Type 1 Sphingosine 1-Phosphate G Protein-Coupled Receptor (S1P₁) Mediation of Enhanced IL-4 Generation by CD4 T Cells from S1P₁ Transgenic Mice

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Sphingosine 1-phosphate (S1P) is a natural lipid mediator that regulates immune cell traffic, Ab production, and T cell cytokine generation by mechanisms that enhance Th2 activities. Responses to S1P are controlled principally by the diverse expression patterns of its receptors in different cells. In T cells, the type 1 (S1P1) and type 4 (S1P4) G protein-coupled receptors are predominant. S1P1 mainly transduces effects on T cell migration and trafficking, whereas S1P4 transduces immunosuppression via its effects on T cell proliferation and cytokine production. Using T cell-specific S1P1 transgenic (TG) mice, we investigated the regulatory effects of the S1P-S1P1 axis on T cell cytokine production. The production of IL-4, but not IL-2 or IFN-γ, was significantly up-regulated >10-fold in activated CD4 T cells from S1P1 TG mice compared with those from wild-type mice. Quantitative real-time PCR analysis revealed that IL-4 up-regulation was initiated at the mRNA level as early as 4 h after T cell activation. The up-regulation of IL-4 mRNA was mediated by c-Maf, Jun B, and Gata3 as demonstrated by increases in their protein expression and DNA-binding activities. In contrast, the expression and DNA-binding activities of T-bet, Foxp3, C-Fos, Jun D, Fra-1, Fra-2, and c-Jun all were identical in wild-type and TG CD4 T cells. Immunological assays showed that increased IL-4 levels induced greater production of IgE. Thus, the S1P-S1P1 axis specifically up-regulates c-Maf, Jun B, and Gata3, which consequently enhance IL-4 production that may lead to a Th2 phenotype. The Journal of Immunology, 2007, 178: 4885–4890.
of stimulated T cells from S1P1 TG mice were only 10–34% of those for WT T cells (6).

In this S1P1 TG mouse model, we found for the first time that IL-4 production by activated CD4 T cells is increased significantly. Increased IL-4 production leads to Th2 bias and increased IgE production. Studies of transcription factors indicate that IL-4 up-regulation is mediated by cooperative effects of c-Maf, Jun B, and Gata3 in S1P1 TG mice.

**Materials and Methods**

**CD4 T cell isolation, culture, and activation**

Mouse CD4 T cells were isolated from splenocytes of 6- to 8-wk-old C57BL/6 WT or S1P1 TG mice using CD4 T cell immunomagnetic beads following the manufacturer’s protocol (Miltenyi Biotec). S1P1 TG mice were made via cloning of S1P1 into an improved CD2 mirinig based vector, which promotes T cell-specific and copy number-dependent expression of gene products (6). S1P1 construct was injected into (C57BL/6 × DBA/2)F1 hybrid embryos and offspring was backcrossed with C57BL/6 for more than seven generations. Mating of the highest expressors of each generation resulted in S1P1 TG mice bearing ∼35 copies of the CD2-S1P1 minigene by and after the F1 generation, as determined by real-time PCR. The expression of S1P1 in TG mice was regularly measured by real-time PCR and Western blotting. To completely eliminate the endogenous S1P1 in S1P1 TG, S1P1 was first neutralized by charcoal/dextran treatment and then residual S1P was degraded by S1P lyase (a gift from Dr. J. D. Saba, Children’s Hospital of Oakland Research Institute, Oakland, CA). New synthesis of S1P was inhibited by adding 3.3 μM (IC50 = 500 nM) sphingosine kinase inhibitor named 2-(p-synapthoyanilino)-4-(p-chlorophenyl)ilthiazole in the culture medium (Calbiochem). Four × 105 cells/ml CD4 T cells from WT and S1P1 TG were seeded at 200 μl/well for indicated times in 96-well plates preplated with 100 μl each of 5 μg/ml anti-CD3 and anti-CD28 mAbs (BioLegend). CD4 T cells were cultured in RPMI 1640 medium containing 3.3 μM sphingosine kinase inhibitor, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 1% S1P lyase-pretreated charcoal-treated FBS. The viabilities of cells were monitored by trypan blue exclusion. Twenty-four and 48 h after TCR activation, the viabilities of CD4 T cells were 91.2 and 85.5%, respectively.

**Real-time PCR quantification of mRNA encoding mouse IL-4**

Total RNA was isolated with an RNeasy minikit (Qiagen) and treated with DNase I (Qiagen). The mRNAs encoding mouse IL-4 and the standard hypoxanthine phosphoribosyltransferase (HPRT) were amplified in triplicate using TaqMan primers and probes (Integrated DNA Technologies) as previously described (7). The Prism 7700 Sequence Detection System with the recommended optimal reagents and conditions provided internal analysis coefficients of variation lower than 4% (Applied Biosystems). The value for each unknown was determined from its optimized threshold cycle (Ct) and normalized with reference to the Ct value of HPRT using the comparative Ct method (User Bulletin 2 of ABI Prism 7700 Sequence Detection System). Relative values for mRNA encoding each IL-4 were assigned by setting that of HPRT at 100. The forward primer sequence is 5’-CGGACATGCAGGAGATG-3’, the reverse primer sequence is 5’-FAM-TGCCAACAGTCCTCAGACCG-3’TAM.

**Western blot analysis**

Nuclear proteins were isolated using a nuclear extraction kit (Pierce). Ten micrograms of nuclear extract per lane was loaded on a 10% SDS-PAGE. Protein-DNA complexes were separated from free oligonucleotides on a polyacrylamide gel. Protein and DNA were transferred to a nylon membrane and incubated with streptavidin-HRP, which provided chemiluminescent signals detected by Kodak Biomax light x-ray film (Eastman Kodak). The c-Maf probe sequence is 5’-GCGAGTGGCCACTCGGACGAT-3’, the unrelated probe sequence is 5’-GGAGGAGGCTGCTT3’TAM.

**FACS analysis**

CD4 T cells were stained with PE-anti-mouse CD44, PE-anti-mouse CD69, FITC-anti-mouse CD25, PerCP-anti-mouse CD4 (BD Biosciences), PE-anti-mouse forkhead box P3 (FoxP3), and FITC-anti-mouse glucocorticoid-induced TNF-like receptor (GITR) Abs according to the manufacturer’s protocols. The labeled cells were analyzed using a FACSScan system (BD Biosciences).

**Assays of active transcription factors**

Active forms of the AP-1 family (Jun B, Jun D, c-Fos, FosB, Fra-1, Fra-2, c-Jun) and Gata3 in nuclear extracts were measured using a TransAM transcription factor assay kit (Active Motif). In brief, nuclear extracts were loaded into wells of a 96-well plate that was precoated with specific oligonucleotides containing a consensus-binding sequence for each transcription factor. After incubation and washing, specific primary Abs were added to recognize each transcription factor. A secondary Ab conjugated with HRP provided chemiluminescence signals detected by Kodak Biomax light x-ray film (Eastman Kodak).

**Chromatin immunoprecipitation (ChIP) assays**

To measure the individual binding activities of Jun B, c-Maf, and Gata3 for the mouse IL-4 gene in CD4 T cells, ChIP assays were conducted with a CHIP-IT enzymatic kit (Active Motif) according to the manufacturer’s protocol. In brief, DNA and protein complexes were cross-linked by paraformaldehyde. The DNA was sheared into small and uniform fragments by enzymatic digestion. Anti-Jun B, anti-c-Maf, or anti-Gata3 Abs (Santa Cruz Biotechnology) was added to precipitate specific transcription factor-DNA complexes. A negative control IgG provided by Active Motif was added to serve as a background control. Input DNA, cross-linked chromatin without immunoprecipitation of specific Abs, was used as a positive control. Following immunoprecipitation, cross-linking was reversed, the proteins were digested by proteinase K, and the DNA was isolated for PCR analyses. Input DNA, specific Ab-precipitated DNA, and the negative control Ab-precipitated DNA were assessed for their levels of IL-4 promoter/enhancer activity. The primer set for measuring Jun B and c-Maf-binding activities was as follows: forward, 5’-CAA TTG GTCT GCA TTT CAC AG-3’ and reverse, 5’-GCT CTG TGT AGC CAG TCA-3’ (239-bp product). The primer set for measuring Gata3-binding activity was as follows: forward, 5’-AGG GCA CTT AAA CAT TGC-3’ and reverse, 5’-ACG CCT AAC AAT TCC-3’ (235-bp product).

**EMSA**

The measurement of binding activities of c-Maf in nuclear extracts was conducted using an EMSA assay kit (Panomics) following the manufacturer’s protocol. In brief, nuclear extracts were incubated with biotinylated oligonucleotides containing a consensus c-Maf-binding site in a buffer. Protein-DNA complexes were separated from free oligonucleotides on a polyacrylamide gel. Protein and DNA were transferred to a nylon membrane and incubated with streptavidin-HRP, which provided chemiluminescent signals detected by Kodak Biomax light x-ray film (Eastman Kodak). The c-Maf probe sequence is 5’-GCGAGTGGCCACTCGGACGAT-3’, the unrelated probe sequence is 5’-GGAGGAGGCTGCTT3’TAM.

**Quantification of cytokine production**

CD4 T cells were seeded at 4 × 105 cells/ml in 96-well plates at 0.2 ml/well and adhered by anti-CD3 and anti-CD28 mAbs for 24 or 48 h. The supernatants were then collected. IL-4, IL-2, and IFN-γ in the supernatants were quantified by ELISA kits (Pierce-Endogen) according to the manufacturer’s protocols.

**Quantification of IgE production**

Mouse peripheral mononuclear cells from spleen were isolated by Ficoll-Hypaque (Amer sham Biosciences) gradient centrifugation. These mononuclear cells were cultured in a 25-cm culture dish at 37°C for 1 h. Non-adherent cells were collected as mixed lymphocytes. Mouse lymphocytes from WT and S1P1 TG mice were seeded at 1 × 107 cells/ml in wells of a 96-well plate precoated with or without 100 μl of 5 μg/ml anti-CD3 and anti-CD28 mAbs (BioLegend). Mixed lymphocytes were incubated with 20 μg/ml pokeweed mitogen (Sigma-Aldrich), 20 ng/ml IL-4, and/or 10 μg/ml anti-IL-4-neutralizing Ab (BioLegend). Mixed lymphocyte culture was seeded in 10 W M S1P in 10% charcoal-treated FBS-RPMI 1640 for 7 days. The supernatants were collected and the IgE concentrations were measured using an IgE ELISA kit (BioLegend) according to the manufacturer’s protocol.

**Statistics**

Experiments were conducted in duplicate or triplicate and repeated three times. The result was expressed as mean ± SD. A two-tailed t test was used to determine the significance of differences (*) p < 0.05, ** p < 0.01.

**Results**

Extensive studies have characterized S1P1, initiation and control of lymphocyte migration and trafficking in lymphoid organs, as well as survival and proliferation. In contrast, far less is known of the
mRNA in CD4 T cells from WT and S1P1 TG mice was quantified. The up-regulation of IL-4 seen in S1P1 TG mice is attributable to signals from the S1P-S1P1 axis. Therefore, as expected, up-regulation of IL-4 mRNA preceded the up-regulation of IL-4 protein expression. Most instances of overexpression of cytokines are due to stimulation of transcription. To investigate the possibility that S1P1-mediated increases in IL-4 occur at the transcriptional level, IL-4 mRNA in CD4 T cells from WT and S1P1 TG mice was quantified by real-time PCR. IL-4 mRNA in S1P1 TG mice was dramatically increased up to 10-fold in S1P1 TG mice compared with WT mice in the presence of 10^{-7} M S1P. The up-regulation of IL-4 was abolished in the absence of S1P. S1P was removed by charcoal/dextran and S1P lyase treatments. CD4 cells were cultured with or without 3.3 μM sphingosine kinase inhibitor, C. The up-regulation of IL-4 was abolished in the absence of S1P. S1P was removed by charcoal/dextran and S1P lyase treatments. CD4 cells were cultured with or without 3.3 μM sphingosine kinase inhibitor. D. The IL-4 up-regulation in CD4 T cells from S1P1 TG mice was rapid after TCR activation. To ensure that the naive cells we used were not preactivated, the expression of T cell activation markers CD44 and CD69 were measured by flow cytometry. Before TCR activation, there was no expression of CD69, and CD44 expression was at a low level in CD4 T cells from WT and S1P1 TG mice. Twenty-four hours after TCR activation, the expression of CD44 and CD69 were significantly up-regulated. The expression of additional T cell activation markers and CD4^+CD25^+ regulatory T cell (Treg) markers also were examined, including CD25, FoxP3, and GITR. Flow cytometry data showed that the expression of CD25, FoxP3, and GITR in naive CD4 T cells from S1P1 TG mice was 1.5- to 1.7-fold higher than those from WT mice at time 0 (Fig. 4). FoxP3 is the best Treg marker and its expression is independent of the status of T cell activation. CD25 and GITR are Treg markers for naive T cells. However, their expression is
up-regulated in activated T cells. High expression levels of all three Treg markers in CD4 T cells from S1P1 TG mice suggests that the S1P-S1P1 axis may promote up-regulation of Tregs. To eliminate the possible effects from different levels of Tregs in WT and S1P1 TG mice, IL-4 production after T cell activation was quantified using CD4+CD25+ T cells free of CD4+CD25- T cells whose purities were analyzed by flow cytometry. As in complete sets of CD4 T cells, after TCR activation, the IL-4 production by CD4+CD25+ T cells from S1P1 TG mice was around 10-fold higher than that of identical cell cultures from WT mice (Fig. 5). These data suggested that freshly isolated CD4 T cells are naive T cells. Higher expression levels of CD25 in CD4 T cells from S1P1 TG mice were due to higher expression of Tregs, as proven by the elevated expression of FoxP3, a Treg marker independent of activation status.

Several transcription factors were investigated to elucidate the mechanism by which IL-4 mRNA expression was up-regulated. The expression of transcription factor proteins was measured by Western blots. The functional binding activity of transcription factors was measured by ELISA, EMSA, and CHIP assays. Jun B and c-Maf are two transcription factors involved in up-regulation of IL-4 in CD4 T cells after their stimulation by vasoactive intestinal peptide and other factors (8). In this study, Jun B expression level and binding activity both were significantly increased after T cell activation in CD4 T cells from S1P1 TG mice (Fig. 6). The peak increase in Jun B expression and binding activity was at 2 h after samples were incubated in 10 M S1P.
FIGURE 7. The protein expression and binding activity of c-Maf were significantly up-regulated in CD4 T cells from S1P1 TG mice. CD4 T cells from WT and S1P1 TG mice were activated for 0–24 h in the presence of 10^{-7} M S1P. The nuclear extracts were isolated to measure c-Maf protein expression and binding activity. A, c-Maf protein was quantified by Western blot. Upper panel, Levels of expression of c-Maf. Lower panel, Levels of expression of Lamin B1, as a loading control. B, EMSA of c-Maf-binding activity using a standard gel electrophoresis method. WT, CD4 T cells from WT mice; TG, CD4 T cells from S1P1 TG mice; TGC, CD4 T cells from S1P1 TG mice incubated with a labeled c-Maf probe and an excess amount of unlabeled self-oligonucleotide; TGU, CD4 cell nuclear extract from WT, CD4 T cells from S1P1 TG mice incubated with a labeled c-Maf probe and an excess amount of labeled unrelated oligonucleotide; TGS, CD4 cell nuclear extract from S1P1 TG mice incubated with anti-c-Maf Ab. CD4: *, p < 0.05; **, p < 0.01 from t test. C, CHIP assay to measure c-Maf-binding activity in CD4 T cells from WT and S1P1 TG mice.

T cell activation. The induction of Jun B expression and binding activity began to decrease 24 h after T cell activation. A similar result was observed for expression and binding activity of c-Maf.

FIGURE 8. The protein expression and binding activity of Gata 3 were significantly up-regulated in CD4 T cells from S1P1 TG mice. CD4 T cells from WT and S1P1 TG mice were activated for 0–48 h in the presence of 10^{-7} M S1P. The nuclear extracts were isolated to measure Gata 3 protein expression and binding activity. A, Gata 3 protein expression was measured by Western blot. Upper panel, Levels of expression of Gata 3; lower panel, levels of expression of Lamin B1, as a loading control. B, ELISA of Gata 3-binding activity was performed with a kit. For Gata 3-binding assays, all ODs were normalized to that of a 5-µg Jurkat nuclear extract, a positive control provided by Active Motif. *, p < 0.05; **, p < 0.01 from t test. C, CHIP assay to measure Gata 3-binding activity in CD4 T cells from WT and S1P1 TG mice.

FIGURE 9. Up-regulation of IL-4 leads to enhanced production of IgE. Mixed lymphocytes from WT and S1P1 TG mice were cultured with 20 µg/ml pokeweed mitogen (PWM), 20 ng/ml IL-4, and/or 10 µg/ml anti-IL-4 Ab in wells of a 96-well plate precoated with or without 100 µl each of 5 µg/ml anti-CD3 and anti-CD28 mAbs in the presence of 10^{-7} M S1P. After a 7-day culture, the supernatants were collected and IgE concentrations were measured by an ELISA kit. Con, Control. (Fig. 7). Gata3 is another transcription factor that could induce the up-regulation of IL-4. Expression of the transcription factor Gata3 is mainly limited to Th2 cells but not Th1 cells (9). In this study, the expression and binding activity of Gata3 were induced only after 24 h of TCR stimulation, which is later than for Jun B and c-Maf (Fig. 8). Other transcription factors we measured included T-bet, FosB, C-Fos, Jun D, Fra-1, Fra-2, and c-Jun. However, these transcription factors were either not activated or activated at a similar level in CD4 T cells from S1P1 TG and WT mice (data not shown). At the mRNA level, IL-4 expression reached a peak at 4 h that was sustained at 24 h after T cell activation (Fig. 2). Therefore, Jun B and c-Maf may be involved principally for the early induction of IL-4 mRNA expression, whereas Gata3 is only recruited for late induction of IL-4 mRNA.

IL-4 is a prominent cytokine of Th2-type cells. Expression of IL-4 biases helper T cells’ differentiation toward the Th2 subtype. IgE is produced by B cells/plasma cells in Th2 cell-mediated humoral immune responses. To investigate the downstream immune functions regulated by the overexpression of IL-4, IgE production in mixed lymphocytes from WT and S1P1 TG mice was examined using pokeweed mitogen, an IL-4-dependent B cell mitogen. In this system, IgE production by B cells depends on exogenously added IL-4 or IL-4 produced by T cells. As shown in Fig. 9, IgE production by lymphocytes from S1P1 TG mice was 4-fold higher than that from WT mice when T cells were activated by anti-CD3 plus anti-CD28 and B cells were activated by pokeweed mitogen (compare the second bar to the sixth bar). This up-regulation was abolished by the addition of 10 µg/ml anti IL-4-neutralizing Ab. In contrast, IgE production was similar in WT and S1P1 TG mice when mixed lymphocytes were cultured in pokeweed mitogen plus 20 ng/ml exogenous IL-4 without T cell activation. Therefore, the overexpression of IL-4 is responsible for the up-regulation of IgE in S1P1 TG mice.

In summary, a high level of S1P1 expression by T cells of S1P1 TG mice led to increased activation of Jun B, c-Maf, and Gata 3, which in turn evoked the overexpression of IL-4 and then up-regulated the expression of IgE.
Discussion

SIP is a multifunctional lysophospholipid with diverse effects on immune cell proliferation, survival, migration, and other functions. Cellular responses to SIP in any physiological environment are regulated principally by the type and level of expression of the SIP GPCRs. The predominant SIP GPCRs of T cells are SIP1, SIP2, SIP1, and SIP1, differ in SIP-binding affinity and signal transduction mechanisms. SIP1 has a mean Kd of 5–8 nM and couples to Go(10), whereas the respective descriptors for SIP2 are 60 nM, and Gi and G12/13 (11, 12). It is important to find out how different responses to SIP are transduced by these two SIP receptors in T cells. SIP1, mainly mediates immunosuppressive effects of SIP on CD4 T cells (2). The SIP-SIP1 axis significantly suppresses proliferation of CD4 T cells and SIP1-transfected model T cells. The SIP-SIP1 axis also suppresses production-secretion of IL-2, IFN-γ, and IL-4, and reciprocally enhances that of IL-10 (2). The SIP-SIP1 axis principally exercises control over migration and trafficking of naive T cells and B cells (5, 6, 13). The effect of SIP on Tregs is also mediated mainly by SIP1 (3). However, effects of the SIP-SIP1 axis on cytokine production have not been elucidated. In this study, we demonstrate for the first time that a hypersensitivity reaction. Many allergic diseases are associated with higher than in WT mice (6). This may augment immediate-type T cells. IL-4 is the major cytokine supporting CD4 T cell differentiation to the Th2 subtype. Our study shows that the overexpressed IL-4 is responsible for augmented production of IgE, which is observed in vivo in SIP1 TG mice (6). In these mice, both serum concentration of IgE and levels of IgE Abs to defined Ag are higher than in WT mice (6). This may augment immediate-type hypersensitivity reaction. Many allergic diseases are associated with a high level of IgE expression. Thus, suppression of IgE production via SIP1 analogs could be a novel treatment for these diseases.

Second, many other IL-4-modulated events may be altered in the SIP1 TG mice due to the overexpression of IL-4. One example would be elevated levels of CD4⁺ CD25⁺ Tregs (16) and the consequent effects on the integrated immune phenotype. It was recently reported that IL-4 can induce the conversion of peripheral CD4⁺ CD25⁺ T cells to CD4⁺ CD25⁺ Tregs (15). Therefore, the high level of IL-4 elicited by the up-regulated SIP1 could lead to a higher than normal level of Tregs in peripheral blood and tissues. Such increased Treg activity may have important effects on both host defense and susceptibility to autoimmune diseases (16, 17). It was recently discovered that IL-4 could induce protection of CD4⁺CD25⁺ T cells from CD4⁺CD25⁺ Treg-mediated suppression (18). Therefore, highly expressed IL-4 may inhibit the suppressive effects of Tregs. In this study, we observed higher expression of Tregs in SIP1 TG mice than in WT mice. The mechanisms of these increased Tregs are currently under investigation in vitro and in vivo. It will be very interesting to examine how these SIP-SIP1 up-regulated Tregs function in mouse autoimmune disease models.

Disclosures

The authors have no financial conflict of interest.

References