

Topical Application of Sphingosine-1-Phosphate and FTY720 Attenuate Allergic Contact Dermatitis Reaction through Inhibition of Dendritic Cell Migration

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Migration of Langerhans cells (LCs) from the skin to the lymph node is an essential step in the pathogenesis of allergic contact dermatitis (ACD). Therefore, inhibition of LC-migration could be a promising strategy to improve this skin disease. Effects of sphingosine-1-phosphate (S1P) and the immunomodulator FTY720 on LC trafficking is not well defined, yet. Thus, we investigated the action of topically administered S1P and FTY720 in a murine model of ACD. Most interestingly, FTY720 as well as S1P inhibited the inflammatory reaction in the elicitation phase of ACD. In the sensitization phase, FTY720, and S1P reduced the weight and cell count of the draining auricular lymph node, as well as immigrated dendritic cells provoked by repetitive topical administration of the hapten. Correspondingly, the density of LCs in the epidermis was higher in FTY720- and S1P-treated mice compared to vehicle treatment. A skin dendritic cell migration assay confirmed the significant inhibition of dendritic cell migration by FTY720 and S1P. These data supply conclusive evidence that the strategy of targeting the migratory response of LCs with locally acting S1P or FTY720 represents an emerging option in the treatment of allergic skin diseases like contact hypersensitivity and atopic dermatitis.

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INTRODUCTION

Sphingosine-1-phosphate (S1P) has been shown to act as a critical signaling molecule and to elicit a variety of partially contrasting cellular effects. The significance of S1P in immune cell regulation became obvious when it was discovered that the immunosuppressive drug FTY720 (Fingolimod) causes lymphopenia through S1P signaling (Mandala *et al.*, 2002). S1P is part of the lipid fraction securing the epidermal permeability barrier. Sphingosine can be cleaved from ceramides, which account for 30–40% of stratum corneum lipids (Herzinger *et al.*, 2007). Sphingosine can then

be phosphorylated by sphingosine kinases to S1P, which binds to a family of G-protein-coupled receptors, named S1P₁–S1P₅. S1P signaling is inactivated irreversibly by a S1P lyase.

FTY720 is a synthetic structural analog of mytiocin, a metabolite of *Isaria cinclarii*. The efficacy of FTY720 has been well established in preclinical transplantation models and also in renal transplantation in humans (Tedesco-Silva *et al.*, 2005). However, in contrast to classical immunosuppressant drugs, FTY720 does not interfere with T-cell proliferation or apoptosis, but induces a severe deprivation in lymphocytes in the blood due to modification of S1P signaling. FTY720 shares structural characteristics with sphingosine and is also a substrate for the sphingosine kinase. Once phosphorylated, FTY720-P acts as an agonist on four of the five known S1P receptors (S1P₁, S1P₃, S1P₄, and S1P₅). However, in contrast to the physiological ligand S1P, FTY720-P is not a substrate of the S1P lyase and is therefore only metabolized by phosphatases (von Wenckstern *et al.*, 2006). This discrepancy is a crucial factor in the altered behavior of FTY720-P toward S1P receptors resulting in a distinct immune modulation by FTY720-P compared with S1P.

SEW2871, another S1P receptor (S1PR) agonist with a low EC₅₀ on the human S1P₁ (~13 nmol l⁻¹) and virtually no action on other S1P receptors induces a transient internalization of S1P₁ after systemic administration comparable to the

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Abbreviations: DC, dendritic cell; LC, Langerhans cell; S1P, sphingosine-1-phosphate; S1PR, sphingosine-1-phosphate receptor; TDI, toluene-2,4-diisocyanate

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natural ligand S1P (Sanna *et al.*, 2004, Gollmann *et al.*, 2008). Mice treated orally with SEW2871 developed acute lymphopenia, which was reversed, when SEW2871 levels in the plasma declined (Sanna *et al.*, 2004). However, a transient increase of blood DC in mice was also noticed (Lan *et al.*, 2005).

It is well described that some central dendritic cell functions (migration, cytokine secretion) can also be modulated *in vitro* by S1P and FTY720 (Renkl *et al.*, 2004; Muller *et al.*, 2005). Dendritic cells play a pivotal role in the initiation and elicitation of allergic diseases. Langerhans cells as well as dermal dendritic cells (DCs), carry haptens (like toluene-2,4-diisocyanate) from the skin through afferent lymphatic vessels to draining lymph nodes, where these haptens, bound to peptides, are presented to T cells, which then become specific T cells (induction phase). When the hapten is painted for a second time, it diffuses through the skin and could be loaded onto Langerhans cells or dermal dendritic cells, which in turn present the hapten to specific T cells. These specific T cells are then activated and respond with cytokine production and activation of further inflammatory cells (elicitation phase; reviewed in: (Krasteva *et al.*, 1999; Kimber *et al.*, 2002)).

It has been demonstrated that the mRNA for the S1P₁, S1P₂, S1P₃, and S1P₄ is expressed on the murine Langerhans cell line XS 52. These data are corroborated by a study on immature and mature murine bone marrow DCs. The mRNA for S1P₂, S1P₃, and S1P₄ have been found on both immature and mature DCs, whereas S1P₁ was only found on mature DCs (Czeloth *et al.*, 2005). However, it was shown that only S1P₁ and S1P₃ receptors are involved in cell migration towards S1P (Czeloth *et al.*, 2005; Radeke *et al.*, 2005).

This study was performed to examine effects of topically administered S1P, FTY720, and SEW2871 on dendritic cell migration in the induction phase as well as the inflammatory response in the elicitation phase in a model of toluene-2,4-diisocyanate (TDI) induced allergic contact dermatitis.

RESULTS

Local lymph node test in the sensitization phase

A repetitive sensitization with 0.5% TDI induced no remarkable ear swelling, indicating that the selected dose is

not irritating (vehicle + TDI treated ears: 2 (±14) µm, S1P + TDI treated ears 3 (±5) µm).

The topical administration of TDI induced a significant increase in local lymph node weight and cell count. This increase is abolished by topical administration of S1P (Figure 1a). A similar effect was observed by topical administration of FTY720, indicating a reduced T-cell stimulation. The S1P₁ agonist SEW2871 had no significant impact on lymph node weight or cell count (Figure 1b).

Dendritic cells in lymph nodes and *in vitro* stimulation

The amount of CD11c/CD40⁺ cells (that is, dendritic cells from skin) in local lymph nodes also increased after TDI sensitization. The amount of skin dendritic cells is reduced to basal levels by topical administration of S1P (Figure 2a). Topical administration of FTY720 also led to a significant decrease in CD11c/CD40⁺ cells in the draining lymph node, whereas SEW2871 had only a minor impact on immigrated DCs (Figure 2b).

Lymph node cells from TDI-sensitized mice stimulated with Con A respond with an increased secretion of IL-6 and IFNγ. This secretion is significantly lower in lymph node cells from S1P- and FTY720-treated animals (Table 1). SEW2871 did not reduce the Con A-induced IL-6 (data not shown) secretion. As SEW2871 also had no impact upon ear swelling in the challenge reaction (not shown), SEW2871 was omitted for subsequent experiments.

Impact of S1P₁ and S1P₃ on dendritic cell migration

SEW2871 had a lower effect on dendritic cell migration through skin compared with S1P and FTY720. SEW2871 acts solely through S1P₁. But as it was demonstrated in antisense oligonucleotide experiments that S1P₃ is also involved in DC migration toward S1P (Radeke *et al.*, 2005), it was tested whether a dual S1P₁/S1P₃ (VPC23019) antagonist displays superior inhibitory effect on DC migration compared with solely S1P₁ (W146) inhibition. Both inhibitors reduced the DC migration towards S1P. However, the dual inhibitor VPC23019 reduced migration to basal control levels and thus showed a significantly higher inhibition of migration compared with the S1P₁ antagonist W146 (Figure 2c).

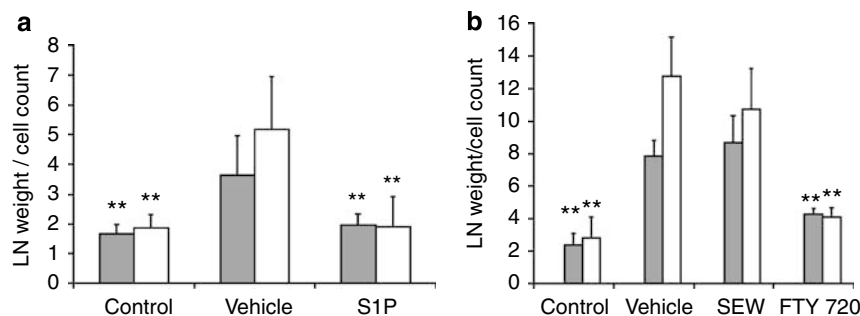


Figure 1. Lymph node weight (mg, gray bars) and cell count ($\times 10^6$ cells, open bars) from the draining lymph nodes of mice 24 h after the third sensitization with TDI. Mice received S1P (100 µg, a) or FTY720 (10 µg) and SEW2871 (b), or vehicle (methanol) over 9 days. From day 7 on, mice received TDI (0.5%) onto the inner and outer surface of the ears. Twenty-four hours after the third sensitization with TDI, mice were killed and the local lymph node was weighed and cells were counted in the lymph node suspension. Untreated controls served as basal values, $n = 6$ each group, $**P < 0.01$ compared to vehicle.

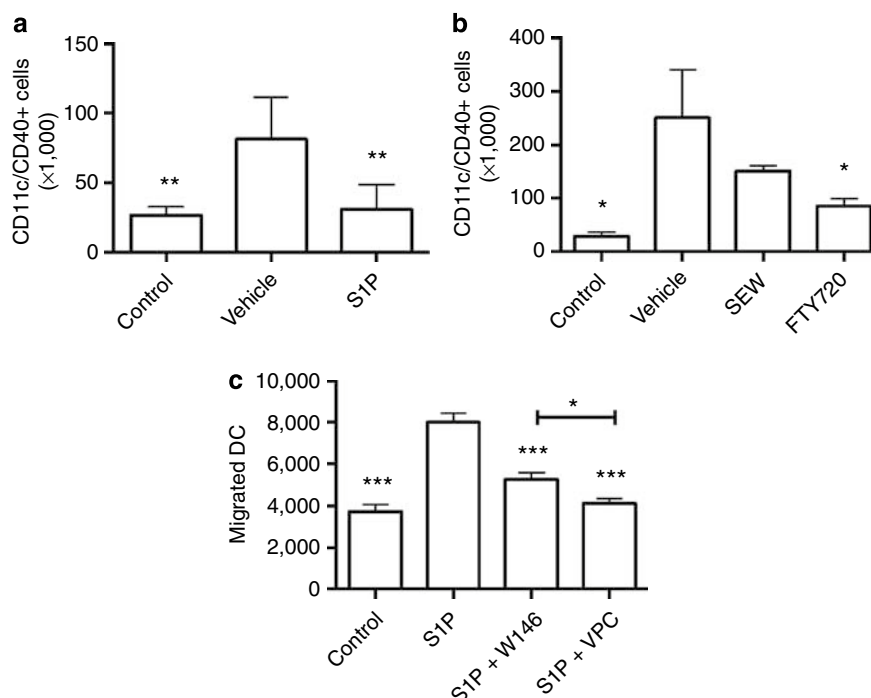


Figure 2. Total number of CD11c/CD40-positive cells (that is, dendritic cells emigrated from skin) in the draining lymph node after third sensitization with TDI. Mice received S1P (100 μ g, **a**) or FTY720 (10 μ g) and SEW2871 (**b**), or vehicle (methanol) over 9 days. From day 7 onwards, mice received TDI (0.5%) onto the inner and outer surface of the ears. Twenty-four hours after the third sensitization with TDI, mice were killed and the local lymph node cells were stained with anti-CD40 and CD11c and the amount of CD11c^{high}CD40^{high} cells were determined by means of flow cytometry, $n=6$ each group, ** $P<0.01$, (**c**): Migration of BM-derived DCs towards S1P. Effects of a S1P₁ (W146) antagonist and a dual S1P₁/S1P₃ (VPC23019) antagonist on DCs migration towards S1P. One of two independent experiments ($n=4$ each group) with similar results, * $P<0.05$, *** $P<0.001$.

Table 1. Local administration of FTY720 and S1P modulates cytokine pattern in draining lymph nodes

	IL-6 (pg ml ⁻¹)	IFN γ (pg ml ⁻¹)
Control	3 ($\pm$ 2)**	181 ($\pm$ 10)*
Vehicle	182 ($\pm$ 108)	978 ($\pm$ 593)
S1P	54 ($\pm$ 37)**	237 ($\pm$ 164)*
Control	4 ($\pm$ 2)**	62 ($\pm$ 17)**
Vehicle	327 ($\pm$ 104)	2271 ($\pm$ 743)
FTY720	52 ($\pm$ 3)**	844 ($\pm$ 227)**

Draining lymph node cells from mice were cultivated 24 h after third sensitization with TDI. Lymph node cells were stimulated *in vitro* with Con A for 48 h. IL-6 and IFN γ were measured in the supernatants by ELISA, Mean ($\pm$ SD) of $n=6$ for each group, experiment was performed two times with comparable results, * $P<0.05$, ** $P<0.01$ compared to vehicle.

MHC class II positive cells in the epidermis

Compared to vehicle, the treatment with S1P resulted in a significant higher cell count of major histocompatibility complex (MHC) class II⁺ cells (that is, Langerhans cells) in the epidermis of TDI-sensitized mice 24 hours after last sensitization (Figure 3a). Topical administration of FTY720 resulted in a similar effect (Figure 3b).

Skin dendritic cell migration assay

Twenty-four hours after last sensitization, the cartilage-free ear halves were cultivated with TDI for 3 days further to determine skin DC migration (Ortner *et al.*, 1996). DC migration was reduced by topical administration of S1P and FTY720, corresponding to the higher density of Langerhans cells observed in epidermal sheets treated with S1P and FTY720 (Figure 4).

Plasma S1P levels after topical administration

To evaluate a possible systemic effect, mice treated for 9 days with S1P were tested for any elevation in S1P levels in plasma. After the last S1P treatment and TDI challenge, mice were killed and S1P levels were detected in plasma. There was a slight, but significant difference in plasma concentration of S1P between vehicle-treated and S1P-treated mice (vehicle-treated mice: 70 ($\pm$ 6) pmol/ml, S1P-treated mice: 102 ($\pm$ 36) pmol/ml).

Lymphocytes in peripheral blood after topical administration of S1P, FTY720, and SEW2871

Although S1P was only slightly elevated in plasma, topical administration of S1P, FTY720, and SEW2871 reduced the number of white blood cells, particularly in lymphocytes (Figure 5).

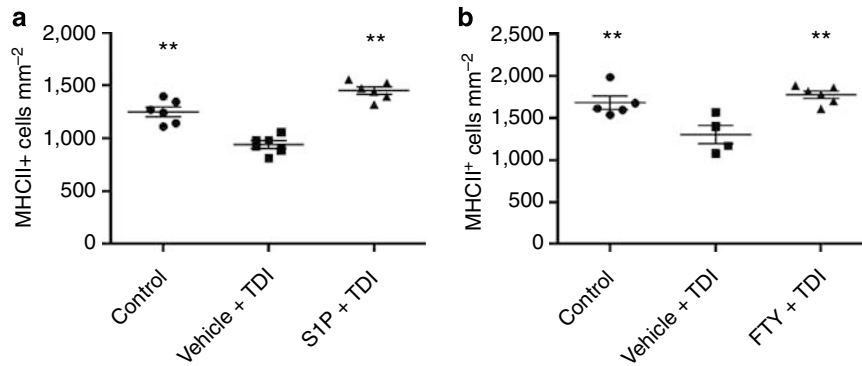


Figure 3. MHC class II⁺ cells in the ear epidermis of mice 24 h after last sensitization with TDI. Mouse ears were treated with S1P (a), FTY720 (b) or vehicle for 9 days. From day 7 to 9, ears were treated topically with TDI (0.5%). Epidermis was stained for MHC class II⁺ cells and density was determined microscopically by means of a calibrated grid, $n=4-6$ per group, ** $P<0.01$.

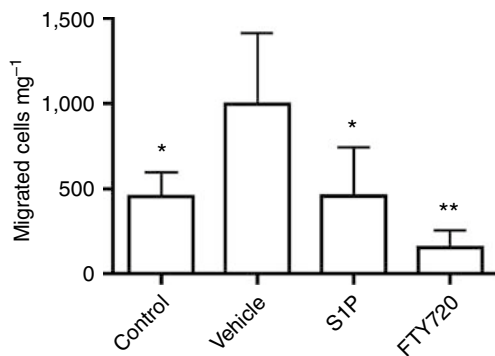


Figure 4. Migration of skin dendritic cells out of mouse ear explants.

Cartilage-free dorsal ear halves were cultivated 24 hours after the third sensitization with TDI for 3 additional days. Explants were transferred daily into new wells. Emigrated cells were pooled for each explant, counted with a hemocytometer and related to the tissue weight. $n=6$ each group, * $P<0.05$, ** $P<0.01$.

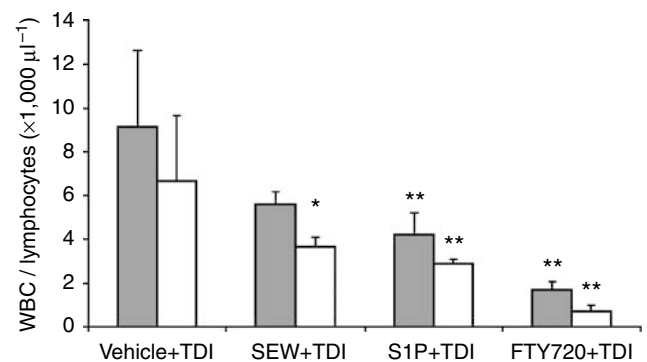


Figure 5. White blood cells (gray bars) and lymphocytes (white bars) of mice treated topically with S1P, FTY720, and SEW2871 or vehicle. Blood cells were counted in heparinized blood collected 24 hours after the third sensitization with TDI. $n=4-6$ per group, * $P<0.05$, ** $P<0.01$.

Impact of topically administered S1P and FTY720 on ear swelling and T-cell influx in the elicitation phase of allergic contact dermatitis

When administered before elicitation with TDI, S1P (100 μg per ear over 9 days) induced a significant inhibition of the ear swelling measured 24 hours after challenge (Figure 6a). Comparable reduction of the inflammatory response was also observed after topical administration of FTY720 (B) for 9 days. Immunohistological evaluation reveals a reduction of CD3⁺ cells in FTY720 and S1P-treated mouse ears (Figures 7 and 8). The staining intensity for MHC II⁺ cells is also reduced in the dermis of S1P- and FTY720-treated ears. However, GR-1⁺ cells (neutrophil granulocytes) are not reduced by treatment with S1PR agonists (Figure 7).

DISCUSSION

The results of this study reveal several crucial aspects: first, topical administration of S1P as well as FTY720 impairs Langerhans cell (LC) migration through the skin. Second, the impaired LC-migration is accompanied by a diminished local lymph node reaction in the sensitization phase and a

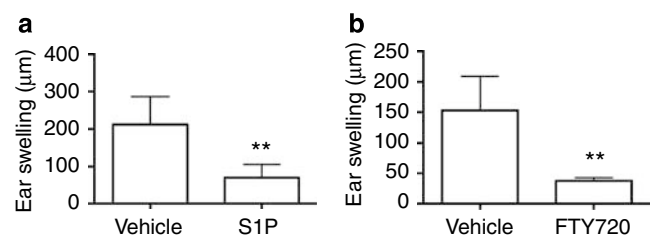


Figure 6. Ear swelling 24 hours after TDI challenge. Mice were sensitized and challenged with TDI as described in Materials and Methods. S1P (a) or FTY720 (b), or vehicle was topically administered for 9 days. On day 9, mice were challenged with TDI. Ear thickness was measured directly before challenge and 24 hours after challenge and ear swelling was calculated, $n=6$ each group, ** $P<0.01$.

diminished inflammatory reaction in the elicitation phase of allergic contact dermatitis.

Dendritic cells (DCs) are pivotal to both the initiation and maintenance phase of allergic inflammatory diseases like atopic dermatitis and allergic contact dermatitis. Thus, it can be postulated that an inhibition of DC (particularly LC) migration and activation can at least partly explain an inhibitory action of immunomodulatory substances. This

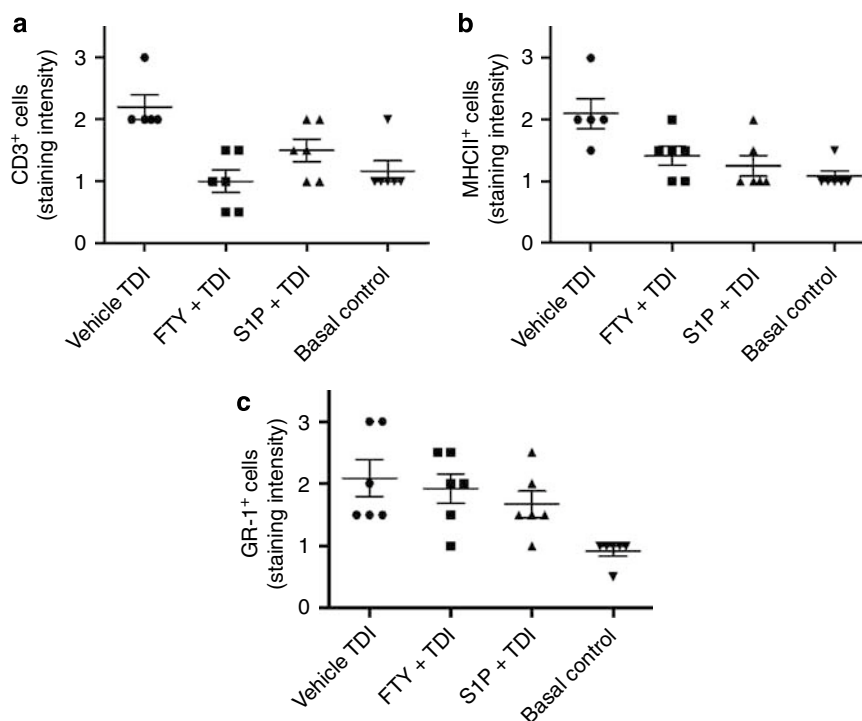


Figure 7. Immunohistological staining of CD3⁺ cells (a), MHC II⁺ cells (b) and GR-1⁺ cells (c) in the epidermis of TDI-challenged mice. Mice were sensitized and challenged with TDI as described in Materials and Methods. S1P, FTY720, or vehicle was topically administered for 9 days. On day 9, mice were challenged with TDI. Ears were stained with anti CD3, anti MHC II and anti-GR-1 antibodies and the staining intensity was evaluated by two persons from 0 (no staining)—3 (strong staining intensity). Mean intensity of each mouse ear is presented, $n = 6$ each group.

has already been demonstrated for immunomodulators like cyclosporine A, tacrolimus, rapamycin, cilomilast, and glucocorticoids (Homey *et al.*, 1998; Panhans-Gross *et al.*, 2001; Chen *et al.*, 2004; Hoetzenecker *et al.*, 2004; Baumer *et al.*, 2005, 2006).

In this study, the anti-inflammatory and immunomodulatory action of locally administered FTY720 and its natural ligand S1P was compared. It was presumed that a high topical administration of sphingosine receptor agonists results in a receptor internalization (mainly S1P₁) and an unresponsiveness of skin dendritic cells (Graler and Goetzl, 2004; Matloubian *et al.*, 2004). The results obtained with the S1P agonist SEW2871 indicate, that not all effects are mediated solely through the S1P₁, or more precisely its internalization. *In vitro* experiments with the S1P₁ antagonist W146 and the S1P₁/S1P₃ antagonist VPC23019 indicate that blocking of S1P₃ is essential for a complete inhibition of DC migration. Our results are supported by the recently published study of Gollmann *et al.* (2008). Intradermal injection of SEW2871 (~20 µg per mouse ear) reduced the migration of DCs to the draining lymph node (even under inflammatory conditions). However, the reduction is not complete. A maximum of approximately 75% inhibition is achieved by intradermal injection of SEW2871 (Gollmann *et al.*, 2008). In addition, an incomplete inhibitory action of SEW2871 can be explained pharmacokinetically, for example, by a low skin penetration of SEW2871.

Most studies concerning the action of FTY720 were carried out by means of a systemic administration of the

S1PR agonist, which results in an inhibition of lymphocyte egress from the lymph nodes and consequently in peripheral lymphopenia (Mandala *et al.*, 2002). For instance, a systemic administration of FTY720 has been demonstrated to prevent spontaneous dermatitis in NC/Nga mice, which are used as a mouse model of atopic dermatitis (Kohno *et al.*, 2004). Very recently it was shown that systemic administration of FTY720 impaired the initiation but not the elicitation phase of allergic contact dermatitis induced by dinitrofluorobenzene (Nakashima *et al.*, 2008). The authors could not find a modulation of skin dendritic cell migration by systemic administration of FTY720. Thus, they conclude that systemically administered FTY720 impairs mainly naive T-cell circulation, which affects the initiation of allergic contact dermatitis. The purpose of this study was to examine effects of topically administered S1PR agonists to reduce possible side effects induced by peripheral lymphopenia. However, long-term treatment with S1P and especially FTY720 also resulted in lymphopenia (Figure 5). Nevertheless, it was intended to study the modulation of dendritic cell function in the skin. Therefore, the substances were administered topically.

Analysis of epidermal sheets demonstrate that, compared with vehicle-treated mouse ears, topical treatment with FTY720 and S1P leads to an inhibition of Langerhans cell migration. This is confirmed by the skin dendritic cell migration assay. The inhibitory effect of FTY720 on skin DC migration has been demonstrated in non-inflamed skin by Czeloth *et al.* (2005) following FITC skin painting after 4 days of systemic administration of FTY720 (Czeloth *et al.*, 2005).

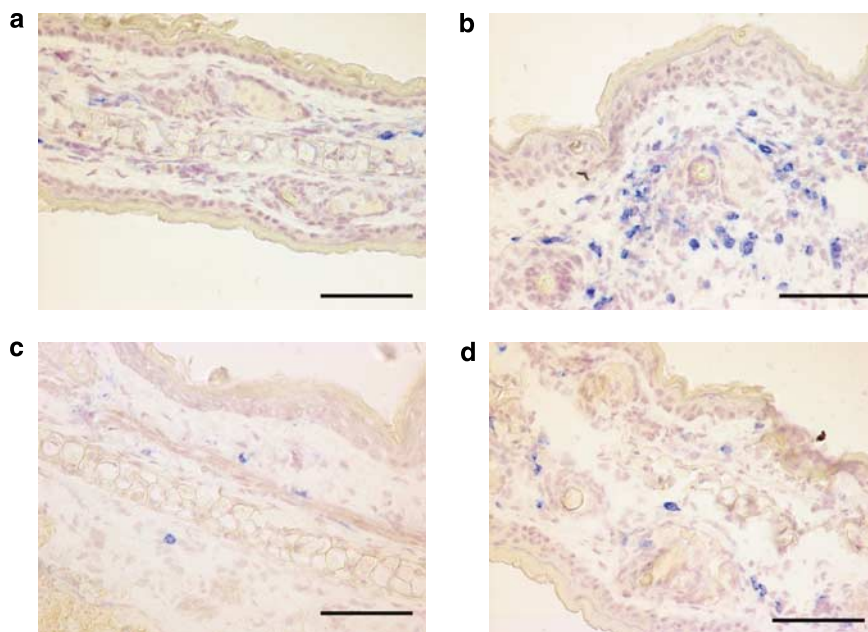


Figure 8. Immunohistological staining of CD3⁺ cells. Mice were sensitized and challenged with TDI as described in Materials and Methods. Vehicle (b), FTY720 (c) or S1P (d) was topically administered for 9 days. On day 9, mice were challenged with TDI. Ears were stained with anti-CD3 as described in Materials and methods. Untreated mouse ears served as basal controls (a), $n=6$ each group, bars represent 100 μm .

It can be concluded that topically administered FTY720 is phosphorylated to FTY720P, which represents the active compound (von Wenckstern *et al.*, 2006).

Comparable to our results, a recent study investigating the lung demonstrated that relatively high concentrations of topically administered FTY720 and S1P can reduce DC migration in lungs accompanied by a reduced allergic inflammatory response (Idzko *et al.*, 2006). However, it is noticeable that DC migration towards other chemokines like CCL19, CCL21 is not affected (Czeloth *et al.*, 2005; Idzko *et al.*, 2006).

In any case, the reduced allergic inflammatory response should not solely be explained by a reduced DC migration but also for example, by a defective T-cell stimulatory effect. For example, the IL-12 secretion of lung DCs was impaired by FTY720 treatment (Idzko *et al.*, 2006), which corresponds to *in vitro* findings for S1P and FTY720 with human monocyte-derived DCs (Idzko *et al.*, 2002; Muller *et al.*, 2005). Our own results show a diminished secretion of IFN γ and IL-6 in Con A-stimulated cells from draining lymph nodes of TDI-sensitized mice after *in vivo* treatment with S1P and FTY720, which also indicates a modulation of the cytokine pattern. This is corroborated by *in vitro* findings: a diminished IFN γ secretion has been demonstrated *in vitro*, when FTY720-treated DCs were added to allogeneic T cells (Muller *et al.*, 2005).

In addition, direct effects of S1PR agonists on keratinocyte function are published. Although most publications indicate an influence of S1P on keratinocyte proliferation (Kim *et al.*, 2004; Schuppel *et al.*, 2008), it was also recently shown that topically administered S1P reduced the epidermal hyperplasia provoked by repeated tape stripping in mice (Hong *et al.*, 2008). Repeated tape stripping is accompanied by a secretion

of several pro-inflammatory cytokines and chemokines like TNF α , IL-1 β , IL-6, IL-8, and histamine (Wood *et al.*, 1992; Amano *et al.*, 2007). Thus, topical administration of S1P might have also an impact on cytokine secretion from keratinocytes. Own preliminary results indicate, that S1P (10 $\mu\text{mol l}^{-1}$) reduces the LPS-induced KC secretion (murine IL-8 homolog) in the murine keratinocyte cell line MSC (data not shown).

Although it was intended to exclude systemic effects of the immunomodulators by topical administration, the observed lymphopenia (Figure 5) and slightly enhanced S1P plasma levels point towards a systemic effect, which can also contribute to the anti-inflammatory action observed in the challenge phase (Figure 6). Interestingly, the accumulation of T cells is vastly reduced in the elicitation phase by topical administration of S1PR agonists (Figures 7 and 8). Furthermore, a reduction of MHC II⁺ cells is apparent, indicating a reduced influx of antigen-presenting cells into the site of antigen challenge. Most striking, this is a cell-specific effect, as neutrophil influx is not affected by topical-administered S1PR agonists (Figure 7).

With regard to allergic diseases like atopic dermatitis it can be postulated that topical administration of FTY720 and S1P may have a benefit and might be superior to systemic administration of S1PR agonists (Nakashima *et al.*, 2008). This is supported by the fact that an enhanced S1P lyase activity has been found in mRNA of lesional skin from atopic dermatitis patients (Seo *et al.*, 2006). Thus, it can be speculated that topical S1P has local immunosuppressive activity in the epidermis and that reduced S1P levels by an enhanced S1P lyase activity in skin might be involved in Langerhans cell activation.

Altogether, the presented data consolidates the strategy of targeting the migratory response of LCs with locally acting S1P or FTY720, hence representing a new option in the treatment of allergic skin diseases like contact hypersensitivity and atopic dermatitis.

MATERIALS AND METHODS

Reagents

Sphingosine-1-phosphate was purchased from York Pharma (Homburg/Ohm, Germany), SEW2871 and FTY720 were purchased from Biozol (Eching, Germany). Toluene-2,4-diisocyanate, lipopolysaccharide (LPS; O127:B8), and concanavalin A (Con A) were obtained from Sigma (Deisenhofen, Germany), ammonium thiocyanate from Riedel de Haën (Hannover, Germany). The S1P₁ antagonist W146 and the S1P₁/S1P₃ antagonist VPC23019 were purchased from Avanti Polar Lipids (Alabaster, AL). Anti-mouse CD 11c (hamster IgG₁, PE-conjugated, HL3), CD40 (rat IgG_{2a}, FITC-conjugated, 3/23), MHC II (I-A/I-E, rat IgG2b), and the corresponding isotype controls were ordered from Pharmingen (Hamburg, Germany). Biotinylated rabbit anti-rat IgG was from DAKO (Hamburg, Germany), carbocyanin 3 from Jackson Immunoresearch Laboratories (PA). For immunohistochemistry, anti-GR-1, -CD3, -MHC II all came from Dianova (Hamburg, Germany), bridging antibodies (rat anti-mouse IgG, Z494) and the APAAP complex (D 488) were obtained from Dako (Hamburg, Germany). Fast Blue was ordered from Sigma. ELISAs for IL-6 and IFN γ were from R&D systems (Wiesbaden, Germany).

Mice

Female BALB/c-mice with an age of 8 weeks (20 g body weight), were obtained from Charles River (Sulzfeld, Germany). The mice were housed in groups of six mice per cage at 22°C with a 12-hours light-dark cycle. Water and a standard diet (Altromin, Lage/Lippe, Germany) were available *ad libitum*. All animal procedures were conducted under experimental license approved by the Bezirksregierung Hannover, Germany (Az. 509.6-42502-03/711).

Induction of sensitization and elicitation in mice

To determine effects of S1P on the sensitization phase, a modified local lymph node assay was performed (Ehling *et al.*, 2005). Over a period of 9 days, mouse ears were treated with 100 μ g S1P dissolved in 80 μ l methanol. Another group of mice received FTY720 (10 μ g in 80 μ l) for 9 days. A third group received SEW2870 (100 μ g in 80 μ l methanol) for 9 days. The dosage for SEW2871 was selected because of the low EC₅₀ (13 nmmol l⁻¹) on the S1P₁ and the quite long elimination half-life after systemic administration (~7 hours; Sanna *et al.* 2004). From day 7 vehicle or S1PR agonist-treated ears were sensitized by application of 20 μ l 0.5 % toluene-2,4-diisocyanate (TDI) in acetone onto the mouse ears. On day 10 ear thickness was measured, the mice were killed and the Lnn. auriculares were prepared. The lymph nodes were weighed and cell count was determined using a hemocytometer (Neubauer, VWR, Darmstadt, Germany).

TDI-induced allergic dermatitis was performed as described earlier (Baumer *et al.*, 2004, 2005). After settling in for 1 week, the abdominal skin of the mice was shaved and depilated with Veet. The abdominal skin was stripped 10 times with adhesive tapes. For active sensitization, 50 μ l 5% TDI in acetone was administered to the stripped epidermis on 4 consecutive days. The allergic reaction was

boosted 21 days later by administration of 50 μ l 0.5 % TDI in acetone onto the shaved abdomen. Starting on day 19, mouse ears were treated with 100 μ g S1P or 100 μ g SEW2871 in 80 μ l methanol for 9 days. Another group of mice received FTY720 (10 μ g in 80 μ l) for 9 days. The allergic reaction was challenged by administration of 20 μ l 0.5 % TDI in acetone onto the mouse ears treated with vehicle or S1PR agonists. The swelling was calculated by a comparison of the ear thickness (cutimeter, Mitutoyo, Neuss, Germany) before and 24 hours after challenge.

Determination of S1P in blood plasma and determination of lymphocytes in blood

Twenty-four hours after TDI challenge, mice were killed, and blood was taken from vehicle-treated and S1PR agonist-treated animals. Blood was collected in siliconized vials coated with heparine. The vials were centrifuged and S1P levels in the plasma were measured using a method recently described by Ruwisch *et al.* (2001). In brief, plasma was diluted in phosphate-buffered saline and mixed with methanol/hydrochloric acid (HCl). As internal standard dihydro-S1P was supplemented. Lipids were extracted by addition of chloroform and 4 M NaCl. For alkalization, 3 N NaOH were added. The organic phase was re-extracted from the aqueous phase with methanol, 1 M NaCl and 3 N NaOH. The aqueous phases were combined, acidified and extracted two times with chloroform. The organic phases were evaporated, and the dried lipids dissolved in 275 μ l methanol/0.07 M K₂HPO₄ (9:1). A derivatization mixture of *o*-phthalaldehyde, ethanol, 2-mercaptoethanol, and 3% boric acid was prepared and adjusted to potassium hydroxide (pH) 10.5 with KOH. Then, 25 μ l of the derivatization mixture was added to the resolved lipids. The derivatives were analyzed by a Merck Hitachi LaChrom 842 HPLC system (Merck Hitachi, Darmstadt, Germany) using a RP18 Kromasil column (Chromatographie Service, Langerwehe, Germany). Separation was carried out using a gradient of methanol and 0.07 M K₂HPO₄. Resulting profiles were evaluated using the Merck system manager software. The recovery of S1P was calculated using dihydro-S1P as standard.

Twenty-four hours after the third sensitization (18 hours after last S1PR agonist treatment), blood was collected in heparine-coated vials and white blood cells and lymphocytes were determined using a flow-cytometry-based automated hematology system with a veterinary software package designed for different species including the mouse (Advia 120, Siemens Medical Solutions Diagnostics, Eschborn, Germany).

Skin dendritic cell migration assay

Twenty-four hours after challenge and determination of ear swelling, the mice were killed by cervical dislocation. The cartilage-free dorsal halves of split mouse ear skin were cultured in 24-well size plates based on a method of Ortner *et al.* (1996). The halves were placed onto tables made of sieves with the epidermal side facing upwards. The tables were transferred daily to new wells with fresh media. Migrated cells from each ear were pooled and counted with a hemocytometer (Neubauer, VWR, Darmstadt, Germany). The viability of the cells was assessed by trypan blue exclusion.

Chemotaxis assay for bone marrow-derived DCs

Bone marrow-derived murine DCs were cultivated with murine GM-CSF according to a standard protocol (Lutz *et al.*, 1999). The

chemotaxis assay was performed in a trans well system as described elsewhere (Renkl *et al.*, 2004). There exists a discrepancy in the literature, whether immature DCs respond to the same extent to S1P as mature DCs (Renkl *et al.*, 2004, Czeloth *et al.*, 2005). We therefore stimulated half of the DCs with LPS (500 ng ml⁻¹) and performed the chemotaxis assay with a 1:1 ratio of immature and mature DCs. A part of the DCs was pre-incubated with 1 μmol l⁻¹ W146 and VPC23019, respectively. The concentration was selected, as both antagonists have K_i values in a low nanomolar range at the S1P₁ (Davis *et al.*, 2005, Sanna *et al.*, 2006). Thus, if the antagonists are administered in excess for blocking S1P₁ (1 μmol l⁻¹), an additional effect by VPC23019 is due to the additional S1P₃ inhibition. Chemotaxis was induced by 1 μmol l⁻¹ S1P (Renkl *et al.*, 2004). Migrated cells were determined 90 minutes after incubation.

Determination of dendritic cells in lymph nodes and *in vitro* stimulation

The auricular lymph nodes were prepared and single-cell suspensions were prepared in phosphate-buffered saline. As it has been reported that DCs in draining lymph nodes, which have migrated from skin are characterized by both CD11c^{high} and CD40^{high} (Ohl *et al.*, 2004), LN cells were stained for CD11c and CD40 or corresponding isotype controls as described earlier (Baumer *et al.*, 2006). The percentage of CD11c and CD40 double-positive cells was determined by flow cytometry (Coulter Epics XL, Beckmann, Krefeld, Germany with Expo 32 ADC Analysis).

For *in vitro* stimulation, 5 × 10⁵ cells from each lymph node were cultured in RPMI and stimulated with 2 μg ml⁻¹ Con A. Forty-eight hours after stimulation, supernatant was harvested and kept at -20°C until determination of IL-6 and IFNγ according to the manufacturer's protocol.

Preparation of epidermal sheets for immunohistochemistry

The preparation and the evaluation of epidermal sheets were described earlier (Baumer *et al.*, 2005). In short, the epidermis was separated from the dermis by means of ammonium thiocyanate. The dendritic cells were detected with monoclonal anti-mouse MHC II. Labeling of the antibodies was visualized by using biotinylated rabbit anti-rat IgG and coupling the antibodies to a fluorochrom (carbocyanin 3) by streptavidin-biotin technique. The density of Langerhans cells was counted under the microscope using a 20-fold magnification and a calibrated grid. Then, 16 randomly chosen areas per ear were analyzed. Vehicle, FTY720, S1P, and untreated control ears were analyzed after sensitization.

Immunohistochemical staining of CD3⁺, MHC II⁺, and GR-1⁺ cells in mouse ears

Cryostat sections (n = 6 each group) of mouse ears (7 μm) were cut 24 hours after challenge with TDI. The sections were stained using the alkaline phosphatase anti-alkaline phosphatase technique (APAAP). The skin was stained for CD3⁺ T cells, MHC II⁺ cells and for neutrophils as described earlier (Baumer *et al.*, 2004). After incubation with the primary antibodies and washing, the slices were incubated with bridging antibodies (rat anti-mouse immunoglobulin G, Z494, Dako, Hamburg, Germany) and after washing, with the APAAP complex (D 488, Dako). Fast Blue (Sigma, Deisenhofen, Germany) served as a substrate for the alkaline phosphatase.

Sections were counterstained with hemalaun. The cell influx was evaluated by a semi-quantitative examination (range: 0 = no staining—3 = strong staining intensity) by two experienced persons. The mean staining intensity of each mouse ear evaluated by the two persons is presented numerically as a scatter box. As this method is not quantitative, no statistics were performed.

Statistical evaluation

All experiments were performed in at least two independent settings with comparable results. Figures are presented as mean (±SD). Statistically significant differences between the drug treatments and controls were assessed by a one-way analysis of variance followed by a *post hoc* test (Dunnett's test). When two groups are compared a Mann-Whitney rank sum test was performed.

CONFLICT OF INTEREST

The authors state no conflict of interest

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REFERENCES

- Amano T, Takeda T, Yano H, Tamura T (2007) Olopatadine hydrochloride accelerates the recovery of skin barrier function in mice. *Br J Dermatol* 156:906–12
- Baumer W, Krekeler S, DeVries VC, Niedorf F, Tschernig T, Kietzmann M (2006) Non-steroidal and steroidal anti-inflammatory drugs vary in their modulation of dendritic cell function in the elicitation phase of allergic contact dermatitis. *Exp Dermatol* 15:322–9
- Baumer W, Seegers U, Braun M, Tschernig T, Kietzmann M (2004) TARC and RANTES, but not CTACK, are induced in two models of allergic contact dermatitis. Effects of cilomilast and diflorasone diacetate on T-cell-attracting chemokines. *Br J Dermatol* 151:823–30
- Baumer W, Szulze B, Weigt H, De Vries VC, Hecht M, Tschernig T *et al.* (2005) Cilomilast, tacrolimus and rapamycin modulate dendritic cell function in the elicitation phase of allergic contact dermatitis. *Br J Dermatol* 153:136–44
- Chen T, Guo J, Yang M, Han C, Zhang M, Chen W *et al.* (2004) Cyclosporin A impairs dendritic cell migration by regulating chemokine receptor expression and inhibiting cyclooxygenase-2 expression. *Blood* 103:413–21
- Czeloth N, Bernhardt G, Hofmann F, Genth H, Forster R (2005) Sphingosine-1-phosphate mediates migration of mature dendritic cells. *J Immunol* 175:2960–7
- Davis MD, Clemens JJ, Macdonald TL, Lynch KR (2005) Sphingosine 1-phosphate analogs as receptor antagonists. *J Biol Chem* 280:9833–41
- Ehling G, Hecht M, Heusener A, Huesler J, Gamer AO, van Loveren H *et al.* (2005) An European inter-laboratory validation of alternative endpoints of the murine local lymph node assay: First round. *Toxicology* 212:60–8
- Gollmann G, Neuwirt H, Tripp CH, Mueller H, Konwalinka G, Heufler C (2008) Sphingosine-1-phosphate receptor type-1 agonism impairs blood dendritic cell chemotaxis and skin dendritic cell migration to lymph nodes under inflammatory conditions. *Int Immunol* 20:911–23
- Graler MH, Goetzl EJ (2004) The immunosuppressant FTY720 down-regulates sphingosine 1-phosphate G-protein-coupled receptors. *FASEB J* 18:551–3
- Herzinger T, Kleuser B, Schafer-Korting M, Korting HC (2007) Sphingosine-1-phosphate signaling and the skin. *Am J Clin Dermatol* 8:329–36
- Hoetzenecker W, Meingassner JG, Ecker R, Stingl G, Stuetz A, Elbe-Burger A (2004) Corticosteroids but not pimecrolimus affect viability, maturation

- and immune function of murine epidermal Langerhans cells. *J Invest Dermatol* 122:673-84
- Homey B, Assmann T, Vohr HW, Ulrich P, Lauerma AI, Ruzicka T *et al.* (1998) Topical FK506 suppresses cytokine and costimulatory molecule expression in epidermal and local draining lymph node cells during primary skin immune responses. *J Immunol* 160:5331-40
- Hong JH, Youm JK, Kwon MJ, Park BD, Lee YM, Lee SI *et al.* (2008) K6PC-5, a direct activator of sphingosine kinase 1, promotes epidermal differentiation through intracellular Ca(2+) signaling. *J Invest Dermatol* 128:2166-78
- Idzko M, Hammad H, van Nimwegen M, Kool M, Muller T, Soullie T *et al.* (2006) Local application of FTY720 to the lung abrogates experimental asthma by altering dendritic cell function. *J Clin Invest* 116:2935-44
- Idzko M, Panther E, Corinti S, Morelli A, Ferrari D, Herouy Y *et al.* (2002) Sphingosine 1-phosphate induces chemotaxis of immature and modulates cytokine-release in mature human dendritic cells for emergence of Th2 immune responses. *FASEB J* 16:625-7
- Kim DS, Kim SY, Kleuser B, Schafer-Korting M, Kim KH, Park KC (2004) Sphingosine-1-phosphate inhibits human keratinocyte proliferation via Akt/protein kinase B inactivation. *Cell Signal* 16:89-95
- Kimber I, Basketter DA, Gerberick GF, Dearman RJ (2002) Allergic contact dermatitis. *Int Immunopharmacol* 2:201-11
- Kohno T, Tsuji T, Hirayama K, Watabe K, Matsumoto A, Kohno T *et al.* (2004) A novel immunomodulator, FTY720, prevents spontaneous dermatitis in NC/Nga mice. *Biol Pharm Bull* 27:1392-6
- Krasteva M, Kehlen J, Ducluzeau MT, Sayag M, Cacciapuoti M, Akiba H *et al.* (1999) Contact dermatitis I. Pathophysiology of contact sensitivity. *Eur J Dermatol* 9:65-77
- Lan YY, De Creus A, Colvin BL, Abe M, Brinkmann V, Coates PT *et al.* (2005) The sphingosine-1-phosphate receptor agonist FTY720 modulates dendritic cell trafficking *in vivo*. *Am J Transplant* 5:2649-59
- Lutz MB, Kukutsch N, Ogilvie L, Rossner S, Koch F, Romani N *et al.* (1999) An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods* 223:77-92
- Mandala S, Hajdu R, Bergstrom J, Quackenbush E, Xie J, Milligan J *et al.* (2002) Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. *Science* 296:346-9
- Matloubian M, Lo CG, Cinamon G, Lesneski MJ, Xu Y, Brinkmann V *et al.* (2004) Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature* 427:355-60
- Muller H, Hofer S, Kaneider N, Neuwirt H, Mosheimer B, Mayer G *et al.* (2005) The immunomodulator FTY720 interferes with effector functions of human monocyte-derived dendritic cells. *Eur J Immunol* 35:533-45
- Nakashima D, Kabashima K, Sakabe J, Sugita K, Kobayashi T, Yoshiki T *et al.* (2008) Impaired initiation of contact hypersensitivity by FTY720. *J Invest Dermatol* 128:2833-41
- Ohl L, Mohaupt M, Czeloth N, Hintzen G, Kiafard Z, Zwirner J *et al.* (2004) CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. *Immunity* 21:279-88
- Ortner U, Inaba K, Koch F, Heine M, Miwa M, Schuler G *et al.* (1996) An improved isolation method for murine migratory cutaneous dendritic cells. *J Immunol Methods* 193:71-9
- Panhans-Gross A, Novak N, Kraft S, Bieber T (2001) Human epidermal Langerhans' cells are targets for the immunosuppressive macrolide tacrolimus (FK506). *J Allergy Clin Immunol* 107:345-52
- Radeke HH, von Wenckstern H, Stoldtner K, Sauer B, Hammer S, Kleuser B (2005) Overlapping signaling pathways of sphingosine 1-phosphate and TGF-beta in the murine Langerhans cell line XS52. *J Immunol* 174:2778-86
- Renkl A, Berod L, Mockenhaupt M, Idzko M, Panther E, Termeer C *et al.* (2004) Distinct effects of sphingosine-1-phosphate, lysophosphatidic acid and histamine in human and mouse dendritic cells. *Int J Mol Med* 13:203-9
- Ruwisch L, Schafer-Korting M, Kleuser B (2001) An improved high-performance liquid chromatographic method for the determination of sphingosine-1-phosphate in complex biological materials. *Naunyn Schmiedeberg Arch Pharmacol* 363:358-63
- Sanna MG, Liao J, Jo E, Alfonso C, Ahn MY, Peterson MS (2004) Sphingosine 1-phosphate (S1P) receptor subtypes S1P1 and S1P3, respectively, regulate lymphocyte recirculation and heart rate. *J Biol Chem* 279:13839-48
- Sanna MG, Wang S-K, Gonzalez-Cabrera PJ, Don A, Marsolais D, Matheu MP *et al.* (2006) enhancement of capillary leakage and restoration of lymphocyte egress by a chiral S1P₁ antagonist *in vivo*. *Nat Chem Biol* 2:434-41
- Schuppel M, Kurschner U, Kleuser U, Schafer-Korting M, Kleuser B (2008) Sphingosine 1-phosphate restrains insulin-mediated keratinocyte proliferation via inhibition of Akt through the S1P(2) receptor subtype. *J Invest Dermatol* 128:1747-56
- Seo EY, Park GT, Lee KM, Kim JA, Lee JH, Yang JM (2006) Identification of the target genes of atopic dermatitis by real-time PCR. *J Invest Dermatol* 126:1187-9
- Tedesco-Silva H, Mourad G, Kahan BD, Boira JG, Weimar W, Mulgaonkar S *et al.* (2005) FTY720, a novel immunomodulator: efficacy and safety results from the first phase 2A study in *de novo* renal transplantation. *Transplantation* 79:1553-60
- von Wenckstern H, Zimmermann K, Kleuser B (2006) The role of the lysophospholipid sphingosine 1-phosphate in immune cell biology. *Arch Immunol Ther Exp* 54:239-51
- Wood LC, Jackson SM, Elias PM, Grunfeld C, Feingold KR (1992) Cutaneous barrier perturbation stimulates cytokine production in the epidermis of mice. *J Clin Invest* 90:482-7