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# How sphingolipids affect T cells in the resolution of inflammation

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The concept of proper resolution of inflammation rather than counteracting it, gained a lot of attention in the past few years. Re-assembly of tissue and cell homeostasis as well as establishment of adaptive immunity after inflammatory processes are the key events of resolution. Neutrophils and macrophages are well described as promoters of resolution, but the role of T cells is poorly reviewed. It is also broadly known that sphingolipids and their imbalance influence membrane fluidity and cell signalling pathways resulting in inflammation associated diseases like inflammatory bowel disease (IBD), atherosclerosis or diabetes. In this review we highlight the role of sphingolipids in T cells in the context of resolution of inflammation to create an insight into new possible therapeutical approaches.

## KEYWORDS

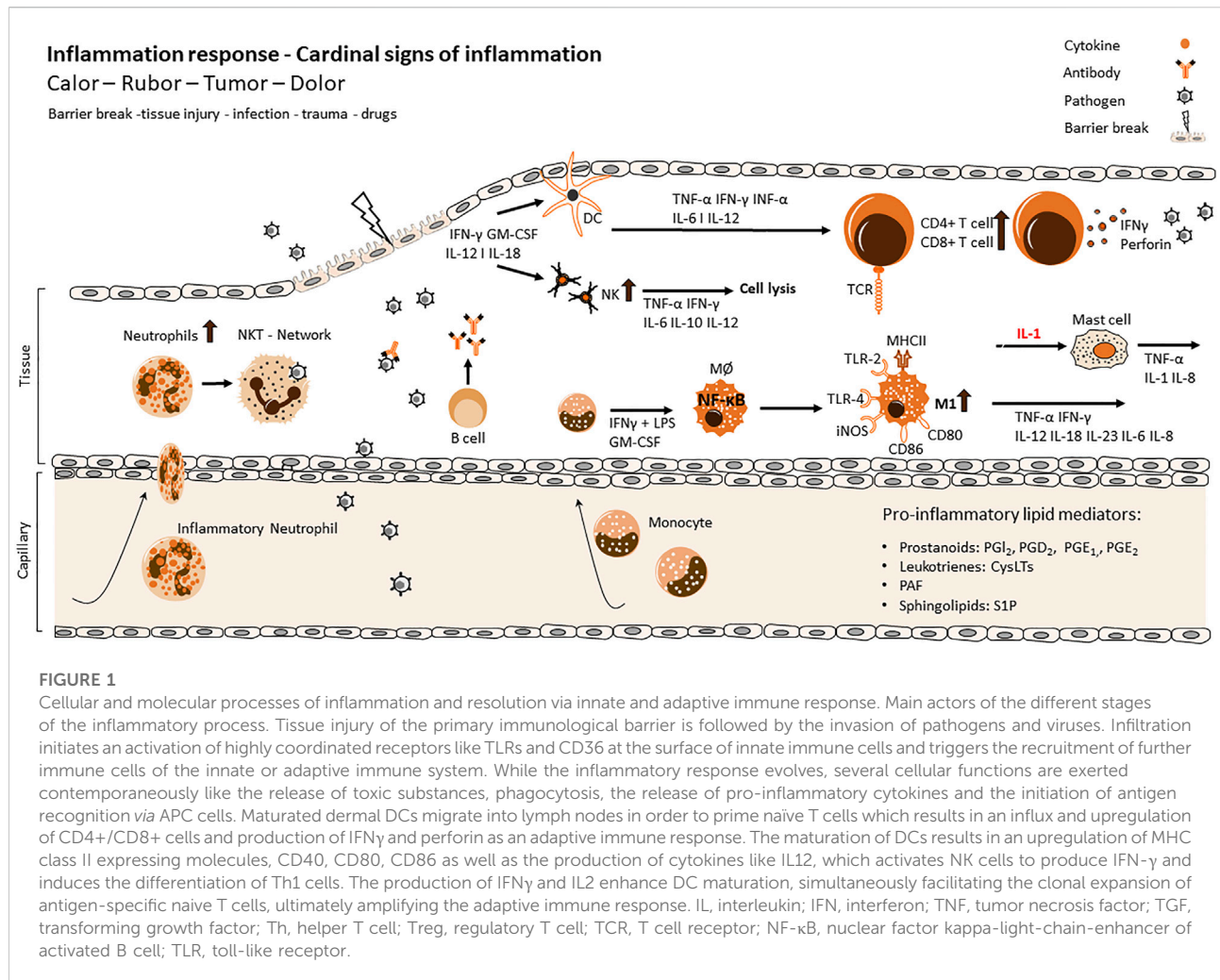
ceramides, gangliosides, S1P, lipid rafts, treg, Th17, bioactive lipids

## The innate and adaptive immune system

The immune system is a large network consisting of an innate and an adaptive part. Both systems work together and intertwine with each other to defend the body from germs or foreign substances (Figure 1) (Yatim and Lakkis, 2015; Hillion et al., 2020). The skin as part of the innate immune system is the first defence against pathogens and protects our body from foreign entries. When pathogens passed this barrier the innate immune system then activates a cascade of signalling pathways and immune cells from both, the innate and the adaptive, immune systems to fight off the danger (Smith et al., 2019).

Pathogens can be neutralized or directly killed by cells of the innate immune system (Macrophages, Natural killer cells etc.), but the scope of function of the innate immune cells goes beyond. Bacterial cell wall components or viral particles are processed by cells of the innate system and presented as antigens to the adaptive immune system. Furthermore, the innate immune cells recruit adaptive immune cells from the blood to the site of inflammation by secretion of chemokines and cytokines.

The adaptive immune system consists of T lymphocytes, B lymphocytes and the antibodies that are produced by the B cells (Buchholz et al., 2016; Bradley and Thomas, 2019; Wang et al., 2020). B cells come from and mature in the bone marrow. They are activated by a subpopulation of T cells (T helper cells) via antigens that bind at their surface immunoglobulin (Ig). Upon activation B cells then release soluble antibodies. The antibody release enables the humoral immune response consisting of pathogen neutralisation,



opsonization and complement activation. The complement system enzymatically facilitates lysis and phagocytosis of pathogens (Merle et al., 2015). Antibodies also prevent bacterial adherence including binding to the pathogen (Forthal, 2014).

T cells derive from the bone marrow and are then moved through the blood for maturation in the thymus. They have a broad spectrum of functions that are determined by their surface expression pattern that divides these cells into different subpopulations (Romagnani, 2014). The activation and maturation of those cells is mediated via the T Cell Receptor (TCR). They have to undergo a checkpoint to ensure only non-self-antigen responsive cells are selected (Fife and Bluestone, 2008). Negative selection of T cells removes self-reactive cells from the body (Palmer, 2003), positive selection assures that only T cells with a TCR that can recognize foreign antigens via MHC stimulation will mature (Klein et al., 2014). This process takes place in the thymus and occurs with cells that already express the TCR and both co-receptors Cluster of Differentiation (CD) 4 and 8.

## Inflammation processes and proper resolution

Under normal circumstances, the process of inflammation is a mechanism to defend the body from foreign danger like pathogens, toxins or damaged cells. Usually this process is managed within days and finished by resolution of this acute inflammation (Varela et al., 2018). Inflammatory cells, such as macrophages, neutrophils, B cells and T cells, are recruited and send to the site of pathogen infiltration to act against the those pathogens to heal the injury and thereby restoring tissue homeostasis (Figure 1) (Schnoor et al., 2016).

Local, acute inflammation in the tissue is characterized by Galen's prominent five properties: rubor (redness), tumor (swelling), dolor (pain), calor (heat) and function laesa (impaired function). These characteristics are triggered by the immune cell response to the infectious or sterile damage. Receptors on the surface of immune cells can recognize

harmful stimuli via different receptors. Infectious stimuli are so called PAMPs (pathogen-associated molecular patterns) that activate a variety of PRRs (pattern-recognition receptors) in immune and non-immune cells, but additionally endogenous signals also known as DAMPs (danger-associated molecular patterns) can be responsible for inflammatory responses (Walsh et al., 2013; Eppensteiner et al., 2019; Zindel and Kubes, 2020; Murao et al., 2021). Toll-like receptors (TLRs) are one class of these PRR. When they get activated, different inflammatory signalling cascades in immune or non-immune cells are triggered, resulting in the translocation of the nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF- $\kappa$ B), activator protein 1 (AP-1) or interferon regulatory factor 3 (IRF3) from cytosol to the nucleus, thereby leading to a transcriptional regulation of the immune response (Kawai and Akira, 2007; Liu W. et al., 2009). The activation of these pathways leads to the release of pro-inflammatory cytokines like Interferon-Gamma (IFN- $\gamma$ ), Interleukin 1 $\beta$  (IL1 $\beta$ ) or IL6 and pro-inflammatory lipid mediators like Platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine), leukotriens, prostanoids or sphingolipids (Lawrence, 2009; Schaubberger et al., 2016; Srivastava and Baig, 2018; Yanai et al., 2018). Interactions of cytokines with their receptors on immune cells activate signalling pathways including NF- $\kappa$ B, mitogen-activated protein kinase (MAPK), januskinase- signal transducers and activators of transcription (JAK-STAT) that promote further cytokine production, proliferation and differentiation of immune cell to specific subtypes (Moens et al., 2013; Fang et al., 2018; Salas et al., 2020). PAF is produced by various cells including neutrophils, eosinophils, endothelial cells and fibroblasts and induces platelet aggregation and leukocyte degranulation and adhesion (Palgan and Bartuzi, 2015). Leukotrienes (Cysteinyl leukotrienes (CystLTs), leukotriene B4 (LTB4), leukotriene C4 (LTC4)) are produced by leukocytes such as macrophages, eosinophils, basophils and are important for leukocyte influx and vascular permeability (Rådmark et al., 2015; Schaubberger et al., 2016). Prostanoids are produced by almost every cell type and are crucial for chemotaxis, activation of immune cells, act on platelet aggregation and vascular smooth muscle cells (Schaubberger et al., 2016). S1P (sphingosine-1 phosphate) is the best known pro-inflammatory sphingolipid and involved in chemotaxis as well as activation of various immune cells (see also chapter 6.4) (Sukocheva et al., 2020).

To restore the pre-inflammation status of the tissue, immune cells must be removed from the inflammatory site, inflammatory signalling processes have to be resolved and chemokine gradients have to be diluted to prevent further migration of immune cells (Figure 2). Chemokines at the site of inflammation are cleaved by proteolysis and sequestration (Ye, 2013; Chongsathidkiet et al., 2018; Tao et al., 2019; Sallenave and Guillot, 2020). Neutrophils are the first cells to be egressed from the tissue (Greenlee-Wacker, 2016). They undergo Fas-ligand induced apoptosis via tumor

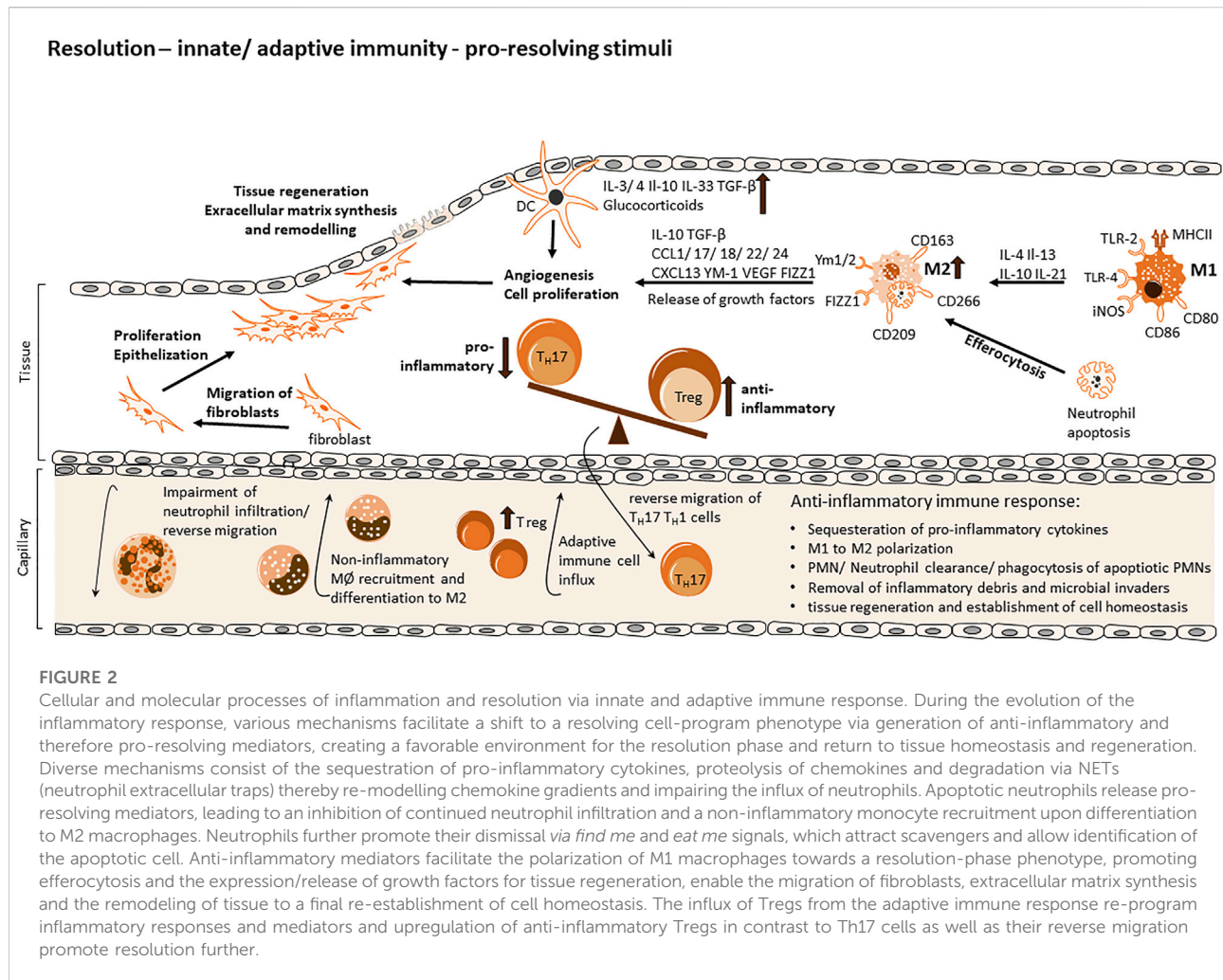
necrosis factor (TNF) receptor interactions that lead to phosphoinositide 3-kinase (PI3K)-activation, they produce reactive oxygen species (ROS) (Salamone et al., 2001; Croker et al., 2011). Macrophages are responsible for the release of Fas-ligand and TNF and the clearance of neutrophils that went apoptotic (Brown and Savill, 1999). During this efferocytosis the pro-inflammatory expression pattern (M1) of macrophages change to an anti-inflammatory pattern (M2) (Kohno et al., 2021). M2 macrophages are characterized by their ability to release immunosuppressive cytokines like IL10, transforming growth factor  $\beta$  (TGF $\beta$ ) and vascular endothelial growth factor (VEGF) (Orecchioni et al., 2019).

Anti-inflammatory signals are beneficial for tissue homeostasis. Stromal cells, macrophages and progenitor or stem cells must work together in order to impede scar formation or fibrosis (Figure 2). TGF $\beta$  released from macrophages promote fibroblast differentiation into myofibroblasts through regulation of ECM remodelling (Caja et al., 2018). VEGF signalling leads to angiogenesis and recovery of normal oxygen supply (Shibuya, 2011). Also mesenchymal stem cells (MSC) affect inflammation by the production of growth factors and chemokines that influence macrophage polarization, maturation and migration (Zhao et al., 2020). MSCs promote phagocytosis of macrophages and their differentiation by effecting the metabolism of the macrophages via a prostaglandin E2 (PGE2) dependant mechanism (Vasandan et al., 2016). Polarization of M1 type macrophages to a M2 type can also be enhanced by MSCs (Liao et al., 2020).

Dysfunction or -regulation of these processes can shift acute inflammation to a chronic status and are the cause of many diseases like inflammatory bowel disease (IBD), chronic airway inflammation, stromal keratitis, rheumatoid arthritis and even cancer.

## T cell subsets

T cells have a broad spectrum of functions. CD8 expressing T cells are also called cytotoxic T cells because of their capability of killing pathogens and cancer cells. Cytotoxic T cells have to be stimulated by antigen-presenting cells (APC) via MHC class I in order to mature (Kratky et al., 2011). This happens in the nature of an infection and causes these cells to proliferate and fight off intracellular dangers (Cox and Zajac, 2010). CD8 cells are capable of direct killing of infected cells via induction of apoptosis in those cells through the secretion of perforin and granzymes (Voskoboinik et al., 2015). Perforins form pores into the target's cell membrane and enable the entrance of granzymes (Osińska et al., 2014). Granzymes are a group of serine proteases that cleave viral and cellular proteins of the infected target cells thereby killing it (Trapani, 2001). Apoptotic target cells are then phagocytized by macrophages (Kourtzelis et al., 2020). Another possibility of target cell killing is the induction of apoptosis via



Fas-FasL interactions (Yamada et al., 2017). Upon Fas activation a signalling cascade is activated and caspase proteases are induced following apoptosis of these cells (Aouad et al., 2004; Sobrido-Cameán and Barreiro-Iglesias, 2018).

CD4 cells are often characterized as T helper cells (Th). Those cells regulate immune and non-immune cells via the production and release of cytokines and recognize antigens via MHC class II molecules. There is a variety of different T helper cell subsets, that is, described by their cytokine release and transcriptional profile. On the one hand, Th1 cells are prominent to produce IFN $\gamma$ , TNF $\beta$ , TNF $\alpha$  and IL2 and they also express the transcription factor T-bet (T-box expressed in T cells) (Viallard et al., 1999; Szabo et al., 2000; Hwang et al., 2005; Solomou et al., 2006; Kisuya et al., 2019; Artham et al., 2020). On the other hand, TH2 cells do not release IFN $\gamma$ , but IL4, IL5 and IL13 and they produce the transcription factors GATA binding protein 3 (GATA-3) and c-Maf (musculoaponeurotic fibrosarcoma) (McKenzie et al., 1998; Kanhere et al., 2012; Paul, 2015; Imbratta et al., 2020). Both subpopulations express

different chemokine receptors, Th1 cells express C-C chemokine receptor type 5 (CCR5) and CXCR3, TH2 cells express CCR4 and CCR8 (Zingoni et al., 1998; Turner et al., 2007; Yoshie and Matsushima, 2015; Watanabe et al., 2020). Th1 cells are mainly responsible for the recruitment and activation of macrophages by IFN $\gamma$  release and TH2 cells activate B cells via IL4 and IL13, but also eosinophils via IL5.

There is one additional major subpopulation among the CD4 effector cells, that is, called Th17 cells. Those cells mainly release IL17A to activate macrophages, endothelial and epithelial cells and fibroblasts, as well as CXCL8 to stimulate neutrophils and exhibit pro-inflammatory functions (Pelletier et al., 2010; Li J. et al., 2013; Brembilla et al., 2013; Tabarkiewicz et al., 2015; Zhang et al., 2021; Muench et al., 2022). RAR-related orphan receptor gamma (ROR( $\gamma$ t)) is a transcription factor, that is, solely expressed by Th17 cells (Steinmetz et al., 2011).

Besides their effector functions in inflammatory processes, there is one specialized CD4 subset, that has a more regulatory function. Regulatory T cells (Tregs) are a subpopulation of

CD4 expressing cells that additionally express high amounts of CD25, the alpha-subunit of the IL2 receptor (Kmieciak et al., 2009). They are also often categorized by their expression of the transcription factor forkhead box P3 (FOXP3) which is a prominent Treg marker (Rudensky, 2011). It is important for both, regulatory and developmental pathways in Tregs. Tregs are known for their immune-suppressive function and behaviour (Wan, 2010). The production of the anti-inflammatory cytokine IL10 is one of the main tasks of Tregs and a key player in suppressive function (Ng et al., 2013). The process of self-tolerance is tightly regulated by Tregs and disturbances in this processes lead to autoimmune diseases (Sakaguchi et al., 2008; Yang et al., 2015). Because of their immunosuppressive function, Tregs are a key mediator in resolution of inflammation. They promote macrophage efferocytosis and modulate monocyte macrophage differentiation thereby driving the resolution of inflammation (Weirather et al., 2014; Proto et al., 2018).

## T cell activation

The TCR is a heterodimer complex that has two TCR chains and six CD3 chains also connected with different components like Co-receptors, kinases and ligands. Most T cells express the  $\alpha$ - and  $\beta$ - TCR chain and are referred to as normal T cells, whereas T cells that express  $\gamma$ - and  $\delta$ - TCR chain receptors are called  $\gamma\delta$ - T cells (Nanno et al., 2007). They only make up 0.5–5% of the T lymphocytes (Carding and Egan, 2002). The variable domains of the  $\alpha$  and  $\beta$ ,  $\gamma$   $\delta$  respectively, are responsible for the specificity of the TCR and distinguish the different antigens presented by the Major Histocompatibility complex (MHC). TCR $\alpha$ /TCR $\beta$  and TCR $\gamma$ /TCR $\delta$  heterodimers form complexes with the CD3 $\gamma/\epsilon$  and/or CD3 $\delta/\epsilon$  heterodimer molecules, as well as CD3 $\zeta$  homodimers (Birnbaum et al., 2014).

T cells can be generally classified via their cell surface molecules CD4 and 8, respectively. Both markers are co-stimulatory proteins of the TCR. They are important mediators of the MHC peptide recognition cascade and they are also important for the maturation process of T cells in the thymus (Tikhonova et al., 2012). They are single molecules with an extracellular and a transmembrane region and an intracellular tail (Leahy, 1995). The intracellular tail enables interaction with the lymphocyte-specific protein tyrosine kinase (LCK) which initiates the signalling cascade upon activation of the TCR (Artyomov et al., 2010). CD4 can recognize MHC class I molecules, whereas CD8 cells bind to MHC class II molecules (Li Y. et al., 2013).

The activation of TCR is only possible when not only CD3 or CD4/8 are stimulated but also CD28 (Linsley and Ledbetter, 1993). It is a cell surface monomeric protein that binds to CD80 and CD86 of APCs in order to activate phosphoinositide-3-kinases (PI3K) (Garçon et al., 2008). PI3K

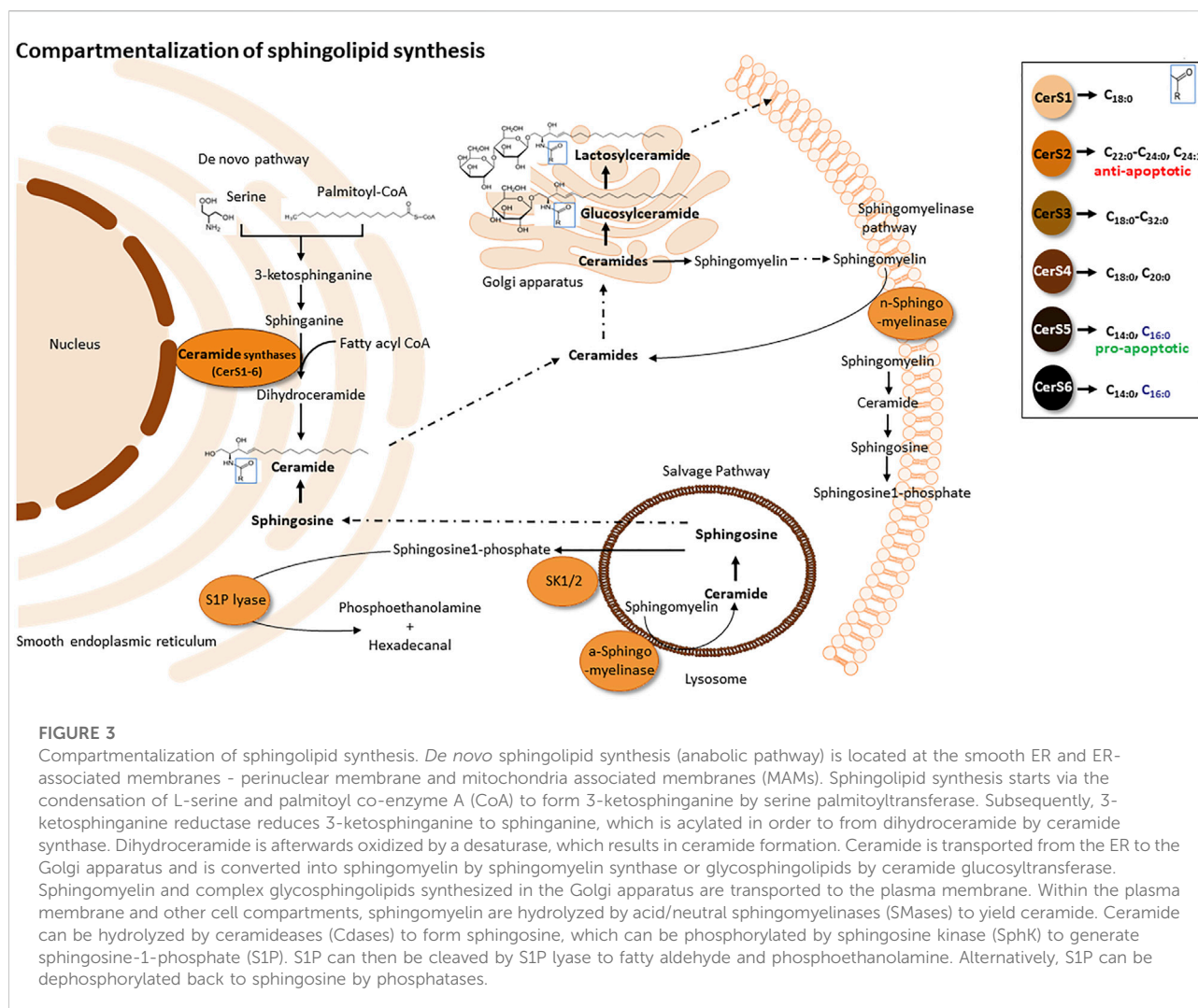
catalyzes the conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3) which then activates phospholipase C- $\gamma$  (PLC- $\gamma$ ) in the TCR activation machinery (Falasca et al., 1998). CD28 also enhances the production of IL4 and IL10 via TCR activation and CD40L binding (Hünig et al., 2010).

Upon stimulation of the TCR CD4/8 mediated LCK activation leads to the cascade of ZAP-70 phosphorylation and subsequent activation of the linker activator of T cells (LAT) (Lo et al., 2018). LAT as well as CD28, activate PI3K to form PIP2, but LAT can also activate PLC- $\gamma$  directly (Balagopalan et al., 2015). Signalling pathways of TCR lead to NF $\kappa$ B, AP-1 and NFAT activation, which then promotes cell proliferation, differentiation and cell survival (Smith-Garvin et al., 2009).

The organization of the TCR's activation takes place in so-called lipid rafts, often also called micro-domains or detergent-resistant membranes (DRM) (Zumerle et al., 2017). Lipid rafts are specialized compartments in the cell membrane with a high density of sphingomyelins, cholesterol and glycosphingolipids (Lingwood and Simons, 2010; Simons and Sampaio, 2011; Regen, 2020). They are cellular signalling hubs, bringing receptors closely together, kinases and second messengers thereby transforming a signal from the membrane to intracellular signalling cascades. The assembly of TCR/CD3, CD28, PKC $\theta$  and LCK is defined as supramolecular activation cluster (SMAC) with a central region (c-SMAC) and a peripheral region (p-SMAC) (Zumerle et al., 2017).

## Insights into sphingolipid metabolism and function

As lipid rafts are characterized as structures enriched in sphingomyelins and glycosphingolipids, the role of sphingolipids (Sph) is very crucial (Bieberich, 2018). Sphs are one of the most important classes of membrane lipids. They owe their name to the mythological sphinx after they were first discovered in brain tissue in the 1870s (Thudichum, 1962). Sphs commonly consist of a sphingoid base, like sphingosine or sphinganine, as backbone, at which via an amid-bound a second fatty acyl chain of different chain length is added. Further, the C1-hydroxygroup could be connected to different charged groups like phosphate, glucose/galactose or phosphocholine, resulting in Ceramide-1-phosphate, Glucosyl-Ceramide (GluCer), Galactosyl-Ceramide (GalCer) or sphingomyelin (SM). GluCer can further be processed to Lactosyl-Ceramide (LacCer) and complex Gangliosides (GMs). A nice overview of the sphingolipid pathway is given in (Rajasagi and Rouse, 2018). The *de novo* synthesis of ceramides takes place in the Endoplasmatic Reticulum (ER) afterwards ceramides are transported to the Golgi apparatus where the synthesis of complex sphingolipids takes place (Figure 3). The first step of



the sphingolipid *de novo* synthesis is mediated by the serine palmitoyltransferase (SPT) which condensates serine and palmitoyl-CoA to generate 3-ketosphinganine, that is, directly reduced to sphinganine by the 3-ketosphinganine reductase. Six isoforms of ceramide synthases (CerS1-6) add a second acyl-chain of different chain length to sphinganine/sphingosine to generate dihydro-ceramides or ceramides, respectively. CerS1 and CerS4 add C18/C20 fatty acids, CerS2 C22-C26, CerS3 C18-C32 and CerS5 and CerS6 use mainly C14/C16 fatty acids as substrate (Figure 3). Ceramide synthases are regulated and expressed tissue specific and each ceramide has definite cellular effects (Brachtendorf et al., 2019 und Wegner et al., 2016). Sphingolipids could also be recycled in the salvage pathway, where sphingosine can be obtained from constitutive degradation of sphingomyelin or glycosphingolipids either at the plasma membrane or in the late endosomes and lysosomes by the action of sphingomyelinases, glycosidases and ceramidases. Sphingosine is then either N-acetylated by CerS to form

ceramides, phosphorylated by sphingosine-kinases to sphingosine-1-phosphate (S1P), or degraded to phosphatidylethanolamine and fatty aldehyde by lyases. S1P regulates many cellular functions via binding to G protein-coupled receptors S1P1-5. Those receptors reside in different cell types and tissues and upon their stimulation trigger signalling cascades for proliferation, survival, migration and vaso-regulative pathways (Schwab and Cyster, 2007; Obinata and Hla, 2019). The impact of membrane located sphingolipids on cells depends on their basic structure. Ceramides, for example, can affect cellular pathways and diseases differentially through the length of their fatty acid chains (Grösch et al., 2012). On the one hand, the amphilic properties of short chain ceramides result in membrane permeability and enable them to translocate freely into the cytosol. On the other hand, long chain ceramides stay tightly bound to the membrane and cannot pass as freely. Very long chain ceramides are responsible for the epidermal barrier function and can be found in the skin (Li et al., 2007). Specific

TABLE 1 Animal studies, showing the importance of sphingolipids in T cells.

Sphingolipid class	Animal model	Treatment	T Cell population	References
-	SCID and <i>rag1</i> <sup>-/-</sup>	DSS (colitis)	Adoptive Treg transfer led to resolution of colitis	Mottet et al. (2003)
-	<i>Foxp3</i> <sup>YFP</sup> - <i>cre</i> ; <i>Ppp2r1a</i> <sup>fllox/fllox</sup>	-	Tregs displayed different metabolic and cytokine profile and did not show immune suppressive activity	Apostolidis et al. (2016)
Sphingomyelins and Ceramides	<i>ASM</i> <sup>-/-</sup>	-	Tregs were elevated and more activated	Hollmann et al. (2016)
Glycosphingolipids	T cell specific <i>Ugcg</i> <sup>-/-</sup>	DSS (colitis)	Treg levels were decreased and the pathogenesis of colitis was more severe compared to control	Komuro et al. (2022)
C22-24 Ceramides	<i>CerS2</i> <sup>-/-</sup>	Ovalbumin (asthma)	TH2 response was impaired and Th17 response was elevated accompanied by higher TCR signalling activity	Shin et al. (2021)
Ceramides	<i>CerS6</i> <sup>-/-</sup>	GVHD	T cell response to alloantigen was impaired and differentiation into Th1 cells was inhibited	Sofi et al. (2017)
Ceramides	<i>CerS5</i> <sup>-/-</sup>	DSS (colitis)	CD8 <sup>+</sup> cells were reduced and TCR signalling impaired	El-Hindi et al. (2020)
Ceramides	T cell specific <i>CerS4</i> <sup>-/-</sup>	DSS (colitis)	Prolonged TCR activation in CD8 cells	El-Hindi et al. (2022)

lipid and protein interactions in membranes are crucial for signal transduction and can be influenced also by the chain length, saturation of the acyl chain or their polar head groups (Quinn, 2014; Uche et al., 2021). Therefore, the biophysical properties of membrane lipids can facilitate or impede receptor signalling.

Sphs control and influence major cellular events such as apoptosis, cell growth and proliferation, signal transduction and differentiation (Cerbón et al., 2018; Xie et al., 2019; Hadas et al., 2020; Jakobi et al., 2020; Loberto et al., 2020; Mignard et al., 2020; Muthusamy et al., 2020; Monasterio et al., 2021; Capolupo et al., 2022). Therefore, a dysregulation of these processes have also an impact on chronic inflammation which we want to highlight in the next sections.

## The impact of sphingolipids on T cells and their role in resolution of inflammation

In this chapter we want to give an overview how T cells affect resolution of inflammation and how sphingolipids can influence these mechanisms. As mentioned above, T cells have different functions in inflammation and can either enhance or contribute to the resolution of inflammatory lesions. While T helper cells seem to increase and sustain chronic inflammation, Tregs display an important factor in the resolution process.

## Role of sphingolipids in treg development and function

Early study showed that the adoptive transfer of Tregs into immune-deficient mice with Th1 and Th2 induced colitis leads to

resolution of inflammation and reappearance of normal intestinal architecture (Table 1) (Mottet et al., 2003). Nowadays, we know that Tregs promote macrophage efferocytosis by production and release of IL13 that activates IL10 production in macrophages (Proto et al., 2018). IL10 release enhances efferocytosis and immunosuppression but can also promote the M1 to M2 macrophage phenotype class switch. As a hallmark of Treg cells the transcriptional program is controlled by Foxp3 that reduces the expression of sphingomyelin synthase 1 (SMS1). This leads to an increase in ceramide level, among others C18-ceramide (Apostolidis et al., 2016). C18-ceramide interferes strongly with the SET protein (originally named inhibitor 2 of protein phosphatase 2 A) which reduces the interaction between SET and PP2A and constraints its inhibitory action on the PP2A complex (Mukhopadhyay et al., 2009). Therefore, Foxp3-mediated suppression of SMS1 results in accumulation of ceramide in Treg cells that leads to activation of the PP2A complex (Apostolidis et al., 2016). PP2A inhibits the mTORC1/AKT pathway in Tregs which is involved in the regulation of cytokine production (IL-2/IL17) and is important for the suppressive function of Treg cells (Apostolidis et al., 2016). Treg specific depletion of PP2A led to a multi-organ, lymphoproliferative autoimmune disorder that manifested in mice by the age of 10–14 weeks (Table 1) (Apostolidis et al., 2016).

Another mechanism how sphingomyelin influences Tregs is described by Hollmann et al. (Hollmann et al., 2016). They demonstrated that in acid sphingomyelinase (ASMase)-deficient mice a higher number of splenic Tregs could be observed in comparison to control mice (Table 1). ASMase is located in endo-lysosomes and catalyzes the hydrolysis of sphingomyelin to phosphorylcholine and ceramide. This enzyme has a pH optimum of 5.0. After certain stimuli ASMase translocates from the lysosome to the plasma

membrane which results in the formation of ceramide-enriched membrane platforms. In T cells ASMase is activated after stimulation of CD28 but not after CD3 stimulation (Boucher et al., 1995). In wt mice ASMase activity is higher in Tregs than in CD4<sup>+</sup>/FoxP3<sup>-</sup>/CD25<sup>-</sup> T cells upon CD28 stimulation. This leads to an increase in ceramide content and higher amount of low lipid order membranes in Tregs. In ASMase deficient Tregs (as well as to a lower amount in CD4<sup>+</sup>/FoxP3<sup>-</sup>/CD25<sup>-</sup> T cells) the percentage of low lipid order membranes is reduced, which goes along with a higher sphingomyelin/ceramide ratio, and facilitates T cell activation (Hollmann et al., 2016). In ASMase knockout Treg cells neither CD25 nor IL-10 were increased in comparison to wt Tregs but the expression of CTLA-4 (Cytotoxic T-Lymphocyte-Associated Protein 4) at the membrane is increased that competes with CD28 for the binding to B7 (CD80/CD86) and additionally removes co-stimulatory ligands from the surface of APCs by transendocytosis (Hollmann et al., 2016). Also in a clinical study it has been shown that treatment of depressive patients with the antidepressant and acid sphingomyelinase inhibitor sertraline leads to an increase in Tregs among CD4<sup>+</sup> T cells which was dependent on CD28 activation (Wiese et al., 2021).

Not only sphingomyelin but also glycosylceramide or gangliosides are important for Treg development. The UDP-glucose ceramide glucosyltransferase (UGCG) is the first enzyme in the process of glycosphingolipid biosynthesis. In IBD patients and in a DSS induced colitis mouse model UGCG activity was decreased, and knockdown of UGCG resulted in Treg decrease and CD4 effector cell increase (Table 1). In line with this, intravenous application of nanoparticles loaded with glucosylceramide (GluCer) into DSS treated mice improved colitis symptoms in these mice and enhanced Treg cells in spleen and colon (Komuro et al., 2022). These data indicate that Treg development and function depends on different sphingolipids that impact activation and differentiation of Tregs at various cell stages.

## The role of gangliosides in Th17 development

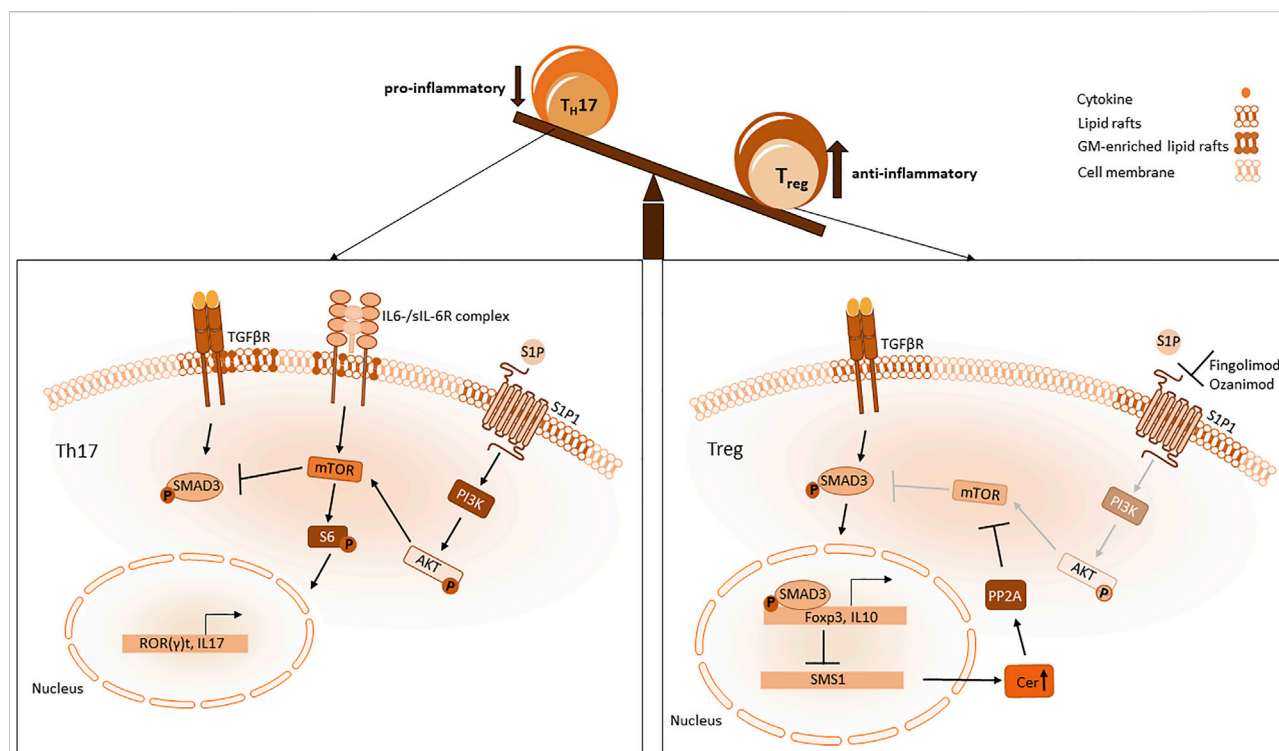
A dysbalance between Th17 and Treg cells that leans toward the pro-inflammatory Th17 phenotype is also one of the hallmarks of inflammation. To resolve the inflammatory site it should also be considered if pro-inflammatory cells could be inhibited by sphingolipids. It has already been shown that the activation of CD4 and CD8 T cells depends on different gangliosides in their membrane (Nagafuku et al., 2012). Especially during the development of Th17 cells from Th0 cells gangliosides (GMs) and LacCer are upregulated and build a specific signature of differentiated CD4<sup>+</sup> Th17 cells (Sen et al., 2021). The important role of GMs during this

differentiation process is supported by the finding that a deficiency of GM3S (GM3 synthase) attenuates the differentiation of CD4<sup>+</sup> T cells to Th17 cells (Zhu et al., 2011). As GMs are enriched in lipid rafts changes in their expression are likely responsible for alterations of lipid raft associated protein functions. The differentiation of Th17 cells is strongly dependent on the IL-6 receptor (Glycoprotein 130, GP130) and TGF- $\beta$  (Figure 4). Both receptors have been shown to be located in lipid rafts and transduce their signal via the STAT and MAPK pathway (Zuo and Chen, 2009; Allen et al., 2014). Allen et al. have shown that Th17 differentiation is dependent on lipid-raft dependent IL-6 responsiveness (Allen et al., 2014). But also TGF $\beta$  receptor signalling and endocytosis is dependent on its localisation into specific lipid rafts at the plasma membrane (Le Roy and Wrana, 2005). Future studies will show if alterations in GM can directly affect IL-6 or TGF $\beta$  signalling in Th17 cells via disrupting their association within lipid rafts.

## The impact of ceramides on TCR activity

T cell activation depends on the assembly of different proteins around the TCR in lipid rafts. Cholesterol binds directly to the TCR $\beta$  chain facilitating nanoclustering of TCRs independently of antigen binding (Molnár et al., 2012). TCR activation is also affected, when the lipid order is disturbed, for example, by abolishment of neutral SMase 2 (Börtlein et al., 2019). But also ceramides of distinct chain length are important for T cell function. As described above, six mammalian CerS are responsible for the acyl chain length of sphingolipids. CerS2 knockout mice were less sensitive against Ovalbumin-induced allergic asthma which was accompanied by an impaired Th2 response and increased Th17 response. Notably, the increased Th17 response was only visible after TCR stimulation, indicating that loss of very-long chain ceramides in CerS2 knockout mice affects TCR signalling in Th17 cells (Table 1) (Shin et al., 2021). However also CerS4, CerS5 and CerS6 affect TCR signalling, although in different ways. Inhibition of CerS6 in T cells prevents the differentiation into Th1 cells and impairs TCR signal transduction which was related to a reduced colocalization of PKC $\theta$  with CD3 at the plasma membrane (Table 1) (Sofi et al., 2017). In CerS5 knockout mice the number of CD3<sup>+</sup>/CD8<sup>+</sup> and Treg cells was reduced in the intra-epithelial lymphocyte fraction of the colon and TCR signalling was impaired (Table 1) (El-Hindi et al., 2020). Instead, depletion of CerS4 in T cells led to a constitutively and prolonged TCR activation (Table 1) (El-Hindi et al., 2022). These data indicate that the molecular composition of the membrane around the TCR is important for proper T cell activation and can be influenced by sphingolipids of various chain length.





**FIGURE 4**

Sphingolipid influence on Th17 and Treg imbalance. In a pro-inflammatory setting Th17 differentiation is mediated through mTOR which is activated by S1P1 and IL6 Receptor signalling. mTOR activates S6 phosphorylation resulting in a Th17 favouring expression pattern with ROR( $\gamma$ t) and IL-17 expression. Blockade of Smad3 activity mediated via S1P1 dependent mTOR pathway inhibits Foxp3 expression. When S1P1 is antagonized, mTOR signalling is diminished and activity of Smad3 mediated through the TGF $\beta$  pathway is sustained. Smad3 enhances Foxp3 expression, which impairs SMS1 transcription resulting in an increased level of ceramides in the cytosol. PP2A interacts with those ceramides and attenuates mTOR signalling.

## The impact of S1P on T cell migration and signalling

S1P is a bioactive lipid mediator, that is, generated ubiquitously. S1P is formed from ceramide, which is recycled from sphingomyelin by sphingomyelinases and then converted to sphingosine by ceramidases. Two isoenzymes, sphingosine kinase 1 and 2 (SphK), phosphorylate sphingosine to S1P. Both enzymes are located differently, SphK1 can be found in the cytoplasm and the cell membrane, whereas SphK2 is located mainly in mitochondria, nucleus and the ER. S1P is exported out of cells by transporters (such as spinster homolog 2 (SPNS2)) and binds to chaperones in the vascular system (Obinata and Hla, 2019). S1P is known to control many cellular processes via binding and signalling to the G-coupled receptors S1P1-5. S1P and S1P1-5 are crucial for inflammatory responses. Especially S1P<sub>1</sub> is important for lymphocyte trafficking and vascular integrity (Schwab and Cyster, 2007). A gradient of S1P is built between lymph organs, tissues and blood. High concentrations of S1P in the thymus and low concentrations in blood inhibits the egress of T cells and low concentrations in the thymus combined

with high concentrations in blood facilitate T cell trafficking out of the thymus. Maintenance of high S1P levels in blood is mediated by SphKs, while low levels of S1P in lymphoid organs is obtained by S1P lyase activity. Binding of S1P to its receptors subsequently leads to an internalisation of the receptors. Co-expression of chemokine receptors can influence S1P receptor 1 surface expression. Expression of CCR7 in T cells, for example, leads T cells into lymphnodes and deletion of CCR7 promotes T cell egress (Debes et al., 2005). Upon TCR stimulation CCR7 expression is downregulated which results in more egress of T cells into the blood. S1P1 is then internalised after S1P exposure and T cells become unresponsive to S1P gradient and migration signals. The internalisation and desensitization of S1P1 is very crucial to the S1P response, incomplete S1P1 internalization can promote Th17 dependent autoimmune neuro inflammation (Garris et al., 2013). S1P1 deletion in Th17 cells could bear a resistance to EAE, while deletion in Tregs led to autoimmunity in a MS model (Eken et al., 2017). In inflamed tissue cells respond to Interferon release with the upregulation of CD69, a membrane-bound, type II C-lectin receptor (Cibrián and Sánchez-Madrid, 2017).

CD69 binds S1P1 and stabilizes a conformation that mimics S1P1 in a ligand-bound state and induces its internalization, resulting in the trapping of naïve T cells in inflammatory tissue (Shiow et al., 2006; Bankovich et al., 2010). S1P1 also delivers an intrinsic negative feedback loop to decrease thymic production of Tregs thereby suppressing Treg activity via Akt-mTOR pathway (Liu G. et al., 2009). mTOR signalling favours Th17 differentiation and suppresses Treg functions by antagonizing important pathways for Treg activity (Liu et al., 2010). Sustained Smad3 activity is important for Foxp3 expression by TGF- $\beta$  signalling in Tregs and is antagonized by mTOR pathway activated by S1P1 (Liu et al., 2010). Activation of STAT3 induces Th17 differentiation and attenuates Treg development (Garris et al., 2013). In summary, S1P does not only impact T cell migration but also affects the development of Tregs and Th17 cells, favouring the development of Th17 (Figure 4).

## Enhancing the resolution process by pharmacological interaction with the sphingolipid pathway

As described above several enzymes of the sphingolipid pathway can influence T-cell function and differentiation and might be new targets for the pharmacological treatment of inflammation.

The repurposing of drugs that are already used for a long time in patient treatment is an auspicious approach to accelerate the approval of drugs for new treatment targets. Inhibition of ASMase by tricyclic antidepressants like amitriptyline, fluoxetine or sertraline is one of these examples (Kornhuber et al., 2008). Amitriptyline accumulates in the late lysosomes (low pH) and inhibits the translocation of ASMase from lysosomes to the plasma membrane which is subsequently inactivated by proteolytic degradation (Beckmann et al., 2014). How this leads to an increase in Tregs in human blood, as mentioned above for sertraline (Wiese et al., 2021), might be explained by the molecular mechanisms that are induced by the inhibition of ASMase. Inhibition of ASMase by amitriptyline or fluoxetine leads to an accumulation of sphingomyelin in lysosomes and Golgi membranes and ceramides in the endoplasmic reticulum. This leads to the induction of autophagy in cells (Gulbins et al., 2018). Th17 cells are highly sensitive to autophagy induction which results in a switch of gene expression with lower IL17 expression level and enhanced Foxp3 expression that converts these cells into Tregs. In accordance with that the Treg phenotype is highly dependent on autophagy and inhibition of autophagy in Tregs leads to increased production of IL17A in these cells (Park et al., 2016; González-Osuna et al., 2022). These data indicate that the induction of autophagy in Th17 cells might be

a trigger to switch the Th17 phenotype to a Treg phenotype thereby promoting anti-inflammatory mechanisms that contribute to the resolution of inflammation. Data from animal studies of cystic fibrosis as well as a clinical phase IIb study indicate that amitriptyline improves lung inflammation and function (Table 2), which might be related to the functional switch of T cells (Becker et al., 2010; Nährlich et al., 2013). These data indicate that tricyclic antidepressants might be new treatment option for chronic inflammatory diseases that are characterized by an unbalance between Th17 and Treg cells.

Although, GM3S seems to be an additional beneficial target to manipulate the equilibrium between Th17 and Tregs, unfortunately inhibition of this enzyme might have severe side effects as mutations in the ST3GAL5 gene (encodes for GM3S) are associated with severe infantile-onset of neurological symptoms such as progressive microcephaly, intellectual disability, dyskinetic movements, blindness and deafness (Inamori and Inokuchi, 2022). Gangliosides are important lipid components of the myelin sheath in the central and peripheral nervous system. So, the application of substances that inhibit GM3S should be cell type specific. As from right now, no specific GM3S inhibitor is on the market (Yamanaka et al., 2020). However, a glycosyl-ceramide synthase (GCS) inhibitor might be a useful alternative. Venglustat is an inhibitor of GCS and originally developed for the treatment of patients with lysosomal storage disease (Gaucher disease type 3, Fabry disease and GM2 Gangliosidosis, Sandhoff disease, see also clinical trials in Table 2). GCS couples the first glycoside to ceramide resulting in GlyCer, that is, the precursor for all other complex gangliosides, as well as for the GM3S. Fabry patients suffer additionally from airway inflammation. Treatment of these patients with an enzyme replacement therapy using recombinant human  $\alpha$ -galactosidase A enzyme, which partial restores the glycosphingolipid level, stabilizes obstructive lung disease and decreased Th17 cells while increase Th1 cells (Odler et al., 2017). As this was only a very small study, further data are needed. The impact of venglustat on the development of Th17 or Tregs has not been investigated in detail so far.

Furthermost progress is made in the pharmacological inhibition of the S1P signaling pathway. Fingolimod (FTY720) is a prodrug, that is, phosphorylated by Sphk2 to its active metabolite fingolimod-phosphate. It is an unselective S1P receptor agonist, with exception of S1P<sub>2</sub>, and after binding to S1P<sub>1</sub> it leads to an internalization and degradation of this receptor. Fingolimod has immunomodulatory effects and is approved for the treatment of multiple sclerosis patients since 2011 (Stepanovska and Huwiler, 2020). Although, Fingolimod shows promising data in animal studies for ulcerative colitis (UC) (Makled et al., 2022), it is not approved for this indication. Instead, Ozanimod, a selective S1P receptor

TABLE 2 Ongoing clinical trials with substances that interfere with the sphingolipid pathway.

Substance	Target	Indication	Clinical phase	Clinical trial No	Status
Amitriptyline	ASMase	Lung inflammation	II	NCT00515229	completed
Venclustat	GCS	Sandhoff disease (Late-onset GM2)	III	NCT04221451	a.n. recruiting
Venclustat	GCS	Fabry's disease	III	NCT05206773	recruiting
Venclustat	GCS	Gaucher's Disease Type III	III	NCT05280548	recruiting
Venclustat	GCS	Gaucher Disease Type 1/3	II	NCT05222906	recruiting
Venclustat	GCS	Gaucher Disease Type 1/3	II	NCT02843035	a.n. recruiting
Fingolimod, Ofatumumab, Siponimod	S1P <sub>1</sub> , S1P <sub>3</sub> , S1P <sub>4</sub> , S1P <sub>5</sub> CD20, S1P <sub>1</sub> , S1P <sub>5</sub>	Multiple sclerosis	III	NCT04926818	recruiting
Etrasimod	S1P <sub>1</sub> , S1P <sub>4</sub> , S1P <sub>5</sub>	Ulcerative colitis	II	NCT04607837	a.n. recruiting
			II	NCT05061446	recruiting
			II	NCT05287126	recruiting
			III	NCT04706793	a.n. recruiting
			III	NCT04176588	recruiting
Etrasimod	S1P <sub>1</sub> , S1P <sub>4</sub> , S1P <sub>5</sub>	Eosinophilic esophagitis	II	NCT04682639	a.n.recruiting
Etrasimod	S1P <sub>1</sub> , S1P <sub>4</sub> , S1P <sub>5</sub>	Crohn's disease	II/III	NCT04173273	recruiting
Ponesimod	S1P <sub>1</sub> , S1P <sub>4</sub> , S1P <sub>5</sub>	Multiple sclerosis	III	NCT03232073	a.n. recruiting
			II	NCT01093326	a.n. recruiting
			n.s.	NCT03500328	recruiting
			IV	NCT03535298	recruiting
Amiselimod	S1P <sub>1</sub>	Colitis	II	NCT04857112	recruiting
Ozanimod	S1P <sub>1</sub> , S1P <sub>5</sub>	Colitis	II/III	NCT05076175	recruiting
			III	NCT03915769	recruiting
Ozanimod	S1P <sub>1</sub> , S1P <sub>5</sub>	Crohn's disease	II/III	NCT05470985	not yet recruiting
			III	NCT03467958	recruiting
Ozanimod	S1P <sub>1</sub> , S1P <sub>5</sub>	Multiple sclerosis	n.s.	NCT05245344	not yet recruiting
				NCT05028634	recruiting
Siponimod	S1P <sub>1</sub> , S1P <sub>5</sub>	Multiple sclerosis	n.s.	NCT04895202	recruiting
			n.s.	NCT05376579	recruiting
			IV	NCT04792567	a.n. recruiting
			IV	NCT04925557	recruiting

ns = not specified.

a.n.recruiting = active not recruiting.

modulator with high affinity to S1P1 and S1P5 is approved for UC patients. A phase III clinical trial shows a significantly higher clinical remission in colitis patients after ozanimod treatment in comparison to placebo treatment (Sandborn et al., 2021). Other selective S1P receptor modulators are in development or used in clinical trials like Etrasimod (selective modulator of S1PR1,4,5, phase III clinical trial in UC patients), Amiselimod (high affinity to S1PR1, clinical trials in Crohn's disease (CD)), Ponesimod (selective modulator of S1PR1,4,5 tested in clinical trial phase II for psoriasis) (Pérez-Jeldres et al., 2021) (Table 2). FTY720-p, siponimod, etrasimod, ponesimod, ozanimod, and amiselimod-p bind to the orthosteric site of S1P<sub>1</sub> and S1P<sub>5</sub>.

They display similar receptor pharmacology and compete for binding at the same site. Therefore, they can be considered interchangeable with one another (Selkirk et al., 2022). Furthermore, current clinical trials compare the safety and efficacy of combination therapy combining fingolimod, siponimod and ofatumumab (anti-CD20 antibody) in pediatric patients with multiple sclerosis (Table 2). A very comprehensive overview about S1PR modulators in clinical studies is given in (Stepanovska and Huwiler 2020).

These data indicate, that the pharmacological interaction with S1P signaling to influence T cell migration or function is a promising approach to treat inflammatory conditions.

## Conclusion

The resolution of inflammation gained a lot of attention in the past few years as an active process of re-establishing tissue-homeostasis and resolving inflammatory processes. Tregs regulate macrophage responses, immunosuppressive cytokine release and restoration of tissue homeostasis whereas pro-inflammatory Th1/2/17 cells promote inflammation. In this review we highlighted how sphingolipids can interfere with the differentiation and activation process of T cells. A particular sphingolipid composition of the membrane seems to be required for the different subsets of T cells and the formation of variable immunological synapses. For this reason sphingolipids influence diverse chronic inflammatory diseases that are driven by Th1 or Th2 cell response, and an imbalance between Th17 and Tregs such as in rheumatoid arthritis (Tsukuda et al., 2012) or IBD patients (Komuro et al., 2022) and are important for the resolution of inflammation. So, in future studies enzymes of the sphingolipid pathway as well as sphingolipids themselves should be considered as new possible targets to boost resolving processes and to dampen pro-inflammatory mechanisms. Ongoing clinical trials with various sphingolipid modulators might shed light on the utility of these substances in different inflammatory diseases. However, to proof if anti-inflammatory effects are mediated by the impact of these substances on T cells, future studies should also investigate the T cell subtypes in blood after treatment of patients with sphingolipid modulators. Additionally, investigating the effect of sphingolipid inhibitors on primary human blood cells *in vitro* using lipidomics, transcriptomics and characterizing released cytokines by cytometric bead array assay (CBA), might give a hint how these substances influence T cell

subtypes and which signaling pathways are involved in the resolution of inflammation.

## Author contributions

JH and SG structured and wrote the paper, JH and NM conceived and designed the figures.

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## Conflict of interest

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