Th17 Augmentation in OTII TCR Plus T Cell-Selective Type 1 Sphingosine 1-Phosphate Receptor Double Transgenic Mice

Mei-Chuan Huang, Susan R. Watson, Jia-Jun Liao and Edward J. Goetzl

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Sphingosine 1-phosphate (SIP) is produced by mast cells and mononuclear phagocytes in immune organs, but not by T cells, is secreted into T cell corridors where it elicits and regulates T cell movements in thymus, lymph nodes, and spleen, and their chemotaxis in nonimmune tissues (1–3). Effects of SIP on T cell migration and trafficking are mediated by the type 1 G protein-coupled receptor, designated SIP$_1$, which is down-regulated briefly by SIP occupancy and persistently by T cell activation (4–6). Investigations of the immune phenotype of a line of S1P$_1$ transgenic (TG) mice, in which T cells express high levels of both mouse and human S1P1 mRNA and of mouse IL-17 mRNA were conducted in an ABI 7700 Sequence Detection System (Applied Biosystems) with the specific primers and human S1P1 in the RNA samples was conducted in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Materials and Methods

**OTII TCR TG (OTII) and T cell-selective S1P$_1$ x OTII DTG mice**

DTG mice were derived by crossing C57BL/6 mice expressing a human S1P$_1$ transgene selectively in T cells (7) with OTII mice in which T cells express $\alpha$- and $\beta$-chains of a TCR specific for peptide 323–339 of OVA in the context of I-A$\beta$ (stock number 004194; The Jackson Laboratory). After three to four generations of breeding, genotyping was begun with PCR using genomic DNA isolated from cut tail tissues and primers for: human S1P$_1$: forward: 5'-TGGTGGGAGGAAGGATGCTACCG-3', reverse: 5'-GGCGGAAGTTATGCTCCC-3'; mouse OTII TCR $\alpha$-chain: forward: 5'-AAAGGGAGAAAGCCTCTCC-3', reverse: 5'-ACACGACGTTCTGGTTC-3'; mouse OTII TCR $\beta$-chain: forward: 5'-GCTGCTGCACGACCTACT-3', reverse: 5'-CACCTACCTACTACAGAGGG-3'.

All mice were bred and maintained in a pathogen-free facility and studies were conducted with procedures approved by the University of California San Francisco Institutional Animal Care and Use Committee.

**Real-time RT-PCR**

RNA samples extracted with Tri-Reagent (Molecular Research Center, Cincinnati, OH) were treated with RNase-free DNase I and isolated on RNeasy columns (Qiagen). TaqMan real-time RT-PCR analyses of mouse and human S1P$_1$ in the RNA samples was conducted in an ABI 7700 Sequence Detection System (Applied Biosystems) with the specific primers and 5'-FAM/3'-TAMRA-labeled probes described (7). Relative levels of expression of mouse and human S1P$_1$ mRNA and of mouse IL-17 mRNA were lower generation of IFN-$\gamma$ than in control OTII mice, prompted analyses of other CD4 T cell cytokines. The most novel resultant discovery was significant SIP enhancement of generation of IL-17 by OVA-stimulated DTG mouse CD4 T cells, but not by control OTII mouse CD4 T cells, which was supported by findings of higher levels of both mRNA encoding IL-17 and of the percentage having intracellular IL-17 in CD4 T cells of DTG than OTII mice.

Th17 Augmentation in OTII TCR Plus T Cell-Selective Type 1 Sphingosine 1-Phosphate Receptor Double Transgenic Mice

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Sphingosine 1-phosphate (SIP) in blood and lymph controls lymphoid traffic and tissue migration of T cells through signals from the type 1 SIPR (SIP$_1$), but less is known of effects of the SIP-SIP$_1$ axis on nonmigration functions of T cells. CD4 T cells from a double transgenic (DTG) mouse express OTII TCRs specific for OVA peptide 323–339 (OVA) and a high level of transgenic S1P$_1$, resistant to suppression by T cell activation. OVA-activated DTG CD4 T cells respond as expected to SIP by chemotactic migration and reduction in secretion of IFN-$\gamma$. In addition, DTG CD4 T cells stimulated by OVA secrete a mean of 2.5-fold more IL-17 than those from OTII single transgenic mice with concomitantly higher levels of mRNA encoding IL-17 by real-time PCR and of CD4 T cells with intracellular IL-17 detected by ELISPOT assays. OVA challenge of sc air pockets elicited influx of more OTII TCR-positive T cells producing a higher level of IL-17 in DTG mice than OTII control mice. Augmentation of the number and activity of Th17 cells by the SIP-SIP$_1$ axis may thus enhance host defense against microbes and in other settings increase host susceptibility to autoimmune diseases. The Journal of Immunology, 2007, 178: 6806–6813.

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3 Abbreviations used in this paper: SIP, sphingosine 1-phosphate; SIP$_1$, type 1 SIP G protein-coupled receptor; WT, wild type; TG, transgenic; DTG, double TG; DTH, delayed-type hypersensitivity; STG, single TG; TNP, trinitrophenol; KLH, keyhole limpet hemocyanin.

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normalized separately using the respective hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene for endogenous loading controls, as described. The levels for unchallenged OTII CD4 T cells (left frame, mouse S1P₁) and unchallenged DTG CD4 T cells (right frame, human S1P₁) were set at 1.0. OVA/APC challenge for 24 h significantly suppressed levels of mRNA encoding mouse S1P₁ ($p < 0.01$ for both OTII and DTG) and human S1P₁ ($p < 0.05$ for the DTG mouse). B, Quantification by TaqMan real-time RT-PCR of mRNA encoding human S1P₁ in T cells from S1P₁ STG and DTG. Unstimulated (naive) CD4 T cells from both STG and DTG were set at 100%. Anti-TCR activation (CD3 + CD28) suppressed human S1P₁ levels in both sets significantly, whereas Ag stimulation (APC + OVA) only suppressed T cells from DTG ($p < 0.01$). C, Western blot quantification of mouse and human S1P₁ in splenic CD4 T cells by rabbit anti-S1P₁ Abs. Each of the six wells received 10 μg of extracted CD4 T cell proteins. Top frame, The left three lanes contain first, proteins from DTG T cells after stimulation by APCs plus OVA (Ag), second, proteins from DTG T cells after stimulation by anti-CD3 plus anti-CD28 Abs (A-TCR), and third, proteins from unstimulated DTG (U). The right three lanes contain samples from OTII T cells in the same order as for the set from DTG T cells. Bottom frame, Immunostaining of the CD44 loading control for the same samples as in the top frame.

**FIGURE 1.** Expression of S1P₁ by CD4 T cells of OTII, STG, and DTG mice. A, TaqMan real-time RT-PCR quantification of mouse and human S1P₁ in splenic CD4 T cells. Each column and bar depicts the mean ± SD of the results of three or more analyses. The levels for unchallenged OTII CD4 T cells (left frame, mouse S1P₁) and unchallenged DTG CD4 T cells (right frame, human S1P₁) were set at 1.0. OVA/APC challenge for 24 h significantly suppressed levels of mRNA encoding mouse S1P₁ ($p < 0.01$ for both OTII and DTG) and human S1P₁ ($p < 0.05$ for the DTG mouse). B, Quantification by TaqMan real-time RT-PCR of mRNA encoding human S1P₁ in T cells from S1P₁ STG and DTG. Unstimulated (naive) CD4 T cells from both STG and DTG were set at 100%. Anti-TCR activation (CD3 + CD28) suppressed human S1P₁ levels in both sets significantly, whereas Ag stimulation (APC + OVA) only suppressed T cells from DTG ($p < 0.01$). C, Western blot quantification of mouse and human S1P₁ in splenic CD4 T cells by rabbit anti-S1P₁ Abs. Each of the six wells received 10 μg of extracted CD4 T cell proteins. Top frame, The left three lanes contain first, proteins from DTG T cells after stimulation by APCs plus OVA (Ag), second, proteins from DTG T cells after stimulation by anti-CD3 plus anti-CD28 Abs (A-TCR), and third, proteins from unstimulated DTG (U). The right three lanes contain samples from OTII T cells in the same order as for the set from DTG T cells. Bottom frame, Immunostaining of the CD44 loading control for the same samples as in the top frame.

**Western blots**

Membrane total proteins were extracted from T cells as described (8) and aliquots containing 10 μg of protein (BCA method; Pierce) were electrophoresed in a precast SDS 4–15% gradient polyacrylamide gel (Bio-Rad). After transfer of the proteins resolved to a nitrocellulose membrane, the blot was incubated with either mouse monoclonal IgG2b anti-S1P₁ Ab (clone 218713; R&D Systems) or rabbit anti-S1P₁ IgG Abs (7) and then developed respectively with HRP-conjugated goat anti-mouse IgG or HRP-conjugated goat anti-rabbit IgG (both from Jackson ImmunoResearch Laboratories) to allow detection of Ag with the ECL substrate system (Amersham Biosciences). To visualize CD44 as a loading control, each blot was stripped and re stained with rat monoclonal IgG2b anti-CD44 Ab (clone IM7; BD Biosciences) and then HRP-conjugated goat anti-rat IgG; the predominant band was ~85 kDa. Prestained protein molecular mass
marketers (Benchmark; Invitrogen Life Technologies) were included in all electrophoretic gels.

Quantification of CD4 T cell surface proteins, chemokinesis, and cytokine production

Mouse spleen and lymph node CD4 T cells were isolated from 6- to 10 wk-old OTII or DTG mice by an immunobead and magnetic column chromatography method at a purity of at least 94% (Miltenyi Biotec). For flow cytometry, the T cells were incubated in PBS with 1% (v:v) FBS, preincubated for 5 min at 4°C with anti-CD3 plus anti-CD28 Abs (a-TCR), or APCs plus OVA (OVA) before responding to medium alone (0), 10^{-7} M S1P (S), or 10^{-5} M CCL21 (C). CCL21 served as a positive control for comparison with chemotaxis mediated specifically by S1P. The significance of differences between chemotactic responses to one stimulus of T cells of any preincubation group and those of the group preincubated in medium alone were calculated by a two-tailed paired Student’s t test and denoted as: +, p < 0.05.

FIGURE 2. Effects of TCR-mediated activation on CD4 T cell chemotactic responses to SIP and CCL21. Each set of three columns and bars depicts the mean ± SD of the results of three studies conducted in duplicate, where the T cells are incubated for 24 h in medium alone (0), with anti-CD3 plus anti-CD28 Abs (a-TCR), or APCs plus OVA (OVA) before responding to medium alone (0), 10^{-7} M S1P (S), or 10^{-5} M CCL21 (C). CCL21 served as a positive control for comparison with chemotaxis mediated specifically by SIP. The significance of differences between chemotactic responses to one stimulus of T cells of any preincubation group and those of the group preincubated in medium alone were calculated by a two-tailed paired Student’s t test and denoted as: +, p < 0.05.

Determination of the concentrations of plasma anti-OVA Abs

For analyses of anti-OVA IgE Ab production, OTII and DTG mice were immunized directly with OVA adsorbed to Al(OH)₃, whereas WT C57BL/6 mice that had adoptively received splenic T cells from OTII and DTG mice were immunized with OVA in Freund’s adjuvant (all isotypes). Groups of eight mice were immunized s.c. in multiple sites of both flanks with 50 µg of OVA peptide (AnaSpec) in CFA or adsorbed to Al(OH)₃ (IgE). Each mouse adoptively receiving T cells was boosted s.c. 14 days later with 50 µg of OVA in IFA (secondary for all isotypes). After 4 days for OVA-Al(OH)₃, and 14 and 21 days for OVA-Freund’s adjuvant, plasma samples were isolated from retro-orbital heparinized venous blood. OVA-specific IgE, IgA, IgG1, IgG2a, and total IgG levels were quantified by ELISAs in 96-well plates. Each sample well was coated with 1 µg of OVA peptide for 16 h at 4°C, washed, blocked with 150 µl of 1 g of BSA/100 ml of PBS, and loaded and incubated for 1 h at 37°C and 16 h at 4°C with 100 µl of one of two optimized dilutions of plasma samples. Bound anti-OVA Abs were detected with HRP-conjugated anti-mouse total IgG, IgG1, IgG2a, and IgE Abs (Bethyl Laboratories) (7). Standard curves were generated with normal mouse total IgG, IgG1, IgG2a, IgA, and IgE (Bethyl Laboratories).

Induction and quantification of cutaneous delayed-type hypersensitivity (DTH)

Groups of six to eight WT mice, that had been irradiated 4 days earlier with 300 rad, received T cells isolated from OTII and DTG mice, and then were immunized with 50 µg of OVA-CFA in multiple flank s.c. spaces. The DTH challenge 6 days later was with 5 µg of OVA-IFA in a total of 15 µl that were injected into the right rear footpad, whereas 15 µl of PBS alone were injected into the left rear footpad as a control. Thickness of rear footpads was quantified 24 and 48 h after the secondary immune challenge using a calibrated digital micrometer with 0.025-mm resolution (Fisher Scientific).

Measurement of tissue infiltration by lymphocytes in a mouse dorsal air-pocket model

OTII and DTG mice, as well WT mouse recipients of T cells from OTII and DTG mice, received a dorsal air pocket by injection of 5 ml of sterile air s.c. Two to three days later, 50 µg of OVA adsorbed to Al(OH)₃ was injected into each air pocket. Air-pocket cells were harvested in 4 ml of Ca^{2+}- and Mg^{2+}-free PBS at 96 h, and characterized by flow cytometry. One to 2 × 10⁷ nonadherent air-pocket cells were cultured for 24 h on a layer of autologous splenic mononuclear leukocytes with OVA, as in the cytokine generation studies. Concentrations of IL-17 in culture supernatants were quantified by ELISA.
Results

Heightened total expression of S1P1 by CD4 T cells of DTG mice compared with that by CD4 T cells of OTII mice

Quantification of mRNA encoding endogenous mouse S1P1 showed similar levels of expression by naive CD4 T cells from spleens of OTII and DTG mice, and similarly near total suppression of mouse S1P1 expression by OVA stimulation of CD4 T cells from both sources (Fig. 1A). Expression of human S1P1 obviously is limited to CD4 T cells of DTG mice, where the high levels of mRNA encoding this S1P1 in naive CD4 T cells is decreased a mean of 74% by OVA stimulation. Similar quantification of S1P1 mRNA in S1P1 single TG (STG) and DTG CD4 T cells indicated that the levels in DTG generally were somewhat higher than in STG. As expected, the level of S1P1 mRNA was suppressed very significantly in T cells from both sources by Ab stimulation of TCR and only in those of DTG T cells by Ag-dependent stimulation (Fig. 1B). The results of analyses of mRNAs were confirmed by those of S1P1 protein in Western blots using a rabbit anti-S1P1 antiserum that binds both human and mouse S1P1 (Fig. 1C). The total amount of S1P1 proteins in extracts of splenic naive CD4 T cells is several-fold higher for DTG than OTII mice. TCR-mediated activation of CD4 T cells from OTII mice by either OVA Ag or anti-TCR Abs eliminates immunochromically detectable S1P1 protein. In contrast, activation of CD4 T cells from DTG mice by either stimulus decreases the amount of, but does not delete, S1P1 protein (Fig. 1C). In fact, the level of S1P1 protein expressed by activated CD4 T cells of DTG mice is a range of 1.4- to 1.8-fold higher than that in naive CD4 T cells of OTII mice.

Functional consequences of differences in expression of S1P1 in splenic CD4 T cells of OTII and DTG mice

The relationships between altered levels of T cell expression of S1P1 and consequently modified T cell chemotactic responses to S1P were examined in detail because this major response of T cells to S1P is mediated solely by the S1P1R (9, 10). Broad activation of CD4 T cells from WT C57BL/6 mice, induced by incubation with adherent anti-CD3 plus anti-CD28 Abs, significantly decreased their chemotactic response to S1P, but not to the chemokine CCL21, as a result of down-regulation of S1P1 (Fig. 2). Further, equivalent activation of CD4 T cells from OTII mice, that also led to nearly complete down-regulation of mouse endogenous S1P1 and selectively decreased chemotaxis to S1P, was achieved by incubation either with adherent anti-CD3 plus anti-CD28 Abs or with APCs plus OVA. In contrast, the lesser decreases in expression of TG S1P1 by CD4 T cells of DTG mice evoked by either adherent anti-TCR Abs or APCs plus OVA were insufficient to suppress significantly their chemotaxis to S1P (Fig. 2). Thus, CD4 T cells from DTG mice, not from WT or OTII mice, maintain functionally relevant levels of expression of S1P1 in the face of TCR-mediated immune activation.

Diminished DTH and enhanced Ab responses in DTG mice

The immune responses of DTG mice to OVA were characterized to permit comparisons with those of T cell-selective S1P1 STG
FIGURE 5. Greater increases in IL-17 generation by OVA-stimulated CD4 T cells of DTG mice than OTII mice. A. In vitro secretion of IL-17 and IFN-γ. Each column and bar represents the mean ± SD of the concentrations of these cytokines detected by ELISA in supernatants of CD4 T cells incubated for 24 h with APCs + OVA and 10⁻⁹ to 10⁻⁶ M S1P (labeled as S9 to S6), 3 × 10⁻⁷ M or 3 × 10⁻⁶ M SEW2871 (labeled as W3–7 and W3–6), or medium alone (control = 100%) (n = 3 in duplicate). Significance of differences in cytokine secretion between S1P or SEW2871-treated T cells and control OVA-stimulated T cells (100%) were calculated by two-tailed unpaired t tests and denoted by: *, p < 0.05. The unstimulated absolute control (100%, shown as dotted line) values for 1.4 × 10⁶ CD4 T cells/well ranged from 17 to 25 ng/ml (OTII) and 18–35 ng/ml (DTG) for IFN-γ and from 1.3 to 3.2 ng/ml (OTII) and 2.7–6.2 ng/ml (DTG) for IL-17. B, IL-17 mRNA in T cells determined by TaqMan real-time RT-PCR. The normalized level of IL-17 mRNA for OTII-derived CD4 T cells incubated for 24 h with APCs and OVA was set at 1.00 for each of three studies. There was a significant difference between the mean (±SD, n = 4) values of IL-17 mRNA for OTII and DTG OVA-stimulated T cells (p < 0.05) when calculated by a two-tailed paired t test. C, ELISpot quantification of IL-17-containing splenic CD4 T cells. Each column and bar depicts the mean ± SD of the percentage of CD4 T cells that are IL-17-positive at 5 days (primary immunization of mice, left frame) and 21 days (adoptive transfer of T cells to WT C57BL/6 mice, right frame) after introduction of OVA. There were significant differences between the mean percentages with intracellular IL-17 of CD4 T cells from OVA-stimulated OTII and DTG mice (*, p < 0.01) when calculated by a two-tailed paired t test.
mice expressing a full range of TCRs. The same protocol of immunization with Ag s.c. followed in 6 days by footpad Ag challenge was used as in studies of DTH in S1P1 STG mice. The recipients were irradiated WT mouse populated by adoptive transfer with CD4 T cells from DTG mice or from OTII mice. DTH reactions to OVA were dramatically lower in recipients of CD4 T cells from DTG mice than in recipients of CD4 T cells from OTII mice (Fig. 3). In contrast, plasma concentrations of anti-OVA Abs were significantly higher in recipients of CD4 T cells from DTG mice than in recipients of CD4 T cells from OTII mice (Fig. 4). The heightened responses to OVA promoted by CD4 T cells of DTG mice were observed for IgG and two IgG subclasses, as well as IgA with different time courses. No IgE Abs were detected either in the experiments or when DTG mice and OTII mice were immunized directly with OVA adsorbed to Al(OH)3. The results of these studies of Ab responses contrast sharply with those of analyses of by the S1P 1-selective synthetic agonist SEW2871 (Fig. 5).

Enhanced generation/secretion of IL-17 by CD4 T cells from DTG mice

The concentration of IFN-γ in supernatants when splenic CD4 T cells from DTG and OTII mice were stimulated with OVA for 24 h was decreased significantly by 10-5 and 10-6 M S1P, as well as by the S1P1-selective synthetic agonist SEW2871 (Fig. 5A). This has been a consistent finding in several settings and is one basis for postulating that the S1P-S1P1 axis mediates a Th1 to Th2 devia- tion. Quite unexpectedly, however, similar concentrations of S1P and SEW2871 also augmented significantly the generation of IL-17 by CD4 T cells of DTG, but not OTII, mice after 24 h (Fig. 5A). CD4 T cells of DTG mice generated ~2-fold more IL-17 than those of OTII mice in the absence of exogenous S1P and the maximal increases in IL-17 with exogenous S1P was 1.8- and 2.5-fold, respectively, at 24 h. The apparent implication that S1P1 transduces enhancement of IL-17 production was supported by the ability of the S1P1-selective antagonist VPC44116 (IC50 = 30 nM) to inhibit the increase in IL-17 generation elicited by S1P (11). Precipitation of replicate suspensions of 3 × 105 CD4 T cells from DTG mice with VPC44116 at 10-6 M (equimolar with S1P) and 3 × 10-6 M (3-fold higher concentration than S1P) for 4 h at 37°C before addition of 10-7 M S1P and further incubation for 24 h suppressed IL-17 secretion by means of 44 and 72%, respectively (mean, n = 2). In two of the studies in which incubations were continued for up to 48 h, the level of S1P augmentation of IL-17 secretion by CD4 T cells of DTG mice was greater than at 24 h. These mean increases were significant at 284 and 322% of control (p < 0.05 for both) at 10-8 and 10-7 M S1P, respectively, whereas parallel mean increments for CD4 T cells of OTII mice were not significant at 126 and 140%. These observations were supported by documentation of a significant increase in the level of mRNA encoding IL-17 when splenic CD4 T cells of DTG, but not OTII mice, were stimulated with OVA (Fig. 5B).

To evaluate the possibility of greater IL-17 generation by OVA-stimulated CD4 T cells in DTG mice than OTII mice in vivo, a s.c. air-pocket model was used where lymphocytes responding to OVA in the pocket are harvested for analyses after 4 days. Significantly more OVA-specific TCR-bearing (DO11.10-positive) T cells migrated into the s.c. space in DTG than OTII mice (left frame, Fig. 6). The generation of IL-17 by these T cells also was significantly greater in DTG than OTII mice (right frame, Fig. 6).

Table I. Effect of higher expression of S1P1 on generation of IL-17 by CD4 T cells stimulated by anti-TCR Abs

<table>
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<th>Concentration of S1P (-log M)</th>
<th>CD4 T Cells</th>
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<td></td>
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<td>252</td>
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a Each value is the mean of the results of two assays conducted in duplicate and expressed as the percentage of control without S1P (100%). Control mean values were 318 and 810 pg of IL-17 per 2 × 105 T cells for WT and S1P1 TG mice, respectively.

Table II. Enhancement of CD4 T cell IL-17 production by S1P introduced at different times of incubation

<table>
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<th>CD4 T Cells</th>
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<td></td>
<td>6</td>
<td>141</td>
</tr>
</tbody>
</table>

a Each value is the mean of the results of two assays conducted in duplicate 24 h after the addition of S1P and expressed as the percentage of control without S1P (100%). Control mean values were 5,082 and 12,642 pg of IL-17 per 2 × 105 T cells of OTII and DTG mice, respectively, for 0 time addition and 1,364 and 4,460 pg of IL-17 per 7 × 105 T cells of OTII and DTG mice, respectively, for 24 time addition of S1P.
Discussion

The immune phenotype of a recently established line of C57BL/6 DTG mice, in which T cells express constitutively the OTII TCR specific for peptide 323–339 of OVA and a high level of human S1P1 that is relatively resistant to down-regulation from T cell activation (Fig. 1), includes some features predictably similar to those of human S1P1 STG mice (7). Chemotaxis of DTG T cells to S1P and the S1P1-specific agonist SEW2871 was not significantly diminished by prior T cell activation (Fig. 2), which did down-regulate S1P1, and thereby suppress S1P chemotaxis of WT and OTII T cells (Figs. 1 and 2). Activation-induced down-regulation of S1P1 in T cells of OTII mice also prevented S1P inhibition of their chemotaxis to chemokines, which was not observed in T cells from DTG mice or S1P1 STG mice (not shown in Fig. 2). The finding of a higher level of influx of DTG T cells than OTII T cells into the s.c. space of an air pocket after local OVA challenge (Fig. 6) is consistent with their lesser loss of chemotactic responses to a cutaneously generated S1P stimulus after TCR-mediated activation. The strikingly reduced DTH reaction to OVA in DTG mice, contrasted with that in OTII mice (Fig. 3), was similar to the difference documented between S1P1 STG mice and WT mice in the same assay system (7). The capacity of TG S1P1 in T cells of either DTG or STG mice to diminish cutaneous DTH initially was attributed to greater S1P suppression of generation of IFN-γ and/or heightened inhibition by S1P of chemotactic attraction by chemokines. Although the latter possibility has not been examined in this system, there clearly is not lower production of IFN-γ by CD4 T cells from DTG mice than by those from OTII mice at equivalent concentrations of S1P (Fig. 5).

The observation that DTG mice mount greater IgG and IgA responses to OVA than OTII mice (Fig. 4), whereas IgG Ab responses to hapten-protein conjugates in STG mice were much lower than in WT mice (7), focused attention on other possible differences in cytokine systems. These investigations led to the discovery of heightened secretion of IL-17 when OVA-stimulated CD4 T cells from DTG mice, but not those from OTII mice, were incubated with 0.1 μM or higher concentrations of S1P or equivalent levels of the S1P1-selective agonist SEW2871 (Fig. 5A). In contrast, IFN-γ secretion by OVA-stimulated CD4 T cells from both sources was suppressed to similar levels by these concentrations of S1P and SEW2871. In these same sets of OVA-stimulated CD4 T cells, levels of mRNA encoding IL-17 were significantly higher in those from DTG mice than OTII mice (Fig. 5B). Further, ELISPOT assays revealed very significantly higher percentages with intracellular IL-17 in OVA-stimulated CD4 T cells from DTG than OTII mice, whether responding T cells were endogenous or adoptively transferred (Fig. 5C). That this augmentation of the IL-17 pathway is relevant in vivo was proven in a s.c. air-pocket model, in which OVA was introduced into the pocket and responding lymphocytes are harvested 4 days later (Fig. 6). The mean quantity of IL-17 produced by T cells from OVA-stimulated pockets in DTG mice was 2.5-fold higher than that from those in OTII mice, which is very significantly higher.

Any postulation of the immune consequences of concurrent suppression of Th1 cells and enhancement of Th17 cells and their respective cytokine products in DTG mice will depend on the distinctive immunological contributions attributable to these major subsets of CD4 T cells. The most novel effects of overexpression of the S1P-S1P1 axis were expansion of the Th17 subset and a resultant increase in secretion of IL-17 (Figs. 5 and 6). Rapidly progressing characterization of Th17 cells suggests that commitment, expansion, maintenance, and activation of this subset are separately dependent on distinct arrays of cytokines (12, 13). Once their mature functions are established, Th17 cells secrete TNF-α and IL-6, the latter of which can promote differentiation of additional Th17 cells, as well as IL-17, that in turn makes broad primary contributions to host defense ranging from promoting macrophage secretion of IL-1, TNF-α, and IL-6 to eliciting keratinocyte secretion of antimicrobial peptides (14, 15). The immune roles of Th17 cells and factors critical for their maintenance similarly range broadly from regional defense against microbes to stimulation of neutrophilic tissue inflammation to initiation of various autoimmune diseases (14, 16). Thus, augmentation by the S1P-S1P1 axis of the numbers and activities of Th17 cells may in some settings enhance host defense against microbes and in other settings increase host susceptibility to autoimmune diseases. The exact roles of the S1P-S1P1 axis in commitment, differentiation, and functional control of Th17 cells are not yet known. Thus, it is entirely possible that high levels of expression of S1P1 act predominantly by increasing host susceptibility to autoimmune diseases and that immunosuppressive drugs, which selectively evoke sustained down-regulation of S1P1, act principally by decreasing the number and activity of Th17 cells (17). Additional studies are needed of the integrated immune effects of enhanced levels and

FIGURE 6. OTII mouse and DTG mouse T cell influx and IL-17 generation in an air-pocket model. Each column and bar depicts the mean ± SD of results of studies of six mice. T cell expression of the OTII mouse TCR marker D011.10 was determined flow cytometrically (left frame) and IL-17 production in 24 h by mixed lymphocytes from air pockets challenged again in vitro with OVA was measured by ELISA (right frame). Statistically significant differences between results for OTII and DTG mouse lymphocytes were calculated by a two-tailed unpaired t test, where *, p < 0.01.
activities of Th17 cells in DTG mice, the autoimmune consequences of augmented Th17 cell effects in DTG mice, and the ability to reverse such Th17-mediated effects by suppressing activity of the S1P-S1P₁ axis.

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