Cellular Physiology and Biochemistry

Cell Physiol Biochem 2014;34:27-44
DOI: 10.1159/000362982
Published online: June 16, 2014
Accepted: May 01, 2014

Karger Open access

1421-9778/14/0341-0027$39.50/0

Review

Sphingosine-1-Phosphate Modulates Dendritic Cell Function: Focus on Non-Migratory Effects *in Vitro* and *in Vivo*

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Key Words
Sphingosine-1-phosphate • Dendritic cells • Fingolimod • IL-12 • Inflammation

Abstract
Dendritic cells (DCs) are the cutting edge in innate and adaptive immunity. The major functions of these antigen-presenting cells are the capture, endosomal processing and presentation of antigens, providing them an exclusive ability to provoke adaptive immune responses and to induce and control tolerance. Immature DCs capture and process antigens, migrate towards secondary lymphoid organs where they present antigens to naive T cells in a well-synchronized sequence of procedures referred to as maturation. Indeed, recent research indicated that sphingolipids are modulators of essential steps in DC homeostasis. It has been recognized that sphingolipids not only modulate the development of DC subtypes from precursor cells but also influence functional activities of DCs such as antigen capture, and cytokine profiling. Thus, it is not astonishing that sphingolipids and sphingolipid metabolism play a substantial role in inflammatory diseases that are modulated by DCs. Here we highlight the function of sphingosine 1-phosphate (S1P) on DC homeostasis and the role of S1P and S1P metabolism in inflammatory diseases.

Introduction

With a major focus on lymphocyte migration, immune modulation by sphingolipids has recently entered the clinics with the approval of the S1P analog fingolimod (Gilenya®, Novartis) for the treatment of relapsing multiple sclerosis [1]. In parallel to extensive investigations into the role of the most prominent sphingolipid family member S1P for lymphocyte migration, a considerable number of investigations analyzed the migratory effects of S1P for dendritic cells (DCs) *in vitro* [2] and *in vivo* [3]. Many of these studies have

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been reviewed by others and by us [4-6]. In contrast to its lymphocyte sequestration effect, interestingly, the very first studies on the role of sphingolipids for DCs already discovered a direct cytokine regulating and maturity modulating influence of S1P [7].

DCs are important in chronic inflammation (autoimmunity / auto-inflammation) and cancer immunology [8-11]. They represent an essential link between innate and adaptive immune system. More effectively than monocytes, macrophages or B lymphocytes their prime task is to take up and process antigens in order to activate $CD4^+$ and $CD8^+$ T lymphocytes. Equipped with the most diverse set of membrane and cytosolic pattern recognition receptors, DCs comprise the capacity to translate microbial, fungal or viral attacks to a specific adaptive Th1, Th2 or Th17 response. In most cases however, in a direct contact with regulatory T cells (Treg) they advance to a regulatory phenotype and abrogate potentially destructive immune reactions. Cancer cells have developed strategies to exploit these latter properties by both, membrane-associated and secretory suppressive factors that finally turn DCs - in addition to M2 macrophages - into a major tumor promoting cell population [12]. Consequently, one of the most promising applications of DCs as cell therapeutic principle is based on adjuvant- and co-stimulation-dependent reversion of tumor tolerogenic DCs into anti-cancerogenically active DCs [13, 14].

To review the modulating influence of sphingolipids on DCs properly another level of complexity regarding DC physiology needs to be considered, i.e. their ontogeny and functional diversity. Only recently the progeny of "true" DCs different from monocytes and macrophages was resolved [8, 10, 11, 15, 16]. Now it appears clear that deriving from pre-pro DCs, specific transcription factors in conjunction with external signals are decisive for the development of different types of immature DCs, conventional DCs (cDCs), plasmacytoid DCs (pDCs) and Langerhans cells (LCs).

On the background of the complex and rapidly evolving characteristics of DC biology, herein we aim to describe the DC-directed immune modulating influence of S1P and S1P analogs as well as of the S1P-generating/metabolizing enzymes, in terms of maturation, differentiation and expansion of DCs. Additionally, we will present published and in part unpublished evidence for a more direct potential of S1P to modify toll-like receptor (TLR)-stimulated DC cytokine profiles and subsequent results of *in vivo* studies related to a direct modification of DC biology.

S1P and fingolimod metabolism in dendritic cells

S1P enzyme expression profiles

The gradient of S1P levels between thymus tissue or secondary lymphatic organs (SLO) and blood stream is generated by the expression profile of S1P-generating and metabolizing enzymes. Especially S1P lyase, which is irreversibly degrading S1P forming hexadecenal and phospho-ethanolamine, is a major regulator of this S1P balance. The enzymes' activity in endothelial cells, which is especially high in endothelial venules in SLO [17], reduces lymphatic tissue S1P concentration to a low nanomolar range. However, some cell types do not express S1P lyase and in combination with the presence of the S1P-generating enzymes sphingosine kinase 1 and 2 (SphK1/2) and the outward transport of S1P, high serum concentrations of S1P are usual. A debate is still ongoing, to which extent erythrocytes [18], platelets [19, 20] or other cell types [21] contribute to this high serum concentrations of S1P of about 0.1-1 µM [22, 23]. Even less is known about the sphingolipid enzyme equipment of immune cells and their capacity to elevate or reduce extracellular or intracellular S1P levels [6].

To address the diverse effects of S1P metabolism on both, development of DCs from bone marrow precursors and on direct inflammatory activation of DCs, it is essential to determine and be aware of the expression profile and distribution of S1P receptors, the sphingolipid enzyme profile, subcellular compartment distribution and the S1P import and export systems. At least partially, the distribution of the S1P receptors 1-5 in different types of DCs, such as cDCs [24], lung DCs [25] and LCs [2] albeit not pDCs, has been investigated

and collectively described in recent reviews [6, 26]. However, with the exception of SphK1, no consistent information is available regarding the expression profile and regulation of other sphingolipid enzymes, i.e. SphK2, S1P phosphatases 1/2 (SPP1/2) and the S1P lyase (sgpl1) in DCs. Given that these enzymes are not randomly distributed within DCs, it is also of great importance to understand, how sphingolipid enzyme substrates and products accumulate in subcellular compartments. Moreover, both, transport mechanisms across the plasma membrane to the extracellular space and facultative nuclear import transporters will affect the distribution of sphingolipids within DCs [27-29].

Distribution of S1P and fingolimod within immune cells

In our studies targeting the role of S1P in DCs we started to develop highly sensitive methods of measuring subcellular compartment-specific concentrations of sphingosine, dihydro-S1P (dhS1P), and of its partial agonist FTY720 (fingolimod) and FTY720-phosphate [30].

The sphingosine-analog FTY720 has been approved for the oral treatment of relapsing multiple sclerosis by the FDA. After the lipophilic FTY720 has entered the cell it is phosphorylated by SphK2 and following cellular export, mimics the effects of S1P on S1P receptors 1, 3, 4, and 5. Due to insufficient information about disposition of FTY720 and FTY720-P we analyzed the membrane penetration and the subcellular distribution of these compounds in splenocytes. For this purpose we used a newly developed method for their quantification utilizing LC-MS/MS [30]. These experiments included a liquid-liquid extraction and injection of the extracted samples into a liquid chromatographic system operating with a tandem mass spectrometer. The transitions per drug were analyzed with a recovery of about 80%. The limitations of this assay were 0.875 ng/ml and 2 ng/ml for FTY720 and FTY720-P. C-17-sphingosine and C-17-sphingosine-1-phosphate served as internal standards. Our measurements clearly indicated a dramatic and compartment-specific accumulation of both substances inside immune cells. Even considering the limitations of such a cell culture system, these findings raise concerns regarding the toxicity of fingolimod *in vivo*. Given the need for a prolonged use of fingolimod for the treatment of multiple sclerosis, these results may influence the discussion about the benefit/risk ratio of the drug. As will be lined out below, immune biological effects are not restricted to partial S1P receptor agonism of phosphorylated fingolimod. Recently, elegant studies proved that lipophilic FTY720 at nanomolar concentrations is indirectly binding to protein phosphatase 2A (PP2A) and affects immune and cancer cell signaling in addition to its interference with S1P signaling [31]. While these non-migratory effects of fingolimod are increasingly acknowledged, it may be challenging to separate intracellular S1P [32] from non-phosphorylated FTY720 effects [31].

Functional enzyme activity assays to address metabolism of S1P and fingolimod

Because amphiphilic metabolites of the sphingolipid family and its analogs are not freely moving across plasma membrane and subcellular compartments, their differential distribution is highly dependent on the subcellular localization and activity of SphKs, S1P phosphatases and the S1P lyase. To further understand the regulation of sphingolipid flow through the cell, the measurement of enzyme mRNA and protein (if possible at all), is not sufficient. Additionally, given the low frequency of blood or splenic DCs we started to optimize functional enzyme activity assays for SphK1 and SphK2 as well as S1P lyase [33-, 35]. In a first approach, we analyzed plain spleen cell suspensions, GM-CSF-/Flt3L-expanded, CD11c-enriched bone marrow cell preparations or DC/LC cell lines. Therefore, we tested two different sphingosine kinase activity assays that either employed a fluorescently labeled sphingosine (NBD-Sph) as substrate or a ^{32}P-labeled gamma-ATP as co-substrate for the kinases. Extensive analysis of the fluorescence-based assay exhibited the methodological problem that the fluorescent dye labeling is “leaky” making the results hard to validate. As shown by others the radioactive-based assay system worked successfully and first data suggest that the reduction of the Th1 inducing capacity of SphK2-/- DCs may be dependent

on an enhanced activity of SphK1 as it was investigated for LPS-stimulated splenocytes [34].

Apart from S1P phosphatases 1 and 2 the final enzymatic degradation of S1P by the S1P lyase will define the intracellular and, following an export via specific transporters, the extracellular concentration of S1P. Therefore, and to elucidate its role in physiological and pathological processes of DC homeostasis, a S1P lyase activity assay based on the determination of the S1P degradation product hexadecenal was established [35]. To this end, a high sensitivity such as mass spectrometry is required because only low levels of hexadecenal exist in biological samples such as serum, plasma and cells. However, hexadecenal is not simply ionisable which is elementary for detection by mass spectrometry via electrospray ionization. Thus, derivatisation of hexadecenal with 2-diphenylacetyl-1,3-indandione-1-hydrazone (DAIH) is an elegant method leading to the formation of an imine structure with good property characteristics for mass spectrometry. An outstanding limit of detection (LOD) of 1 fmol hexadecenal per sample can be achieved with this novel method. To implement this method for the measurement of the S1P cleavage product in biological matrices, the amount of hexadecenal in plasma was examined for the first time indicating that 2.4 nM of the aldehyde is present in this natural sample. Moreover, S1P lyase activity was modulated in cells by the use of 4-deoxypyridoxine, which is a competitive inhibitor of pyridoxal-5-phosphate, an essential cofactor of this enzyme. Mass spectrometry analysis after DAIH derivatisation revealed a significant decrease of hexadecenal compared to untreated cells, which allows the detection of S1P lyase activity also in DCs in response to immune modulatory stimuli [35].

S1P and analogs modify development of DC subtypes from common precursors

In the bone marrow the initiation of DC development can be traced back to a very early point of separation from monocyte and macrophage myeloid lineage [8, 10, 11, 15, 16]. Only very recently sophisticated studies employing single cell cloning and transcription factor tracing confirmed that common dendritic cell precursors (CPD) give rise to both, pDC and pre-cDC due to specific activity of Zbtb46, Batf3 and other transcription factors [15, 36]. Still a matter of debate, GM-CSF-expansion of myeloid-dendritic precursors may also drive development of cells with DC-like properties that might be indistinguishable from Zbtb46 pre-DCs [11, 37].

Due to their novelty, there are no studies available that analyze the role of S1P for DC development based on the new concept of their progeny. Encompassing the earliest experimental work on sphingolipids and DCs, only very few published studies evaluate the influence of S1P, FTY720 or ceramides on the differentiation of murine or human DC types in the expansion phase [38]. Supplemented by our own studies with a GM-CSF-based expansion protocol of 'cDC-like' cells from murine bone marrow (Fig. 1), in this section we will delineate the sparse knowledge about the phenotypical modifications of S1P on immature DCs and on their subsequent reaction following TLR stimulation (Fig. 2).

Because S1P functions as an important regulator of cell proliferation and survival during inflammatory conditions [39], it is potentially able to influence DC development. As shown by Martino et al., GM-CSF, IL-4 and high concentrations of S1P led to the differentiation of a monocyte-derived DC population lacking CD1a molecules on the surface [40]. Those cells were not able to produce IL-12p70 after TLR4 stimulation with LPS. However, they produced high amounts of TNFα and IL-10. Accordingly, these cells were, in comparison to $CD1a^+$-DC generated from S1P-untreated monocytes, not capable of stimulating allogeneic T lymphocytes. Similarly, Idzko et al. demonstrated that presence of S1P during stimulation of human peripheral blood mononuclear cells (PBMCs) with LPS led to decreased concentrations of IL-12p70 in the supernatants compared to the controls stimulated solely with LPS [7]. Comparably, Renkl et al. demonstrated that S1P treatment of maturing DC limited their capacity to initiate Th1 responses [41].

In own studies focusing on two major DC-secreted cytokines, IL-12p70 and IL-23, which are interesting to us, because they share one common protein subunit p40 but play

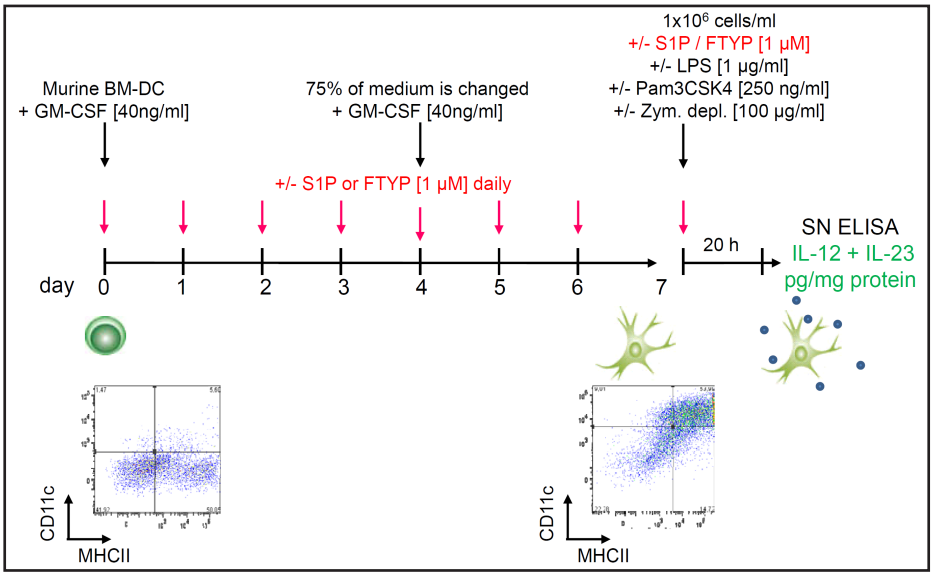

Fig. 1. Experimental protocol to analyze the role of sphingolipids for the development of murine bone marrow-derived DCs (BM-DCs) and the subsequent influence on TLR-dependent DC activation; SN=supernatant.

an opposite role in chronic inflammation and tumor development, we obtained a similar reduction on IL-12p70 secretion upon TLR4 stimulation [34]. More importantly, our data revealed that S1P had differential effects on developing DCs depending on its absence or presence during early maturation (Fig. 1, 2). When S1P was present during the early differentiation phase as well as during stimulation with LPS/TLR4, decreased IL-12p70 concentrations were detectable in the supernatants. However, when S1P was only present during the differentiation phase of the DCs but not at the time of TLR stimulation, no decrease of IL-12p70 was detected. Interestingly, we could show that addition of FTY720-P instead of S1P during early differentiation of DCs led to a smaller decrease of IL-12p70 production during the following stimulation with S1P and LPS. Since FTY720-P was shown to lead to an internalization and degradation of $S1PR_1$ [42, 43], we suggest that in those cells less $S1PR_1$ was present to mediate a S1P-dependent effect on DC maturation. Zeng et al. postulated a FTY720-induced anergic phenotype switch of DCs, especially upon endotoxin activation [35]. Besides impaired phagocytotic, endocytotic and specific antigen presentation abilities observed in the FTY720-treated bone marrow-derived DCs (BM-DCs), they found a down-regulation of pro-inflammatory cytokines such as IL-6, TNF-α, IL-12p70 and MCP-1 in LPS-activated mature cells.

In additional studies, we further confirmed that the S1P axis plays a major role in DC maturation. Stimulation of Flt3-L-differentiated DCs or splenocytes deficient for SphK1 ($SphK1^{-/-}$), the major S1P-producing enzyme, increased maturation of DCs as observed by an enhanced production of IL-12p70 [34]. Interestingly, addition of either S1P or $S1PR_1$-specific analogs to the supernatants of the cells abolished the increase of IL-12p70 production in SphK1-deficient DCs. In addition, we found that the $S1PR_1$-specific ligand CYM5442 reduced IL-12p70 production [34]. In line with these data, Franchi et al. showed that inhibition of sphingosine kinases during LPS-stimulation caused extensive accumulation of ceramide, the precursor of S1P, and a related increase of death rate of the respective cells [44].

These modulating effects of S1P are not restricted to LPS/TLR4 signaling. In own studies, permanently increased S1P concentrations present during the differentiation/expansion phase of DCs caused increased IL-23 production following to stimulation with the TLR-2/1 agonist Pam3CSK4 (Fig. 2). Likewise, decreased IL-23 levels were measured within BM-DCs cultures of $SphK1^{-/-}$ cells treated with FTY720-P during differentiation. Thus, the observed increase of IL-23 releasing potential was also dependent on SphK1. Interestingly, this S1P-dependent differentiation of 'IL-23-DCs' seemed to be limited to TLR-2 signaling since dectin-1 stimulation showed no sensitivity towards S1P pre-treatment.

It appears that S1P is a major modulator of both, DC maturation and DC activation. With respect to long-term modulation of inflammatory processes and subsequent cancerogenesis,

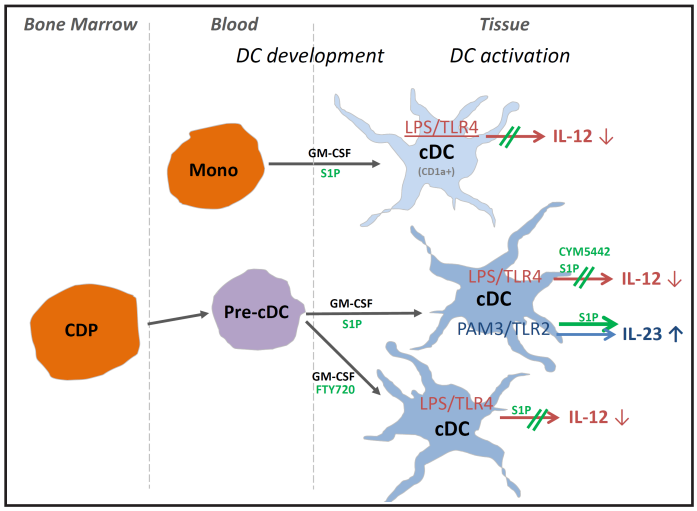

Fig. 2. S1P affects DCs differentially, dependent on its continuous presence during expansion phase of DC precursors to immature DCs versus its direct interference with DC cytokine expression during TLR-dependent activation. Unlike FTY720, S1P, present during BM-DC expansion, S1P presence favored development of TLR2-elicitable "IL-23-DCs", whereas following LPS/TLR4 stimulation it reduces IL-12p70 release. FTY720 present during maturation, decreased the effects of S1P on IL-12 reduction.

the considerable early influence of S1P during differentiation on the subsequent cytokine secretion profile of DCs is especially interesting. While our data warrant confirmation, in the setting of BM-DCs expansion recent findings regarding the interplay of IL-6, SphK1 and $S1PR_1$ in inflammation-dependent cancerogenesis may support our findings [45]. Like IL-6, IL-23 is activating the STAT3 signaling pathway, which according to Lee et al. stabilized phospho-STAT3 due to a reduction of SOCS3 activity by the S1P-$S1PR_1$ axis [45].

Taken together, and in line with earlier observations of the Goetzl group [46, 47], unlike FTY720-P, S1P might be able to mediate a development of DCs towards a cell population augmenting a Th17 response and rather attenuating a Th1 immune response.

S1P directly modulates functional activity of dendritic cells

S1P influences antigen uptake and presentation by dendritic cells

One of the events starting the specific function of DCs after an infectious challenge is triggering of several TLRs leading to cytokine and chemokine secretion, chemokine receptor expression and migration [48]. At the same time, and bridging their function to the adaptive immune response, DCs are the most potent antigen uptake and presenting cells with the ability to activate T and B cells. Therefore, it is of interest to evaluate whether sphingolipids possess an influence on the ability to capture antigens [27].

In an immature stage, DCs are able to take up antigens via at least three different mechanisms. As professional antigen presenting cells (APCs), they are able to capture antigens by receptor-mediated endocytosis as they possess distinct surface receptors such as C-type lectin receptors. A second receptor mediated process is phagocytosis, involving the vesicular internalization of solids such as bacteria. Finally, an efficient uptake of soluble antigens can also be mediated via macropinocytosis, which includes the arrangement of endocytotic vesicles formed by a cytoskeleton rearrangement and fusion of distinct regions of the cell surface. This mechanism allows screening of a large volume of extracellular fluid for antigens. Our experiments indicated that at least the process of macropinocytosis is modulated by the sphingolipid S1P [27].

In particular, S1P effectively diminishes the ability of DCs to capture antigens via macropinocytosis. It has been well established that macropinocytosis is mediated by a modulation of the PI3K activity, which regulates the Rho-family GTPases, including Rac1 and Cdc42, for actin cytoskeletal rearrangements. Indeed, S1P is able to reduce PI3K activity of DCs in a dose-dependent manner. Moreover, it is of interest that the inhibitory effect of S1P on macropinocytosis and Akt activity is mediated by the S1P-receptor subtype $S1PR_2$. This has been clearly indicated by pharmacological approaches and abrogation of this receptor subtype in DCs. In this manner it is of interest that down-regulation of $S1PR_2$ is not only connected with a decreased S1P-mediated action but also improves the basal endocytotic

capacity of the antigen presenting cells suggesting an autocrine modulation of endocytosis by S1P.

Thus, presumably DCs continuously produce and release S1P to the extracellular environment, thereby regulating DC homeostasis such as antigen uptake. The question remains how intracellular formed S1P is secreted from DCs to act in an autocrine manner. Although some clearly arranged reviews about S1P transporters in general have been published [49, 50], knowledge about S1P transport in immune cells, particularly in DCs is rather scarce. The transporter ABCA1 has been identified to be crucial for S1P transport in astrocytes [51], platelets and erythrocytes [52]. The spinster homolog 2 (spns2) is predicted to be required for S1P signaling in zebrafish and inhibition of the transporter in CHO cells led to an augmented release of S1P [53, 54]. However, a recent publication defining the role of endothelial spns2 for S1P levels in the vascular system, failed to detect this specific transporter in myeloid cells [28]. Interestingly, it seems likely that the ABC transporter ABCC1, which plays a crucial role in the translocation of S1P in mast cells and fibroblasts [55, 56] is crucial for DC function as well. This transporter, and also ABCC4 and ABCB1, has been described to be essential for migration and differentiation of DCs [57] indicating that specifically S1P transportation possesses a fundamental role in DCs to fulfill important physiological functions.

In addition to our study lined out above, Ocaña-Morgner et al. demonstrated that SWAP-70, a molecule localized at DC membranes that promotes surface localization of peptide-loaded MHCII, is important for S1P-induced endocytosis and migration [58]. SWAP-70-deficient BM-DCs are not capable to activate RhoA in response to S1P treatment. In wild type mice, RhoA is activated by $S1PR_3$ after association with $G\alpha_i$ and $G\alpha_{12/13}$. Three signals are delivered from the DCs to activate T cells: signal 1 is the presentation of e.g. MHCII peptides on the cell surface, which can also occur after *in vitro* treatment with LPS [59]. The second signal is the expression of co-stimulatory molecules CD40, CD80 and CD86, thus, shifting DCs from the immature into the mature status. Since we work mainly on GM-CSF-differentiated BM-DCs our own data provide an elevation of these molecular markers after the differentiation phase [33]. In particular, expression of MHCII was induced up to 60%, CD86 raised to 42% and the marker for cDCs, CD11c, to 20% after GM-CSF-treatment. Finally, the signal 3 implies the secretion of inflammatory cytokines to promote priming and differentiation of T cells [60] as for instance, the cytokine IL-12p70 is responsible for the differentiation of $CD4^+$ and $CD8^+$ T cells into Th1 effector cells as mentioned above.

Modulation of TLR-dependent cytokine profiles

Distinct TLRs coupled by different adaptors to downstream signaling components lead to a separate induction of the subunits IL-12p40 and IL-12p35, which in combination built IL-12p70. However, as mentioned before, IL-23 also shares the p40 subunit. Others and we recently demonstrated that SphK1-delivered extracellular S1P inhibits the production of IL-12p70 in DCs via $S1PR_1$ [34, 61]. In order to determine whether SphK1 and its product S1P are pro- or anti-inflammatory, in our experimental setting we used spleen cells and Flt3-ligand-differentiated BM-DCs of WT and $SphK1^{-/-}$ mice to determine cytokine profiles [34]. We showed that the absence of SphK1 augmented the LPS-induced IL-12p70 but none of the other inflammatory cytokines such as IL-6 or IL-10. In detail, we measured both subunits of the IL-12p70 on mRNA level, the IL-12p35 and IL-12p40 and could observe that IL-12p35 concentration as the limiting factor for IL-12p70 expression was augmented in $SphK1^{-/-}$ DCs compared to WT DCs after treatment with LPS. This TLR4 ligand enhanced also the SphK1 expression and the total SphK activity in WT cells. Importantly, we did not observe a compensatory elevation of SphK2 mRNA in SphK1-deficient cells. To further address the S1P receptor specificity, we confirmed that IL-12p70 release in SphK1-deficient DCs was reduced, when we supplemented S1P exogenously. $S1PR_1$-specific antagonists, SEW2871 and CYM5442, alleviated these S1P effects again. Further confirming that G(i)-coupled signaling downstream of $S1PR_1$ is important for the selective regulation of IL-12p70 production, pertussis-toxin abolished the S1P effects [34]. Thus, this rapid IL-12p70-negative feedback

loop in inflamed DCs is dependent on the simultaneous SphK1-$S1PR_1$ activation and favors adaptive Th17 differentiation.

While Schaper et al. confirmed our findings that S1P decreased IL-12p70 production; disparately they determined a reduced expression of IL-23 after LPS-induced exogenous S1P-pre-treatment of BM-DCs and in a co-culture system of BM-DCs with keratinocytes. Also different from our studies, they attributed this combined down-regulation to an effect of S1P on the shared subunit p40 [61]. Moreover, DCs treated with LPS and S1P showed an augmented IL-27 release. Mechanistically this S1P effect was dependent on ERK signaling, as shown by specific stress kinase inhibitors. The differences regarding S1P effects on BM-DC cytokine release are possibly due to disparate procedures of cell generation. While in our system we expanded BM-DCs with Flt3-ligand or with 40 ng/ml of GM-CSF for a period of seven days, in the above mentioned work the investigators used 20 ng/ml GM-CSF for 9 days of cultivation. On day 3 of differentiation, in both protocols the medium was changed and fresh Flt3-ligand or GM-CSF was added. Additionally and thus different from our protocol, Schaper et al. performed an accessory change of medium on day 6 and 8. Either way, similar to our own studies, the modulation of cytokines by extracellular S1P was suggested to be $S1PR_1$-dependent as indicated by specific receptor agonists. According to these authors, the study implies a more general anti-inflammatory DC function by the action of extracellular S1P that might prevent diseases like atopic dermatitis or psoriasis.

In addition to our previous publication [34], showing an increase of IL-12p70 protein release in SphK1-deficient splenocytes, the secretion of IL-12p70 was significantly diminished in $SphK2^{-/-}$ splenocytes and BM-DCs after LPS treatment compared to WT cells [34]. These results suggest that, in contrast to SphK1-derived S1P, SphK2-delivered S1P may enhance inflammation towards a Th1-response via augmented IL-12p70 excretion. Interestingly, TGFβ as a well-known activator for SphK1 [2, 5, 62] and furthermore PGE2 reduced IL-12p70 even in SphK1-deficient splenocytes. While these findings need confirmation they indicate that TGFβ and PGE2 may affect the signaling pathways towards IL-12 independent of SphK1. Moreover, SphK2 may not be involved in their activities, because the expression level of SphK2 was not changed in SphK1-deficient cells implying that both enzymes do not compensate for each other (also see [33, 34]). In a lung injury model, SphK1 has been reported to be rapidly up-regulated within 6 hours following LPS treatment, whereas SphK2 increased gradually over 24 hours [63]. Strongly supporting our *in vitro* observations, these authors showed that *in vivo* overexpression of SphK1 reduced and SphK2 overexpression enhanced LPS-induced lung injury. The study of Yoshimoto et al. showing that SphK2 exclusively associates with the cytoplasmic region of the IL-12Rbeta1 might offer an explanation for a positive SphK2/IL-12 interaction [64]. Knock-down of SphK2 in a Th1 cell clone reduced IL-12-induced IFN-γ production, further implying that SphK2 is positively modulating IL-12p70-inducing signal pathways. To understand the divergent roles of sphingosine kinases 1 and 2 better, specific inhibitors would be necessary especially for the analysis *in vivo*. SKI has been introduced as an unspecific inhibitor for both isoenzymes. In an earlier study, Jung et al. [65] found that this inhibitor blocked co-stimulatory molecules on DCs and decreased LPS-induced IL-12p70 production. Furthermore, SKI inhibited the IFN-γ production by T cells. Given the differential effects of SphK1 and SphK2 on these DC parameters, the data of Jung et al. suggest a more pronounced blockade of SphK2 by SKI.

It is known from the literature that during inflammation, among others, the sphingosylphosphorylcholine (SPC) accumulates inside the cells [66]. Exposure of SPC to human DCs caused a rapid intracellular calcium increase. Furthermore, the authors suggested an SPC-induced elevation of the MHCII cell surface receptor, HLA-DR, and of the co-stimulatory molecules CD86 and CD83. Importantly, SPC augmented IL-12 and IL-18 production and ameliorated the T cell priming ability of DCs. Therefore, we have to consider also SPC as a possible stimulatory factor of IL-12 increase in DC and Th1-mediated immune response. And, different from our study, Renkl et al. demonstrated that extracellular S1P reduces the IL-12 production only in human but not in mouse dendritic cells [40]. Moreover, extracellular S1P increased the IL-10 production of human DC but did not alter the murine

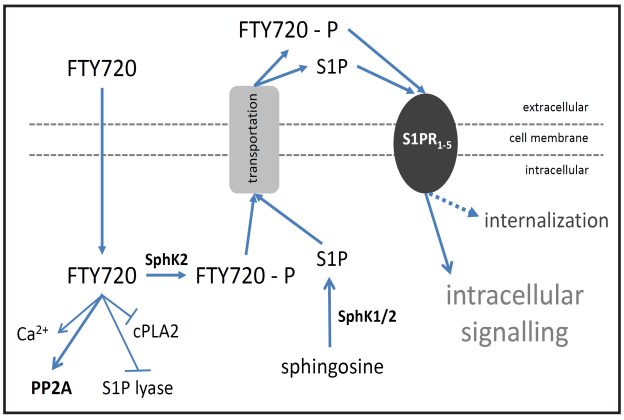

Fig. 3. Possible non-migratory effects of the S1P analog FTY720-phosphate and its parent drug FTY720 (fingolimod) in DCs and lymphocytes. Lipophilic FTY720 may pass immune cell membranes with low energy consumption and exert biologic effects e.g. specifically activating protein phosphatase-2A (PP2A; [31, 91]. However, following its phosphorylation exclusively by sphingosine kinase-2 due to its amphiphilic properties requires active transport mechanisms to exert its S1P analog functions via S1P receptors 1, 3-5 (Drawing modified from [70]).

DC cytokine release. While these data are in part controversial to later investigations (see above), nevertheless they stress possible differences of murine and human DCs in response to S1P. Another publication on human monocyte-derived DCs described a reduced IL-12 and elevated IL-10 production when cells were matured in the presence of FTY720 or FTY720-P [67]. Co-culture of T cells and FTY720 or FTY720-P-treated DCs shifted to Th2-differentiation rather than Th1- differentiation by a changed cytokine release. Therefore, S1P and FTY720 seemed to act in an opposite manner on DCs [68]. Along these lines, S1P protected tumor cells and immature DCs from natural killer (NK) cell-mediated lysis whereas FTY720 did not. The protective effect of S1P was accomplished by elevating the expression of HLA-I and HLA-E on DCs and in contrast, FTY720 blocked this expression. S1P protected tumor cells from NK cell-induced cell lysis via the $S1PR_1$ on tumor cells. To avoid the side effects of FTY720 and FTY720-P currently several derivatives of FTY720 are produced and tested. In this context, Seo et al. reported an unexpected immune-inducing function for a sphingosine analog and FTY720 derivative, AAL-R [69]. AAL-R was shown to increase TLR7-mediated DC responses via induction of MHCI, of co-stimulatory molecules and of type I IFN expression. This new established immune stimulatory action of AAL-R was dependent on type I IFN signaling and moreover was found to induce the activation of p38 MAPK to signal type I IFN production.

To further clarify the role of S1P and fingolimod on immune cell signaling we focused on the IL-33-induced IL-2 production by a T cell line [70]. These T cells are an EL4 6.1 thymoma cell line transduced with a receptor for IL-33, the ST2. The results revealed that the IL-33-induced IL-2 expression was significantly diminished by FTY720, FTY720-P and its newly synthesized analogs in a dose-dependent manner. When OVA-TCRtg spleen cells were co-incubated with LPS and FTY720, we observed an impairment of the IL-17 and IFN-γ cytokine secretion. In addition, the inhibited LPS-induced IFN-γ production by FTY720 in OVA-TCRtg spleen cells was rescued by an inhibitor for PP2A, ocadaic acid, suggesting PP2A as a target for FTY720 (Fig. 3). Thus, immune modulation towards a regulatory T cell response may prevail and has to be considered during treatment with fingolimod. While in parallel to our experiments a direct interaction of FTY720, unlike FTY720-P, with PP2A has been confirmed in tumor cells [31], our data definitely argue for further investigations into the FTY720/PP2A interaction in DCs, because FTY720 dramatically accumulates inside immune cells.

Furthermore, exposure of human DCs towards apoptotic debris failed to promote DC-induced cytotoxicity towards living tumor cells [71]. Instead, regulatory T cells, which co-expressed CD39 and CD69, were indirectly activated by S1P derived from apoptotic cells. Thus, S1P was suggested to act via $S1PR_4$ on DCs in order to induce IL-27 production which at the end suppresses the cytotoxicity towards tumor cells. In line with these findings, the new potent and selective $S1PR_4$ agonist 3-(2-(2,4-dichlorophenoxy)ethoxy)-6-methyl-2-nitropyridine was recently identified from a high-throughput screening [72]. Thus, a novel tool is provided to understand the mechanisms underlying the immune responses of DCs via $S1PR_4$. From studies on $S1PR_4$-lacking mice it is already known that DCs reduce their

capacity to induce Th17-differentiation [73]. Interestingly, the deficiency does not affect lymphocyte numbers or the structure of lymphocyte organs, but elevated the Th2-mediated immune responses *in vivo*. Of importance, in the $S1PR_4$-deficient mice a reduced pathology was detected in a murine model of dextran sulfate sodium-induced colitis.

Summarizing the *in vitro* data, S1P and FTY720 are crucially albeit differentially involved in DC activity and subsequent T lymphocyte differentiation. Assembling the limited knowledge about the role of continuously present extracellular S1P it may be assumed that DCs will support an IL-6, IL-23, STAT3-dependent Th17 inflammatory profile, while at least in part Th1 is attenuated (e.g. IL-12p70). Conversely, at least in the majority of the studies, FTY720-P is reducing Th17- and Th2-dependent reactivity. Further studies are warranted into the differential effects of FTY720 and FTY720-P regarding Th1 autoimmunity given the distinct effects of FTY720 on PP2A. Collectively though, these *in vitro* findings of non-migratory S1P effects on DCs fit quite seamlessly to *in vivo* studies in inflammation (see below) and moreover in cancerogenesis [45].

Role of S1P for DCs *in vivo*: inflammation

Early evidence of non-migratory therapeutic effects of S1P and analogs from animal models

Both, in human chronic inflammatory disease and in animal models of inflammation the prominent role of DCs is well established. Thus, when transferring the information gathered from *in vitro* studies of the sphingolipid influence on DCs to the *in vivo* pathophysiology, one would expect a possibly subtle but significant modulation by S1P-mimicking or -blocking compounds.

Indeed, already very early on when the major therapeutic activity of FTY720 was solely attributed to its $S1PR_1$-dependent lymphocyte sequestration effect, others and we raised initial evidence for direct modification of the DC immune profile by FTY720 [25, 74, 75].

In the OVA-asthma model Idzko et al. observed that local treatment with FTY720 reduced this Th2-type pathology by reducing the number of lung DCs reaching the mediastinal lymph nodes [7, 25]. Of note, and in line with our findings in an ulcerative colitis model (see below [74]) they described an additional direct inhibitory effect of FTY720 on the activation of naïve T cells, their differentiation to asthma-driving Th2 cells and especially their capacity to release IL-4, -5 and -13 [25]. Along similar lines several recent publications of Marsolais et al. worked out a concept of local therapeutic principles of $S1PR_1$ agonists in viral or other lung diseases that is independent of systemic lymphopenia [76-78]. They also suggested a reduced DC secretory activity locally and reduced migration of DCs to the lung draining lymph nodes with subsequent beneficial effects for lung inflammation.

Two former studies strongly augmented our interest in non-migratory therapeutic activities of sphingolipids and their analogs. First, we studied the effect of FTY720 in Th2-mediated, oxazolone-induced colitis model in mice [74]. Above the finding that treatment with FTY720 prominently reduced the clinical and histopathologic severity of ulcerative colitis in this model, surprisingly, we found that the effects of FTY720 were accompanied by a significant reduction of the key effector Th2 cytokines IL-13, IL-4 and IL-5. Strikingly, FTY720 inhibited GATA3 and IL-33 receptor ST2 expression [74]. These data could not be explained by alterations of lymphocyte migration and were confirmed by *ex vivo* experiments. Secondly, we investigated into non-migratory activities of FTY720 in the Th1-mediated TNBS colitis model [75]. The therapeutic effects of FTY720 were associated with a down-regulation of the classical DC cytokine IL-12p70 and a subsequent reduction of Th1 cytokines. In addition to these DC targeted effects of FTY720 we also raised evidence that FTY720 treatment possibly enhanced Treg activity by showing an up-regulation of FoxP3, IL-10, TGFβ, and CTLA4 [75].

As lined out above, Schulze et al. described a distinct role of $S1PR_4$ expressed by DCs on differential T lymphocyte response that did not affect T cell proliferation but reduced IL-17 production. Correspondingly, this group applied several classical *in vivo* models, such as contact hypersensitivity and DT hypersensitivity typically driven by Th1- or Th2-directed immune activity [73]. In the Th2 FITC-hypersensitivity model applied to $S1PR_4$-deficient

animals they observed an enhanced ear swelling after a challenge at 24 and 48 h. Conversely, the classic Th1-dependent DT hypersensitivity response using a hapten footpad injection was stronger in WT than in $S1PR_4$-deficient mice. In a third Th2 *in vivo* model, allergen-evoked levels of cytokines were increased in both, WT and $S1PR_4$-deficient bronchial alveolar lavage cells. However, selectively IL-17 levels were reduced in the $S1PR_4$-deficient mice. To resolve these mixed *in vivo* findings, i.e. less inflammation in Th1- and enhanced inflammation in Th2 models, the investigators directly analyzed cytokine secretion of LPS treated BM-DCs *in vitro*. They found no alteration of IL-12p70 but a lower expression of IL-6 in $S1PR_4$-deficient BM-DCs. Taken together, the mixed results in various Th2 and Th1 *in vivo* models may be based upon a diminished IL-6 production of $S1PR_4$-deficient DCs with a subsequent mitigation of Th17 lymphocyte development that favored a Th2 pathology. On the other hand, at least in *in vivo* models the $S1PR_4$-deficiency reduced a synergism of Th17- and Th1-driven pathology [73]. This data deliver a strong argument for non-migratory immune deviating effects of S1P that are dependent on a divergent regulation of DC cytokine profiles by $S1PR_1$ versus $S1PR_4$ signaling. While these studies are not controlled for compensatory S1P action via other S1P receptors (see below), they warrant further investigations specifically into the role of $S1PR_4$ on DCs.

S1P and dendritic cells in inflammatory skin diseases

The most prominent antigen presenting cells in the skin are LCs, a subset of DCs that reside in the epidermis and modulate immune responses. They pick up antigens that have penetrated into the skin and transfer this information to the next lymph nodes. LCs exhibit the distinct function of endocytosis and are phenotypically distinguished by the presence of the intracellular organelles named Birbeck granules. In addition to LCs there exist dermal DCs (dDCs), which represent another subtype of DCs in the skin and contribute to an effective antigen uptake. The dDCs are phenotypically different from the LCs but also possess the ability to stimulate naive T cells. Thus, it has been clearly demonstrated that in the deficiency of LCs, dDCs can maintain the immunological function against penetrated antigens. Both, LCs and dDCs, are the cutting edge in innate and adaptive immunity in the skin. As DCs have a fundamental function in the triggering of inflammatory skin diseases, current investigation pointed out that SPLs modulate DC-mediated immune response *in vivo*.

Indeed, recently it has been shown that topical application of S1P is beneficial in the treatment of atopic dermatitis (AD) [79]. AD is a multifaceted skin disease characterized by erythematous lesions, pruritus and xerosis. These features are the result of a disturbed epidermal barrier. Indeed, several mutations in genes encoding proteins that are responsible for the maintenance of the skin barrier function have been described. Consequently, the entrance of antigens into the skin is enhanced leading to a communication of these antigens with the epidermal and dermal DCs. Due to the fact that S1P regulate DC homeostasis such as antigen capture at least *in vitro* [27], it was of interest whether a topical application is also able to modulate DC function within the skin. For this purpose, an *in situ* experiment was generated visualizing the uptake of dextran as endocytotic tracer by LCs. Immunohistochemical examination of epidermal sheets indicated an efficient uptake of the model tracer by epidermal DCs. Most interestingly, there was a pronounced variance concerning the endocytotic capacity of LCs depending whether mice were administered with S1P. Topical administration of the bioactive sphingolipid significantly diminished endocytosis of LCs [79].

These data suggest that topical application of S1P can be beneficial in the treatment of inflammatory skin diseases such as AD. Contact hypersensitivity is a well-established murine animal model to investigate immunological processes of AD and to examine the potency of immune modulators in this skin disorder. In this model, the topical administration of S1P was analyzed. The immune modulatory function of the sphingolipid was monitored in the sensitization as well as in the elicitation phase. In the sensitization phase, S1P diminishes the weight and the cell count in the local auricular lymph nodes. In fact, the number of DCs within the lymph node was reduced in response to a S1P application suggesting a diminished DC

activation within the skin. In agreement, the cytokine profile in the auricular lymph nodes was modulated after topical administration of S1P. A significantly reduced secretion of the cytokines IL-6 and IFN-gamma was observed. In congruence with the decreased cell counts of DCs in the lymph node, immune histochemical examination of treated skin indicated that the LCs remained in the epidermis. Besides its beneficial role in the initiation of the immune response, S1P also showed an anti-inflammatory action in the elicitation phase of contact dermatitis as a reduced number of T cells in the skin was detected [79].

In this light, it is of interest that a dysregulation of S1P homeostasis has been discussed in the pathogenesis of AD. As already mentioned, S1P can be degraded either via dephosphorylation to sphingosine or via an irreversible cleavage by S1P lyase into hexadecenal and phosphoethanolamine. Indeed, a disturbed S1P level due to a dysregulation of S1P lyase has been discovered in human and canine atopic skin lesions [80-82]. In this manner, the S1P levels in canine atopic lesions are drastically reduced compared to healthy dogs [76]. These data are in agreement with the increased S1P lyase activity measured by mRNA expression in lesional skin from human and dogs [81, 82].

A further inflammatory skin disease is psoriasis vulgaris, which is characterized by erythematous papules and scaly plaques occurring at the skin surface. Additionally, the skin disease is characterized by an infiltration of T cells [83]. Besides the involvement of genetic alterations in the initiation and progression of psoriasis, it is still a question of discussion whether the origin of the disease has an epithelial or an immunological nature. However, several lines of evidence suggest that initiation of psoriasis is due to a disturbed immune regulation [80]. In this context, it has been shown that expression levels of IL-1, TNF-α, IL-12p70, IL-17, IL-22, and IL-23 are elevated in psoriatic skin leading to novel pharmacological therapies using monoclonal antibodies directed against these cytokines [85, 86].

As already mentioned S1P is able to modulate cytokine expression such as IL-23 and IL-12, suggesting that topical administration may be beneficial not only in AD but also psoriasis vulgaris. Therefore, S1P was tested in the Imiquimod (IMQ) model [87]. Topical treatment with IMQ, an agonist for TLR7 and TLR8, has recently been identified to induce psoriasis-like skin inflammation leading to acanthosis and parakeratosis [88]. Concerning the cytokine expression it has been shown that the occurrence of these pathological features is critically dependent on the IL-23/IL-17 axis [88]. In this novel model, topical administration of S1P was examined indicating that S1P reduced the IMQ-induced epidermal hyperproliferation of the ear skin. Moreover, there was also a remarkable reduction of ear swelling and a moderate decrease of inflammatory cell invasion after S1P treatment. These data suggest that topical application of S1P might be a new option for the treatment of mild to moderate psoriasis lesions [87]. However, it is still not well elucidated how IL-17-producing T cells are activated in the IMQ-treated mice, especially in the view of the participation of skin DCs. Most recently it has been shown, that a reduced ear swelling and decreased levels of IL-23 occurred in an IMQ-induced psoriasis model using LC-depleted mice. These results suggest that LCs are required for generation of psoriasis like lesions in response to IMQ in mouse models and that the beneficial role of S1P in this skin disease is at least in part due to an influence on LC homeostasis. In light of the crucial function of S1P on DCs it is not astonishing that S1P metabolism is dysregulated in psoriatic skin lesions. Indeed, it has been found that an increased degradation of S1P occur in psoriatic skin due to an upregulated expression of the S1P phosphatase 2 [89].

Summary and Outlook

Remarkable progress has been made in the last decade in illuminating the role of DCs in innate and adaptive immunity. In this context, it has been shown that the major sphingolipid metabolite S1P plays a central role in maintaining the homeostasis of DCs. S1P is the ligand for a family of five G-protein coupled receptors, named $S1PR_1$-$S1PR_5$, which mediate distinct signaling pathways. The significance of S1P in immune cell regulation became first obvious

when it had been discovered that the immune modulator fingolimod causes lymphopenia via $S1PR_1$ signaling. Indeed, fingolimod has recently entered the clinics for the treatment of multiple sclerosis. However, the action of S1P and fingolimod is not limited to T cell migration and function; both, S1P and the partial agonist fingolimod also influence the development of DCs from precursor cells and directly modulate functions of DCs.

The knowledge about the influence of S1P and even more of the different sphingolipid enzymes on the development of different DC types from early precursors is sparse. Nevertheless, in line with the very early reports about DCs and S1P, recent experiments suggested that during DC expansion from bone marrow progenitors the "survival effect" of increased S1P levels may be associated with the development of an "IL-23-prone" DC with attenuated capacity to induce Th1-like response. While these studies are in line with S1P data during STAT3/Akt-signaling, they are still preliminary and need to be verified more thoroughly.

One key event after an immunological challenge is the stimulation of divergent TLRs leading to the secretion of specific cytokines and chemokines. Moreover, antigen capture is a fundamental process that is mediated by DCs as well. The sphingolipid S1P modulates both, cytokine profiling and antigen capture. Concerning antigen uptake it has been figured out that S1P effectively decreases the aptitude to capture antigens via the $S1PR_2$ subtype. Taking a look on the cytokine profiling, it seems likely that S1P induces an IL-6, IL-23, and STAT3-dependent Th17 inflammatory profile of DCs, while Th1 responses are decreased by a reduction of IL-12p70 expression. On the contrary, FTY720 seems to reduce Th17- and Th2-dependent reactivity, but the Th1 autoimmunity inducing IL-12p70 may be not affected. Further studies are necessary to discern a direct interaction of non-phosphorylated FTY720 with the protein phosphatase 2A from these S1P receptor-dependent activities of FTY720-P.

With respect to the therapeutic application of the gathered *in vitro* knowledge, one has to be very careful, since so far only the partial agonist fingolimod was introduced into the clinic successfully. However, at least in part these *in vitro* data of non-migratory S1P and fingolimod functions on DCs are in congruence with *in vivo* studies in inflammation. First of all, in addition to its lymphocyte sequestration activities, fingolimod's success in patients with multiple sclerosis fits quite well to its therapeutic effects on the DC-dependent and disease-driving Th17 and Th2 pathologies. Interestingly, FTY720 was less successful in the prevention of transplantation rejection, a pathology more likely driven by Th1 reactions. In support of a therapeutic activity of fingolimod in allergic, Th2-prone, inflammatory conditions we have collected several animal studies herein, e.g. ulcerative colitis and allergic lung diseases. As reflected by trials with specific $S1PR_1$ receptor antagonists, S1P antibodies or recently, even S1P degrading, recombinant S1P lyase dimers [90], these beneficial effects of fingolimod are more likely to be based on its S1P antagonistic properties or even S1P-independent intracellular effects [91].

Distinct from fingolimod, systemically available S1P as a direct agonist may support IL-6- and IL-23-dependent inflammation and possibly reduce IL-12p70. However, opening up a new niche, the topical application of S1P appeared to be beneficial in DC-relevant inflammatory diseases such as AD and psoriasis vulgaris. Indeed, animal models of both diseases indicated that a topical administration of S1P is accompanied by an improvement of the diseases in both inflammatory models.

In summary, this review restricted to non-migratory effects of S1P and its analogs on DC function *in vitro* and *in vivo* has highlighted distinct and thus far less acknowledged immune biologic aspects. While it remains quite challenging, a better understanding of tissue-, cell- and even subcellular compartment-specific S1P regulation is a promising area for the development of new immune modulators. Given the pleiotropic S1P physiology, to our opinion one of the keys for the development of clinically successful drugs in this area will include S1P receptor or sphingolipid enzyme specificity strictly combined with an optimal target-specific delivery.

Abbreviations

S1P (sphingosine-1-phosphate); SphK (sphingosine kinase); S1PR (S1P receptor); DC (dendritic cell); Th1/2/17 (T helper 1/2/17); cDC (conventional DC); pDC (plasmacytoid DC); SphK1/2 (sphingosine kinase-1/2); SPP1/2 (S1P phosphatases-1/-2); Sgpl1 (S1P lyase); dhS1P,(dihydro S1P); GM-CSF (granulocyte macrophage colony stimulating factor); LC (Langerhans cells); IL (interleukin); APC (antigen presenting cells); dDC,(dermal DC); AD (atopic dermatitis); WT (wild type) TLR(toll-like receptor); BM-DCs (bone marrow-derived DCs); MHC I/II (Major Histocompatibiliy Complex I/II); SPC (sphingosylphosphorylcholine).

Disclosure Statement

The authors declare no conflict of interest.

Acknowledgements

While we made an effort to include recent and relevant publications regarding the role of sphingolipids for DC biology, we definitely had to restrict ourselves due to a limitation of space. We apologize, if we left out any important findings and contributions. We like to thank Martina Herrero San Juan for her excellent technical support. This work was supported by the priority program 1267 “sphingolipids - signals and disease” from the German Research Foundation (DFG), grant RA 525/10-1 to HHR and by the SFB1039 ‘signalling by fatty acid derivatives and sphingolipids in health and disease’.

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