



Published in final edited form as:

Nature. 2014 June 5; 510(7503): 58–67. doi:10.1038/nature13475.

sphingolipid metabolites in inflammatory disease

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Abstract

Sphingolipids are ubiquitous building blocks of eukaryotic cell membranes. Progress in our understanding of sphingolipid metabolism, state-of-the-art sphingolipidomic approaches and animal models have generated a large body of evidence demonstrating that sphingolipid metabolites, particularly ceramide and sphingosine-1-phosphate, are signalling molecules that regulate a diverse range of cellular processes that are important in immunity, inflammation and inflammatory disorders. Recent insights into the molecular mechanisms of action of sphingolipid metabolites and new perspectives on their roles in regulating chronic inflammation have been reported. The knowledge gained in this emerging field will aid in the development of new therapeutic options for inflammatory disorders.

In the 1880s, the neurochemist J. L. W. Thudichum presciently named the brain lipid ‘sphingosine’ after the Sphinx, owing to its enigmatic chemical nature¹. Sphingosine and its relatives continue to surprise and confound us today. These fatty amino alcohols are the backbone of a ubiquitous class of eukaryotic lipids, the sphingolipids, which include ceramide (*N*-acyl-sphingosine), sphingomyelin, and hundreds of different glycosphingolipids (Fig. 1a). Sphingolipids, like glycerolipids, are reservoirs of bioactive metabolites of profound importance to myriad cell signalling and pathological functions. The sphingolipid metabolites, ceramide, ceramide-1-phosphate (C1P), and sphingosine-1-phosphate (S1P) are emerging as important signalling molecules that regulate cell growth, survival, immune cell trafficking, and vascular and epithelial integrity, and are particularly important in inflammation and cancer^{2,3}.

The past decade has seen an explosive advancement in the field of sphingolipid signalling based on the convergence of several key aspects. First, most of the regulatory proteins and enzymes involved in sphingolipid metabolism and the receptors for S1P have been cloned. This allowed the generation of knockout mice, yielding insights into the physiological functions of sphingolipid metabolites. Second, the advent of advanced mass spectroscopic techniques has brought the ‘omics’ revolution to sphingolipids, allowing for the simultaneous analysis and quantification of multiple species. Third, specific agonists and antagonists of S1P receptors and inhibitors of signalling enzymes were developed. The chief

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The authors declare no competing financial interests.

development among these was the discovery of FTY720 (fingolimod), a sphingosine analogue that alters immune cell trafficking and is already being used in the clinic for the treatment of multiple sclerosis⁴.

These are exciting times for the field and research continues apace. Several sphingolipid signalling protein structures have been solved, allowing for rational drug design. This Review will focus on the function of three key bioactive sphingolipids: ceramide, C1P and S1P, and their roles in inflammation. Although this is a normal physiological response to harmful stimuli such as infection, unchecked inflammation can lead to numerous pathophysiological states, including oedema, asthma, inflammatory bowel disease and associated cancer, and autoimmune disorders such as multiple sclerosis and rheumatoid arthritis. Sphingolipid metabolites play crucial parts at multiple stages of these disorders, and new mechanistic perspectives on their actions will be discussed. We will also highlight how knowledge gained in this relatively new field will aid in the development of therapeutic options for inflammatory disorders.

Sphingolipid metabolism

Sphingolipids are essential lipids consisting of a sphingoid backbone that is *N*-acylated with various fatty acids to form many ceramide species, which can have hundreds of distinct head groups. As this Review is mainly concerned with sphingolipid signalling and inflammatory diseases, we will focus on mammalian sphingolipids and their metabolism. Sphingolipid synthesis starts in the endoplasmic reticulum (ER) with the condensation of serine and palmitoyl coenzyme A (CoA) by serine palmitoyltransferase (SPT), the rate-limiting step, forming 3-ketosphinganine (Fig. 1b). SPT mutations can shift substrate preference to alanine, producing neurotoxic deoxysphingolipids that cause hereditary sensory and autonomic neuropathy type 1 (ref. 5). SPT activity is negatively regulated by ORMDL proteins⁶, and a genetic variant that increases ORMDL3 expression has been linked to increased risk of asthma⁷. Reduction of 3-ketosphinganine forms dihydrosphingosine that is then *N*-acylated by one of six ceramide synthases (CerS1–CerS6), each using specific acyl chains, typically with saturated or mono-unsaturated fatty acids with 14 to 26 carbons, forming dihydroceramides that are subsequently dehydrogenated to ceramides by dihydroceramide desaturase. Metabolism of ceramide to sphingomyelin and a vast array of complex glycosphingolipids mainly occurs in the Golgi (Fig. 2). ER to Golgi transport of ceramide is mediated by ceramide transfer protein (CERT) for sphingomyelin synthesis⁸, or by vesicular transport for glucosylceramide synthesis. Ceramides can also be phosphorylated in the Golgi by ceramide kinase (CERK) to form C1P, a rare species. Glycosphingolipid synthesis requires the transfer of glucosylceramide to the *trans*-Golgi network (TGN) by four-phosphate adaptor protein 2 (FAPP2), which also regulates vesicular trafficking from the Golgi to the plasma membrane⁹.

Sphingolipids have a rapid turnover and their levels are controlled by the balance between synthesis and degradation in multiple compartments². Sphingolipids are degraded in lysosomes by glycosidases or acid sphingomyelinases that remove the head groups to form ceramides. Deacylation of ceramide by ceramidases is the only pathway known to generate sphingosine. Sphingosine can be recycled back to ceramide, and 50% or more of

sphingosine molecules follow this reutilization pathway, which has a significant role in sphingolipid homeostasis². Sphingosine can also be phosphorylated by one of two sphingosine kinases, SphK1 and SphK2, forming S1P, a pleiotropic bioactive metabolite and key intermediate in the sphingolipid-to-glycerolipid metabolic pathway¹⁰.

S1P can then either be dephosphorylated by phosphatases, including two S1P-specific, ER-localized phosphatases (SPP1 and SPP2), or irreversibly degraded by S1P lyase (SPL) to phosphoethanolamine and hexadecenal that can be incorporated into glycerolipids. The fatty aldehyde dehydrogenase ALDH3A2 converts hexadecenal to hexadecenoate, which is utilized for the formation of palmitoyl-CoA in glycerolipid synthesis, revealing the connection between sphingolipid and glycerolipid homeostasis¹¹. ALDH3A2 is non-functional in Sjögren–Larsson syndrome, a disease characterized by ichthyosis and intellectual disability, suggesting that the accumulation of S1P degradation products contributes to the pathogenesis¹¹.

The S1P gradient

S1P concentrations are high in blood and lymph, and low in tissues, presumably due to the higher activity of S1P degrading enzymes in tissue. The high level of blood S1P has important homeostatic functions in the maintenance of vascular integrity, and the S1P gradient is crucial for immune cell trafficking. S1P in blood is rapidly turned over¹², suggesting robust mechanisms for its synthesis inside cells and its transport outside. Both SphK1 and SphK2 contribute to circulating pools of S1P. Erythrocytes¹³ and vascular endothelial cells¹⁴ are the main sources of blood S1P, and lymphatic endothelial cells are the main sources for lymph S1P¹⁵. S1P is exported out of vascular and lymphatic endothelial cells by the specific transporter Spns2, which is not present in platelets or erythrocytes, suggesting the existence of other transporters in these cells^{16,17}. Although ceramide, which is also abundant in plasma¹⁸, is associated with very-low-density lipoprotein and low-density lipoprotein, S1P in plasma is bound to both albumin and apolipoprotein M (apoM), which preferentially associates with high-density lipoprotein. However, only high-density lipoprotein containing apoM–S1P is required to preserve endothelial barrier integrity¹⁹. ApoM produced by the liver influences plasma S1P levels by enhancing S1P biosynthesis for secretion by hepatocytes, demonstrating that the liver is involved in S1P dynamics and that apoM delivers S1P to extrahepatic tissues^{20,21}.

Sphingolipid signalling

Multiple stimuli affect sphingolipid biosynthesis and metabolism by regulating key enzymes in a spatial and temporal manner, leading to the restricted production of ceramide, C1P and S1P². For example, pathogens, oxidative stress and cytokines activate acid or neutral sphingomyelinases to generate ceramide in specific compartments². Sphingosine generated by ceramidase is another sphingolipid metabolite involved in cell signalling²² (see Review by Platt on page 68). However, its role in inflammatory responses is not yet clear^{23,24}. Some inflammatory mediators also activate SphK1 by extracellular signal regulated kinase (ERK)-mediated phosphorylation, which promotes SphK1's translocation to the plasma membrane, at which its substrate, sphingosine, is localized and/or generated, resulting in transient

elevations in S1P levels^{3,25}. Although these studies were mainly carried out with cultured cells, the development of high-sensitivity mass spectrometry methods has led to demonstrations of changes in levels of bioactive sphingolipids being reported in specific tissues from mouse inflammatory disease models²⁶⁻²⁹ and, in a few cases, in human samples^{30,31}. More studies are needed to rigorously correlate bioactive lipids with disease stage. Understanding what bioactive sphingolipids do and how they signal is crucial for our understanding of their functions in inflammatory responses.

Ceramide

Despite the involvement of ceramide in numerous biological processes, only a few of its direct targets have been described. The best examples are ceramide-activated serine/threonine protein phosphatases, including PP1, PP2A and PP2C^{32,33}. PP2A is found in complex with SET (also known as I2PP2A), which inhibits PP2A function. Ceramide binds to SET, relieving its inhibitory actions³³. Activation of PP2A that leads to dephosphorylation of Akt, a potent promoter of cell survival, could partly explain the pro-apoptotic actions of ceramide. In addition, PP1 could be the target of ceramide generated at the plasma membrane³². Stimulation of the atypical protein kinase PKC ζ by ceramide, probably concomitant with its membrane recruitment, also leads to inhibition of Akt³⁴.

Another concept that has been subject to renewed interest is the formation of ceramide-enriched membrane microdomains or platforms as a mechanism by which ceramide transduces intracellular signalling. Alterations in membrane domains, owing to the elevation of ceramide and its tendency to self-associate, influence membrane composition and interactions of lipids or signalling proteins. These ceramide-rich platforms have been implicated in a variety of signalling cascades in immune cells, including activation of B cells, bacterial pathogen infections and release of cytokines during infection; they are also especially important in the induction of apoptosis³⁵.

C1P

C1P is formed on the TGN by CERK. Here, C1P activates cytosolic phospholipase-A2 α (cPLA2 α)³⁶, the enzyme that releases the eicosanoid precursor arachidonate. Although early concerns regarding cPLA2 α activation by C1P were raised³⁷, others have shown that not only does C1P bind cPLA2 α through an RxRH motif in its amino-terminal CaLB (also known as C2 lipid-binding domain) and recruit it to the membrane at which its phospholipid substrates reside, C1P also stimulates its activity and therefore has been implicated in eicosanoid production^{36,38}. Conversely, C1P has been shown to inhibit ADAM17 (also known as tumour necrosis factor (TNF)-converting enzyme, or TACE), the major metalloprotease responsible for cleaving pro-TNF to release the active inflammatory form, probably by binding to one or more similar tribasic motifs³⁹.

S1P

The many diverse roles of S1P in innate and adaptive immunity, including immunosurveillance, immune cell trafficking and differentiation, immune responses and endothelial barrier integrity are mediated by its binding to one of five G-protein-coupled receptors, named S1PR1– S1PR5 (refs 3, 40, 41). Downstream signalling of these receptors

is complex as they are differentially expressed in immune and endothelial cells and couple to a varied set of heterotrimeric G proteins. For example, activation of S1PR1 generally promotes cell migration and the egress of T and B lymphocytes from lymphoid tissues⁴², whereas S1PR2 inhibits motility to promote retention of B cells in germinal centres⁴³. S1P produced inside cells by activation of SphK1 can be secreted by SPNS2 or promiscuous ABC transporters^{16,17,44}. S1P, in turn, signals through its receptors in a paracrine and/or autocrine manner, termed 'inside-out' signalling. However, the crystal structure of S1PR1 suggests that S1P must slide laterally within the plane of the bilayer to access the binding pocket⁴⁵, raising the question of whether S1P needs to be secreted for its autocrine actions.

Much less is known about the intracellular targets of S1P. Within the past decade, new intracellular targets of S1P have been characterized. S1P formed by SphK1 in response to TNF or interleukin-1 (IL-1) binds to TNF receptor-associated factor 2 (TRAF2) and cellular inhibitor of apoptosis 2 (cIAP2), respectively, and enhances their lysine-63-linked polyubiquitylation activities^{46,47}. In response to IL-1, which has a pivotal role in autoinflammatory diseases, cIAP2 and SphK1 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 that recruit mononuclear cells to sites of sterile inflammation⁴⁷. Thus, in addition to canonical S1P receptor signalling, these new intracellular functions of S1P might be involved in the immune and inflammatory responses of these potent cytokines. Furthermore, S1P in the nucleus, formed by SphK2 or by inhibition of SPL, binds and inhibits the histone deacetylases HDAC1 and HDAC2, linking sphingolipid metabolism to inflammatory and metabolic gene expression⁴⁸⁻⁵⁰. Further studies are needed to solidify these new concepts.

Spingolipids in endothelial biology and function

The vascular endothelium forms a barrier that lines blood vessels and plays an important part in cardiovascular homeostasis and blood flow, and in controlling the passage of leukocytes into and out of the bloodstream. Ceramide and S1P have opposing effects on vascular endothelium functions: whereas increased ceramide leads to endothelial barrier dysfunction⁵¹, S1P is crucial for maintenance of vascular integrity⁵²⁻⁵⁴ (Fig. 3a).

Pro-inflammatory cytokines, oxidative stress and injurious insults, such as platelet activating factor (PAF), TNF and lipopolysaccharide, generate excess ceramide in endothelial cells linked to increased vascular permeability and dysfunction (Fig. 3a). Generation of ceramide is both stimulus- and context-specific, and is due to the activation of acid or neutral sphingomyelinase, or increased *de novo* biosynthesis. Ceramide triggers several pathways that induce endothelial cell death, including activation of caspases, or PP1 or PP2A^{2,55}, and increasing mitochondrial permeability by forming ceramide-enriched platforms capable of translocating proteins. Moreover, PAF-induced formation of ceramide microdomains drives endothelial nitric oxide synthase (eNOS) activation and contributes to barrier dysfunction⁵⁶. Ceramides have also been linked to growth arrest, cytoskeleton rearrangements, oxidative stress and senescence of endothelial cells². Thus, ceramides regulate important endothelial cell functions that are thought to be responsible for the pathogenesis associated with

vascular dysfunctions, including emphysema, sepsis and acute respiratory distress syndrome.

Using animal models of acute and chronic inflammation, it has been convincingly demonstrated that plasma S1P limits disruption of vascular endothelial monolayers and reduces oedema⁵⁷ (Fig. 3a).

S1P activates endothelial S1PR1, leading to enhanced Rac-dependent cytoskeleton rearrangements, contacts between cells and the matrix, adherens junction assembly and barrier integrity^{3,41}.

Lymphocytes circulate through lymph nodes for immune surveillance, entering at high endothelial venules (HEVs) — specialized blood vessels. Until recently, it was not known how HEVs allow lymphocyte transmigration, which increases during immune responses, and maintain vascular integrity. A study demonstrated that podoplanin expressed on HEV fibroblastic reticular cells binds and activates platelet C-type lectin-like receptor-2 (CLEC2)⁵⁸. Activation of CLEC2 on extravasated platelets leads to the release of S1P in the perivenular space of HEVs. S1P, in turn, enhances vascular endothelial (VE)-cadherin expression for maintenance of the integrity of HEVs during immune responses⁵⁸. It is likely that the S1P–S1PR1 axis is also involved in regulating the integrity of HEVs.

During inflammation, infiltrating immune cells and the production of pro-inflammatory cytokines increase endothelial permeability. S1P signalling attenuates the increased permeability induced by the inflammatory mediators histamine and PAF⁵⁷. It is still not clear whether levels of S1P in plasma are reduced in systemic inflammation or sepsis. However, because levels of the S1P carrier protein apoM are decreased in patients with sepsis and systemic inflammatory response syndrome⁵⁹, it is possible that S1P plasma levels are reduced in these conditions and thus contribute to vascular leakage.

Although S1PR2 does not regulate basal permeability, it protects against acute vascular barrier disruption and lethality after antigen challenge or PAF administration⁶⁰. Endothelial S1PR2 inhibits Akt activation, suppressing eNOS and NO generation, and protects against the disassembly of adherens junctions by decreasing S-nitrosylation of β -catenin and thereby vascular leakage^{41,60}. By contrast, during endotoxaemia, endothelial S1PR2 enhances vascular permeability and expression of pro-inflammatory and pro-coagulation molecules by activating Rho and p38 SAPK signalling pathways⁶¹. Together, these observations suggest that S1PR1 and S1PR2 have specific roles in preventing or triggering vascular leakage, probably by activation of distinct signalling pathways. For example, S1PR1 mediates G_i-dependent Rac and Akt activation, whereas S1PR2 inhibits them and stimulates Rho^{3,41}. Alternatively, the S1P receptors might be temporally and spatially localized and upregulated during inflammation.

An unexpected function of endothelial S1PR1 was discovered using S1PR1-specific agonists that suppressed the cytokine storm and recruitment of innate immune cells to the lung during influenza infection⁶². The efficacy of S1PR1 agonists was a result of the inhibition of common pathways downstream of multiple innate sensing pathways that are important for interferon production⁶³. A better understanding of how S1P receptors and their

downstream signalling are regulated and how they affect the vascular system could provide the basis for developing improved therapies for vascular disorders.

Sphingolipids and inflammation

During inflammation, innate and adaptive immune cells enter sites of infection or injury and the activation of cytokine networks that follows helps to protect the host. Sphingolipid metabolites have key roles in the regulation of both trafficking and functions of immune cells^{42,64}, and there are indications that sphingolipid metabolism and S1P receptors might be altered by inflammation.

S1P drives the differentiation of many types of immune cells, inducing changes in their functional phenotypes and regulating production of pro-inflammatory cytokines and eicosanoids. These topics will not be covered here as they have recently been extensively discussed (see refs 3, 42, 64 and 65 for authoritative reviews). In particular, S1P has emerged as a central regulator of lymphocyte egress. Although the S1P–S1PR1 axis regulates trafficking and migration of most types of immune cells (such as T and B lymphocytes, haematopoietic progenitors, macrophages, dendritic cells, neutrophils, mast cells, natural killer T cells and osteoclasts^{3,42}), the regulation of T- and B-lymphocyte trafficking is the best understood and will be used to illustrate the primary mechanisms involved.

S1P and lymphocyte trafficking

S1P concentrations in blood and lymph are much greater than in tissues, and lymphocyte egress from the thymus or secondary lymphoid tissues is dependent on the cells sensing this gradient through activating S1PR1 (ref. 42). When ready to leave the thymus, T lymphocytes upregulate S1PR1 (ref. 66) and exit in response to the S1P gradient (Fig. 3b). S1PR1-dependent Rac activation is required for *trans*-endothelial migration of T cells and lymph node egress⁶⁷. After entering the blood, high S1P levels desensitize S1PR1, owing to phosphorylation by guanine nucleotide-binding protein coupled receptor kinase-2 (GRK2)⁶⁸. Coordinated action of selectins, integrins and especially the retention signals of the CCL21 chemokine receptor CCR7 are required for lymphocytes to enter the lymph nodes through HEVs⁴². T lymphocytes sample the environment randomly — cortical sinus probing is followed by S1PR1-dependent entry⁶⁹ and then flow into the efferent lymph containing high S1P15 (Fig. 4b). During inflammation, lymphocyte retention is increased by the loss of S1PR1 expression, its internalization and degradation. After several rounds of division, newly generated effector cells upregulate S1PR1, lose the CCR7 retention signal and then exit into circulation and travel to locations of injury and inflammation⁴². The immune system co-opts the S1P–S1PR1 axis to determine the establishment of resident memory T cells, a distinct lineage that are embedded in non-lymphoid tissues and that mediate protective immunity. Regulation of the transcription factor KLF2 and its target gene *Slpr1* in mice by cytokines determines whether CD8⁺ T cells re-enter circulation or become tissue resident⁷⁰.

In addition to regulating the egress of B cells, the S1P–S1PR1 axis plays a part in the positioning of marginal-zone B cells in the spleen⁷¹. These B cells are the first line of

defence against systemic blood-borne antigens. Levels of S1P are high in the splenic marginal zone, causing desensitization of S1PR1 on resident B cells that allows them to migrate into the follicles in response to the follicular-dendritic-cell-produced chemokine CXCL13 (ref. 42). B cells in the marginal zone shuttle constantly between this zone and follicles, depending on the S1P gradient. This allows for the rapid delivery of antigens from the blood to the follicles⁷¹. However, in germinal centres in which mature B cells proliferate, differentiate and switch antibody class production, S1PR2 activation and its downstream effectors Gα12, Gα13 and p115RhoGEF counteract signalling by Akt, decreasing survival and responses to follicular chemoattractants, thus helping to focus the cells to the follicle centre⁴³. It remains to be seen whether this gradient of S1P exists between the germinal centre and the follicle boundary. The remaining major challenges are to understand how this gradient is produced and how to measure S1P pools in local environments. For S1P to be sensed during egress, its levels in tissue interstitial fluid would be expected to be very low to ensure that B-cell S1PR1 could respond to higher concentrations at the luminal surfaces of egress sites. However, although interstitial levels are generally assumed to be due to high S1P degrading activity in tissues, so far it has not been possible to directly measure interstitial fluid S1P levels. It is anticipated that this will be accomplished soon with the improved sensitivity of newer mass spectrometers.

S1P could be pro- or anti-inflammatory

The inflammatory role of S1P formed in immune cells by SphK1 or SphK2 is still unclear. This conundrum began when inflammatory responses were examined in *Sphk1* and *Sphk2* knockout mice. Whereas some studies have reported that colonic inflammation²⁶ and synovial inflammation in TNF-induced arthritis⁷² are reduced in *Sphk1* knockout mice, others have reported that these mice have normal acute and chronic inflammatory responses⁷³. The retraction of a paper that suggested SphK1 had a role in regulating pro-inflammatory responses in a mouse sepsis model was concerning⁷⁴, and there is no evidence that inhibiting SphK1 or deleting its gene will reduce sepsis. Although *Sphk2* knockout mice have normal neutrophil functions, in a model of bacterial lung infection these mice have accelerated disease progression⁷⁵. Similarly, neuroinflammation⁷⁶ and lung inflammatory injury⁷⁷ induced by lipopolysaccharide were increased by deletion of *Sphk1*.

It is possible that S1P formed by SphK1 or SphK2 has distinct functions and responses to stimuli in specific types of cells and tissues. This could also depend on the expression of S1P receptors and their specific downstream targets. Also, whereas deletion of *Sphk1* decreases S1P levels in blood, deletion of *Sphk2* increases it^{26,78}, probably due to upregulation of *Sphk1* expression and an increase of S1P in some tissues but not others⁷⁸. Changes in levels of S1P in the circulation and in tissues could affect immune-cell differentiation and functional phenotypes⁶⁴. Production of S1P is spatially and temporally regulated and its physiological and inflammatory outcomes are context-, tissue- and source-dependent. Future studies with conditional knockout mice could clarify the inflammatory roles of S1P and the kinases that produce it in specific tissues.

Sphingolipids may mediate inflammatory signalling by TNF

TNF, a key pro-inflammatory cytokine and therapeutic target in inflammatory diseases, stimulates sphingolipid metabolic enzymes including sphingomyelinases, ceramidase and SphK1 (refs 2, 79). Several sphingolipid metabolites have been suggested to be involved in TNF signalling and chemokine production based on studies of reduction or overexpression of these enzymes. For example, cells that are deficient in acid sphingomyelinase or acid ceramidase have decreased production of CCL5 induced by TNF, possibly due to regulation of sphingosine levels²³, whereas knockdown of the gene encoding neutral sphingomyelinase regulates adhesion molecules independently of NF- κ B activation⁷⁹. The effects of deletion or knockdown of *SphK1* have yielded conflicting results. Initially, overexpression of a dominant-negative SphK1 mutant⁸⁰ or SphK1 downregulation^{81,82} reduced NF- κ B activation, production of cytokines and chemokines, and induction of COX2 and PGE2 production in response to TNF. More recently, S1P formed by TNF-mediated activation of SphK1 was suggested to be crucial for TRAF2-mediated K63 polyubiquitylation of RIP1, a key step in NF- κ B activation⁴⁶. However, studies have now found that downregulation of SphK1 had no effect on TNF-induced NF- κ B activation but enhanced CCL5 expression through the p38 MAPK pathway, whereas downregulation of SphK2 reduced CCL5 expression without affecting NF- κ B⁸³. Moreover, TNF-mediated NF- κ B activation and cytokine expression in murine macrophages that lack both SphK1 and SphK2 were not altered but these cells had increased sphingosine and ceramide levels and autophagy²⁴. Clearly, resolving these discrepancies requires further study; however, there are several intriguing possibilities. The interconversion of sphingolipid metabolites is rapid, and fluxes of sphingolipids depend on the cell type; thus, distinct bioactive sphingolipids could be increased by TNF and their targets could be cell-type dependent. The outcome of inside-out signalling by S1P induced by TNF, as observed in certain cell types³, would depend on the expression of S1P receptors and their downstream effectors. It is also possible that deletion of sphingosine kinases induces compensatory mechanisms. Moreover, TNF-induced inflammatory responses are not only regulated by NF- κ B but are also controlled by crosstalk between NF- κ B with other context-dependent pathways. Finally, TNF signalling leading to NF- κ B and cytokine and chemokine production is now appreciated to be more complex than was originally suggested⁸⁴. The recent discovery of RIP1-dependent and -independent activation of the early and late phases of I κ B kinase (IKK), which are differentially regulated by low and high doses of TNF that lead to the expression of distinct sets of NF- κ B target genes⁸⁴, suggests a potential explanation for the divergent findings on the role of SphK1 and S1P. Exploring these possibilities might reconcile contradictory observations and shed new light on the inflammatory roles of S1P.

Ceramides and inflammation

During host-pathogen defence, pattern recognition receptors, including Toll-like receptors (TLRs) and Nod-like receptors (NLRs), expressed by sentinel cells, macrophages and dendritic cells, bind microbial products to initiate innate immune responses and prime adaptive immunity. Integration of data from sphingolipidomics and transcriptomics of macrophages treated with lipopolysaccharide, the ligand for TLR4, revealed that several enzymes in sphingolipid metabolism and their products, particularly ceramide, were

coordinately elevated^{85,86}. Several intriguing ideas have been proposed to explain how ceramide links TLR4 and other members of this family to inflammation (Fig. 4). In macrophages, increased *de novo* ceramide biosynthesis is required for autophagosome formation⁸⁶, which is thought to have key roles in innate immunity⁸⁷. A question still to be answered is whether changes in autophagy owing to ceramide contributes to immunity and/or inflammation. Disruption of membrane microdomains in *Cers2* knockout mice, which are unable to make very-long-chain ceramides, prevented TNF-R1 internalization and downstream signalling⁸⁸.

Another area that has received much attention is the connection between low-grade chronic inflammation and the pathogenesis of obesity and metabolic dysfunction⁸⁹. A recently uncovered mechanistic link between increased inflammation and obesity is the activation of TLR4 by saturated fatty acids, leading to transcriptional activation of ceramide biosynthetic genes, including those that encode SPT and specific CerS isoforms, in an IKK- β -dependent manner²⁹. Interestingly, although not required for TLR4-dependent inflammatory cytokine induction, elevated ceramide production was required for TLR4-dependent insulin resistance, owing to inhibition of Akt²⁹, the activation of which is necessary for stimulated glucose transport⁹⁰ (Fig. 4). Likewise, *CerS2* knockout mice are glucose intolerant, owing to the inability of the insulin receptor to translocate to the disrupted membrane platforms, preventing Akt activation⁹¹. Together, these studies implicate ceramide as a mediator linking lipid-induced inflammatory pathways and insulin resistance.

The NLR family member NLRP3 inflammasome is activated by diverse non-microbial danger-associated molecular patterns (DAMPs) derived from damaged cells, and induces inflammation by increasing IL-1 β and IL-18 secretion. In obese mice, lipotoxicity-associated ceramide was shown to increase activated caspase-1 in a Nlrp3 inflammasome-dependent manner in macrophages and adipose tissue⁹². This is the first suggestion that the Nlrp3 inflammasome senses increases in intracellular ceramide (Fig. 4)^{92,93}. Moreover, age-related increased thymic ceramides also leads to Nlrp3 inflammasome-dependent caspase-1 activation⁹⁴, suggesting that this form of ceramide elevation is responsible for the collapse of the thymic stromal cell microenvironment and subsequent decreased production of naive T cells and lower immune surveillance in the elderly⁹⁴.

Ceramides and adipokines

An intriguing potential link has surfaced between sphingolipid metabolism and the pleiotropic actions of the adipocyte hormone adiponectin. By binding to its receptors AdipoR1 or AdipoR2 adiponectin decreases inflammation and increases insulin sensitivity⁹⁵. AdipoR1- and AdipoR2-stimulated ceramidase degraded ceramide to sphingosine, which was converted into the pro-survival S1P; evidence indicates that ceramidase activation initiates many adiponectin actions (Fig. 3)²⁸. It is unclear whether AdipoR1 and AdipoR2 have intrinsic ceramidase activity, although a recent report suggests that AdipoR1, but not AdipoR2, induces caveolin-dependent recruitment of neutral ceramidase⁹⁶.

Subsequent studies demonstrated that the adiponectin–ceramide axis is also involved in fibroblast growth factor-21 (FGF21)-mediated regulation of carbohydrate and lipid

metabolism, and energy utilization. The beneficial effects of FGF21 were abolished in adiponectin-null mice, which were resistant to FGF21-induced decreases in ceramide and increases in energy expenditure⁹⁷. Adiponectin also mediated the FGF21-induced decrease in production of liver pro-inflammatory cytokines in obese, diabetic mice⁹⁸. Experiments are needed to determine which adiponectin-mediated effects depend mainly on decreasing ceramide signalling and which depend on increasing S1P signalling. Interestingly, thiazolidinediones — agonists for the nuclear receptor PPAR- γ , widely used for management of type 2 diabetes — also induce FGF21 and adiponectin, and decrease ceramide in plasma and the liver⁹⁷. These FGF21 and thiazolidinedione effects bring ceramides into the spotlight for research on new therapeutics for obesity-induced inflammation and metabolic disorders.

New insights on the role of C1P in inflammation

C1P is a relatively new addition to the group of bioactive sphingolipid metabolites involved in inflammation. C1P activates cPLA2 α ³⁶, which releases arachidonic acid in the rate-determining step in eicosanoid production. As eicosanoids drive the pathogenesis of many inflammatory disorders, understanding the role of C1P in this process is crucial. An intriguing example of the latter was the characterization of a unique C1P-specific transfer protein called CPTP³⁸. Depletion of CPTP, which is present in the cytosol and associated with the TGN and plasma membrane, increases C1P in the Golgi and nucleus, but decreases it in the plasma membrane. This suggested that CPTP controls levels of C1P produced by CERK in the Golgi by transferring it to the plasma membrane, thereby suppressing cPLA2 α activity and reducing arachidonic acid release and eicosanoid production³⁸. The C1P that CPTP transfers to the plasma membrane is probably rapidly degraded by lipid phosphatases to ceramide. Another intriguing observation from this study was that changes in C1P levels directly correlated with changes in sphingosine and S1P, raising the possibility that C1P might be a precursor of S1P that is mediated by, yet to be characterized, deacylase activity³⁸. Global CERK inactivation has only modest effects on inflammatory responses, in contrast to cPLA2 knockout, and further studies are necessary to address this discrepancy. Nevertheless, acute downregulation of CERK using short interfering RNA did reduce eicosanoid production in a mouse model of airway hyper-responsiveness⁹⁹.

The concept of immunomodulation by C1P has become increasingly intricate as other studies have shown that C1P also negatively regulates inflammatory cytokine production. C1P produced by CERK regulates TNF processing and secretion by direct inhibition of TACE³⁹. In addition, exogenous C1P was found to suppress lipopolysaccharide mediated production of TNF, IL-6, IL-8 and IL-1 β in human peripheral blood mononuclear cells¹⁰⁰. Although exogenous C1P can stimulate macrophage migration by a pertussis-toxin-sensitive GPCR¹⁰¹, a cell surface C1P receptor has never been identified. It is also unclear what biological effects, if any, are regulated by circulating C1P.

Therapeutic interventions in inflammatory disorders

Much of the clinical interest in sphingolipid metabolites and their signalling was spurred by the discovery of the mechanism of action of the immunosuppressant drug FTY720 (ref. 102). *In vivo*, SphK2 is mainly responsible for the phosphorylation of FTY720 to FTY720-

P, an S1P mimetic and agonist for all S1P receptors except S1PR2. Binding of FTY720-P to S1PR1 leads to its downregulation, thereby effectively acting as a functional S1PR1 antagonist. Down-modulation of S1PR1 induces the retention of lymphocytes in lymphoid organs, termed lymphopenia, because the receptor is required for their exit into the blood up the S1P gradient; this prevents adaptive immune surveillance of tissues, and thus can ameliorate autoimmune diseases.

Autoimmune diseases

Thousands of patients have been treated with FTY720 for the relapsing-remitting form of multiple sclerosis. In experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, FTY720 prevented pathogenic T cells committed to T helper (T_H) 1 and T_H17 lineages from crossing the blood–brain barrier into the central nervous system⁴. Down-modulation of S1PR1 in orectal cancer astrocytes by FTY720 reduced astrogliosis¹⁰³, a hallmark of multiple sclerosis, and promoted remyelination and recovery of nerve conduction. These are likely explanations for the significant decrease in inflammatory markers and brain atrophy after FTY720 treatment of patients with multiple sclerosis⁴.

FTY720 and other next-generation S1PR1-modulating drugs are now in various stages of clinical trials for the treatment of a variety of immune diseases. One of the side effects of these modulating drugs is the transient reduction of heart rate by the activation of GIRK potassium channels downstream of cardiomyocyte S1PR1 (ref. 104), but with careful monitoring of patients after the first dose this should not prevent their use. FTY720 also induces pulmonary vascular leakage in mice by inducing degradation of S1PR1 (ref. 105). Receptor pharmacology predicts that competitive S1PR1 antagonists should not have this adverse effect; however, they could reduce barrier function, owing to S1PR1 blockade. So far, S1PR1 antagonists that are effective in various disease models, including EAE, have not been tested in the clinic. Even with FTY720's efficacy, the fact that it is well tolerated in patients and the convenience of oral administration, improvements could still be made. A deeper knowledge of S1P and its receptors, and an understanding of the beneficial and adverse side effects of targeting S1P receptors could lead to the development of even better treatments.

Rheumatoid arthritis

Rheumatoid arthritis is an autoimmune disease characterized by inflammation in movable joints. High levels of S1P have been found in the synovial fluid of patients with rheumatoid arthritis³⁰; however, conflicting data were reported in different mouse rheumatoid arthritis models regarding the involvement of SphK1. Although *Sphk1* knock-out mice showed normal responses in models of collagen-induced arthritis⁷³, synovial inflammation in TNF-induced arthritis was reduced⁷². Hence, this area of research needs to be re-examined.

Lymphopenia-inducing drugs such as FTY720 have potential as rheumatoid arthritis therapeutics, FTY720 was protective in a mouse model of rheumatoid arthritis¹⁰⁶; however, there are no current clinical trials. But, phase II clinical trials are ongoing to target S1P signalling by a completely different mode based on the finding that inhibition of SPL with the food dye THI (2-acetyl-4-tetrahydroxybutylimidazole) induces lymphopenia by

elevating the levels of S1P in lymphoid tissues, disrupting the S1P gradient lymphocytes use for egress¹⁰⁷. Subsequent studies of genetically deficient SPL mice revealed that they also had a disrupted S1P gradient and lymphopenia. On the basis of THI structure, a second compound, LX2931, was developed that was effective in a mouse model of rheumatoid arthritis and is now in phase II trials¹⁰⁸. It remains to be seen whether direct inhibitors of SPL are clinically useful.

Inflammatory bowel disease

Inflammatory bowel disease (IBD), a result of chronic inflammation of the gastrointestinal tract, is due to abnormal host immune responses to the intestinal microbiome¹⁰⁹. Millions of people world-wide have ulcerative colitis and Crohn's disease, the major types of IBD. Studies using common acute and chronic epithelial injury colitis models have underscored a role for the SphK1–S1P–S1PR1 axis in IBD. Knockout of the gene encoding SphK1 or specific SphK1 inhibitors greatly reduce colitis severity, cytokine production and systemic inflammation^{26,78}. In agreement, S1P is elevated during colitis and SphK1 expression is increased in patients with ulcerative colitis²⁶. Several studies have also shown that FTY720 and other pharmacological agents that target S1PR1 are very effective in rodent models of colitis^{78,110,111}, and several second-generation S1PR1 modulators are currently in clinical trials for patients with ulcerative colitis.

Persistent inflammation of the colon increases the risk of developing colorectal cancer¹¹². There is increasing evidence from mouse models that SphK1 and S1P may be a link between chronic intestinal inflammation and the development of colitis-associated cancer (CAC), and that both are important for colon cancer development and progression^{78,113,114}. An intracellular role for SphK1 in intestinal adenoma cell proliferation and polyp size was shown by deletion of SphK1 in *Apc^{Min/+}* mice, a model of intestinal neoplasia¹¹³. Upregulation of SphK1 during colitis and CAC in mice leads to NF- κ B-dependent production of IL-6, robust activation of the transcription factor Stat3, and subsequent upregulation of its target gene, *S1pr1*. FTY720 impeded the SphK1–S1P–S1PR1 axis, suppressing the NF- κ B–IL-6–Stat3 vicious feed-forward amplification loop and CAC⁷⁸. Thus, it seems that FTY720 and second-generation S1PR1 modulators should be considered for the treatment of inflammation-driven cancers.

Asthma

Sphingolipid metabolites contribute to the pathogenesis of asthma, an increasingly prevalent chronic airway inflammation characterized by intermittent airflow obstruction and increased mucus production. S1P levels are elevated in the lungs of patients with allergic asthma. In mice, S1P is involved at multiple stages of the asthmatic response: it promotes the contraction of airway smooth muscle cells, the induction of airway hyper-reactivity by S1PR1 and/or S1PR3 (refs 115, 116), and regulates activation and functions of mast cells, eosinophils and dendritic cells^{27,117}. Inhalation of an SphK1 inhibitor, or FTY720, mitigated asthma in rodent models^{27,117}. *De novo* ceramide synthesis has also been implicated in human asthma based on the association of a 17q21 single nucleotide polymorphism (SNP) with increased expression of ORMDL3 (ref. 7), a negative regulator of SPT, the first enzyme in sphingolipid synthesis^{6,118}. Patients with this SNP are at increased risk of

developing childhood asthma as well as virus-induced respiratory wheezing¹¹⁹. However, how ORMDL3 is involved in asthma remains a matter of debate. It has been shown that allergens upregulate ORMDL3 expression, and overexpression of ORMDL3 increased expression of asthma-associated chemokines and metalloproteases and the unfolded protein response (UPR), suggesting that ORMDL3 and asthma may be linked through the UPR pathway¹²⁰. ORMDL3 also promotes eosinophil trafficking, recruitment and activation¹²¹. By contrast, although ceramide is elevated in murine models of asthma¹²², decreased *de novo* sphingolipid biosynthesis enhances bronchial reactivity by affecting intracellular magnesium homeostasis in the absence of inflammation¹²³. Although these studies functionally link *de novo* sphingolipid biosynthesis to asthma, many questions remain unanswered, presenting challenging opportunities for research.

Future perspectives

The study of bioactive sphingolipids in inflammation is only now coming to the foreground. As always in science, answering one question leads to many more, and this is especially true of these enigmatic sphingolipids. Clearly, more work is required to understand how inflammation regulates bioactive sphingolipid levels, the activities of the enzymes that control their levels and the proteins they signal through, and how this shapes the immune response. Although around a dozen extracellular and intracellular targets of signalling sphingolipids have been identified, it is unlikely that we have found them all, based on their pleiotropic effects. This is especially important for understanding the functions of ceramide in inflammation as only a few direct targets have been confirmed and how it regulates all inflammasomes remains a mystery. The complexity of sphingolipid metabolism and the ‘sphingolipidome’, and the fast interconversion of bioactive sphingolipids make this task even more daunting. This endeavour goes hand-in-hand with current systems-biology research, and it will be greatly aided by advanced sphingolipidomics — which can quantitatively measure changes in sphingolipids bound to their protein targets — combined with the development of conditional and tissue-specific sphingolipid-metabolic-enzyme knockout mice. Moreover, current sphingolipidomic technology for simultaneous quantification during inflammation of multiple sphingolipid species, for example, ceramides with different fatty acids, will be particularly helpful as these signalling molecules could have distinct and, sometimes, even opposing functions. Such a diverse array of bioactive sphingolipids could provide immune cells with a large toolkit to fine-tune specific inflammatory responses. Finally, improvement of matrix-assisted laser desorption ionization (MALDI) technology may, in the future, allow imaging of the S1P gradient in tissues such as the lymph nodes, the thymus and the spleen; this is crucial for our understanding of the role of the S1P– S1PR1 axis in lymphocyte trafficking.

Given the success of FTY720 for treatment of multiple sclerosis, it is hoped that next-generation S1PR1 modulators will find even wider therapeutic uses in other inflammatory disorders — as has been suggested by pre-clinical data. Progress in targeting other S1P receptors will increase our basic knowledge of their functions and how S1P can induce pro- and anti-inflammatory responses in different cell types, and will enable the rational design of more selective and potent inhibitors of immune responses. There are ongoing clinical trials with other interventions that target bioactive sphingolipids, such as Sphingomab, a

monoclonal antibody that neutralizes S1P; ABC294640, which inhibits SphK2; and amitriptyline, which inhibits acid sphingomyelinase, and perhaps they will turn out to be useful for inflammatory disorders. Although such advances for other sphingolipid metabolites are still to be made, now that X-ray crystal structures of sphingolipid metabolic enzymes are available and carriers and targets of bioactive metabolites are gradually being elucidated, it is expected that drugs will be developed to interfere with specific sphingolipid-mediated functions, which are important for the pathogenesis of human inflammatory disease. ■

Acknowledgments

We thank S. Milstien for critically reading the manuscript. This work was supported by grants R37GM043880, R01AI500941 and R01CA61774 from the US National Institutes of Health. We apologize to those whose work was not cited owing to space constraints.

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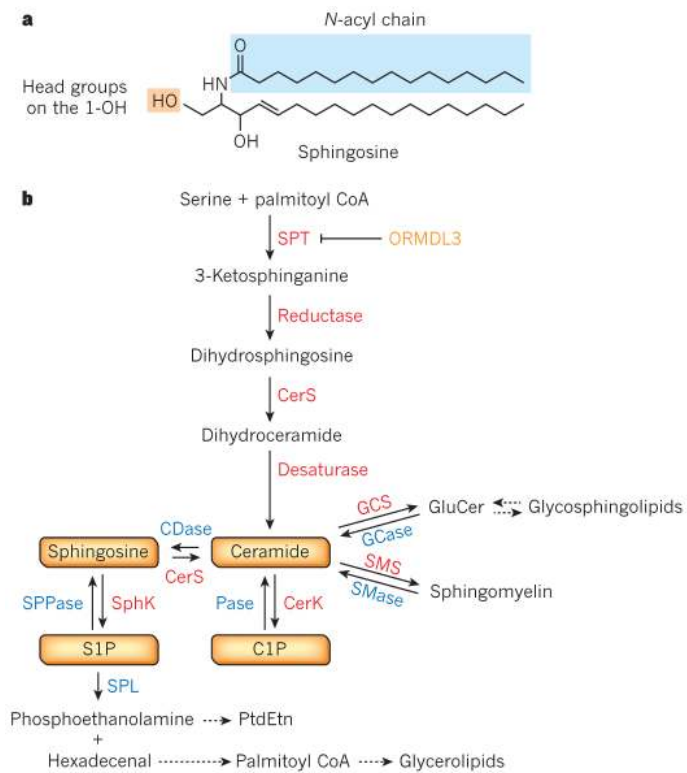


Figure 1. Spingolipid metabolism and interconnected bioactive spingolipid metabolites
a, Structure of ceramide, with a sphingosine backbone. **b**, *De novo* spingolipid biosynthesis starts with the condensation of palmitoyl coenzyme A (CoA) and serine by serine palmitoyltransferase (SPT), an enzyme that is negatively regulated by ORM1-like protein 3 (ORMDL3). This is followed by a series of reactions (catalysed by enzymes in red) leading to formation of ceramide and subsequent formation of sphingomyelin and glycosphingolipids. Ceramide can be metabolized to other bioactive spingolipid species, phosphorylated by ceramide kinase (CERK) to ceramide-1-phosphate (C1P), or hydrolysed to sphingosine, which is then phosphorylated to sphingosine-1-phosphate (S1P) by sphingosine kinases (SphKs). S1P can be degraded by phosphatases to sphingosine or by the lyase (SPL) that cleaves it to phosphoethanolamine and hexadecenal, which are subsequently reincorporated into glycerolipid metabolic pathways. For simplicity, degradative enzymes (blue) for reutilization of spingolipids in the salvage pathway are included but these reactions take place in different subcellular compartments (see Fig. 2). CDase, ceramidase; CerS, ceramide synthase; GCase, glucosylceramidase; GCS, glucosylceramide synthase; Pase, phosphatase; PtdEtn, phosphatidylethanolamine; SMase, sphingomyelinase; SMS, sphingomyelin synthase; SPPase, sphingosine phosphate phosphatase.

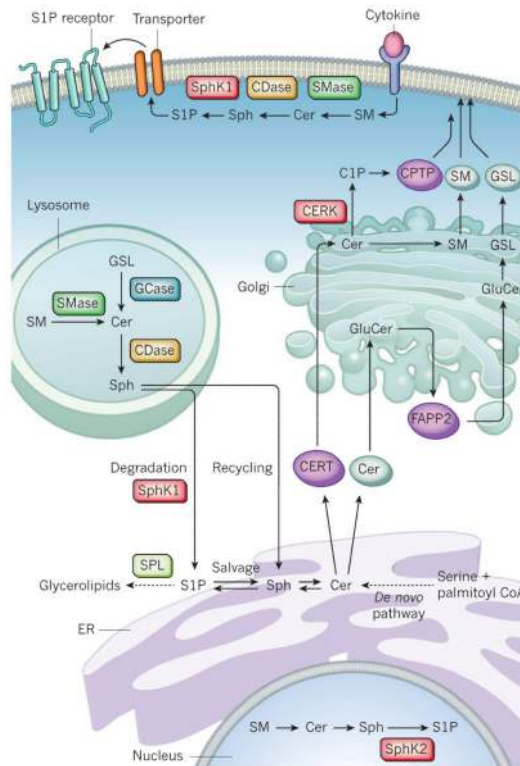


Figure 2. Subcellular compartmentalization of sphingolipid metabolism

De novo ceramide (Cer) synthesis takes place in the endoplasmic reticulum (ER). Cer is delivered by ceramide transport protein (CERT) or vesicular transport to the Golgi for synthesis of ceramide-1-phosphate (C1P) (by ceramide kinase, CERK), sphingomyelin (SM), and glucosylceramide (GluCer). Four-phosphate adaptor protein 2 (FAPP2) then transports GluCer to the *trans*-Golgi for biosynthesis of complex glycosphingolipids (GSLs). SM and GSLs are delivered to the plasma membrane by vesicular transport and C1P by a C1P-specific transfer protein (CPTP). For signalling at the plasma membrane, sphingomyelinase (SMase), ceramidase (CDase), and sphingosine kinases (SphK) produce the bioactive metabolites Cer, sphingosine (Sph) and sphingosine-1-phosphate (S1P), respectively. S1P is then transported across the membrane. Membrane sphingolipids are internalized by the endocytic pathway and in the lysosome they are degraded by acidic forms of SMase, glycosidase (GCase) and CDase. The Sph formed can be metabolized to glycerolipids after phosphorylation by SphKs (probably SphK1) and cleavage by S1P lyase (SPL) or reutilized for sphingolipid synthesis in the salvage pathway. In the nucleus, SphK2-produced S1P inhibits histone deacetylases.

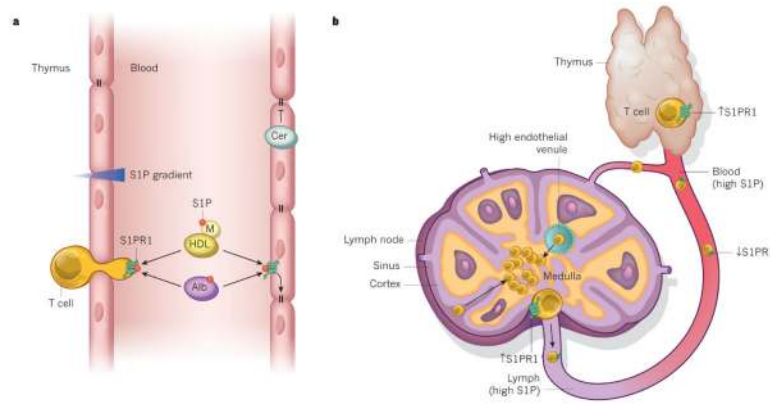


Figure 3. S1P and S1PR1 control lymphocyte trafficking and vascular integrity

a. The sphingosine-1-phosphate (S1P) level in lymphoid tissues, such as the thymus, is low compared with the blood, forming an S1P gradient (shaded red) that attracts lymphocytes and promotes S1PR1-dependent egress into the blood. In the blood, S1P is bound to albumin (Alb) and apolipoprotein M (M) in high-density lipoprotein (HDL), and signalling by S1PR1 maintains endothelium barrier function by promoting cell–cell interactions (right). Conversely, ceramide (Cer) can promote barrier leakage. **b.** When T cells are ready to exit the thymus and enter the blood, S1PR1 is re-expressed so that T cells can respond to the chemotactic effect of high S1P levels in circulation. When T cells re-enter the blood, S1P downregulates S1PR1. Signals that control lymphocyte entry through high endothelial venules and retention in lymph nodes are not shown. Lymph node egress into lymphatic vessels also requires S1PR1. Increased lymphoid tissue S1P, by SPL inhibition, or in the presence of S1PR1 modulators such as FTY720, blocks egress of lymphocytes by disruption of the S1P gradient or desensitization of S1PR1 on T cells, respectively.

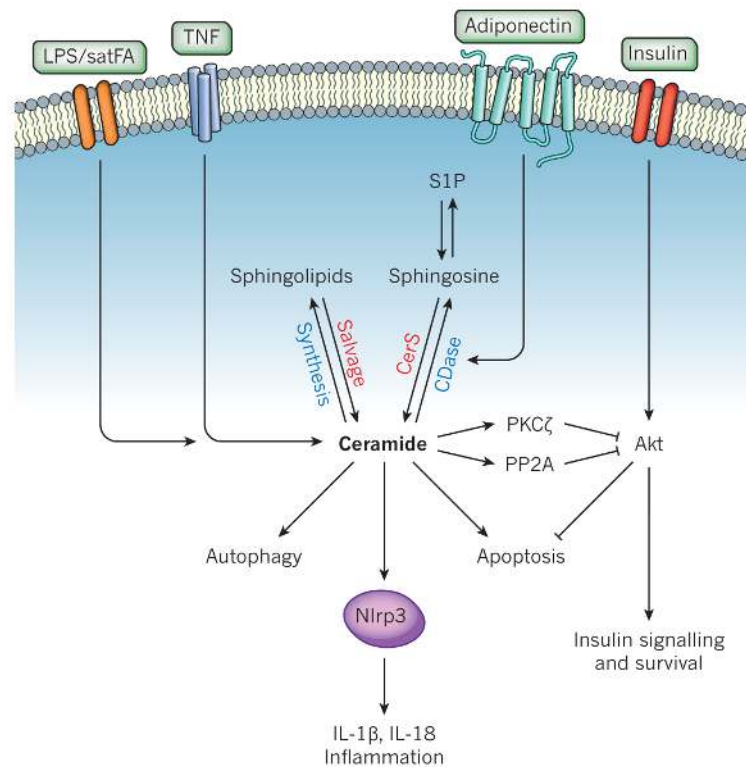


Figure 4. Ceramide at the nexus of obesity and inflammation

Ceramide is generated in response to obesity signals (such as saturated fatty acids, satFA), lipopolysaccharide (LPS) or pro-inflammatory cytokines (such as TNF) by enhancement of *de novo* biosynthesis or sphingolipid recycling. Ceramide can then signal to a variety of pathways that promote inflammation and the deleterious effects of obesity. Ceramide can bind to and inhibit SET/I2PP2A (not shown), leading to the activation of PP2A, which dephosphorylates and inhibits Akt, blocking many of the actions of insulin signalling and leading to insulin resistance, and decreased cell survival; activate PKC ζ , also leading to the inhibition of Akt; induce autophagy, which can influence innate immune responses; activate the Nlrp3 inflammasome to promote the processing and secretion of the pro-inflammatory cytokines IL-1 β and IL-18; and induce apoptosis. Binding of adiponectin to its receptors stimulates ceramidase (CDase), degrading ceramide to sphingosine, which is then phosphorylated to sphingosine-1-phosphate (S1P). Insulin stimulates Akt. It should be noted that this diagram is not meant to imply that all of these events occur in the same cell. CerS, ceramide synthase.