

# Roles of Ceramides and Other Sphingolipids in Immune Cell Function and Inflammation

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## Abstract

Ceramides are bioactive sphingolipids that support the structure of the plasma membrane and mediate numerous cell-signaling events in eukaryotic cells. The finding that ceramides act as second messengers transducing cellular signals has attracted substantial attention in several fields of Biology. Since all cells contain lipid plasma membranes, the impact of various ceramides, ceramide synthases, ceramide metabolites, and other sphingolipids has been implicated in a vast range of cellular functions including, migration, proliferation, response to external stimuli, and death. The roles of lipids in these functions widely differ among the diverse cell types. Herein, we discuss the roles of ceramides and other sphingolipids in mediating the function of various immune cells; particularly dendritic cells, neutrophils, and macrophages. In addition, we highlight the main studies describing effects of ceramides in inflammation, specifically in various inflammatory settings including insu-

lin resistance, graft-versus-host disease, immune suppression in cancer, multiple sclerosis, and inflammatory bowel disease.

## Keywords

Ceramides · Immune cells · Sphingolipids · Inflammation · Disease

## Abbreviations

3KS	3-keto-sphinganine
acyl-CoA	fatty acyl-coenzyme A
aSMase	acid sphingomyelinase
ATM	adipose tissue macrophages
C1P	ceramide 1-phosphate
CerS	ceramide synthases
CNS	central nervous system
COX-2	cyclooxygenase-2
CTL	cytotoxic T lymphocytes
CXCR2	C-X-C motif chemokine receptor type 2
DAG	diacylglycerol
DC	dendritic cells
DSS	dextran sulfate sodium
EAE	autoimmune encephalomyelitis
ERK	extracellular signal-regulated kinases

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fMLP	N-formylmethionine-leucyl-phenylalanine
GalCer	galactosylceramides
G-CSF	granulocyte-colony stimulating factor
GI	gastrointestinal
GVHD	Graft-Versus-Host Disease
HIV	human immunodeficiency virus
IBD	Inflammatory Bowel Disease
IFN $\gamma$	interferon gamma
IL-	interleukin
iNOS	inducible nitric oxide synthase
IRS-1	insulin receptor substrate-1
JNK	c-Jun N-terminal kinase
LacCer	lactosylceramide
LipC6	nanoliposome-loaded C <sub>6</sub> -ceramide
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
M-CSF	macrophage-colony stimulating factor
MDSC	myeloid-derived suppressor cells
MS	Multiple Sclerosis
NETs	neutrophil extracellular traps
Nlrp3	Nod-like receptor pyrin domain-containing-3
NO	nitric oxide
PI3K	phosphatidylinositol 3 kinase
PKB	protein kinase B
PKC $\zeta$	protein kinase C zeta
PRR	pattern recognition receptors
ROS	reactive oxygen species
S1P	sphingosine 1-phosphate
Sa	sphinganine
SK	sphingosine kinase
SLs	sphingolipids
SMase	sphingomyelinase
So	sphingosine
SPT	serine palmitoyltransferase
TAM	tumor-associated macrophages
TCR	T cell receptor
TEM	tumor microenvironment
TGF $\beta$	transforming growth factor beta
TLRs	toll-like receptor
TNF $\alpha$	tumor necrosis factor alpha
T <sub>reg</sub>	regulatory CD4 <sup>+</sup> T cells

## 15.1 Introduction

Ceramides are sphingolipids (SLs) that along with sterols and glycerolipids constitute the “fluid” part of the plasma membrane of eukaryotic cells. Ceramides are biologically active metabolites of the SL family, composed of a sphingoid base that mainly consists of the 18-carbon amino alcohols sphinganine (Sa) or sphingosine (So) covalently bound to a long fatty acyl side-chain [1, 2]. Conjugation of various headgroups to ceramide leads to the production of sphingomyelin (choline phosphate group), ceramide 1-phosphate (C1P) (phosphate group), glucosylceramide (glucose), galactosylceramides (GalCer) (galactose), or diverse glycolipids of the ganglioside and globoside families (addition of various saccharides) [1, 3, 4]. Apart from the differences in the headgroups, the variations in the number of carbons of the sphingoid base, the length of the fatty acyl side-chain, and location of double bonds lead to the diversification of ceramide structure and biological function [5].

In eukaryotic cells, ceramide generation occurs via three main pathways: *de novo* synthesis, sphingomyelin hydrolysis, and the salvage pathway [6, 7]. In the *de novo* biosynthetic pathway, ceramide synthesis is first mediated by serine palmitoyltransferase (SPT) that transfers serine to fatty acyl-coenzyme A (acyl-CoA), leading to the generation of 3-keto-sphinganine (3KS), which is then reduced by 3KS reductase producing the saturated amino alcohol Sa. Sa is later *N*-acylated through the action of any of the 6 identified ceramide synthases (CerS) forming dihydroceramide which is finally converted to ceramide via the action of dihydroceramide desaturases [8–10]. The second major reaction that results in ceramide production is sphingomyelin hydrolysis. In this reaction ceramides are generated via hydrolysis of the phosphocholine head group from sphingomyelin by sphingomyelinase (SMase) enzymes [11, 12]. The third major pathway by which ceramide is produced is

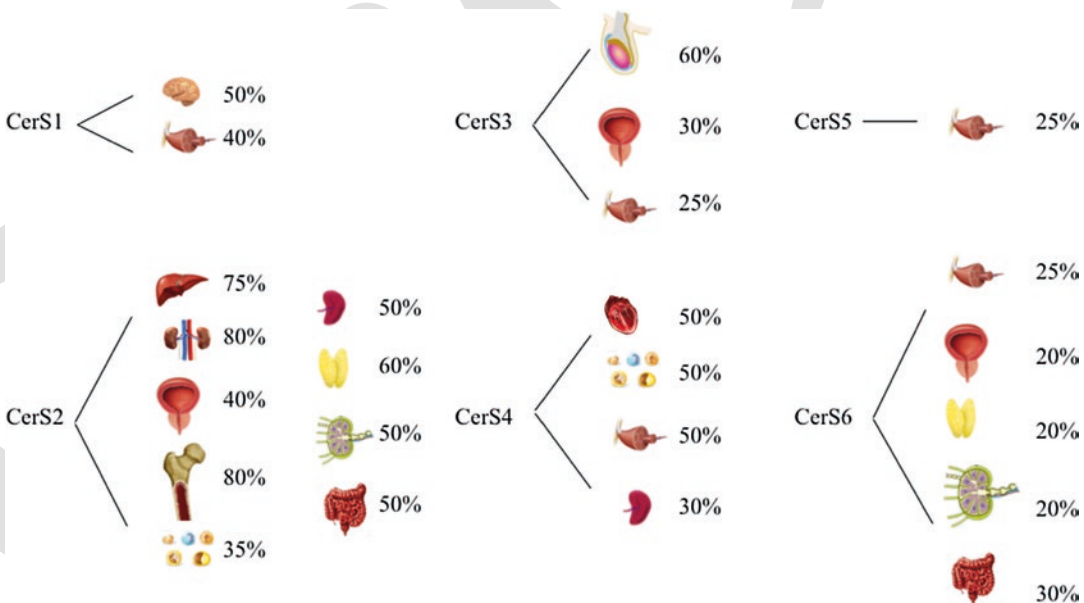
the SL recycling or salvage pathway. In this pathway, complex SLs are catabolized to So which is subsequently *N*-acylated to ceramide. A more complex network of enzymes involving SMases, ceramidases, and CerS are implicated in the pathway [13].

In mammals CerS family consists of 6 enzyme isoforms, named CerS1-6 [9, 14, 15]. Different CerS are distributed differentially in cells and tissues in the body (Fig. 15.1) [14, 16]. All CerS transfer acyl-CoA of variable lengths to the amine group of a sphingoid base [2]. Each CerS has a higher specificity towards the transfer of acyl-CoA of a certain length [9]. CerS1 shows specificity towards the transfer of C<sub>18</sub>-CoA [17]; CerS2 is specific to C<sub>20-26</sub> [16]; CerS3 acylates sphingoid bases with very long-chain fatty acyl CoAs (>26) [18]. CerS4 is specific for the transfer of C<sub>18-22</sub> acyl CoAs [19], whereas the preferred substrates for CerS5 and CerS6 are C<sub>14-18</sub> CoAs [20], and C<sub>14-16</sub> CoAs [16], respectively. Even though the specificity of CerS towards different CoAs has been well established, little is known about the CerS regions that determine such specificity, because the crystal structure of different CerS has not been solved. However, it

has been recently reported by Tidhar et al., that 11 key amino acid residues might be critical in determining the acyl chain length specificity of CerS2, CerS4, and CerS5 [21].

Functional CerS are important players for the wellbeing of cells. Loss of CerS has led to abnormalities in mouse models (Table 15.1): CerS1 knockout mice suffered from reduced ganglioside levels and Purkinje cell loss leading to impaired behavioral and motor development [17, 22]. CerS2 knockout mice developed hepatocarcinoma and cerebral degeneration [28–30]. In addition, CerS3 loss resulted in disruption of the skin barrier and spermatogenic arrest [18, 31]. While CerS4 deficient mice developed alopecia [32] due to destabilization in epidermal stem/progenitor cell homeostasis [35]. Interestingly, CerS5 loss led to improved adipose tissue health and function after consumption of high fat diet [20]. Deficiency of CerS6 led to behavioral abnormalities and abnormal clasping of the hind limbs in mice [34].

Ceramides are the simplest SLs composed of two hydrophobic tails and a simple rather than complex hydrophilic head, consisting of a hydroxyl group [36]. Because ceramides are



**Fig. 15.1** Tissue distribution of CerS isoforms in mice. Tissue distribution of all CerS isoforms based on mRNA levels [14, 16]. The percentage next to organ pictures represents the relative amount of each CerS isoform in that organ

**Table 15.1** Cellular abnormalities resulting from CerS deficiency

Deficient CerS isoform	Abnormality <sup>a</sup>	Reference(s)
CerS1	Purkinje cell loss; motor development impairment	Ginkel et al. [17] and Zhao et al. [22]
CerS2	Delayed liver regeneration after partial hepatectomy	Jin et al. [23]
	Increased intestinal permeability	Chen et al. [24]
	Pheochromocytoma	Park et al. [25]
	Impaired neutrophil migration	Barthelmes et al. [26]
	Enhanced liver tumorigenesis	Chen et al. [27]
	Cerebral degeneration, myelin sheath defects	Imgrund et al. [28] and Pewzner-Jung et al. [29, 30]
CerS3	Skin barrier deformation	Jennemann et al. [18]
	Blocked spermatogenesis	Rabionet et al. [31]
CerS4	Altered lipid composition in skin; alopecia	Ebel et al. [32]
CerS5	Improved glucose homeostasis and adipose tissue health following high fat diet <sup>a</sup>	Gosejacob et al. [20]
CerS6	Protection against the development of colitis <sup>a</sup>	Scheffel et al. [33]
	Behavioral problems	Ebel et al. [34]

<sup>a</sup>Abnormality may not necessarily be detrimental to cells, it may be beneficial, but still it is a change from normal conditions

hydrophobic and usually composed of a C<sub>18</sub> sphingoid base and a long C<sub>14–26</sub> N-linked-fatty acyl group, these lipids are embedded in the membrane making it harder to study their physiological functions. For this reason, more permeable ceramide analogs with short chains have been used in the laboratory (C<sub>2</sub>, C<sub>6</sub>, and C<sub>8</sub>) to better understand ceramide biological function [37]. Nevertheless, numerous studies have been performed on the biophysical effects of ceramides in membranes ([30, 38–40]; Stiban et al.

[41]). In all, ceramides are not mere structural components in membranes. Along with So, sphingosine 1-phosphate (S1P), C1P and lyso-sphingomyelin, ceramides have been implicated as bioactive lipids [42] that act as second messengers and regulate cellular functions including apoptosis and stress responses [43], tumor cell death and metabolism [44], and cytokine signaling and inflammation [45, 46]. Interestingly, numerous evidences point to the ability of ceramides to self-assemble into protein-permeable channels [47] in artificial membranes [48, 49], mitochondria [50–53] and lysosomes [54] that are upstream to caspase-dependent apoptotic cell death.

Despite the enormous progress in understanding the effects of ceramides in regulating key events in cellular biology, their role in regulating immune cell function and inflammatory diseases has only gained momentum in the last two decades. Herein, we discuss the main research findings describing the roles of ceramides in regulating the function of various immune cells, including dendritic cells (DC) and neutrophils, and the modulation of T cell function and macrophages in different disease settings. In addition, we will address the main findings highlighting ceramide function in various inflammatory diseases.

## 15.2 Ceramides and Other SLs in Immune Cell Function

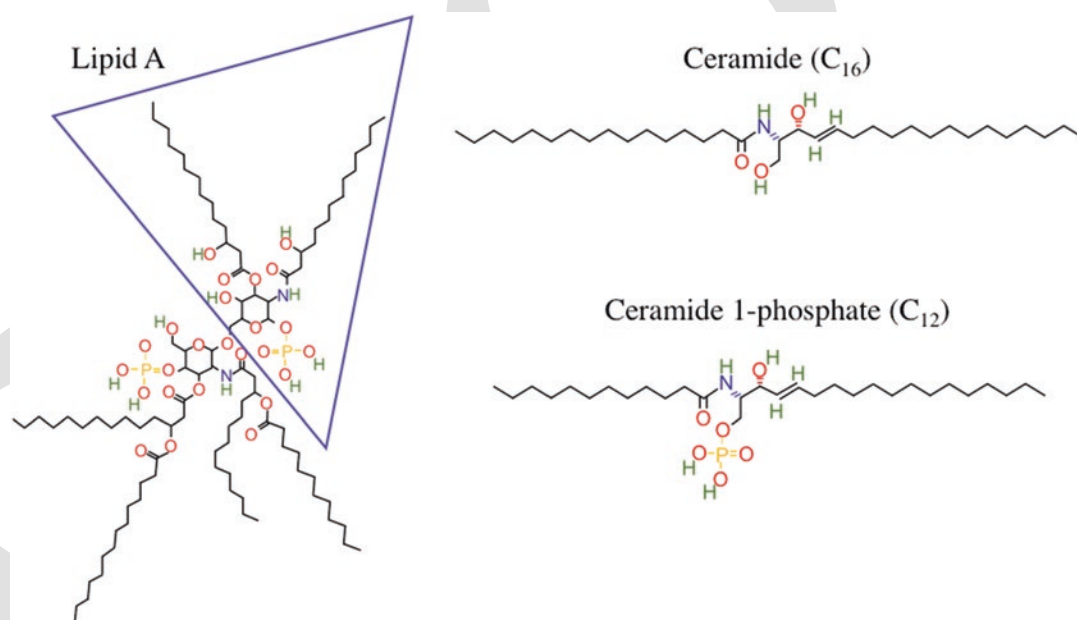
### 15.2.1 Dendritic Cells

Unraveling the mechanisms of DC development, differentiation, maturation, antigen uptake, processing, and presentation have lied in the core of immunology research since their discovery by Steinman more than four decades ago [55, 56]. The main function associated with DC is linking the innate and adaptive immune responses through the uptake of foreign antigens and subsequent presentation to T cells in order to mount effective inflammatory and immune responses. In early studies, the role of ceramides

in regulating DC function was initially associated with induction of apoptosis by  $C_2$ -ceramide [57]. Similarly, induction of DC apoptosis was linked to increased accumulation of ceramides in DC cultured with tumor supernatants, subsequently leading to down regulation of the following survival signaling pathways, phosphatidylinositol 3 kinase (PI3K), Akt kinase, Bcl- $x_L$ , and NF- $\kappa$ B [58]. However, induction of ceramide accumulation in DC by factors that cause DC maturation including, CD40L, interleukin 1 $\beta$  (IL-1 $\beta$ ), tumor necrosis factor alpha (TNF $\alpha$ ), and the gram-negative bacterial endotoxin lipopolysaccharide (LPS) did not induce DC apoptosis [59, 60]. These controversial findings were later reconciled by Franchi and colleagues demonstrating that ceramide-induced cell death in DC is only exacerbated in the absence of serum, while in the presence of serum and LPS, DC survival was achieved by the action of cellular ceramidases that deacylate ceramides to So, thus, preventing ceramide accumulation and DC apoptosis [61].

The study of ceramides in DC gained further attention due to the structural resemblance between the toxic component of LPS, lipid A, and ceramide (Fig. 15.2) [62]. It was suggested that LPS mediates its function by mimicking ceramides since, similar to LPS inducing DC maturation,  $C_2$ -ceramide reduces micropinocytosis and antigen presentation to T cells by DC [60]. However, while both LPS and  $C_2$ -ceramide induce c-Jun N-terminal kinase (JNK), only LPS activates extracellular signal-regulated kinases (ERKs) and NF- $\kappa$ B. In addition, LPS stimulates the production of ceramides regardless of whether macrophages are genetically responsive or unresponsive to LPS [63]. Therefore, this further supported that LPS exerts its function by inducing ceramide accumulation and not by interacting with ceramide-producing enzymes or as a structural mimic of ceramide.

Moreover, ceramides play important roles in DC during viral infections. GalCer on epithelial cells binds the human immunodeficiency virus (HIV)-1 envelope glycoproteins gp120 [64, 65]



**Fig. 15.2** Structural similarity between Lipid A of LPS and ceramides. Two representative ceramides are included for comparison, the most abundant  $C_{16}$ -ceramide and the phosphorylated  $C_{12}$ -ceramide 1-phosphate. The blue triangle identifies the comparable

structure to ceramides. The 2D structures were obtained from PubChem and were redrawn to increase resolution. PubChem CID: 9877306 (Lipid A), 71314646 ( $C_{16}$ -ceramide) and 5283580 ( $C_{12}$ -ceramide 1-phosphate).



and gp41 [66, 67]. In addition, GalCer is essential in the formation of membrane lipid rafts that allow for the internalization of HIV-1 through endocytosis and transcytosis [68]. Interestingly, GalCer is present in monocyte-derived immature DC and human primary DC isolated from mucosa, suggesting that DC mediates HIV-1 transfer to its target cells through GalCer [69]. Ceramides also stabilized the membrane for measles virus entry in DC, as Dendritic Cell-specific intercellular adhesion molecule-3-grabbing non-integrin ligation on DC results in sphingomyelinase activation and subsequent ceramide accumulation in DC exposed to either mannan or measles virus [70].

The role of ceramides was further extended beyond viral entry in DC. It has been demonstrated that local administration of a ceramide analog C<sub>8</sub>-ceramide causes the induction of DC maturation, secretion of the pro-inflammatory cytokines IL-12p70 and TNF $\alpha$ , and enhanced virus-specific T cell responses in murine models of chronic lymphocytic choriomeningitis virus clone 13 and influenza virus [71].

Collectively, these findings demonstrated that DC apoptosis, maturation, and antigen presenting capacity, are finely tuned by the action of endogenous ceramides or treatment with exogenous ceramide analogs. In addition, ceramides are key structural components of lipid rafts in DC required for binding, uptake, and internalization of viruses. These steps are key for initiating DC response allowing for viral internalization, virus peptide processing, and presentation to T cells that subsequently kill virus-infected cells. Since these findings strongly suggest that ceramides are key effectors of DC function, it is important to note that future studies elucidating the role of ceramides on the various DC subsets (e.g. plasmacytoid DC, migratory classical DC, tissue resident classical DC) could be of great therapeutic relevance in numerous immunologic diseases.

### 15.2.2 Neutrophils

Neutrophil extravasation, migration, production of cytokines and superoxide, and formation of

neutrophil extracellular traps (NETs) in response to inflammatory stimuli are crucial for mounting an effective frontline immune response against invading microorganisms, especially extracellular pathogens [72]. Since the isolation of the free long-chain base So from human neutrophils [73], the role of ceramides in mediating and regulating neutrophil function has gained considerable traction. This was particularly due to early studies linking TNF $\alpha$  signaling, a potent inducer of superoxide and apoptosis in neutrophils [74], to ceramide function [75]. In HL-60 human promyelocytic leukemia cells, TNF $\alpha$  causes early sphingomyelin hydrolysis and ceramide production [76]. In addition, in a cell-free system both sphingomyelin content and ceramide concentration were reported to be increased in response to TNF $\alpha$  [77]. It was therefore hypothesized that ceramides might regulate neutrophil function in response to TNF $\alpha$ . However, later studies have proposed that ceramides activated a negative feedback loop to inhibit superoxide production in human neutrophils. It has been shown that ceramides not only did not mediate TNF $\alpha$ -induced superoxide production in human neutrophils [78] but also C<sub>2</sub>-ceramide inhibited the 20:4 (n-6)-mediated superoxide formation in human neutrophils [79]. C<sub>2</sub>-ceramide also inhibited respiratory burst of N-formylmethionine-leucyl-phenylalanine (fMLP)-stimulated adherent neutrophils [80–82]. Modulation of neutrophil response to polarity in response to a chemotactic agent such as fMLP has been shown to be dependent on neutral sphingomyelinase activity that converts sphingomyelin to ceramide through the modulation of Rac1/2/RhoA GTPases [83]. These combined results suggested that induction of ceramides might delay the response of neutrophils to TNF $\alpha$  to allow for neutrophil extravasation and migration prior to superoxide production.

Ceramides have also been implicated in the modulation of phagocytosis and migration in neutrophils. C<sub>2</sub>-ceramides reduced phagocytosis of IgG-opsonized erythrocytes by fMLP-activated neutrophils through the inhibition of MAP kinase activation and tyrosine phosphorylation of ERK1 and ERK2 [84]; and by inhibiting

phospholipase D (PLD) function required for phagocytosis [85, 86]. Similarly, in COS-1 monkey kidney immortalized cells transfected with FcγIIA receptor, inhibition of ceramide synthesis led to enhanced phagocytosis [87]. On the other hand, chemotaxis, phagocytosis and NETs formation in fMLP-stimulated neutrophils were enhanced by the selective estrogen receptor antagonist, tamoxifen, through induction of ceramide accumulation, and subsequently protein kinase C zeta (PKCζ) activation [88]. These conflicting findings might suggest the opposing roles of various ceramides on neutrophil function.

A more direct role of ceramides is also prevalent in neutrophils. Ceramides are pro-apoptotic metabolites whose concentration in cells rises prior to the execution of the apoptotic pathway. Neutrophil apoptosis is mediated by C<sub>16</sub>- and C<sub>24</sub>-ceramides via caspase activation. This is correlated with the ability of granulocyte-macrophage colony-stimulating factor, a neutrophil survival factor, to reduce the accumulation of ceramides in neutrophils [89]. During early neutrophil apoptosis ceramide is generated by acid SMase (aSMase). In aSMase  $-/-$  mice, neutrophil apoptosis is delayed compared to WT mice [90]. In an anti-microbial setting, *Pseudomonas aeruginosa* release pyocyanin that induce reactive oxygen species (ROS), which subsequently activates mitochondrial SMase, therefore, increasing mitochondrial ceramide levels and inducing cytochrome *c* release from mitochondria. This initiates cell death in neutrophils [91]. In addition, the enzyme sphingomyelin synthase, which mediates the transfer of choline phosphate to ceramide from phosphatidylcholine, leads to the production of sphingomyelin and diacylglycerol (DAG) [92, 93] and mediates neutrophil killing of fungus *Cryptococcus neoformans* [94].

Nevertheless, the aforementioned negative regulation by ceramides was not reported upon stimulation with the glycosphingolipid, lactosylceramide (LacCer). In neutrophils, LacCer compose more than two thirds of the glycolipid molecules in the plasma membrane and is mainly associated with a pro-inflammatory phenotype [95]. This large composition was later demon-

strated to be not only relevant in supporting the membrane structure but also as a transducer of cellular signals. *In vitro* studies have revealed that LacCer enhances the upregulation of the integrin, CD11b, on neutrophil surface, inducing neutrophil adherence to endothelium, and mediating the production of ROS through activation of NADPH oxidase [96]. NADPH oxidase activation in neutrophils results from the association of LacCer in lipid rafts with the Src family kinase Lyn, and subsequent activation of PI3K, mitogen-activated protein kinase (MAPK) and PKC [97]. In addition, LacCer with long-chain fatty acids (C<sub>24</sub>) are required for the coupling of Lyn to LacCer lipid rafts resulting in the production of superoxides and induction of migration in neutrophils [98–100]. Association of Lyn with the LacCer containing C<sub>24</sub>-fatty acid chain is necessary for the phagocytosis of mycobacteria by neutrophils. Interestingly, LacCer-enriched lipid rafts are modulated by pathogenic bacteria to evade neutrophil-mediated killing. For instance, *Mycobacterium tuberculosis* prevented phagolysosome formation in neutrophils through binding of bacterial mannose-capped lipoarabinomannan to LacCer rafts in neutrophils [101].

In addition, inhibition of inflammation in neutrophils has also been associated with SL biology. Ceramides inhibit immune cell responses by binding to CD300f inhibitory receptor in sepsis [102], LPS-induced skin inflammation [103], allergic responses [104], and experimental colitis [105]. Interestingly, disruption of ceramide-CD300f binding induces neutrophil infiltration in sepsis and skin inflammation [102]. Ceramide metabolites have also been implicated in the modulation of neutrophil function. For instance, chemotactic migration of neutrophils in response to IL-8 and fMLP is inhibited by S1P, an immediate metabolite of ceramide [102]. Moreover, IL-8 gene expression and secretion was shown to be induced by S1P in lung H441 epithelial cells [107]. Similarly, C1P dampens inflammation in a model of LPS-induced lung inflammation. Particularly, C<sub>16</sub>-C1P and synthetic C<sub>8</sub>-C1P analog inhibit NF-κB activation in neutrophils and LPS-mediated IL-8 production [108].

These observations make it difficult, at a first glance, to conclude whether ceramides induce or inhibit neutrophil function. It is important to note that neutrophils are short-lived innate immune cells that sense extracellular pathogens early on during infection. Therefore, early on during activation, it is possible that ceramide induction in neutrophils leads to a delay in the production of TNF $\alpha$  allowing for neutrophil extravasation. However, it is not yet clear whether, as neutrophil activation progresses, a certain threshold of ceramides is required to turn on the apoptotic program in neutrophils. Data using ceramide analogs must be interpreted with caution, since the use of ceramide analogs might not correspond to the physiologic concentration of ceramide during infection. Nevertheless, the importance of understanding ceramide biology in neutrophils has started to attract more attention, especially in studies describing ceramide inhibitory role in multiple models of inflammation including models of sepsis, skin inflammation, allergic responses, and experimental colitis. Thus, these results may possibly be opening new doors for the inclusion of novel ceramide inhibitors that dampen neutrophil activation in diseases in which neutrophils are the main cause of pathophysiology. The effects of different SLs on neutrophil function are summarized in Table 15.2.

### 15.2.3 Macrophages

As the name implies, macrophages or the ‘big eaters’, along with DC and monocytes, compose the mononuclear phagocytic system [109, 110]. Due to their phagocytic nature, macrophages have the capacity to engulf debris, dead cells, and foreign pathogens [110, 111]. The heterogeneity and plasticity of macrophages enable these cells to mount a spectrum of pro-inflammatory or anti-inflammatory responses to various stimuli [111, 112]. Due to their vast distribution throughout the body, understanding macrophage biology has been a high pursuit since their description by Metchnikoff in the late 1800s. It is therefore not surprising that such a cell type with high membrane activity has recently gained considerable attention from lipid biochemists and immunologists alike [113, 114].

Macrophages recognize pathogen-associated molecular patterns through pattern recognition receptors (PRR), including toll-like receptors (TLRs) [115, 116]. The role of ceramides in modulating the ability of macrophages to sense and respond to microbes has been documented. Global lipidome analysis revealed that ceramides accumulated in all cellular membranes of activated macrophages in response to TLR4 stimulation with Kdo2-lipid A [117–119]. Furthermore, ceramide production was induced in macro-

**Table 15.2** SLs in Neutrophil Function

SL	Function	Modulation	Reference(s)
Ceramide	Superoxide production	Inhibition	Robinson et al. [79], Nakamura et al. [82], Ahmed and Berridge [80], and Fuortes et al. [81]
	Extravasation and migration	Activation	
	Phagocytosis	Inhibition	Suchard et al. [84], Hinkovska-Galcheva et al. [85], Suchard et al. [86]
	Chemotaxis	Activation	Corriden et al. [88]
	Apoptosis	Activation	Manago et al. [91]
	Inflammation	Inhibition	Izawa et al. [102], Shiba et al. [103], Izawa et al. [104], and Matsukawa et al. [105]
LacCer	Adherence to endothelium	Activation	Arai et al. [96]
	ROS production	Activation	Iwabuchi and Nagaoka [97], Chiricozzi et al. [98], Iwabuchi et al. [99], and Sonnino et al. [100]
	Migration	Activation	
S1P	Chemotaxis	Inhibition	Kawa et al. [106]
C1P	Inflammation	Inhibition	Baudiss et al. [108]



phages stimulated with TLR4 in combination with palmitate, suggesting that lipotoxic conditions induce *de novo* ceramide synthesis in macrophages. Increased ceramide production was also associated with induced TNF $\alpha$  and IL1 $\beta$  production by macrophages [150]. In addition, palmitate and LPS synergistic effect was also shown to induce NLRP3 inflammasome activation, resulting in induced IL-1 $\beta$  and IL-18 production [121] in a manner independent of the SPT subunit Sptlc2 [122]. Interestingly, ceramide accumulation has also been implicated in macrophage response to oxidative stress in a TLR2-dependent manner [123]. In the aforementioned study, ceramide accumulation led to suppression of mitochondrial respiration shifting the use of the citric acid cycle metabolites to the production of the antioxidant glutathione.

Ceramide accumulation has also been associated with induced cell death in macrophages [124]. It has been demonstrated that apoptosis in adipose triglyceride lipase-deficient macrophages is mediated by increased C<sub>16</sub>-ceramide concentrations [125]. Interestingly, withdrawal of macrophage-colony stimulating factor (M-CSF) from bone marrow-derived macrophages resulted in enhanced SPT activation and ceramide production prior to apoptosis [126]. This finding was further supported by the partial rescue of aSMase-deficient macrophages following M-CSF withdrawal, suggesting that apoptosis was at least in part mediated by ceramides. Additionally, ceramide accumulation was shown to mediate apoptosis in macrophages exposed to a lipotoxic diet consisting of saturated fatty acids [127].

Altogether, these studies suggest a crucial role for ceramides in modulating macrophage function and metabolism early and late during infection. The ability of ceramides to induce downstream signaling for the production of pro-inflammatory cytokines following an infection, allows macrophages to persist and orchestrate activation of immune cells that subsequently produce survival factors such as M-CSF. Once the infection is cleared and macrophage survival factors are no longer high, ceramides mediate macrophage apoptosis winding down the cycle of immune cell activation.

## 15.3 Ceramides and Other SLs in Inflammatory Diseases

Most of what is known today about the roles of ceramides and other SLs in modulating immune cell responses arises from a plethora of studies performed by lipid biochemists around the world. In the current era of lipidomics and targeted lipid therapeutics, lipid biology and metabolism have also started to attract many immunologists worldwide. In this section we present the main studies highlighting the roles of SLs in some major inflammatory diseases.

### 15.3.1 Insulin Resistance

Insulin Resistance refers to the impaired response of cells to insulin, resulting in a reduced uptake of glucose and glucose accumulation in the bloodstream. Insulin resistance is the main hallmark of type 2 diabetes mellitus and is highly associated with induction of cardiovascular disease, obesity, and various types of cancer [128]. Early studies established a strong link between ceramide accumulation and insulin resistance by demonstrating that SLs inhibited glucose transport into 3T3-L1 mouse adipose fibroblasts [129] and that bacterial SMase and the short-chain ceramides, C<sub>2</sub> and C<sub>6</sub>, reduced insulin-induced tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) downstream the glucose transporter, GLUT4, in insulin-sensitive rat hepatoma Fao cells [130]. Interestingly, inhibition of IRS-1 was later demonstrated to be mediated by TNF $\alpha$  via induction of tumor necrosis factor receptor and SMase activity [131]. However, inhibition of IRS-1 activity by ceramides seems to be a result of protein kinase B (PKB) activity rather than direct inhibition of IRS-1 function [132–136]. Further investigation of the mechanisms of PKB inhibition by ceramides demonstrated that C<sub>2</sub>-ceramide inhibited nuclear translocation of Akt1 [137], reduced phosphorylation of serine 473 in PKB [138], inhibited translocation of Akt/PKB to the plasma membrane and promoted dephosphorylation of Akt/PKB by protein phosphatase 2A [139–141].

Recent studies have also demonstrated induction of PKR/JNK activation [142, 143].

Further investigations aimed to determine whether ceramides induce insulin resistance. Analysis of muscle biopsies from insulin intolerant obese individuals revealed an increased accumulation of ceramides [144–147]. In addition, exogenous C<sub>2</sub>-ceramide induced apoptosis in skeletal muscle myotubes, reducing the cell capacity to uptake glucose, a process that can be reversed by the CerS inhibitor, fumonisin B<sub>1</sub> [148, 149]. In muscle cells, C<sub>2</sub>-ceramide also mediated insulin resistance by inhibiting Rac activation, thus reducing GLUT4 translocation to the plasma membrane in response to insulin [150]. These studies suggested that targeting ceramide accumulation could protect muscles against insulin resistance. In line with this hypothesis, Bruce and coworkers demonstrated that overexpression of sphingosine kinase 1 (SK1) led to reduced ceramide accumulation and insulin resistance in mice given high-fat diet [151]. Inhibition of CerS and ceramide synthesis with the S1P analog, FTY720 [152], also reduced insulin tolerance in mice on high-fat diet [153]. Furthermore, in obese rodents, blockade of *de novo* ceramide synthesis with the SPT inhibitor, myriocin, improved glucose tolerance [128, 154].

Since CerS are required for *de novo* ceramide generation, several studies have focused on the role of CerS in inducing insulin tolerance. Overexpression of CerS1, CerS2, CerS4, CerS5, and CerS6 in L6 myotubes induced ceramide production; however, none of the CerS was able to inhibit insulin signaling [139]. Given the opposing role of CerS2 and CerS6 in inflammation, current studies have been focusing on the role of these CerS in insulin resistance *in vivo*. CerS2 haploinsufficient mice have altered patterns of ceramide acylation, leading to reduced levels of very-long-chain ceramides and increased levels of long-chain C<sub>16</sub>-ceramide as a compensatory mechanism leading to insulin resistance when fed with high-fat diet [155]. In agreement with these findings, CerS6 expression and levels of C<sub>16</sub>-ceramide are induced in the adipose tissue of obese humans. Moreover, CerS6- and CerS5-

deficient mice fed with high-fat diet are protected from obesity, glucose intolerance, and insulin resistance suggesting that targeting CerS6 could be beneficial for the treatment of obesity and type 2 diabetes [20, 156]. It is worth noting that the products of CerS5 (C<sub>16</sub>-ceramide) and CerS2 (C<sub>24</sub>-ceramide) antagonized each other's ability to form channels in mitochondrial outer membranes to induce apoptosis [157]. This may be another mechanism by which different CerS affect insulin resistance and tolerance differentially.

Ceramide accumulation has been linked to inflammatory pathways mainly involving signaling events downstream of TLR4. More specifically, Holland et al., was the first to provide a link between lipotoxicity and inflammation in the induction of insulin intolerance [158]. Particularly, saturated fatty acids induced *de novo* ceramide synthesis via TLR4 activation, which also altered the metabolic program of skeletal muscle, inducing insulin resistance. However, Galbo et al. showed that lipid-induced insulin resistance is a result of increased accumulation of DAG and induced DAG-PKCε signaling rather than induced TLR4-ceramide pathway [159]. These conclusions were based on the observation that knockdown of TLR4 or the adaptor protein MyD88 prevented hepatic steatosis in mice fed with a saturated fat diet through reduction of appetite but not hepatic insulin signaling. When mice were given saturated fat by oral gavage, loss of TLR4 or MyD88 did not protect mice from hepatic insulin resistance. Interestingly, another link between ceramides and inflammatory pathways in insulin tolerance involves stimulation of the Nod-like receptor pyrin domain-containing-3 (Nlrp3) inflammasome [160]. In obese mice, ceramides activated Nlrp3 inflammasome and IL-1β secretion through caspase-1 activation in adipose tissue macrophages in a Nlrp3-dependent manner. This induction subsequently led to T cell activation. However, this study does not exclude the possibility that inducers other than ceramides present in the diet induced caspase-1 activation. Further studies are required to establish whether ceramides are truly sensed by PRR in immune cells.

### 15.3.2 Graft-Versus-Host Disease

Graft-versus-host disease (GVHD) is a major complication of allogeneic hematopoietic stem cell transplantation. It is an inflammatory response mediated primarily by donor T cells, resulting in destruction of host tissues including skin, liver, and gastrointestinal (GI) tract [161]. The role of ceramides in mediating T cell cytotoxic function has only been recently explored in two studies of mouse models of GVHD. Rotolo et al. reported that aSMase in the host mediated GVHD [162]. Adoptive transfer of allogeneic T cells to aSMase-deficient hosts reduced morbidity and mortality. This induction in survival resulted from reduced inflammatory responses, cytokine storm, CD8+ T cell proliferation and activation, and apoptosis of host hepatocytes, skin and intestinal cells. Interestingly, aSMase is required for the formation of ceramide-rich platforms on target cells for cytotoxic T lymphocytes (CTL) efficient killing. Further investigation of requirement of ceramide bioactive function in donor T cells by Sofi and colleagues demonstrated that CerS6-deficient donor T cells reduced GVHD [163]. In addition, T cells lacking CerS6 had an aberrant T cell receptor (TCR) signal transduction due to the reduction of tyrosine phosphorylation and CD3-PKC $\theta$  colocalization required for T cell proliferation and response to pro-inflammatory cytokines, particularly interferon gamma (IFN $\gamma$ ). Furthermore, inhibition of CerS6 with its specific inhibitor, ST1072 [164], reduced T cell proliferation and IFN $\gamma$  production. This could also be a result of reduced IL-2 secretion by aSMase-deficient T cells upon TCR stimulation as previously reported [165]. Moreover, aSMase has also been described to be required for proper TCR signaling downstream CD3/CD28 activation in CD4+ T cells, since blockade of aSMase bioactivity with imipramine impaired PLC $\gamma$ 1, JNK, ERK, Akt, and mTOR phosphorylation downstream TCR [166]. More studies are required to further elucidate the roles of the aSMase and lipid metabolism in driving T cell activation and its role in GVHD pathogenesis.

### 15.3.3 Immune Suppression in the Tumor Microenvironment

Immunotherapy has recently been employed as a novel strategy to eradicate tumors. During tumor development, immune cell responses include massive tumor infiltration and production of cytokines in an attempt to contain tumor growth and kill tumor cells. If not contained, tumor growth leads to the establishment of a tumor microenvironment (TEM) characterized by induced hypoxia [167] and lactic acid accumulation leading to suppression of effector CD8+ CTL [168]. CD8+ T cell responses are inhibited by suppressive immune cells of various types that initially infiltrated the tumor to contain growth but reversed its effector phenotype in the TEM. Among these suppressive immune cells are tumor-associated macrophages (TAM), myeloid-derived suppressor cells (MDSC), and regulatory CD4+ T (T<sub>reg</sub>) cells [111]. Recently, it has been appreciated that tumor cells gain advantage over immune cells by altering their glucose and lipid metabolism. Despite many studies describing the effects of alterations in glucose metabolism in immune cell function, only recently has similar alterations in immune cell lipid metabolism been considered [169].

S1P has been implicated in the polarization of macrophages to an M2 anti-inflammatory phenotype in the TEM. During tumor progression, apoptotic cells are released in the TEM serving as a source for S1P [170]. Interestingly, S1P released from engulfed apoptotic cells by macrophages induces macrophage survival through the activation of PIRK, ERK/2, and Ca<sup>2+</sup> [171] and polarization to an M2 phenotype through the inhibition of inducible nitric oxide synthase (iNOS) and reduction of TNF $\alpha$  and IL-8 production [172–175]. Knock down of the S1P-producing enzyme, sphingosine kinase 2 (SK2) in MCF-7 breast carcinoma cells delayed tumor growth in a xenograft model in nude mice by promoting macrophage polarization to an M1 pro-inflammatory phenotype characterized by increased expression of nitric oxide (NO), TNF $\alpha$ , IL-12, and major histocompatibility

complex class II and reduced expression of IL-10 and CD206, which are markers associated with macrophage suppression [176].

Modulation of TAM function by ceramides has been also achieved in a mouse model of hepatocellular cancer through the administration of nanoliposome-loaded C<sub>6</sub>-ceramide (LipC6) [177]. LipC6 exerts its modulatory function by acting as a ROS scavenger, therefore inducing macrophage polarization from an M2 to an M1 phenotype and resulting in reversal of CD8<sup>+</sup> T cell exhaustion and induction of CD8<sup>+</sup> cytotoxic function against tumor cells. The effect of ceramides has also been explored in suppressive MDSC in mice bearing CMS4-metastatic tumors. Treatment of tumor-bearing mice with acid ceramidase inhibitor, LCL521, reduced MDSC accumulation in tumors without reducing tumor growth. In addition, LCL521 treatment led to an increased accumulation of C<sub>16</sub>-ceramide that resulted in cathepsin-mediated cell death of tumor and MDSC-like J774 cells [178]. Suppression of CD8<sup>+</sup> T cell function in tumors also results from suppression mediated by T<sub>reg</sub> cells. To our knowledge, there have been no studies reporting the effect of ceramides on T<sub>reg</sub> cell function in tumor-bearing mice. However, recent studies have described the effect of ceramide accumulation on T<sub>reg</sub> cells in healthy WT and aSMase-deficient mice. T<sub>reg</sub> cell frequency and suppressive function are induced in aSMase-deficient mice [179]. In addition, this was associated with increased percentage of induced T<sub>reg</sub> (iT<sub>reg</sub>) cells from aSMase<sup>-/-</sup> CD4<sup>+</sup> T cells treated with transforming growth factor beta (TGFβ) and IL-2. Moreover, aSMase product C<sub>6</sub>-ceramide induced a Th17-associated cytokine in CD4<sup>+</sup> T cell hampering T<sub>reg</sub> cell induction, suggesting that aSMase is a negative regulator of T<sub>reg</sub> cell development [180]. The relevance of these findings remains to be further explored in models of inflammation for further development of lipid-targeted therapies.

### 15.3.4 Multiple Sclerosis

Multiple sclerosis (MS) is a chronic inflammatory disease that results in demyelination due to autoim-

mune responses against myelin in the central nervous system (CNS) [181]. In experimental autoimmune encephalomyelitis (EAE), *de novo* synthesis of C<sub>16</sub>-ceramide by CerS6 is required for the production of pro-inflammatory TNFα and iNOS in macrophages in response to IFNγ [182]. Upregulation of CerS6 in EAE led to further speculations on its role in driving MS. Interestingly, upregulation of CerS6 and TNFα mRNA expression was found to be higher in females compared to male littermates in spontaneous relapsing remitting EAE [183]. This increase was correlated with the ability of females to initiate anti-inflammatory responses during the course of the disease, suggesting that CerS6 could promote anti-inflammatory responses. This observation was later confirmed with experiments showing that EAE progression is worsened in CerS6 knockout mice [184]. In addition, EAE was enhanced in chimeric mice in which only leukocytes were CerS6-deficient, providing further evidence that leukocytes lacking CerS6 drive the exacerbated phenotype. Moreover, expression of genes driving leukocyte migration and CNS infiltration (e.g. CCL2, CCL5, CXCL2) was increased, especially in CerS6-deficient neutrophils due to increased C-X-C motif chemokine receptor type 2 (CXCR2) in response to granulocyte-colony stimulating factor. On the contrary, CerS2-deficient mice had delayed development of EAE. This delay in disease onset was correlated with the reduced expression of CXCR2 in CerS2-deficient neutrophils [26]. In addition, the induction of ceramides was also associated with disease development in EAE, since aSMase deficiency protected mice from disease [185]. Collectively, these findings suggest that CerS6 and CerS2 have opposing roles in driving disease progression in EAE.

Excitingly, the S1P receptor agonist, fingolimod FTY720, was FDA approved in 2010 as a first-line therapy for the treatment of MS [186, 187]. FTY720 is phosphorylated *in vivo* by SK to form FTY720-P, which binds with a high affinity to S1P receptors and competes with its natural ligand, S1P. Therefore, FTY720 functionally antagonizes S1P by strongly binding to S1PR leading to internalization and inhibition of the receptor [188–190]. By modulating the S1P receptor, fingolimod prevented autoreactive S1P



expressing inflammatory T cells from exiting the lymph nodes, therefore, inhibiting CNS infiltration by autoreactive T cells. Subsequently, this resulted in reduced destruction of myelin sheath surround the axons of nerve cells [188–191]. This is a perfect example of the potential outcome of modulating lipid metabolism in immune cells driving forward the era of lipid therapeutics.

### 15.3.5 Inflammatory Bowel Disease

Inflammatory Bowel Disease (IBD) is a group of diseases, including ulcerative colitis and Crohn's disease, characterized by chronic inflammation of gastrointestinal tract. Pathology is mediated by leukocytes infiltration and massive production of pro-inflammatory cytokines leading to intestinal damage [192, 193]. The first link between SL metabolism and IBD originated from studies showing that TNF $\alpha$ , a cytokine that induces the generation of S1P and synthesis of cyclooxygenase-2 (COX-2) [194, 195], was induced in patients with IBD [192, 196]. In addition, TNF $\alpha$  blockade alleviated clinical symptoms in mouse models of disease [197, 198]. Since ceramides also induced the activation of MAPK, a key inducer of inflammatory responses [199], follow up studies demonstrated that inhibition of ceramide accumulation by targeting SMase or S1P receptor diminished clinical manifestations of disease in mouse models of colitis. For example, in a dextran sulfate sodium (DSS) model of colitis, inhibition of SMase with a sphingomyelin analogue-7 reduced the formation of ceramide, levels of cytokines, and intestinal injury [200]. In addition, administration of FTY720 reduced disease symptoms in a mouse model of colitis, associated with a reduced production of the pro-inflammatory cytokine IL-12p70 and Th1 cytokines, and upregulation of CD4<sup>+</sup> Foxp3<sup>+</sup> suppressive T<sub>reg</sub> cells [201]. Furthermore, administration of a new S1P receptor agonist, KRP-203, resulted in reduced lymphocyte infiltration and production of pro-inflammatory Th1 cytokines in the colonic mucosa [202]. Similarly, chemical and genetic inhibition of SK1 that is responsible for the production of S1P reduced manifestations of colitis,

COX-2 expression, and neutrophil infiltration [203–205]. In support of these findings, high SK1 expression has also been reported in intestinal mucosa of patients with ulcerative colitis [205]. Currently, modulators of S1P receptor, such as ozanimod (RPC1063) and etrasimod (APD334) are being tested in clinical trials for the treatment of IBD [206, 207], further emphasizing the importance of investigating the role of SL metabolism in this disease setting.

Along with inhibition of S1P receptor function, modulation of CerS has also been explored in IBD, particularly CerS2 and CerS6. In a mouse model of colitis, loss of CerS2 destabilized the epithelial barrier and tight junction in the intestinal membrane leading to increased intestinal permeability. CerS2-deficient mice have reduced expression of junctional adhesion molecule A [208] and tight junction protein ZO-1 [209]. This outcome is associated with reduced levels of very-long acyl chain ceramides (C<sub>24</sub>) and increased levels of long-chain sphingoid bases and C<sub>16</sub>-ceramides [208]. These findings further suggest that CerS2 is protective against colitis as opposed to EAE in which CerS2-deficient mice are protected [26]. Interestingly, further exploration of the role of CerS6 in colitis has led to opposing conclusions. Scheffell et al. reported that the transfer of CerS6-deficient CD4<sup>+</sup> T cells is protective in colitis [33]. On the contrary, Helke et al. showed that in a model of DSS-induced colitis, loss of CerS6 exacerbates inflammation [210]. Interestingly, pathology in CerS6-deficient mice is not a result of reduced intestinal permeability but is associated with enhanced neutrophil infiltration. However, more studies are needed to further elucidate the role of CerS in various models of colitis and its implementation in the development of CerS targeted therapies in humans with IBD.

## 15.4 Conclusions and Future Directions

While it has been thoroughly reviewed in other cells, the roles of ceramides in immune cells have not been proportionally presented. In this chapter we attempted to summarize the significant effects



of ceramides in immune cells and immune diseases. Numerous studies have highlighted the importance of ceramides in a variety of pathways necessary for the development, function, and metabolism of immune cells. Ceramide accumulation has been described as a key step mediating and regulating various immune cell functions, including regulation of immune cell responses to viruses, bacteria, and other foreign pathogens, migration, phagocytosis, and cytokine production. For instance, ceramides have been implicated in the modulation of responses to LPS, cytokine production, antigen presentation, and viral entry in DC; control of migration, cytokine and superoxide production, and formation of NETs in neutrophils; and response to TLR stimulation, inducing apoptosis, and regulating oxidative stress in macrophages. Moreover, ceramide functions extend to T cells, stabilizing the T cell receptor complex and mediating T cell responses during inflammation. Ceramide metabolites and upstream regulators including CerS in immune cell function have also been thoroughly described in the literature, further emphasizing the important role that ceramides play during inflammation.

Investigating the roles of ceramides and other SLs as modulators of the biology and metabolism of immune cells has unraveled new avenues in targeting ceramides in life-threatening inflammatory diseases that negatively impact the life of millions around the globe. For instance, recently, the FDA approved the CerS inhibitor, FTY720, as the first-line therapy for MS patients. This certainly has raised an interest among immunologists and lipid biochemists alike in developing novel inhibitors to target ceramide metabolism in various models of inflammation in the laboratory to hopefully translate such findings in the clinic.

It is also strictly important to consider the multiple effects of a plethora of ceramide species on the function of a certain immune cell or different cells when targeting ceramides in disease. Despite the encouraging routes that ceramide research has taken, further studies and collaborations between scientists of multidisciplinary fields are still needed to elucidate the mecha-

nisms of ceramides in immune cell function and other cell types. Since diversity of ceramides, similar to other membrane and bioactive lipids, arises from the different possible combinations of head groups and/or degree of saturation, the effect of each type of SL and ceramide on every immune cell opens endless possibilities in targeting various ceramides to retune immune cell function in cancer, transplantation, diabetes, MS, IBD and many other diseases.

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