor in MS-like disease [26]. These independent results from studies of MS are consistent with our data in M/R stroke models, which also showed astrocyte activation, $S1P_1$ downregulation, and the protective effect of FTY720. However, it still remains unclear that S1P receptors with altered expression levels in ischemic brain actually function as pathogenetic factors, which may be tempting to be pursued as a further study.

It is clear that S1P signaling is important for the pathogenesis of cerebral ischemia. This study suggests a possible downregulation of S1P signaling by FTY720, but it is unclear that FTY720 indeed reduces the signaling. It is presumed that the functional antagonism of FTY720 on $S1P_1$ may be involved in this neuroprotection. In addition, a recent report demonstrates that FTY720 also causes the downregulation of $S1P_3$ [50], as it does for $S1P_1$. In this study, $S1P_3$ was downregulated at mRNA level in the ischemic brain, so it may be possible that FTY720-mediated functional antagonism on $S1P_3$ may contribute to neuroprotection. Therefore, it would be tempting to find pathogenetic role of $S1P_1$ or $S1P_3$ in cerebral ischemia employing genetic or pharmacological tools to study loss of function in the future.

Neuroinflammation that is featured by the activation of neuroglia, such as microglia or astrocytes, in the brain is an important event contributing to brain damage in both initial and recurrent stroke [1, 3–10]. There are several reports on the anti-inflammatory role of FTY720 in microglial cells [29, 51] through as yet unidentified receptor subtype(s). In activated microglia exposed to lipopolysaccharide, FTY720 reduced activation of inflammation-associated signaling molecules [51] and production of proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α [29]. In activated astrocytes, FTY720 reduced TNF-α-induced ceramide formation [52] even with contrasting results that it did not affect IL-6 production in normal or activated human fetal astrocytes [53]. In this study, FTY720 also reduced neuroglial activation in the ischemic brain. It still remains to elucidate the specific receptor subtypes involved in neuroglial activation. Neuroinflammation is closely linked to blood brain barrier (BBB) disruption and, moreover, S1P signaling has important roles in regulating the BBB, possibly via 2 subtypes of S1P receptors ($S1P_1$ and $S1P_2$) [54]. It has been reported that S1P signaling modulates BBB integrity, with contrasting roles depending on receptor subtypes: activation of $S1P_1$ is linked to enhanced BBB integrity [55–57] while $S1P_2$ is linked to increased BBB permeability [58–60]. Moreover, FTY720 was reported to have protective effects on BBB damage through the activation of $S1P_1$ [61]. Therefore, the neuroprotective effect of FTY720 observed in this study may be due to its function on the BBB through $S1P_1$ because BBB disruption is also pathogenic in the ischemic brain. However, it is of note that S1P-stimulated responses, including S1P microinjection-induced glial cell activation and potentiated brain damage in the ischemic brain, are attenuated by FTY720 exposure. These results suggest that FTY720 might reduce S1P signaling rather than enhance it, but the exact role of S1P receptors, especially $S1P_1$, in the ischemic brain still remains unclear.

5. Conclusions

Results from the study of both MS and stroke support S1P receptor-mediated signaling as relevant to these diseases through effects not only on neuroinflammation but also through direct CNS effects involving neuroglial activity, with particular relevance to recurrent stroke through the ability of locally microinjected S1P to potentiate M/R stroke damage. Our data are consistent with the primary effects on $S1P_1$ and astrocytes; however the role of other S1P receptors and involved cell types in cerebral ischemia awaits future clarification. These data support the actions of S1P receptor modulators for the treatment of stroke, in both the immune system and within the CNS itself, which has therapeutic and mechanistic relevance through targeting these components by brain nonpenetrant versus penetrant compounds.

Conflict of Interests

The authors have no conflict of interests to declare.

Acknowledgments

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Research Article

Chemical Hypoxia Brings to Light Altered Autocrine Sphingosine-1-Phosphate Signalling in Rheumatoid Arthritis Synovial Fibroblasts

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Emerging evidence suggests a role for sphingosine-1-phosphate (S1P) in various aspects of rheumatoid arthritis (RA) pathogenesis. In this study we compared the effect of chemical hypoxia induced by cobalt chloride ($CoCl_2$) on the expression of S1P metabolic enzymes and cytokine/chemokine secretion in normal fibroblast-like synoviocytes (FLS) and RAFLS. RAFLS incubated with $CoCl_2$, but not S1P, produced less IL-8 and MCP-1 than normal FLS. Furthermore, incubation with the $S1P_2$ and $S1P_3$ receptor antagonists, JTE-013 and CAY10444, reduced $CoCl_2$-mediated chemokine production in normal FLS but not in RAFLS. RAFLS showed lower levels of intracellular S1P and enhanced mRNA expression of S1P phosphatase 1 (SGPP1) and S1P lyase (SPL), the enzymes that are involved in intracellular S1P degradation, when compared to normal FLS. Incubation with $CoCl_2$ decreased SGPP1 mRNA and protein and SPL mRNA as well. Inhibition of SPL enhanced $CoCl_2$-mediated cytokine/chemokine release and restored autocrine activation of $S1P_2$ and $S1P_3$ receptors in RAFLS. The results suggest that the sphingolipid pathway regulating the intracellular levels of S1P is dysregulated in RAFLS and has a significant impact on cell autocrine activation by S1P. Altered sphingolipid metabolism in FLS from patients with advanced RA raises the issue of synovial cell burnout due to chronic inflammation.

1. Introduction

Rheumatoid arthritis (RA) is a chronic systemic disorder that causes destruction of joints through inflammation and proliferation of the synovial membrane [1, 2]. In RA, the synovial tissue lining the joints becomes inflamed. In comparison with the normal synovial membrane, which is normally 1-2 cell layers thick, RA synovial tissue is hypertrophic and invaded by an excess of various leukocytes including neutrophils, T cells, macrophages, and monocytes [3]. This recruitment of leukocytes is likely to be mediated by selective chemotactic factors, such as interleukin-8 (IL-8) that recruits neutrophils and T cells, and monocyte chemotactic protein-1 (MCP-1) that recruits monocytes, into the synovium [4, 5]. A role for IL-8 [6, 7] and MCP-1 [8, 9] in these processes has been highlighted. The synthesis of chemokines in RA may be dependent, at least in part, on the production of inflammatory cytokines, such as IL-1β and tumor necrosis factor-α (TNF-α) [4], by the hypertrophic synovium and activated leukocytes. The complex cascade of production of chemokines, cytokines, and tissue-remodelling enzymes associated with leukocyte recruitment plays a role in synovial cell proliferation and joint erosion in RA [1, 2, 10]. Eventually, the thickened synovial membrane decreases capillary density and the oxygen tension in the joint [11–13]. Severe reduction of mean oxygen pressure in the RA synovium compared to that of healthy joints correlates with severity of inflammation [14–16]. The hypoxic RA joint environment in turn affects

a host of genes involved in angiogenesis, apoptosis, cellular metabolism, matrix degradation, and inflammation [17]. Hypoxia drives vascular endothelial growth factor (VEGF) expression leading to angiogenesis [18–20]. The expression of cyclooxygenase-2 (COX-2) [21], matrix metalloproteinases (MMPs) [22], stromal cell-derived factor 1 [23], IL-6 and IL-8 [22, 24], and migration [25, 26] and proliferation of synovial fibroblasts as well [27], are exacerbated in response to hypoxia.

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid implicated in various pathological processes through binding to and activation of five G protein-coupled receptors designated as $S1P_{1-5}$ [28]. Intracellular S1P is transported outside cells and gains access to cognate receptors for autocrine or paracrine signalling [28, 29]. The steady state level of intracellular S1P is regulated through synthesis by two sphingosine kinases (SphK1 and SphK2) and degradation either via dephosphorylation by S1P phosphatases (SGPP1 and SGPP2) or irreversible cleavage by S1P lyase (SPL) [30]. Moreover, S1P exported outside cells is dephosphorylated back to sphingosine by lipid phosphate phosphatases (LPPs), thereby attenuating its effects on the activation of surface receptors [31]. Alteration in the enzymes involved in S1P synthesis and catabolism may mediate many pathological states including arthritis (reviewed in [28, 32]).

Fibroblast-like synoviocytes from RA patients (RAFLS) express $S1P_1$, $S1P_2$, and $S1P_3$ receptors [33]. RAFLS stimulation with S1P promotes the synthesis of cytokines/chemokines, COX-2 expression and release of prostaglandin E2 (PGE2), and cell migration, proliferation, and survival as well [33, 34]. SphK activation and high S1P levels have been reported in the synovium and synovial fluids of patients with RA [34–36]. Studies suggest a role for S1P in the pathophysiology of RA since SphK1 deficiency and blockade of $S1P_1$ receptors attenuate collagen-induced arthritis in mice [37, 38]. Though SphKs can be activated by TNF-α and IL-1β to generate S1P, new evidence suggests a potential link between S1P and hypoxia in cancer and cardiovascular diseases [39, 40]. In this study we evaluated the impact of chemical hypoxia induced by $CoCl_2$ on chemokine synthesis by normal FLS and RAFLS. We report that the blockade of $S1P_2$ or $S1P_3$ receptors attenuates $CoCl_2$-mediated IL-8 and MCP-1 secretion in normal FLS but not in RAFLS. Furthermore, we provide evidence that low levels of intracellular S1P in RAFLS attenuate the $S1P_2$ and $S1P_3$ receptor-dependent synthesis of chemokines under conditions of chemical hypoxia.

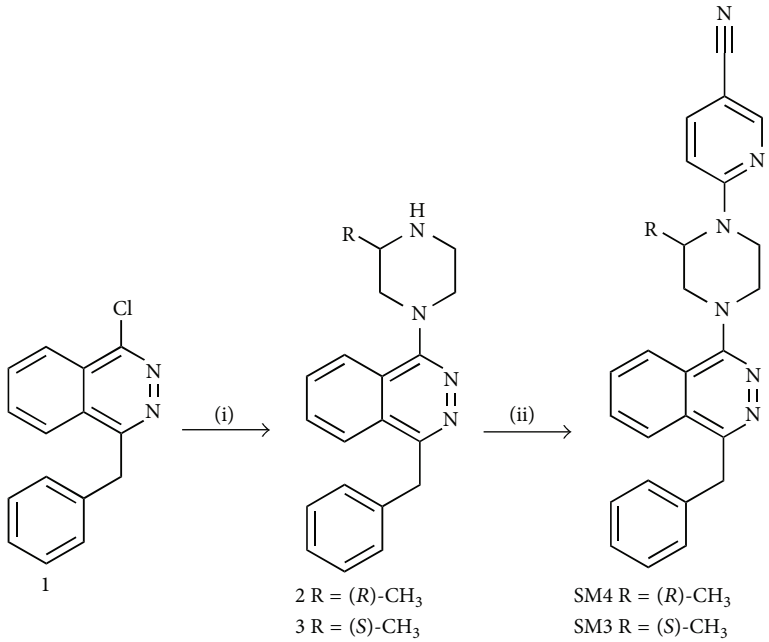
2. Materials and Methods

2.1. Reagents. Cobalt chloride ($CoCl_2$) was from Sigma Aldrich (Oakville, ON, Canada). S1P was purchased from Biomol (Plymouth Meeting, PA, USA). Human IL-8 and MCP-1 ELISA (Enzyme-Linked Immunosorbent Assay) kits were purchased from BioSource International Inc. (Camarillo, CA, USA) and R&D Systems (Minneapolis, MN, USA), respectively. The $S1P_2$ and $S1P_3$ receptor antagonists (JTE-013 and CAY10444) were from Cayman Chemical (Ann Arbor, MI, USA). The S1P assay kit was from Echelon Biosciences (Salt Lake City, UT, USA). SYBR Green JumpStart Ready Mix kits were obtained from Sigma (Oakville, ON, Canada). TRIzol reagent and Superscript II were purchased from Life Technologies (Burlington, ON, Canada). Anti-SGPP1 and SPL antibodies were from Novus Biologicals (Oakville, ON, Canada) and R&D Systems (Minneapolis, MN, USA), respectively. Anti-PI3 kinase p85 (06-195) was purchased from Upstate Biotechnology Associates (Billerica, MA, USA). The Proteome Profiler Human Cytokine Array (panel A) was bought from R&D Systems (Minneapolis, MN, USA). Cell culture reagents were from Wisent Inc. (St-Bruno, QC, Canada).

2.2. Synthesis of SPL Inhibitor. Starting chemicals and solvents were purchased from Sigma Aldrich (Oakville, ON, Canada) and Alfa Aesar (Ward Hill, MA, USA). A Biotage initiator system was used for microwave heating. Nuclear magnetic resonance (NMR) spectra were collected on a Bruker Avance III 400 MHz spectrometer with chemical shifts referenced to residual solvent peaks as secondary reference for ^{1}H and ^{13}C spectra. Crude products were purified using a Sg100c (Teledyne Isco) flash chromatographic instrument.

Compounds SM4 (SPL inhibitor) and SM3 (the inactive enantiomer) (Figure 1) were prepared as previously described [41] and as shown in Scheme 1. Briefly, the substitution of the chlorine of the commercially available 1-benzyl-4-chlorophthalazine (1) with (*R*)-methylpiperazine or (*S*)-methylpiperazine followed by a second substitution of the chlorine of 6-chloronicotinonitrile with compound 2 or 3 gives us the desired compounds SM4 and SM3. The ^{1}H NMR of compounds 2, 3, SM4, and SM3 were identical to those reported previously [41].

2.3. Cell Treatment and Viability. Human primary FLS were isolated from articular synovia of donors with RA (RAFLS) or without history of arthritis (normal FLS). Patients from whom synovial specimens were obtained were diagnosed based on the criteria developed by the American College of Rheumatology Diagnostic Subcommittee for RA [42] and underwent arthroplasty. FLS were isolated by sequential enzymatic digestion as described previously [43]. Briefly, FLS were released by sequential enzymatic digestion with 1 mg/mL pronase for 1 h, followed by 6 h with 2 mg/mL collagenase at 37°C in DMEM supplemented with 10% FBS, 1% sodium pyruvate, 100 U/mL penicillin, and 100 mg/mL streptomycin. Released cells were incubated for 1 h at 37°C in tissue culture flasks allowing the adherence of nonfibroblastic cells possibly present in the synovial preparation. The cells were cultured in DMEM supplemented with 10% FBS and antibiotics at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Semiconfluent cells were starved with serum-free medium for 24 h before treatment. At the moment of cell treatment, the culture medium was replaced with fresh serum-free medium containing various concentrations of the tested compounds as indicated below. Cells were used between passages 3 and 9. Propidium iodide (PI) was used to evaluate the viability of RAFLS by flow cytometry.



Scheme 1: Reagents and conditions: (i) (2): Na_2CO_3, (*R*)-methylpiperazine, dioxane, 100°C, 48 h, 91%; (3): Na_2CO_3, (*S*)-methylpiperazine, dioxane, 100°C, 48 h, 88%; (ii): Na_2CO_3, 6-chloronicotinonitrile, DMF/dioxane, 180°C, microwave (SM4: 11%, SM3: 16%).

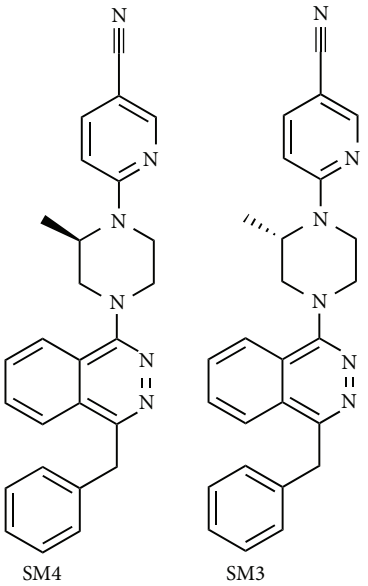


Figure 1: Structures of SM4 and SM3.

Cells were detached using Accutase cell detachment solution and incubated with PI (5 mg/mL). PI negative RAFLS were considered viable.

2.4. IL-8 and MCP-1 ELISA. FLS (5×10^4 cells/well) were plated in 24-well plates and serum starved for 24 h prior to stimulation with 200 μM $CoCl_2$ or 5 μM S1P for an additional 24 h. Where indicated, cells were pretreated for 30 min with 5 μM of the selective $S1P_2$ receptor antagonist JTE-013 and/or selective $S1P_3$ receptor antagonist CAY10444, prior to stimulation with $CoCl_2$ or S1P. To evaluate the effect of SPL inhibition on $CoCl_2$-mediated chemokine secretion, cells were treated with the SPL inhibitor SM4 (or the inactive enantiomer SM3) for 24 h in the absence or the presence of $CoCl_2$ and/or sphingosine. Cell culture supernatants were collected and stored at −80°C until the ELISAs were performed. IL-8 and MCP-1 in all samples were monitored in triplicate, according to the manufacturer's protocol. Optical densities were determined using a SoftMaxPro40 plate reader at 450 nm. The results were compared with a standard curve that was generated using known concentrations (pg/mL) of the chemokines. The detection limit of IL-8 and MCP-1 ELISA was 12.5 pg/mL and 15.625 pg/mL, respectively. Data are expressed either as pg/mL or as the percentage of chemokines secreted relative to the appropriate controls.

2.5. Quantitative Real-Time PCR. FLS (5×10^5 cells) were plated in 6-well plates and serum starved for 24 h prior to stimulation with or without 200 μM $CoCl_2$ in serum-free medium for various times. Total cellular RNA was extracted using TRIzol reagent according to the manufacturer's instructions. RNA (1 μg) was reverse-transcribed using random priming and the Superscript II Reverse Transcriptase system. Real-time PCR was performed to assess the expression of SGPP1, SGPP2, and SPL and their regulation by $CoCl_2$. The following sets of primers were used: SGPP1 forward (5′-GCCGCTGGCAGTACCCT-3′) and reverse (5′-AATAGAGTGCATTCCCATGTAAATTCT-3′); SGPP2 forward (5′-TTCAGAACATCCCACCACTCACCA-3′) and reverse (5′-TTCCTGGTGACCACCTTGAACCAT-3′); and SPL forward (5′-GCCAGAGAGTTTATGGTCAAGGTT-3′) and reverse (5′-CAACTTGTCTTGAATCTTACGACC-AA-3′). The ribosomal protein RPLP0 mRNA was used

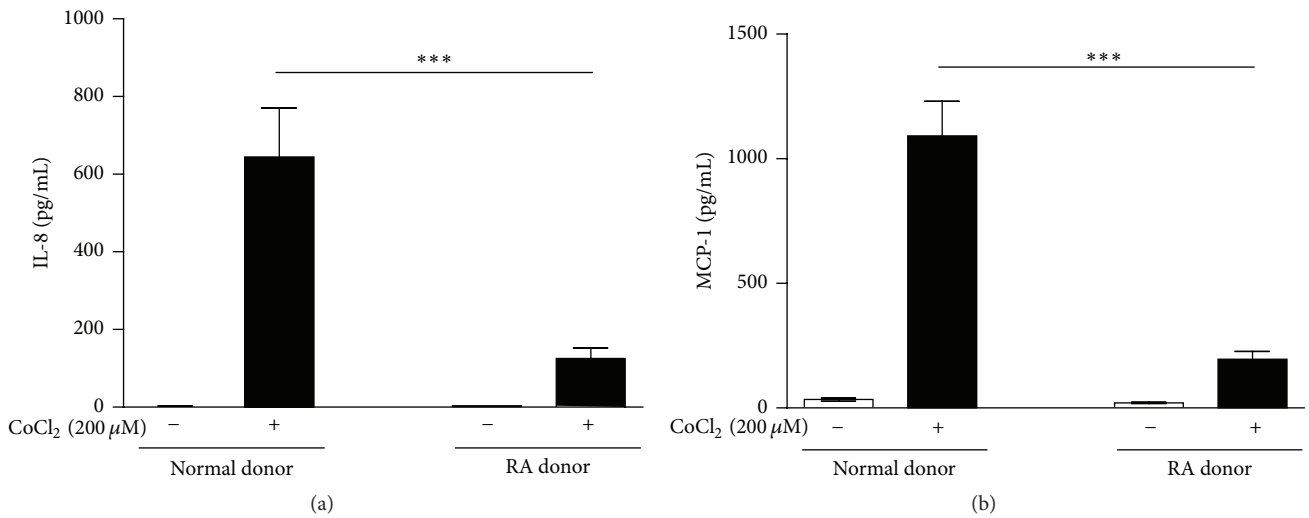


Figure 2: Effect of $CoCl_2$ stimulation on IL-8 and MCP-1 secretion in normal FLS and RAFLS. Human primary FLS from normal ($n = 4$) and RA ($n = 4$) donors were incubated with 200 μM $CoCl_2$. The amounts of IL-8 (a) and MCP-1 (b) released in the supernatants were monitored 24 h after stimulation. The data are the means ± SE from four experiments (4 different donors) performed in triplicate (3 independent experiments). For statistical comparative analyses, we compared RA to normal FLS treated with $CoCl_2$. $^{***}p < 0.001$.

as an internal PCR control. RPLP0 primer sequences were as follows: forward (5′-GTTGTAGATGCTGCC-ATTG-3′) and reverse (5′-CCATGTGAAGTCACTGTGC-3′). Amplicon expression in each sample was normalized to its RPLP0 content. The thermal cycling conditions were as follows: 95°C (initial denaturation, 3 min) followed by 40 cycles of 95°C (denaturation, 15 sec), 54°C (annealing, 20 sec), and 72°C (extension, 20 sec).

2.6. Western Blot. Cells were exposed to 200 μM $CoCl_2$ for various times (0–48 h) and lysed in boiling sample buffer [50 mM Tris/HCL (pH 6.8), 10% (v/v) glycerol, 50 mM DTT, and 4% (v/v) SDS] for 7–10 min. Equal amounts of protein were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to methanol-soaked Immobilon PVDF membranes (Millipore Corporation, Bedford, MA, USA). Primary antibody incubation was performed either overnight at 4°C (anti-SGPPL, SPL) or 1 h at 37°C (anti-PI3 kinase p85). The membranes were then washed three times and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Membranes were washed three times and antibody-antigen complexes were revealed using Western Lightening ECL^+ according to the manufacturer's instructions (Perkin Elmer Life Sciences, Woodbridge, ON, Canada).

2.7. S1P ELISA. FLS from 2 normal and 2 RA donors were cultured up to 80–85% confluence in 75 cm^2 flasks and serum starved for 24 h. Cells were lysed in 400 μL of lysis buffer provided with the S1P ELISA kit. Protein concentration was measured by the BCA method and S1P in cell lysates (1 : 10 in delipidated human serum) was monitored according to the manufacturer's instructions.

2.8. Cytokine/Chemokine Profiling Analysis. RAFLS were treated with the SPL inhibitor SM4 for 24 h in the absence/presence of $CoCl_2$ and sphingosine. Cell culture supernatants were collected and stored at −80°C until the Proteome Profiler Human Cytokine Array (panel A) was performed.

2.9. Statistical Analysis. Unless otherwise stated, experiments were performed three times for each donor and results presented are expressed as mean ± SE or as representative studies. All statistical analyses were performed using Prism 4.0 software. Statistical significance of the difference between samples of two different treatments was determined by t-test (two-tailed p value). For multiple comparisons, statistical significance was determined by one-way ANOVA, Dunnett's multiple comparison test. p values less than 0.05 were considered statistically significant.

3. Results

3.1. Chemokine Secretion by Normal FLS and RAFLS in Response to Hypoxic Stress. To mimic hypoxia, FLS were incubated with $CoCl_2$, a chemical inducer of hypoxia-inducible factor-1 (HIF-1) [44]. The effect of chemical hypoxia on chemokine synthesis was assessed using ELISA assays and $CoCl_2$-dependent secretion of IL-8 and MCP-1 by normal FLS and RAFLS was compared (Figure 2). Small amounts of IL-8 (<3 pg/mL) (Figure 2(a)) and MCP-1 (<35 pg/mL) (Figure 2(b)) were produced by both normal FLS and RAFLS cultured under normoxic conditions. When incubated with $CoCl_2$, normal FLS released significantly larger amounts of IL-8 (644.3 ± 125.9 pg/mL) and MCP-1 (1092 ± 138.6) than RAFLS with similar passage number (125.7 ± 26.5 pg/mL

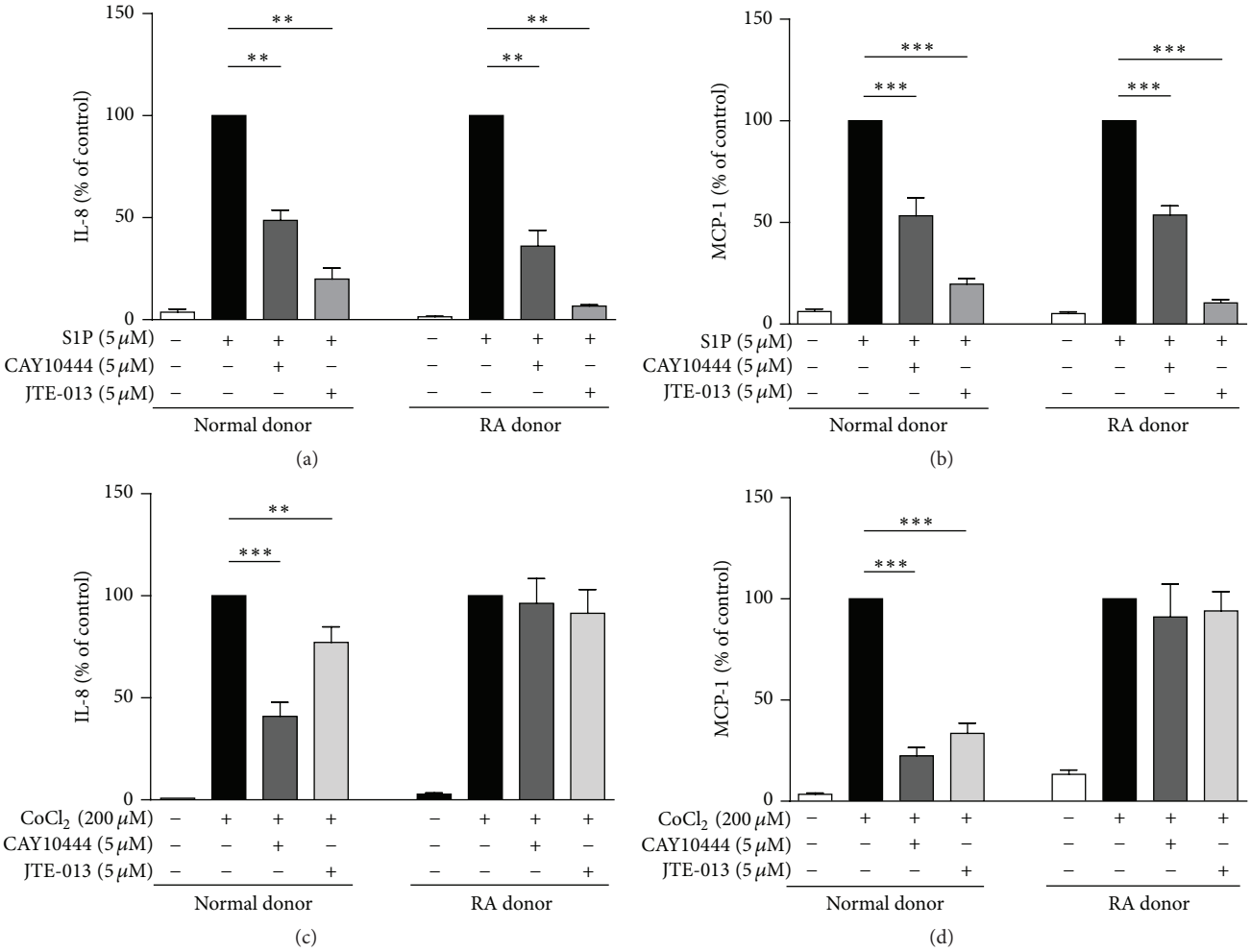


Figure 3: Differential involvement of S1P receptors in S1P- and $CoCl_2$-mediated chemokine secretion by normal FLS and RAFLS. Human primary FLS from normal ($n = 4$) and RA ($n = 4$) donors were incubated with 5 μM S1P (a, b) or 200 μM $CoCl_2$ (c, d). Where indicated, cells were pretreated with $S1P_3$ antagonist CAY10444 (5 μM) or $S1P_2$ antagonist JTE-013 (5 μM) for 30 min before stimulation with S1P or $CoCl_2$. The amounts of chemokines released in the supernatants were monitored after 24 h. Data are expressed as percentage of chemokine production induced by S1P (a, b) or $CoCl_2$ (c, d). The data are the means ± SE from four experiments (4 different donors) performed in triplicate (3 independent experiments). For statistical comparative analyses, the samples stimulated with S1P (a, b) or $CoCl_2$ (c, d) were compared to those stimulated in the presence of CAY10444 or JTE-013, respectively. $^{**}p < 0.01$; $^{***}p < 0.001$.

for IL-8 and 195.3 ± 31.9 for MCP-1) ($p < 0.001$). In both control FLS and RAFLS there was a similar trend of decreased synthesis of IL-8 and MCP-1 in response to $CoCl_2$ with increased number of cell passages (data not show).

3.2. S1P Receptor(s) Dependency of Chemokine Secretion in Normal FLS and RAFLS. S1P regulates a variety of cellular processes through binding to G protein-coupled receptors [45]. We previously reported a role for $S1P_2$ and $S1P_3$ in S1P-mediated IL-8 secretion in RAFLS [33]. As expected, the addition of S1P to normal FLS and RAFLS stimulated the secretion of IL-8 and MCP-1. The amounts of IL-8 and MCP-1 released by normal FLS and RAFLS in response to S1P were not statistically different (149.0 ± 28.62 versus 126.9 ± 14.3 pg/mL for IL-8 ($p = 0.47$) and 800.3 ± 116.4 pg/mL versus 546.5 ± 69.42 pg/mL for MCP-1 ($p = 0.10$)). Under these conditions the $S1P_3$ antagonist CAY10444 and the $S1P_2$ antagonist JTE-013 significantly decreased S1P-induced IL-8 by 51.3 ± 5.0% ($p < 0.01$) and 80.1 ± 5.4% ($p < 0.01$) in normal FLS and by 63.9 ± 7.8% ($p < 0.01$) and 93.3 ± 0.6% ($p < 0.01$) in RAFLS, respectively (Figure 3(a)). CAY10444 and JTE-013 also reduced S1P-mediated MCP-1 secretion by 46.7 ± 8.9% ($p < 0.001$) and 80.3 ± 2.7% ($p < 0.001$) in normal FLS and that of RAFLS by 46.4 ± 4.5% ($p < 0.001$) and 89.6 ± 1.6% ($p < 0.001$), respectively (Figure 3(b)). Similarly, the incubation in normal FLS with CAY10444 and JTE-013 in combination with $CoCl_2$ reduced IL-8 secretion by 59.0 ± 6.8% ($p < 0.001$) and 22.0 ± 7.5% ($p < 0.01$) and that of MCP-1 by 77.6 ± 4.2% ($p < 0.001$) and 66.4 ± 5.0% ($p < 0.001$), respectively (Figures 3(c) and 3(d)). In contrast, the production of chemokines by RAFLS incubated with $CoCl_2$ was not inhibited by the $S1P_3$ or the $S1P_2$ receptor antagonist

Table 1: S1P content in normal FLS and RAFLS.

	S1P content (pmol/mg of protein)
Normal FLS	
Donor #1 (S3618)	64.5 ± 1.5
Donor #2 (S3739)	273.0 ± 21
RAFLS	
Donor #1 (37158A1-S)	19.5 ± 1.5
Donor #2 (87546A1-S)	22.5 ± 1.5

Cell lysates from human primary FLS of normal ($n = 2$) and RA ($n = 2$) donors were prepared. S1P content in cell lysates (50 μg protein) was measured using the S1P assay kit from Echelon Inc. according to the manufacturer's instruction.

(Figures 3(c) and 3(d)). The percentage of PI positive cells treated with 200 μM $CoCl_2$ together with 5 μM CAY10444 and 5 μM JTE-013 for 24 h was identical to that of untreated cells (1.25 ± 0.15% versus 1.4 ± 0.3% for normal FLS treated with $CoCl_2$/CAY10444 versus untreated, 1.15 ± 0.15% versus 1.4 ± 0.3% for normal FLS treated with $CoCl_2$/JTE-013 versus untreated; 1.2 ± 0% versus 1.0 ± 0.1% for RAFLS treated with $CoCl_2$/CAY10444 versus untreated, and 1.15 ± 0.25% versus 1.0 ± 0.1% for RAFLS treated with $CoCl_2$/JTE-013 versus untreated), indicating that inhibition of chemokine synthesis was not mediated by a cytotoxic effect of these compounds.

3.3. Intracellular Levels of S1P in Normal FLS and RAFLS. The response of normal FLS and RAFLS to exogenously added S1P and inhibition of chemokine secretion by the $S1P_3$ and $S1P_2$ receptor antagonists provide evidence for functional S1P receptors in both types of FLS. On the other hand, inhibition of $CoCl_2$-dependent chemokine synthesis by the S1P antagonists in normal FLS but not in RAFLS points toward alteration of an autocrine positive feedback loop driven by S1P. This could be due to impaired steady levels of intracellular S1P and/or export outside cells. To gain insight into the possible mechanisms we monitored the intracellular levels of S1P in normal FLS and in RAFLS. As shown in Table 1 the basal level of intracellular S1P was more elevated in normal FLS as compared to RAFLS.

3.4. Regulation of the Expression of the S1P Degradation Enzymes by $CoCl_2$ in Normal FLS and RAFLS. Decreased steady state levels of intracellular S1P in RAFLS could be due to altered production of S1P by SphKs, increased degradation by S1P phosphatases (SGPP1 and SGPP2) or S1P lyase (SPL), and/or a combination of the two mechanisms. In this study we focussed on the impact of $CoCl_2$ on S1P phosphatases and SPL gene/protein expression in normal FLS and in RAFLS. Quantitative real-time PCR (qPCR) analyses highlighted the expression of SGPP1 and SPL mRNA in cells (Figure 4(a)). SGPP2 mRNA was not detected with the primers we designed for this study (data not shown). As shown in Figure 4(a), SGPP1 and SPL mRNA were ~1.5- and 1.53-fold more abundant in RAFLS than in normal FLS ($p < 0.05$). Moreover, incubation with $CoCl_2$ decreased SGPP1 mRNA levels by 34.7 ± 2.5% ($p < 0.001$) and 64.8 ± 6.2% ($p < 0.05$) and those of SPL mRNA by 45.9 ± 3.0% ($p < 0.001$) and 67.7 ± 6.9% ($p < 0.01$) in normal FLS and RAFLS, respectively (Figure 4(a)). Decreased expression of SGPP1 was confirmed at the protein level in RAFLS with a 48.8 ± 13.1% decrease in SGPP1 protein ($p < 0.05$) after treatment with $CoCl_2$ for 48 h (Figure 4(b)). SPL protein levels in RALFS were not significantly reduced by $CoCl_2$ as estimated by immunoblotting (Figure 4(b)).

3.5. Effect of SPL on $CoCl_2$-Mediated Chemokine Secretion by Normal FLS and RAFLS. To determine whether the levels of intracellular S1P in FLS may impact its transport outside cells and access to its cognate receptors for autocrine signalling we incubated the cells with $CoCl_2$ in the presence or absence of a SPL inhibitor [46, 47]. When normal FLS and RAFLS were incubated with $CoCl_2$ in combination with increasing concentrations of the SPL inhibitor SM4 there was a trend towards increased secretion of IL-8 and MCP-1 (Figure 5 and data not shown). However, even with 3 μM SM4, the highest concentration tested, the increase in chemokine synthesis was not significant compared to cells treated with $CoCl_2$ alone (data not shown). Since the addition of sphingosine to cell line or primary cell cultures has been shown to provide a source of intracellular S1P that is susceptible to degradation by SPL [46, 47], we evaluated the impact of exogenously added sphingosine in combination with the SPL inhibitor on $CoCl_2$-mediated chemokine synthesis. Figure 5 shows that the inhibition of SPL in the presence of sphingosine significantly increased $CoCl_2$-induced chemokine secretion in RAFLS (Figures 5(b) and 5(d)) and in normal FLS as well (Figures 5(a) and 5(c)). In RAFLS SM4 increased the secretion of IL-8 and MCP-1 by 232 ± 23.8% ($p < 0.001$) and 158.7 ± 10.7% ($p < 0.05$), respectively, while in normal FLS SM4 increased IL-8 and MCP-1 secretion by 243.3 ± 73.4% ($p < 0.05$) and 368.5 ± 109.7% ($p < 0.01$), respectively. No significant increase in chemokine synthesis was observed when cells were incubated with $CoCl_2$ in the presence of sphingosine without the SPL inhibitor, with the SPL inhibitor but without sphingosine, or with the inactive enantiomer SM3. The Proteome Profiler Antibody Array confirmed in RAFLS that inhibition of SPL in combination with sphingosine increases $CoCl_2$-mediated IL-8 secretion and possibly that of other cytokines such as IL-6 and IL-23 (Figure 5(e)). When RAFLS were treated with the SPL inhibitor in the presence of sphingosine (Figure 5(f)), $CoCl_2$-mediated secretion of IL-8 and MCP-1 becomes sensitive to inhibition by the $S1P_3$ receptor antagonist CAY10444 (46.9 ± 10.3% and 55.3 ± 4.0% decrease, $p < 0.01$, resp.) and the $S1P_2$ receptor antagonist JTE-013 (45.9 ± 15.9% and 23.5 ± 7.0% decrease, $p < 0.01$, resp.).

4. Discussion

FLS are key effector cells in RA. They spread arthritis to unaffected joints [48] and their altered phenotypes in RA have been associated with changes in signalling cascades, apoptotic responses, and the expression of adhesion molecules as well as matrix-degrading enzymes [49, 50]. The cell microenvironment plays an essential role in determining

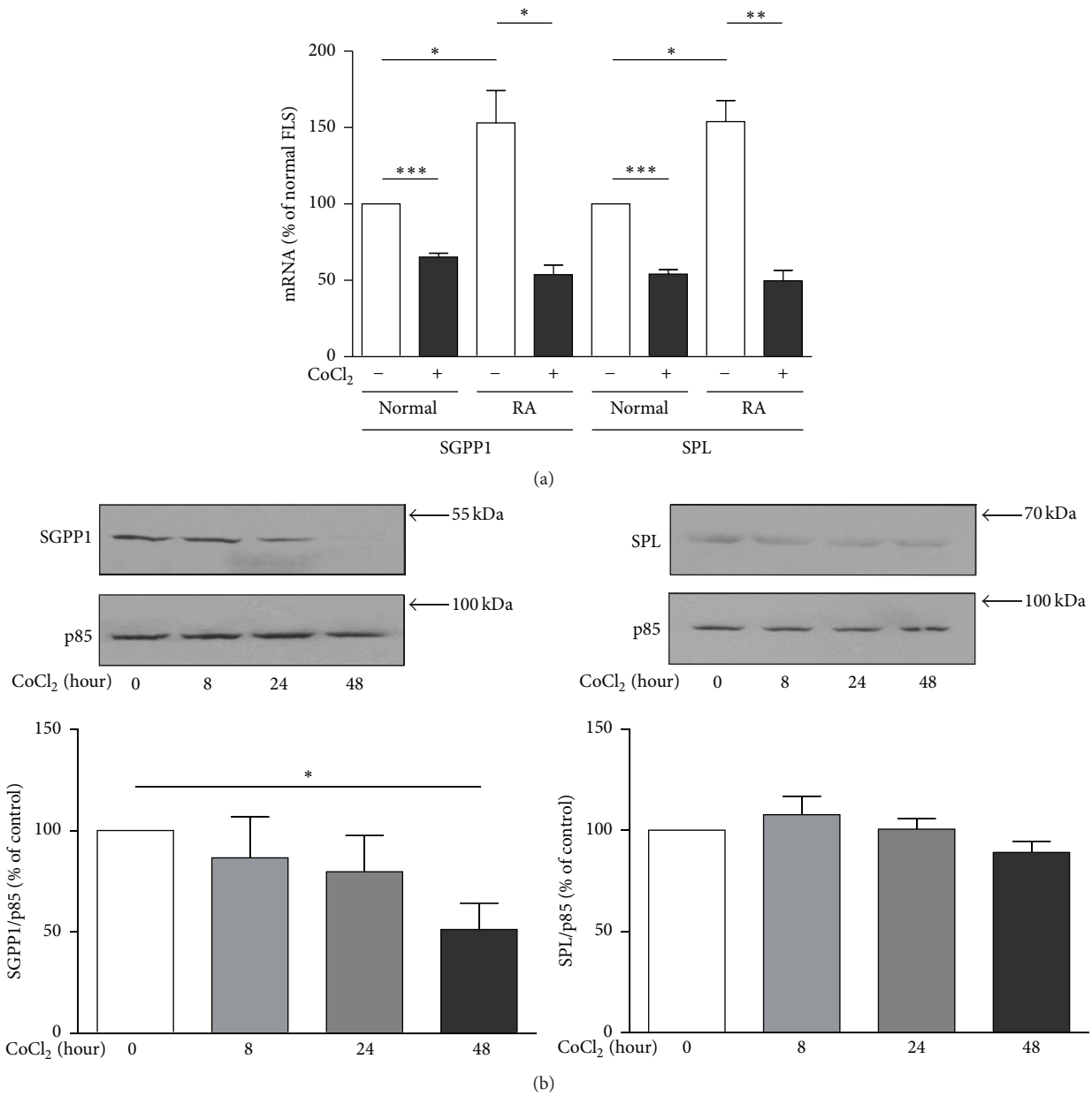


Figure 4: Differential expression of SGPP1 and SPL in normal FLS and RAFLS. Human primary FLS from normal ($n = 4$) and RA ($n = 4$) donors were incubated with or without 200 μM $CoCl_2$ for 24 h. Total RNA was extracted for quantitative PCR analyses and RPLP0 was used as an internal control and data normalized to that of normal FLS (a). The data are the means ± SE from four experiments (4 different donors) performed in triplicate (3 independent experiments). For statistical analyses, we compared the cells stimulated with $CoCl_2$ to those without $CoCl_2$, or normal FLS to RAFLS. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Human primary FLS from RA patients ($n = 3$) were incubated with 200 μM $CoCl_2$ for up to 48 h (b). Proteins from whole cell lysates were prepared for Western blot. Total PI3-kinase p85 subunit was used as a control for protein loading. Data presented are from a representative blot (upper panel) or the means ± SE from three experiments (lower panel). For statistical comparative analyses, the samples stimulated with $CoCl_2$ at 0 h were compared to those treated for indicated times. $^{*}p < 0.05$.

cell phenotype and phenotypic and metabolic characterization of those changes will further our understanding of the pathogenesis of RA. Herein, we report novel characteristics of RAFLS that distinguish these cells from their normal counterparts: (1) RAFLS are less prone to release IL-8 and MCP-1 in response to the hypoxia mimetic $CoCl_2$; (2) $CoCl_2$-mediated chemokine production is, at least in part, due to autocrine activation of S1P receptors in control FLS but not in RAFLS; (3) expression of SGPP1 and SPL mRNA is elevated whereas intracellular levels of S1P are reduced in RAFLS when compared to normal FLS; (4) whereas $CoCl_2$ reduces SGPP1 mRNA and protein expression, the combination of the hypoxic-like stress, sphingosine, and inhibition of SPL is required to enhance chemokine/cytokine synthesis and

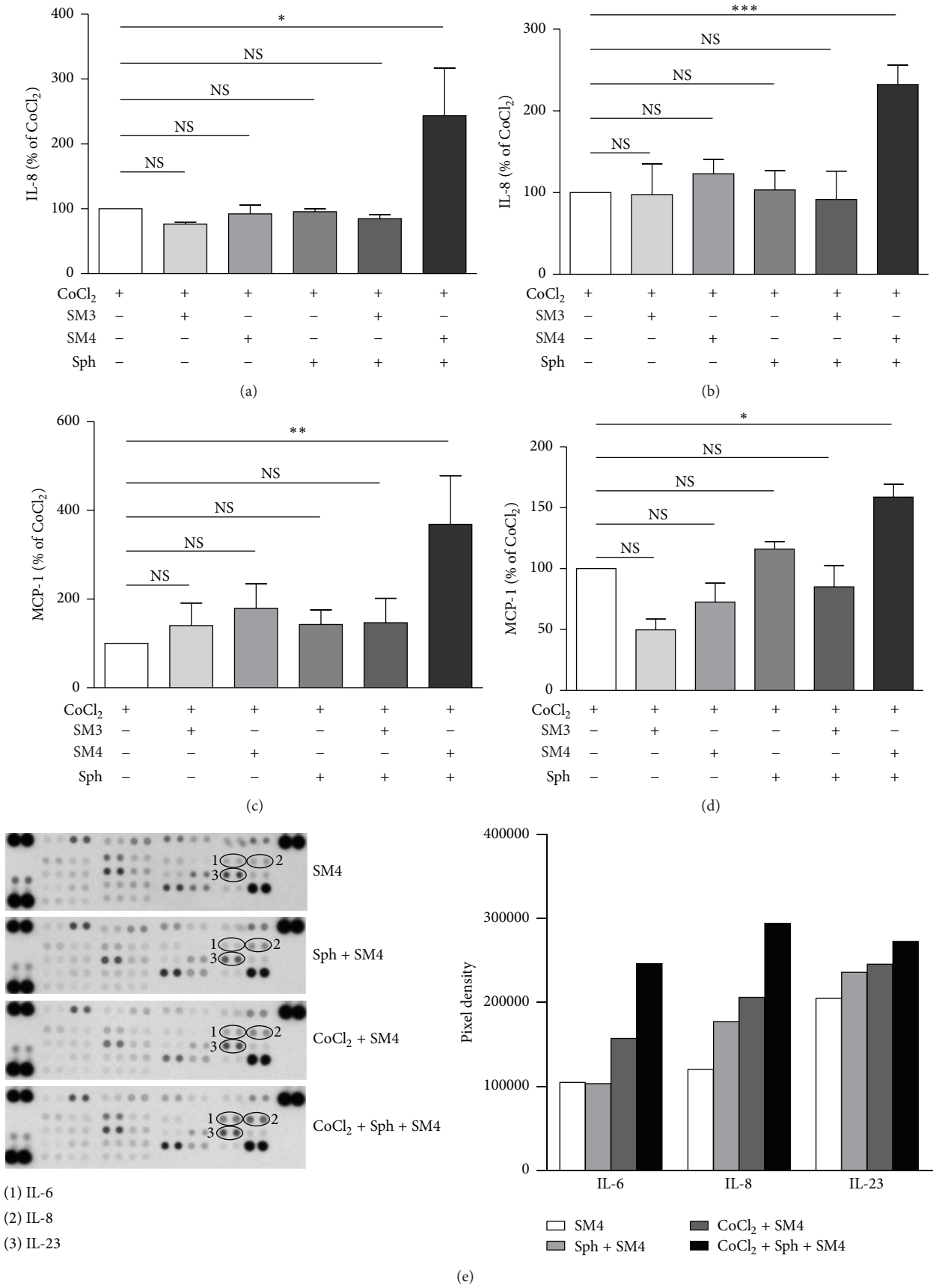


Figure 5: Continued.

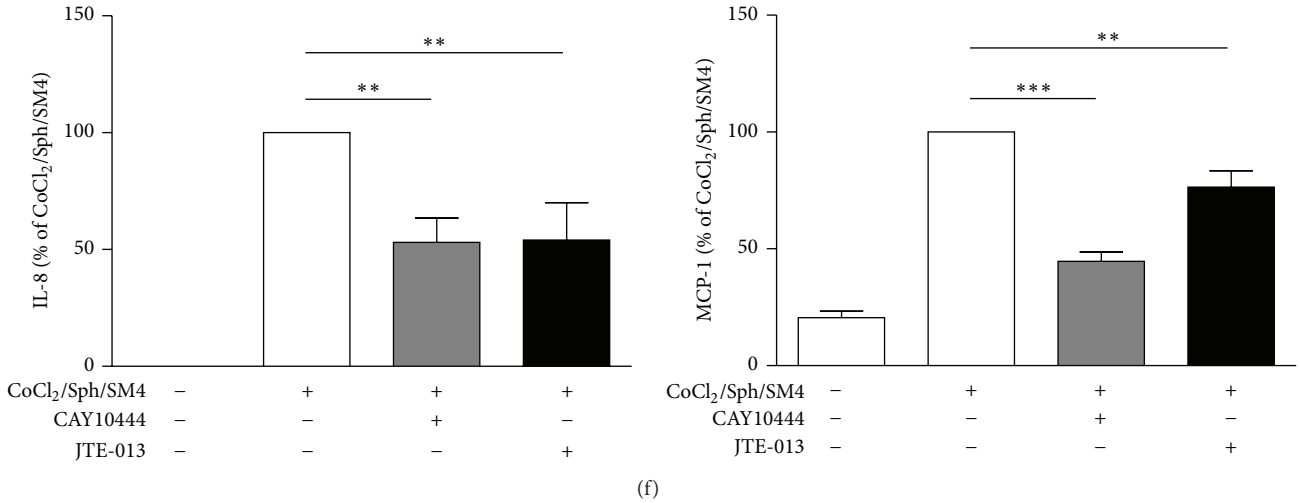


Figure 5: Impact of SPL inhibition on $CoCl_2$-mediated chemokine/cytokine secretion in normal FLS and RAFLS. Human primary FLS from normal (a, c) and RA (b, d, e, f) donors were incubated with 200 μM $CoCl_2$ in the presence of SPL inhibitor SM4 (3 μM) or the inactive analog SM3 (3 μM) and sphingosine (1 μM) for 24 h. Where indicated, cells were pretreated with $S1P_3$ antagonist CAY10444 (5 μM) or $S1P_2$ antagonist JTE-013 (5 μM) for 30 min before stimulation with $CoCl_2$ in combination with sphingosine (Sph), SM4, or SM3. The data are the means ± SE from three experiments. For statistical comparative analyses, chemokine levels in the samples stimulated with $CoCl_2$ were compared to that of other samples (a–d) or chemokines produced by cells stimulated with $CoCl_2$ in combination with Sph and SM4 were compared to those produced by cells incubated with the S1P receptor antagonists prior to cell stimulation (f). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Cytokine/chemokine secretion in RAFLS supernatants was analyzed using Proteome Profiler Human Cytokine Array panel A (e). Circled pairs of duplicate spots represent one cytokine/chemokine.

to restore a positive autocrine feedback loop of chemokine synthesis depending on S1P receptor activation in RAFLS. The data suggest that sphingolipid metabolism is altered in RAFLS collected from patients with advanced RA.

Hypoxia was reported to potentiate the expression of inflammatory cytokines, MMPs, and VEGF in RAFLS stimulated with TLR ligands [51]. Moreover, hypoxia has been shown to induce the expression of IL-8 mRNA in RAFLS [24]. In agreement with those findings we report that the hypoxia mimetic agent $CoCl_2$ stimulated IL-8 and MCP-1 production in normal FLS and RAFLS. Surprisingly, the amounts of IL-8 and MCP-1 released by RAFLS incubated with $CoCl_2$ were less than those produced by normal FLS, indicative of altered molecular pathways regulating chemokine synthesis in RAFLS. RAFLS phenotypic changes are possibility related to genetic/epigenetic determinants and genetic mutation due to chronic exposure to a hypoxic inflammatory environment [52]. Indeed, the expression of many genes involved in immune and inflammatory function is differently regulated by hypoxia in normal FLS and RAFLS [53]. The proinflammatory chemokines/cytokines MCP-2, MIP-2α, MIP-2β, and IL-12A for instance are downregulated whereas the anti-inflammatory mediators CD300a and AMPD3 are upregulated by hypoxia in RAFLS [53].

Upregulation of SphK1 expression and activation by hypoxia has been linked to increases in intracellular and extracellular S1P levels [54]. Previous studies have highlighted the expression of $S1P_1$, $S1P_2$, and $S1P_3$ receptors in RAFLS [33, 34]. High expression of $S1PR_1$ in RA synovial tissue was observed in the synovial lining, vascular endothelial cells, and mononuclear cells when compared to osteoarthritis and normal synovial tissues [34]. In vitro S1P induces RAFLS migration, expression of cytokines/chemokines and COX-2, prostaglandin synthesis, and cell proliferation and survival [33, 34]. S1P receptors expressed by RAFLS have redundant functions. In a wound-closing assay S1P induced RAFLS migration through $S1P_1$ and $S1P_3$ receptors [33]. On the other hand, S1P stimulated the secretion of numerous cytokines/chemokines (IL-8, IL-6, MCP-1, and RANTES) through $S1P_2$ and $S1P_3$ receptors. In the present study we provide evidence that the mechanism by which $CoCl_2$ induces the secretion of chemokines is, at least in part, through autocrine activation of $S1P_2$ and $S1P_3$ receptors in normal FLS. Although RAFLS express functional $S1P_2$ and $S1P_3$ receptors, $CoCl_2$-mediated chemokine synthesis was not reduced by S1P receptor antagonists. This was related to low levels of intracellular S1P in RAFLS since incubation of cells with an inhibitor of SPL and sphingosine, a condition that has been shown to increase intracellular amounts of S1P and its release by various cells [46, 47], restores autocrine signalling through $S1P_2$ and $S1P_3$ receptors in RAFLS stimulated with $CoCl_2$.

S1P synthesis requires the concerted action of ceramidase and sphingosine kinases and once formed, S1P is either metabolized to hexadecenal and ethanolamine phosphate by SPL or recycled to sphingosine by S1P phosphatases [30]. Upregulation of SGPP2 has been detected in samples of skin lesions from patients with psoriasis, a chronic inflammatory skin disease [55]. Other studies investigating sphingolipid metabolism have shown that oxygen deprivation in microendothelial cells resulted in reduced SPL activity [56] and that adipocytes respond to hypoxia by downregulating

SPL expression [57]. In this study we provide evidence for increased expression of SGPP1 and SPL mRNA in RAFLS, suggesting that the lower level of intracellular S1P in these cells is possibly driven by a hypercatabolic state. Targeting $S1P_1$ receptor with a selective antagonist [38] or with the sphingosine analogue FTY720 [58, 59] and pharmacological inhibition of SPL in mice [60], all decreased the development of collagen-induced arthritis (CIA). The anti-inflammatory properties of these compounds are associated with abnormal B and T cell maturation and lymphocyte egress from lymphoid organs due to local S1P gradient breakdown or $S1P_1$ receptor degradation [38, 61, 62]. Whereas inhibition of SPL may have a beneficial effect through targeting lymphocyte trafficking from lymphoid organs, we suggest that inhibition of SPL may have adverse inflammatory effects by increasing the steady state levels of intracellular S1P, S1P export, and synthesis of proinflammatory chemokines/cytokines through autocrine/paracrine activation of $S1P_2$ and $S1P_3$ receptors. Allende et al. recently reported that SPL deficiency in mice promotes an inflammatory response [63].

A few studies have evaluated S1P levels and S1P metabolizing enzymes in RA synovial biopsy. For example, expression of SphK2 and elevated levels of S1P were detected in the synovium and synovial fluids of RA patients [34–36]. Animal models have been used to evaluate the role of S1P in inflammatory arthritis. In the CIA model, administration of a nonspecific inhibitor of SphKs or of a siRNA to silence SphK1 markedly suppressed cartilage and bone erosion, synovial hyperplasia, and leukocyte infiltration into the joint compartments [36]. While SphK1 activity is proinflammatory, SphK2 has an opposite role since the silencing of this enzyme in mice promotes CIA-mediated synovitis [64]. However, depending on the animal models of arthritis employed, studies with KO mice have produced conflicting information. Whereas SphK1 deficiency has been reported to reduce synovial inflammation and bone erosions in human TNF-α transgenic mice, which spontaneously develop inflammatory arthritis [37], SphK2 deficiency has no impact on disease severity and progression [65]. Our preliminary data suggest that $CoCl_2$ induces SphK1 expression in normal FLS whereas SphK1 seems to be less prone to upregulation by $CoCl_2$ in RAFLS (data not shown). Further characterization is underway to determine whether altered expression and/or activation of Sphks contribute to reduced steady state levels of intracellular S1P in RAFLS.

In summary, the results of this study suggest that the sphingolipid metabolism involved in the production and/or release of S1P under hypoxic-like conditions is altered in RAFLS. Decreased steady state levels of intracellular S1P in RAFLS were associated with reduced production of chemokine/cytokine and autocrine activation of $S1P_2$ and $S1P_3$ receptors in response to chemical hypoxia. Our data provide new insights into the mechanisms that may regulate inflammation and possibly joint destruction in advanced cases of RA.

Conflict of Interests

The authors have declared no conflict of interests.

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