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Fingolimod targeting protein phosphatase 2A differently affects IL-33 induced IL-2 and IFN-γ production in $CD8^+$ lymphocytes

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Abbreviations

EAE: experimental autoimmune encephalomyelitis, **FTY720**: fingolimod, **MS**: Multiple Sclerosis, **MOG**: myelin oligodendrocyte glycoprotein, **IL-33**: interleukin-33, **JC virus**: John Cunningham virus **pb**: plate bound, **PML**: progressive multifocal leukoencephalopathy, **PP2A**: protein phosphatase 2a, **sb**: soluble, **S1P**: sphingosine-1-phosphate, **S1PR**: sphingosine-1-phosphate-receptor, **SphK**: sphingosine kinase

Abstract

Multiple Sclerosis patients are treated with fingolimod (FTY720), a prodrug that acts as an immune-modulator. FTY720 is first phosphorylated to FTY720-P and then internalizes sphingosine-1-phosphate-receptors, preventing lymphocyte sequestration. IL-33 is released from necrotic endothelial cells and contributes to MS severity by coactivating T cells. Herein we analyzed the influence of FTY720, FTY720-P and S1P on IL-33 induced formation of IL-2 and IFN-γ, by using IL-33-receptor overexpressing EL4 cells, primary $CD8^+$ T cells and splenocytes. EL4-ST2 cells released IL-2 after IL-33 stimulation that was inhibited dose-dependently by FTY720-P but not FTY720. In this system, S1P increased IL-2, and accordingly, inhibition of S1P producing sphingosine kinases diminished IL-2-release. In primary $CD8^+$ T cells and splenocytes IL-33/IL-12 stimulation induced IFN-γ, which was prevented by FTY720 but not FTY720-P, independently from intracellular phosphorylation. The

inhibition of IFN-γ by non-phosphorylated FTY720 was mediated via the SET/protein phosphatase 2A (PP2A) pathway, since a SET peptide antagonist also prevented IFN-γ formation and the inhibition of IFN-γ by FTY720 was reversible by a PP2A inhibitor. While our findings directly improve the understanding of FTY720 therapy in MS, they could also contribute to side effects of FTY720 treatment, like progressive multifocal leukoencephalopathy, caused by an insufficient immune response to a viral infection.

Introduction

Multiple sclerosis is an autoimmune disorder, characterized by the destruction of the myelin sheath by auto-reactive immune cells, leading to astrogliosis, demyelination and increasing disability. Most patients are diagnosed between 20 and 40 years, with a 2:1 female to male ratio [1]. Fingolimod [2-amino-2-(2-(4-octylphenyl)ethyl)-propane-1,3-diol; FTY720] is an immune-modulating prodrug derived from the natural compound myriocin and was approved in 2010 as the first oral treatment for relapsing-remitting MS. Its phosphorylated derivative is an analog of the bioactive sphingolipid sphingosine-1-phosphate (S1P), which is involved in cellular processes such as cell cycle, apoptosis or immune modulatory processes such as migration or the regulation of cytokine expression [2, 3]. Extracellular lipophilic FTY720 or sphingosine easily passes the membrane of immune cells to the intracellular space. Here, sphingosine is phosphorylated by the sphingosine kinases (SphK) SphK1 and SphK2 to S1P, whereas FTY720 is phosphorylated by SphK2 to fingolimod-phosphate (FTY720-

P) [4]. Once phosphorylated, the amphiphilic nature of FTY720-P or S1P prevents their free passage across membranes. Consequently, they have to be exported out of cells by specialized transporters (mainly members of the ABC transporter family are discussed such as ABCC1 or SPNS2) [5, 6]. Only then, both S1P and FTY720-P may mediate their effects on extracellular domains of the S1P receptors in the plasma membrane. S1P binds and activates five known G-protein-coupled S1P receptors S1PR1 – S1PR5 (EC_{50} 5 – 10 nM [7]) with a subsequent single or multiple activation of Gαi, GαQ or Gα12/13 pathways [8]. FTY720-P exhibits a sufficient affinity to activate S1PR1, 3, 4 and 5 [9]. Upon binding of S1P or FTY720-P the receptors internalize, but the internalization is prolonged upon FTY720-P-binding compared to S1P-binding. FTY720-P-occupied receptors are not readily recycled but ubiquitin-dependently degraded inside the cell to a greater extent, reducing the total number of receptors being recycled [1]. The prolonged internalization of S1PR1 by FTY720-P represents the general accepted mode of action of FTY720-treatment in MS. It leads to an inhibition of central memory T-cell egress from secondary lymphoid organs and results in peripheral lymphocyte depletion. Side effects of FTY720 treatment in MS are e.g. bradycardia and second-degree atrioventricular block which is thought to be mediated by agonistic effects of FTY720-P on the S1PR3 receptor [10]. Additionally, FTY720 treatment does not result generally in a higher incidence of infections, but especially higher incidences of lower-respiratory tract infections could be observed [11]. Additionally, cases of FTY720-treated patients with John Cunningham (JC) virus infection leading to progressive multifocal leukoencephalopathy (PML) and cases of haemophagocytic syndrome (HPS) were reported [11]. HPS is a result of an uncontrolled cytokine release from NK and T cells by different stimuli, particularly the Epstein-Barr virus. However, FTY720 is of course an immune-modulatory drug, but the current mechanism of action of FTY720-treatment does not mechanistically explain these side effects [11, 12].

In addition to the extracellular S1P receptors, more recently intracellular effects of non-phosphorylated FTY720 have been described, like e.g. the activation of the protein phosphatase 2a (PP2A) by FTY720 but not FTY720-P [13, 14]. Here, non-phosphorylated FTY720 bound a hydrophobic pocket of the PP2A inhibitor SET which led to SET inactivation and subsequent PP2A activation. So far, this mechanism has been prominently discussed for the anti-cancer activities of non-phosphorylated FTY720, because the activation of PP2A enhances necrosis, apoptosis and cell death [15, 16]. Along these lines, a specific SET peptide antagonist OP449 was developed, which potently inhibited the growth of malignant B-cells [17]. Furthermore, FTY720 but not FTY720-P is known to bind and inhibit the cytosolic phospholipase A2 (cPLA2), which is the rate-limiting enzyme for the biosynthesis of eicosanoids. Consequently, FTY720 attenuated the antigen induced secretion of PGD_2 in mast cells [18].

Interleukin-33 (IL-33) is a member of the IL-1 family of cytokines and is constitutively expressed in endothelial cells and stored in the nucleus. Bioactive IL-33, escaping caspase 3/7-dependent inactivation is released from necrotic cells and signals as an "*alarmin*" through ST2-IL-1-RacP-TIR, IRAK1/4 and Myd88 as downstream components [19, 20]. An involvement of IL-33 and its receptor ST2 was proposed in MS. In human MS patients, IL-33 protein was elevated in plasma, highly expressed in peripheral leukocytes and released in MS plaques [21]. In a myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ peptide-induced autoimmune encephalomyelitis (EAE) mouse model of MS, IL-33 and its receptor was markedly elevated in the spinal cord of mice and an anti-IL-33 antibody inhibited the onset and severity of EAE, whereas administration of IL-33 worsened the disease by inducing IFN-γ and IL-17 [22]. IL-33 drives both, T_H1 and T_H2 i mmunity, which is dependent on the cell type and the local cytokine milieu. As originally described, IL-33 robustly stimulated T_H2

cytokines like IL-5 and IL-13 [23]. Moreover, IL-33 induced IL-2, the classical lymphocyte and NK cell growth or T_{reg} activating factor [24]. The blockade of IL-2 receptor (IL-2R) signaling with an αIL-2R antibody (*Daclizumab*) is currently under investigation and passed the clinical phase III trial for the treatment of MS [25]. IL-33 is also involved in T_H1 immunity by e.g. enhancing T_H1 NK cell function or by enhancing the $CD8^+$ T-cell antigen-specific tumor immunity and IFN-γ formation in a human papilloma virus (HPV)–associated model for cancer immunotherapy [26, 27] While enhanced lysis of tumor cells by IL-33 costimulated $CD8^+$ T cells may be beneficial for cancer patients, in MS patients, this particular activity of IL-33 may worsen MHC I-restricted astrogliosis. Recently it was confirmed that $CD8^+$ T cells are present in human MS lesions, outnumber $CD4^+$ T cells and sequencing of their TCR revealed a limited number of T-cell clones in human MS plaques. The latter data suggested that $CD8^+$ T-cell clones mediate direct MHC-I/ antigen-specific cytotoxic effects [28, 29]. Furthermore IL-33 is known to induce IFN-γ formation from $CD8^+$ T cells or NK cells [30, 31]. IFN-γ promotes a T_H1 cytokine milieu for $CD8^+$ T cells or can drive Fas-mediated apoptosis of oligodendrocytes itself [31, 32].

Besides the accepted mode of action for FTY720-treatment, our group gathered increasing evidence for alternative immune-modulating properties of FTY720, FTY720-P and the S1P/S1PR axis in the recent years. We have shown that SphK1-mediated S1P/S1PR1 signaling specifically counteracted the LPS-induced IL-12p70 production in immune cells of the spleen [2]. Extensive *in vivo* studies showed that FTY720 ameliorated T_H1 and oxazolone-induced T_H2 colitis. Already our 2007 investigation in T_H2 colitis clearly demonstrated that FTY720 inhibited the IL-33-induced production of T_H2 cytokines *ex vivo* [33, 34]. Interestingly, FTY720 but not FTY720-P was specifically able to inhibit the LPS-induced IFN-γ and IL-17 production in transgenic ovalbumin (OVA)-TCR-specific $CD4^+$

and $CD8^+$ spleen cells (OT-II/OT-I), while having no effect on other cytokines [13]. These findings raise the question, whether the beneficial effects of fingolimod treatment in MS are restricted to an inhibition of the S1PR1-mediated lymphocyte egress, but also due to direct effects on the cytokine production of immune cells. Herein we will demonstrate that the IL-33/ST2-TIR-pathway is regulated by S1P, FTY720 and FTY720-P, which not only suggests additional benefit for MS patients treated with FTY720 but may also partly explain undesired side effects of FTY720 therapy, such as reactivated viral infections.

Results

FTY720-P and S1P differently regulate the IL-33-induced IL-2 formation in EL4-ST2 cells

The murine lymphoma cell line EL4 was first used in the 1980s for studying IL-1β signaling, by the detection of IL-1β induced IL-2 production into the medium supernatant [35]. In the same cells, IL-33 signaling was analyzed about 20 years later, because the IL-1- and the ST2-receptor share the same downstream components [19]. Herein we used stable ST2-transduced EL4 cells as a model for IL-33 induced immune cell activation. As shown in figure 1 A, the cells produced IL-2 as a functional result of IL-33 stimulation, which was enhanced by the addition of different concentrations of ionomycin. The calcium ionophor increased IL-2 formation by co-activation of TCR-dependent kinases (e.g. calmodulin/calcineurin), but itself failed to induce IL-2 in a concentration up to 1000 nM [36]. EL4-ST2 cells express the S1P receptors S1PR2, S1PR4 and S1PR5, as shown with a semi-quantitative RT-PCR in figure 1 B. To analyze the effect of the S1PR-axis on IL-33 mediated responses, we

stimulated the cells with either IL-33 alone (Figure 1 C) or in combination with ionomycin (Figure 1D) and co-incubated different concentrations of S1P, FTY720 and FTY720P. Interestingly, the natural S1PR agonist S1P was able to increase IL-2 formation under both experimental conditions by up to 31 % (Figure 1 C, D). In contrast, the functional S1PR-antagonist FTY720-P inhibited IL-2 formation by 27 % at the highest concentration. However, non-phosphorylated FTY720 failed to inhibit IL-2 formation and increased IL-2 in coincubation with ionomycin at 1 µM.

Inhibition of sphingosine kinases inhibits IL-33 induced IL-2 formation

Since S1P was able to increase IL-2 formation, we were interested if inhibition of S1P-producing sphingosine kinases *vice versa* inhibits IL-2 formation. Therefore, we utilized the SphK1-selective inhibitor PF543 (IC_{50} *in vitro* 2 nM), the SphK1/2-dual inhibitor SKI-II (IC_{50} 0.5 – 1.1 µM) and the SphK2 selective inhibitor ABC294640 (K_i 9.8 µM) [37, 38]. In EL4-ST2 cells stimulated with IL-33 alone, PF543 did not inhibit IL-2 formation; however, SKI-II significantly attenuated IL-2 formation at high concentrations (Figure 2 A). With coincubation of ionomycin, PF543 reduced IL-2 and SKI-II inhibited IL-2 even stronger as in the absence of the calcium ionophore (Figure 2 B). The SphK2 selective inhibitor ABC294640 inhibited IL-2 formation very potently at higher concentrations under both experimental conditions (Figure 2 C, D). Since PF543 and SKI II revealed different potencies in inhibiting IL-2 release with or without ionomycin, we further investigated whether IL-33 or ionomycin regulated the expression of sphingosine kinases by qRT-PCR. We determined a 5-7 fold upregulation of SphK1 but not SphK2 exclusively by ionomycin (Figure 2 E, F). IL-33 alone had no effect on kinase expression. Since inhibition of SphK1 resulted in diminished IL-2 formation during ionomycin co-stimulation, we anticipated a specific interaction of SphK1 with the IL-33-mediated IL-

2 production. However, since the dual and the SphK2 selective inhibitor showed effects in both settings, we further hypothesized that SphK2 may also play a role for IL-33 signaling.

IL-33 induces IFN-γ formation in splenocytes and CD8$^+$ T cells

To further analyze immune-modulating properties of FTY720, FTY720-P and S1P, we investigated the influence of IL-33 stimulation on primary murine splenocytes and CD8$^+$ T cells (for purity see Supporting Information Figure 1). Without co-activation, IL-33 failed to induce IFN-γ, IL-2, IL-17 or TNFα (data not shown). However, IL-33 stimulation slightly augmented IFN-γ formation from soluble (sb) αCD3 TCR-stimulated splenocytes and plate bound (pb) αCD3 stimulated CD8$^+$ T cells (Figure 3 A, C). In coincubation experiments with IL-12, IL-33 stimulated a very prominent formation of IFN-γ (Figure 3 B, D). IFN-γ formation was also detected by coincubation of the structural related IL-1β with IL-12 (Supporting Information Figure 2). Microscopically, pooled splenocytes or CD8$^+$ T cells revealed no typical signs of TCR activation following IL-33/IL-12 stimulation (Supporting Information Figure 3), i.e. typical "cluster formation" that may be attributed to the absence of IL-2 in IL-33/IL-12 stimulated cells. No formation of IL-17 or TNFα was detectable in cellular supernatants (data not shown). We therefore chose a combination of IL-33 and IL-12 stimulation for our subsequent experiments and analyzed the influence of S1P receptor signaling especially on IFN-γ formation.

FTY720 but not FTY720-P inhibits IL-33/IL-12 induced IFN-γ from splenocytes and CD8$^+$ T cells

After settling optimal stimulatory conditions for our cells, we further analyzed immune-modulatory effects of the S1PR-axis on IL-33/IL-12 induced IFN-γ formation (Figure 4). Splenocytes contain numerous immune cells which produce IFN-γ by appropriate stimuli, like natural killer (NK) cells, innate lymphoid cells (ILC) or $CD8^+$ T cells. All of these express different sets of S1P receptors [8]. $CD8^+$ T cells predominantly express S1PR1 and S1PR4 [39]. Coincubation of S1P with IL-33/IL-12-stimulated cells led to an inhibition of IFN-γ formation by 24 % in $CD8^+$ T cells, an effect only apparent at the highest concentration. However, this effect was not seen in pooled splenocytes (Figure 4 A, B). The IL-33/IL-12 induced IFN-γ formation in splenocytes and $CD8^+$ T cells was unaffected by FTY720-P (Figure 4 C, D). Surprisingly, non-phosphorylated FTY720 prominently abrogated IFN-γ release of splenocytes and $CD8^+$ T cells by up to 86 % (Figure 4 E, F).

Activation of the protein phosphatase 2A mediates the inhibition of IFN-γ by FTY720

While S1P and FTY720-P exhibited only minor effects on IL-33/IL-12 induced IFN-γ formation, we were interested how mechanistically non-phosphorylated FTY720 mediated its effect. Extracellular FTY720-P or S1P are not able to pass the membrane of immune cells because of their amphiphilic properties. To analyze if FTY720 or FTY720-P have additional intracellular targets besides the extracellular S1P receptors, we used SphK2 -/- splenocytes. FTY720 is predominantly phosphorylated to FTY720-P by SphK2 which is prevented in SphK2 -/- splenocytes [40]. As expected, SphK1 but not SphK2 mRNA expression was detectable by qRT-PCR in SphK2-/- splenocytes, confirming the complete knockout of the enzyme (Figure 5 A). Interestingly, FTY720 was still able to inhibit IL-33/IL-12 induced IFN-γ formation in the absence of SphK2 (Figure 5 B). Consequently, FTY720 must mediate its effect by an intracellular target, independent from FTY720-P and the extracellular

S1P receptors. FTY720 but not FTY720-P was shown to antagonize SET, which subsequent led to PP2A activation [14]. To test, whether FTY720 also mediates its effect on IFN-γ formation by this pathway, we utilized the SET peptide inhibitor OP449 [17, 41]. Interestingly, OP449 led to a comparable inhibition of IL-33/IL-12 induced IFN-γ formation by $CD8^+$ T cells and completely abrogated IFN-γ formation at 1 μM (Figure 5 C). To further prove that FTY720 inhibits IFN-γ formation via SET/PP2A, $CD8^+$ T cells were stimulated with FTY720 in coincubation with the PP2A inhibitor okadaic acid (Figure 5 D). While non-phosphorylated FTY720 reduced IFN-γ formation, rising concentrations of the PP2A inhibitor okadaic acid restored this effect, while having minor effects on IFN-γ itself. This indicates that within the cytosolic compartment non-phosphorylated FTY720 inactivated SET which subsequently led to PP2A activation. Coincubation with the PP2A inhibitor okadaic acid counteracted this effect and IFN-γ formation returned to base line.

Discussion

Since its approval for the clinic in 2010, fingolimod is a widely used drug for the treatment of relapsing-remitting MS. The prolonged internalization of S1PR1 by FTY720-P, resulting in inhibition of lymphocyte egress and lymphopenia is widely accepted as a mode of action [1]. While it was shown that alarmins like IL-33 contribute to MS severity, by e.g. inducing IFN-γ and IL-17 in an EAE mouse model for MS, we were interested whether the therapeutic effects of FTY720 are at least partly due to a direct immune-modulatory and immunosuppressive effect of FTY720 and FTY720-P [22].

S1P and FTY720-P differently modulate the IL-33 induced formation of IL-2

IL-2 is a double-edged sword in immunity, either promoting activation and proliferation of effector T cells, or inducing regulatory T cells in the absence of other cytokines [42]. In MS, IL-2 contributes to disease-progression; therefore, an αIL-2R antibody (*Daclizumab*) showed promising beneficial effects in a clinical phase III trial [25]. We first analyzed the effect of the IL-33 induced formation of IL-2 in ST2 transduced EL4 cells (Figure 1 A). This cell line expresses S1PR2, S1PR4 and S1PR5 on mRNA level (Figure 1 B) which has been previously confirmed by our group with another set of primers [13]. The expression pattern of S1P receptors is in line with the expression of NK- and B-cell markers of EL4 cells, with S1PR4 expressed by B cells and S1PR5 highly expressed by NK cells [8, 43]. In EL4-ST2 cells, S1P further enhanced IL-2 formation whereas FTY720-P inhibited IL-2 following stimulation with IL-33 with or without TCR co-activation (Figure 1 C, D). While S1P is the endogenous agonist of S1P receptors, FTY720-P is a partial S1P receptor antagonist for all S1P receptors, except S1PR2. Therefore, we hypothesize that the agonistic effect of S1P on IL-33 induced IL-2 formation may be mediated by agonistic effects on S1P receptors, while the inhibitory effects from the FTY720-P is mediated by its partial antagonistic activities. FTY720 was not inhibiting IL-2 in both experimental conditions, although we determined an inhibition of IL-33 induced IL-2 in the same cells stimulated with 5 ng/ml IL-33 in one of our previous publications [13]. Thus, FTY720 can inhibit IL-2 formation exclusively, when the cells are stimulated with very low concentrations of IL-33, indicating a low turnover rate from FTY720 to FTY720-P in EL4-ST2 cells.

Inhibition of sphingosine kinases inhibits IL-2 formation of EL4-ST2 cells

While S1P revealed agonistic activities on IL-2 formation, we were interested if inhibition of sphingosine kinases would - *vice versa* - inhibit IL-2 release. Therefore, we employed the SphK1-selective inhibitor PF543, the SphK1/2 dual inhibitor SKI-II and the SphK2-dual inhibitor ABC294640. SKI-II but not PF543 was able to inhibit IL-2 in IL-33 stimulated EL4-ST2 cells, which most likely was due to the dual inhibition of both kinases by SKI-II (Figure 2 A). In the presence of ionomycin PF543 abrogated IL-33-induced IL-2 expression and SKI-II inhibited IL-2 even stronger (Figure 2 B). We conclude that the enhanced capacity of both inhibitors to dampen IL-2 formation is due to an induction of SphK1 activity by ionomycin (Figure 2 E, F). The SphK2-selective inhibitor ABC294640 inhibited IL-2 at higher concentrations, which is in line with the K_i of 9.8 μM and in line with the results of the SphK1/2 dual inhibitor SKI II. These results consistently demonstrate that the IL-33 induced formation of IL-2 in EL4-ST2 cells was increased by S1P, and correspondingly blocked by inhibition of the S1P producing kinases. Ionomycin costimulation caused, besides activating TCR-dependent kinases, also a positive feedback-loop by the SphK1/2-S1P-S1PR axis which enhanced IL-2 formation. Along these lines, SphK1 activity was shown to increase in αCD3 TCR activated T_H1 DO11.10 T cells by Yang and colleagues [44]. However, they found an inhibition of IL-2 formation by SphK1 overexpression, although they missed to measure the expression of S1P receptors of DO11.10 cells. According to our understanding the effects of S1P are definitely dependent on the expression of S1P receptors and associated G protein signaling.

IL-33 synergized with TCR-dependent and independent stimuli to induce IFN-γ

We were further interested whether the S1P receptor axis has comparable effects on the cytokine formation of primary murine splenocytes and $CD8^+$ T cells. Therefore, we first focused on the

induction of IFN-γ and IL-2 in both cell types by stimulation with IL-33 together with an αCD3 antibody or IL-12 (Figure 3). IFN-γ was slightly induced by IL-33 in αCD3 costimulated $CD8^+$ T cells and splenocytes, even if in the latter case statistical significance was not achieved (Figure 3 A, C). While IL-33 was first described to induce T_H2 immunity, it also induces IFN-γ release especially from $CD8^+$ T and NK cells [30, 31]. Particularly, in $CD8^+$ T cells the IL-33 receptor ST2 was demonstrated to be regulated in a T-bet dependent manner, the master transcription factor for T_H1 immunity [31]. Interestingly IL-2 was not further increased by αCD3/IL-33 stimulated splenocytes and $CD8^+$ T cells. Costimulation of IL-33 together with IL-12 revealed a prominent formation of IFN-γ without formation of IL-2 (Figure 3 B, D). As expected, no obvious signs for TCR activation had been detected under these stimulatory conditions, like adherence or cluster formation all of which in agreement with an absence of IL-2 (Supporting Information Figure 3). As nicely phrased by Freeman and colleagues, such TCR-independent cytokine stimulations allow $CD8^+$ T cells to act as "*sentinels*" and promote a T_H1 cytokine milieu with huge amounts of IFN-γ [45]. Because TCR activation is missing, this stimulation is not accompanied by an additional induction of IL-2.

FTY720 inhibits IFN-γ formation via the SET/PP2A pathway

To analyze the effect of S1P receptor signaling on IFN-γ formation, we focused on the IL-33/IL-12 costimulation for our subsequent experiments. Here, S1P and FTY720-P had only minor effects on IFN-γ formation (Figure 4 A-D), but interestingly non-phosphorylated FTY720 was prominently inhibiting IFN-γ in splenocytes and $CD8^+$ T cells (Figure 4 E-F). Our group already investigated that FTY720 but not FTY720-P was specifically able to inhibit the Ovalbumin/LPS-induced IFN-γ formation in transgenic ovalbumin $CD8^+$-spleen cells (OT-II) which was reversible by okadaic acid

[13]. While preparing this manuscript the group of Ntranos showed that FTY720 also inhibited the αCD3/αCD28 induced production of IFN-γ and cytotoxicity of $CD8^+$ T cells *in vitro* and *in vivo* [46]. These results indicate that the effect of FTY720 is conserved for IFN-γ and not dependent on the stimulus. Non-phosphorylated FTY720 will not reach high nanomolar concentrations in the blood of MS patients, but in a previous study we analyzed the subcellular distribution of FTY720 and FTY720-P in immune cells of the spleen and showed that both substances dramatically accumulate in these splenocytes several hundredfold [40]. Thus to outline consequences of our data, the inhibition of IFN-γ by FTY720 could be beneficial but on the other hand bears additional risks. It is well known, that T_H1 immunity and IFN-γ contributes to the clearance of intracellular pathogens and as noted in the introduction, FTY720 treated patients showed

specific side-effects caused by viral infections. The additional effect of FTY720 on IFN-γ formation could also contribute to the latest cases of FTY720-treated patients with JC virus infection, leading to PML or other virus-related side-effects [11, 46].

The effect of FTY720 on IFN-γ was independent of SphK2, which mediates the phosphorylation from FTY720 to FTY720-P and reversible by the PP2A inhibitior okadaic acid. The specific SET peptide antagonist OP449 also inhibited IFN-γ formation comparable to FTY720 (Figure 5 A - D). Furthermore PP2A and its inhibitor SET are already known to regulate IL-18/IL-12 induced IFN-γ formation in human NK cells. Here, SET-downregulation and subsequent PP2A activation inhibits IFN-γ release and vice versa [47, 48]. SET/PP2A was also shown to regulate IFN-γ formation by murine $CD8^+$ T cells. IFN-γ mRNA expression was associated with increased SET mRNA expression. Along these lines, IL-18/IL-12 induced IFN-γ production by murine $CD8^+$ was inhibited by forskolin,

a known PP2A activator which also decreased p-STAT4 levels [49]. Since FTY720 and not FTY720-P is known to bind a hydrophobic pocket of SET which led to SET inactivation and PP2A activation, our data indicate that FTY720 inhibits IFN-γ via this pathway. Downstream of SET, active PP2A may then dephosphorylate and inhibit IFN-γ-related transcription factors, e.g. IFN-γ related pSTAT1 and pSTAT4 [49, 50]. The group of Ntranos demonstrated, that the FTY720-mediated IFN-γ inhibition of αCD3/αCD28 stimulated $CD8^+$ T cells was rescued by arachidonic acid supplementation, indicating a possible involvement of the cytosolic phospholipase A2 (cPLA2) [46]. Interestingly, cPLA2 appeared to be inhibited by non-phosphorylated FTY720 and by dephosphorylation through PP2A [18, 51]. Therefore, in principle interference of FTY720 with SET/PP2A and cPLA2 may contribute to the observed effects here. However, while cPLA2 is highly expressed and active in phagocytes, e.g. monocytes, its expression especially in T cells is neglectable or at least controversially discussed [52].

Taken together we found a strong regulation of the IL-33 pathway by FTY720, FTY720-P and S1P, depending on the cell type and the production of effector cytokines. EL4-ST2 cells, a possible model of T_H2 and T_{regs}, produced IL-2 after IL-33 stimulation which was further enhanced by S1P and inhibited by FTY720-P as well as by inhibition of sphingosine kinases. In primary $CD8^+$ T cells and splenocytes, IL-33 together with IL-12 or αCD3 induced IFN-γ. This was slightly inhibited by S1P in $CD8^+$ T cells and prominently suppressed by FTY720 and not FTY720-P via the SET/PP2A pathway. While our findings directly improve the understanding of FTY720 therapy in MS, they strongly support the development of alternative sphingolipid analogs in autoimmunity. So far, sphingosine analogs have been preferentially optimized for their S1PR specificity, e.g. to prevent S1PR3-dependent bradycardia [10]. Based on our data, the capacity of sphingosine analogs to additionally

modulate the immune response through S1PR-independent intracellular targets should be taken into consideration when discussing further analogs and their risk-benefit ratio.

Materials and Methods

Material

Sphingosine-1-Phosphate (Avanti Polar Lipids, Alabaster, USA), FTY720 and FTY729-P (Novartis, Basel, Switzerland), SKI II (Calbiochem, Darmstadt, Germany), PF543 (Merck, Darmstadt, Germany), ABC294640 (ActiveBiochem, Maplewood, USA), okadaic acid (Alexis Biochemicals, Redfern, Australia) and OP449 (kindly provided by Michael Vitek, Oncotide Pharmaceuticals, Durham, USA) were diluted in dimethylsulfoxide (DMSO), H_2O or PBS for stock solutions. Interleukins were purchased from PeproTech (Hamburg, Germany) and diluted in PBS/0.1 % BSA. An αCD3 antibody (clone 145-2C11) was purchased from eBioscience (Frankfurt, Germany). Cell culture plates and flasks were purchased from Greiner bio-one (Frickenhausen, Germany). All other cell culture reagents were purchased at the highest purity and cell culture grade from Sigma (Steinheim, Germany), Life Technologies (Darmstadt, Germany) and Invitrogen (NY, USA) if not stated otherwise.

Cultivation of cell lines and primary cells

EL4-6.1 cells, stably transduced with the IL-33 receptor ST2 (EL4-ST2) were kindly provided by Michael U. Martin (Immunology, Giessen [19]) and cultivated in RPMI with 5% FCS, 100 IU/ml penicillin and 100 IU/ml streptomycin. Female C57BL/6 wild type mice were purchased from Janvier Labs (Saint Berthevin Cedex, France). Female SphK2-deficient (SphK2-/-) mice backcrossed to the C57BL/6 background, were bred at *mfd Diagnostics* (Mainz, Germany) under specific pathogen-free conditions. Whole spleens were dissected, gently homogenized with a glass tissue homogenizer (OMNI International, USA) and after erythrolysis cultured in Iscove‘s medium supplemented with 5% FCS (PAA Laboratories), 2mM l-glutamine, 100 IU/ml penicillin, 100 g/ml streptomycin, 15 µM Ciprofloxacin, 1mM sodium pyruvate, 100 µM nonessential amino acids and 50 µM 2-ME in an humidified incubator (5 % CO_2 and 37 °C). $CD8^+$ T cells were isolated out of splenocytes with an EasySep™ Mouse $CD8^+$ T-cell enrichment kit (Stemcell Technologies, Köln, Germany) according to the manufacturer’s instructions and cultured in RPMI with 10 % FCS (PAA Laboratories) and additives for splenocytes listed above. T cells were routinely checked for their purity by αCD3 (arm. hamster, IgG1, FITC; BD Biosciences, Heidelberg, Germany) and αCD8 (rat, IgG2a, APC; R&D, Minneapolis, USA) staining and analysis with a FACS Canto II flow cytometer (BD Biosciences, Heidelberg, Germany) (Supporting Information Figure 1).

Stimulation of EL4-ST2 cells, splenocytes and $CD8^+$ T cells

0.25 x 10^6 EL4-ST2 cells/ml were stimulated for 24 hours and 0.5 x 10^6 $CD8^+$ T cells/ml were stimulated for 44 h. 1 x 10^6 splenocytes/ml were seeded out in 12-well plates and left untreated for 24 h with a subsequent stimulation for 44 h. For stimulation of $CD8^+$ T cells with 1µg/ml plate bound

(pb) αCD3, the antibody was coated o/n at 4° C in 96-u-well plates. Then 1 x 10^6 $CD8^+$ T cells/ml were stimulated for 44 hours. Viability of cells was analyzed by trypan blue staining with a T20 automated cell counter (Bio-Rad, München, Germany). IL-33 dependent IL-2 or IFN-γ production into the medium supernatant was quantified with ELISA kits (R&D, Minneapolis, USA) according to the manufacturer's instructions.

RNA isolation, synthesis of cDNA and quantitative real time PCR

Cells were pelleted and RNA isolated with a peqGOLD Total RNA kit (peqlab, Erlangen) due to the manufacturer instructions. Equal RNA amounts were transcribed into cDNA by reverse transcriptase with a Precision nanoScript Reverse Transcription Kit (Primerdesign, Southampton, England) executed with a standard RT-PCR program (65 °C, 5 min, 55°C, 20 min, 75°C, 15 min). Expression analysis in a semi-quantitative PCR was performed in 50 µl reactions using 2 µl of cDNA, 5 u/µl Platinum *Taq* polymerase (Life Technologies, Darmstadt, Germany), 10 mM dNTPs, 50 mM $MgCl_2$, 10 pmol/µl sense- and anti-sense primer (Table 1) and H_2O in 27–33 cycles of 94°C (1 min), 60°C (1 min) and 72°C (2 min). PCR fragments were separated on 12% polyacrylamide gels in Tris-acetate-EDTA buffer and visualized by Gelred staining (Biotium, Hayward, USA). For quantitative real time PCR the 5`FAM marked testing probes of CsnK2a2 and FBX038 as house-keeping genes (Primer Design, Southampton England) respectively SphK1 and SphK2 (Applied Biosystems, Californien, USA, Mm00448841_g1, Mm00445021_m1 and Mm00772700_m1, final concentration: 250 nM) were supplied as complete reaction mixtures. The qRT-PCR were done in triplicates for every sample with 5 µl Precision FAST 2x qPCR MasterMix (Primer Design, Southampton England), 3.5 µl H_2O, 0.5 µl 5`FAM marked testing probes and 1 µl cDNA with the following program: 1x 95°C, 5 min,

afterwards alternating three seconds with 95°C and 30 seconds with 60°C (40x). Not regulated expression of CsnK2a2 and FBX038 RNA genes were employed to create a normalized ratio and to determine the increase of mRNA expression in comparison to untreated cells as a control.

Statistics

Statistical analyses were performed with GraphPad Prism 5.0 software (La Jolla, CA/USA) using one-way ANOVA with Dunnett's posttest with ‚ns' for $p > 0.05$, * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$. In some cases the results are shown as relative concentrations to compensate inter-assay differences due to e.g. preparation, batch of ACK puffer, age and genotype of mice.

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Florian Ottenlinger performed the experiments, wrote the manuscript, performed statistics and designed the figures. Anja Schwiebs, Kathrin Pfarr, Annika Wagner and Christoph Mayer helped by designing, planning and performing experiments and by writing the manuscript. Sophia Grüner performed experiments for the revision. Michael Martin supplied us with EL4-ST2 cells. Stephanie Bourdy kindly optimized the EL4-ST2 bioassay and Alexander Koch supplied RT-PCR-primer for the S1P receptors and Spk2-/- mice. Michael Vitek (Oncotide Pharmaceuticals, Durham, USA) kindly provided OP449. J.M. Pfeilschifter supplied basic lab equipment. H.H. Radeke had the idea, designed

and closely supervised all experiments, checked all data in detail and wrote and finalized the manuscript. We thankfully acknowledge the excellent technical support of Martina Herrero San Juan. We also acknowledge the financial support of the Else Kröner-Fresenius-Graduiertenkolleg and the Translational Research Innovation Pharma (TRIP) funded by the Else Kröner-Fresenius Stiftung. This work was further 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 'signaling by fatty acid derivatives and sphingolipids in health and disease'.

Conflict of interest

The authors haven no financial conflict of interest.

Table 1: Primer pairs for semi-quantitative RT-PCR

Gene	Sense [5′–3′]	Antisense [5′–3′]	Length [bp]
S1PR1	TTCTCATCTGCTGCTTCATCATCC	GGTCCGAGAGGGCTAGGTTG	117
S1PR2	TTACTGGCTATCGTGGCTCTG	ATGGTGACCGTCTTGAGCAG	107
S1PR3	TGCCCTTCTCTATGTCACC	TCTTTCCACCTCAACTCCC	139
S1PR4	CTGTCAGGGACTCGTACC	CGTGAAGAGCAGACTGAAG	105
S1PR5	GAGGTTATTGTCCTTCACTAC	AAGAGCACAGCCAAGTTC	140
18S	GAAACGGCTACCACATCCAAG	CGGGTCGGGAGTGGGT	59

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Figure 1: FTY720-P but not FTY720 inhibits the IL-33 induced IL-2 formation in EL4-ST2 cells. (**A**) EL4-ST2 cells were incubated with the annotated concentrations of IL-33 and ionomycin. The supernatants were collected after 24 h for the measurement of IL-2 (**B**) Representative PAA-gel showing S1P receptor expression in EL4-ST2 cells on mRNA level. Data shown are representative of 2 independent experiments, confirmed by qRT-PCR with a different set of primer [13]. (**C**/**D**) FTY720, FTY720-P or S1P was incubated for 24 h on EL4-ST2 cells stimulated with (**C**) 20 ng/ml IL-33 or (**D**) 5 ng/ml IL-33 + 250 nM Ionomycin. (**C** and **D**) A normalized ratio was calculated with the unstimulated control as a reference to compensate for interassay differences (indicated by the dotted line). (**A**, **C** and **D**) Data are shown as mean ± SD and are pooled from n = 4 – 7 independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$; one-way ANOVA with Dunnett's posttest.

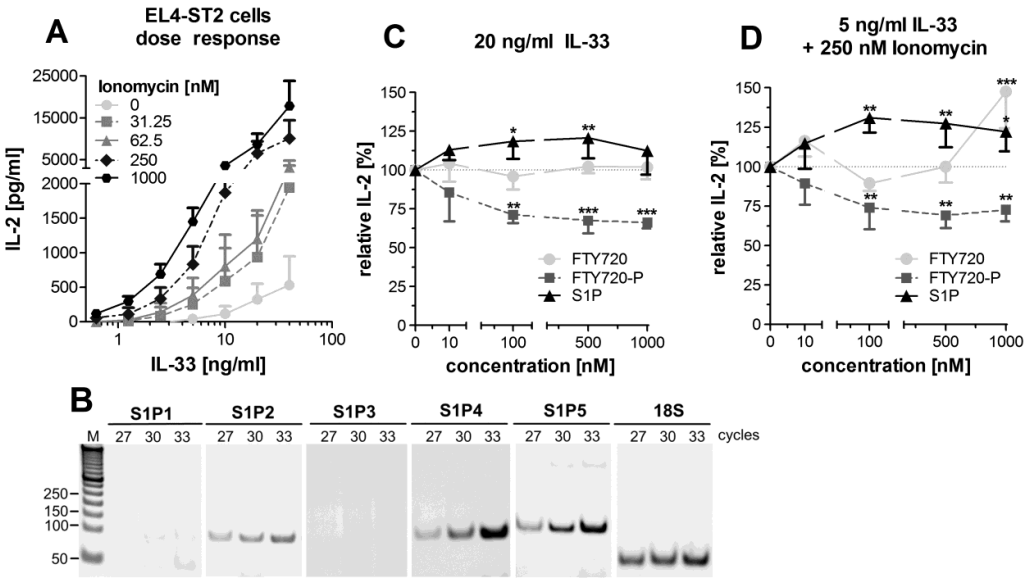

Figure 2: The SphK1/2-S1P axis modulates IL-2 formation in IL-33 stimulated EL4-ST2 cells. (**A-D**) EL4-ST2 cells were stimulated with (**A/C**) 20 ng/ml IL-33 or (**B/D**) 5 ng/ml IL-33 + 250 nM Ionomycin and were incubated with the sphinogosine kinase inhibitors PF543 (SphK1-selecitve), SKI II (SphK1/2-dual) and ABC294640 (SphK2 selective). Viability of cells stimulated with SKI II and ABC294640 was examined by Trypan blue staining using an automatic cell counter, which revealed no cytotoxic effects (data not shown). (**E, F**) EL4-ST2 cells were stimulated with the indicated concentrations of IL-33 and Ionomycin, RNA was isolated and the expression of SphK1 and SphK2 was determined by qRT-PCR. (**A-F**) Data are shown as mean ± SD and are pooled from (**A-D**) 4 – 7 and (**E, F**) 3 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; one-way ANOVA with Dunnett's posttest.

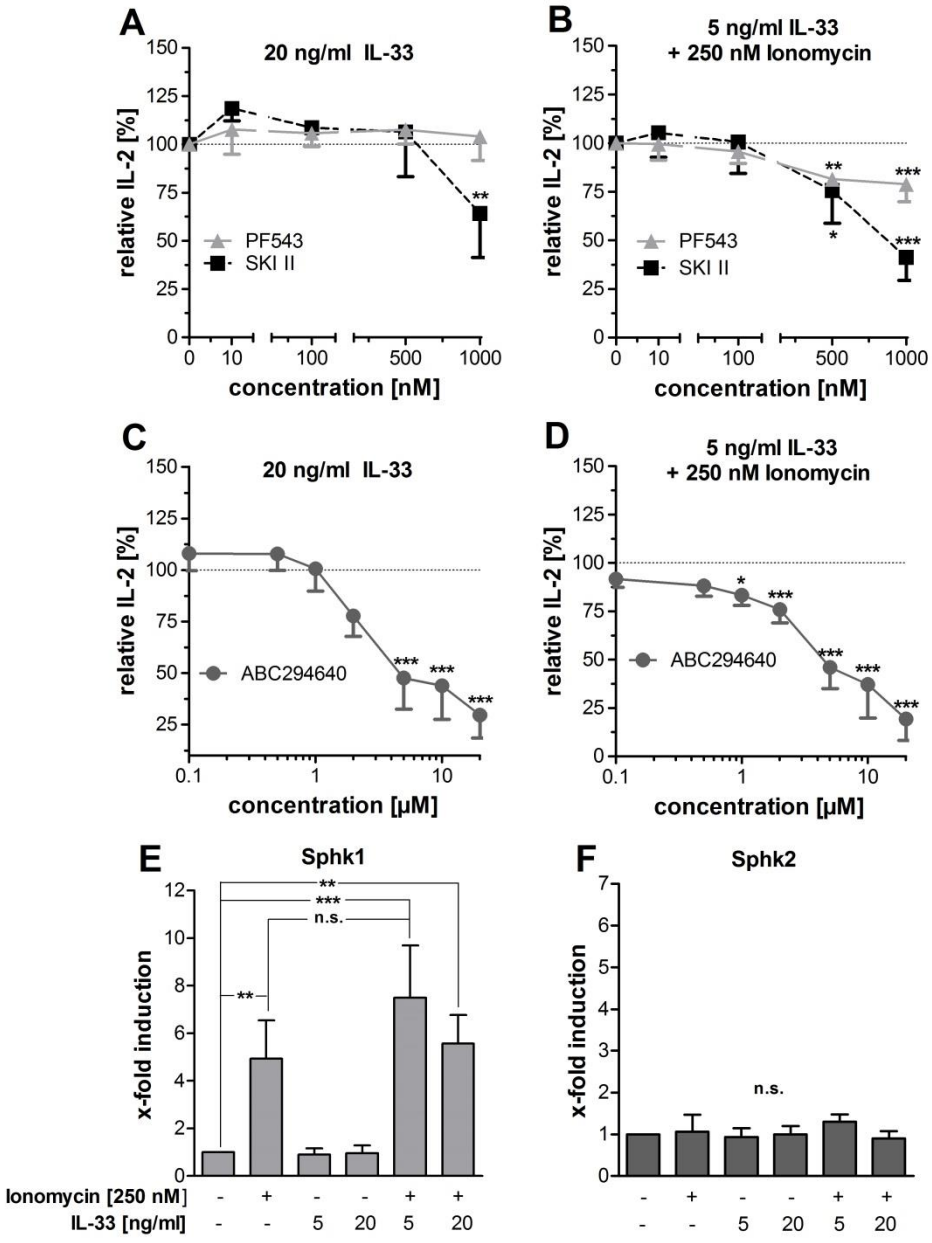

Figure 3: IL-33 synergizes with αCD3 and IL-12 to induce IFN-γ but not IL-2 in pooled splenocytes and CD8$^+$ T cells. (**A-D**) Primary murine splenocytes and CD8$^+$ T cells were stimulated with rising concentrations of IL-33 in the presence of (**A**) 1 µg/ml soluble (sb) (**C**)

1 µg/ml plate bound (pb) αCD3 antibody or (**B/D**) 5 ng/ml IL-12. The supernatant was collected after 44 h for the measurement of IL-2, IFN-γ, IL-17 or TNFα. IL-17 and TNFα formation was below the detection limit and is not shown. The purity of $CD8^+$ T cells was routinely checked by flow cytometry (Supporting Information Figure 1). (**A-D**) Data are shown as mean ± SD and are pooled from 3 independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$; one-way ANOVA with Dunnett's posttest.

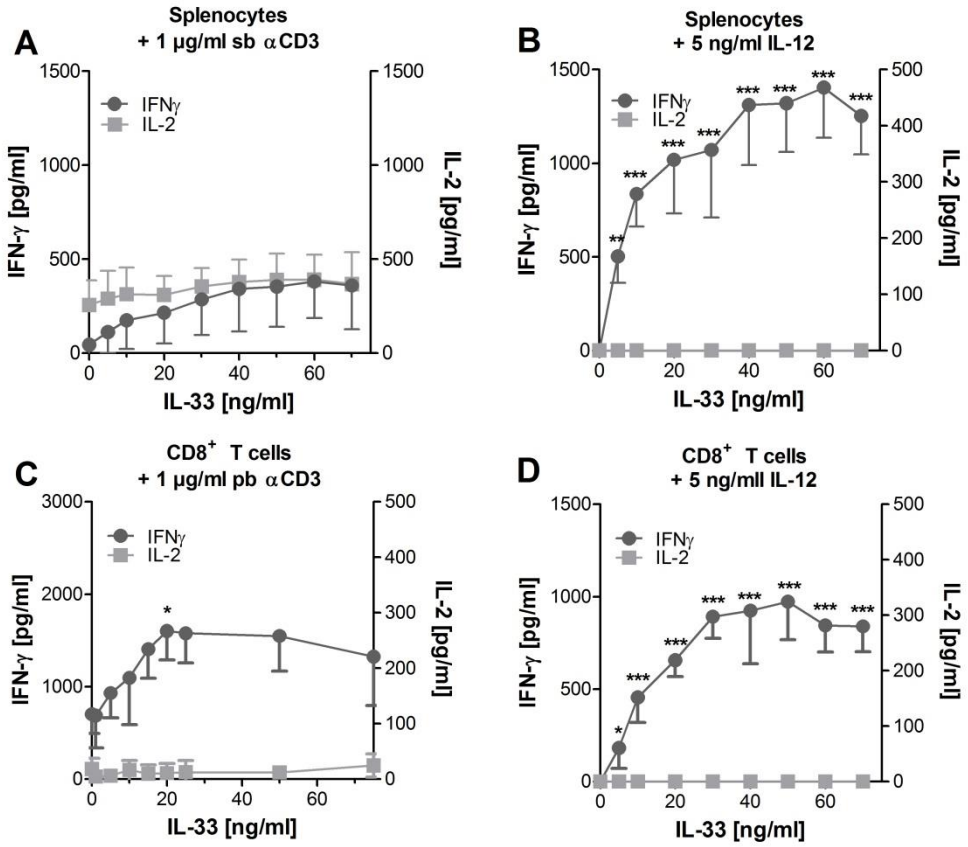

Figure 4: FTY720 but not FTY720-P inhibits the IL-33/IL-12 induced IFN-γ formation of primary murine splenocytes and $CD8^+$ T cells. (**A-F**) Primary murine splenocytes and

CD8$^+$ T cells were stimulated with IL-33/IL-12 and rising concentrations of (**A**/**B**) S1P, (**C**/**D**) FTY720-P or (**E**/**F**) FTY720. The supernatant was collected after 44 h for the measurement of IFN-γ. A normalized ratio was calculated with the unstimulated control as a reference to compensate for interassay differences (indicated by the dotted line). (**A**-**F**) Data are shown as mean ± SD and are pooled from 3-6 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; one-way ANOVA with Dunnett's posttest.

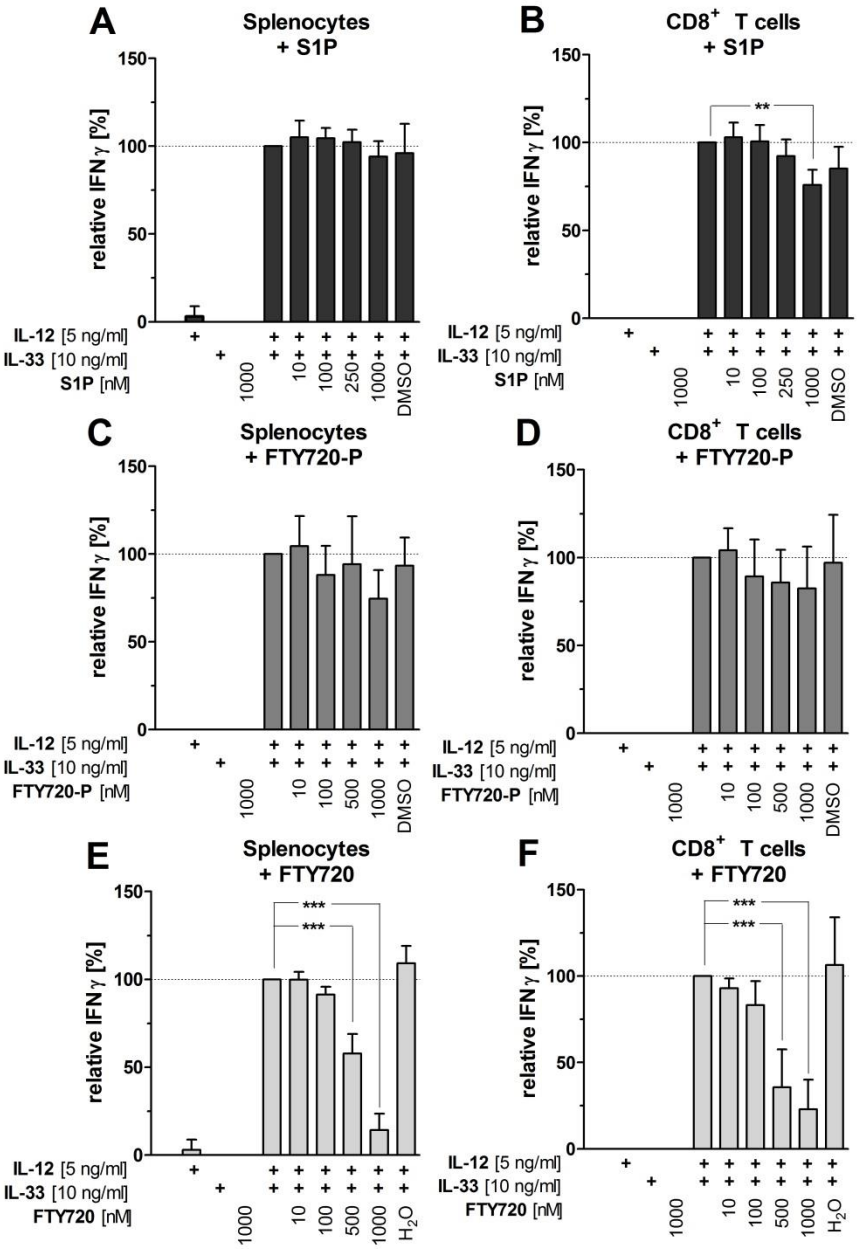

Figure 5: The inhibition of IL-33/IL-12 induced IFN-γ formation by FTY720 is mediated via the SET/PP2A pathway. (**A**) RNA from unstimulated WT and SphK2-/- splenocytes were isolated and the SphK1 and SphK2 mRNA expression was determined by qRT-PCR. (**B**) Primary murine splenocytes from SphK2-/- mice were stimulated with IL-33/IL-12 and rising concentrations of FTY720. (**C**) Primary WT $CD8^+$ T cells were stimulated with IL-33/IL-12 and rising concentrations of the SET peptide inhibitor OP449. (**D**) Primary WT $CD8^+$ T cells were stimulated with IL-33/IL-12, 500 nM FTY720 and rising concentrations of the PP2A inhibitor okadaic acid. (**B**-**D**) The supernatant was collected after 44 h for the measurement of IFN-γ. A normalized ratio was calculated with the unstimulated control as a reference to compensate for interassay differences (indicated by the dotted line). (**A**-**D**) Data are show as mean ± SD and are pooled from (**A**) 2 (WT) and 3 (SphK2-/-), (**B**/**D**) 3 and (**C**) 3 – 6 independent experiments. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$; one-way ANOVA with Dunnett's posttest.