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*J Immunol* 2003; 171:1542-1555; doi: 10.4049/jimmunol.171.3.1542
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Role of Regulator of G Protein Signaling 16 in Inflammation-Induced T Lymphocyte Migration and Activation

Eric Lippert,* David L. Yowe,§ Jose-Angel Gonzalo,§ J. Paul Justice,‡ Jeremy M. Webster,§ Eric R. Fedyk,§ Martin Hodge,§ Cheryl Miller,§ Jose-Carlos Gutierrez-Ramos,§ Francisco Borrego, † Andrea Keane-Myers,‡ and Kirk M. Druey1*

Chemokine-induced T lymphocyte recruitment to the lung is critical for allergic inflammation, but chemokine signaling pathways are incompletely understood. Regulator of G protein signaling (RGS)16, a GTPase accelerator (GTPase-activating protein) for Gα subunits, attenuates signaling by chemokine receptors in T lymphocytes, suggesting a role in the regulation of lymphocyte trafficking. To explore the role of RGS16 in T lymphocyte-dependent immune responses in a whole-organism model, we generated transgenic (Tg) mice expressing RGS16 in CD4+ and CD8+ cells. rgs16 Tg T lymphocytes migrated to CC chemokine ligand 21 or CXC chemokine ligand 12 injection sites in the peritoneum, but not to CXC chemokine ligand 12. In a Th2-dependent model of allergic pulmonary inflammation, CD4+ lymphocytes bearing CCR3, CCR5, and CXCR4 trafficked in reduced numbers to the lung after acute inhalation challenge with allergen (OVA). In contrast, spleens of sensitized and challenged Tg mice contained increased numbers of CD4+CCR3+ cells producing more Th2-type cytokines (IL-4, IL-5, and IL-13), which were associated with increased airway hyperreactivity. Migration of Tg lymphocytes to the lung parenchyma after adoptive transfer was significantly reduced compared with wild-type lymphocytes. Naïve lymphocytes displayed normal CCR3 and CXCR4 expression and cytokine responses, and compartmentation in secondary lymphoid organs was normal without allergen challenge. These results suggest that RGS16 may regulate T lymphocyte activation in response to inflammatory stimuli and migration induced by CXCR4, CCR3, and CCR5, but not CCR2 or CCR7. The Journal of Immunology, 2003, 171: 1542–1555.

Chemokines orchestrate coordinated movement of T lymphocytes through lymphoid organs and promote their migration to sites of inflammation. Distinct patterns of chemokine receptors are expressed in naive and activated T cell populations, and gene-targeting experiments have implicated a role for specific chemokines and their receptors in T cell activation, differentiation into Th1 or Th2 subtypes, and inflammatory responses mediated by either of these subpopulations (1–5). Asthma is a Th2-mediated disease in which CD4+ lymphocytes migrate to the lung parenchyma upon allergen exposure and secrete proinflammatory cytokines (IL-4, IL-5, and IL-13), which leads to recruitment and activation of eosinophils and mast cells and subsequent lung inflammation and injury (4–7). In murine asthma models, Th2 migration to the lung may be dependent on, among others, CCR3, CCR4, CCR5, CCR6, CCR8, and CXCR4 (8–13).

The signaling pathways that mediate chemokine-induced trafficking are not well understood. Although chemokine receptors couple to Gαq and Gα12/13, chemotaxis is not evoked by receptors coupled exclusively to Gαq (14). However, despite the necessity for a Gαq-coupled receptor, Gαq activation is not required for chemotaxis, suggesting that Gβγ may initiate the cascade that induces directed cell migration (14, 15). Nonetheless, thymic emigration (16), transendothelial migration of lymphocytes into lymph nodes (1, 17), and Ag-induced recruitment of lymphocytes to the lung (18) are blocked by pertussis toxin (PTX),2 which inactivates Gα/ Go proteins. Consistent with a role for Gαq signaling in T cell activation, Gαq-deficient T lymphocytes produce higher levels of proinflammatory cytokines in the gut, which is associated with inflammatory colitis (19, 20). Whether this abnormality is related directly to Gαq function in T lymphocyte migration is unknown.

Recently, endogenous molecules poised to regulate chemokine responses have been described: the regulator of G protein signaling (RGS) proteins, which are GTPase-activating proteins (GAPs) for Gα and Gαq subunits of heterotrimeric G proteins (21, 22). RGS GAP activity is predicted to attenuate G-protein-coupled receptor (GPCR) responses including chemotaxis, and RGS overexpression inhibits chemotactic and proadhesive responses in lymphocyte cell lines in vitro (23, 24). T lymphocytes express several RGS proteins, including RGS2 (25), RGS3 (26), RGS4 (27), RGS14 (28), and RGS16 (29, 30). rgs16 mRNA is increased in T cells after IL-2 stimulation, whereas rgs2 mRNA is down-regulated by IL-2 (29). Deficiency of RGS2, which, like RGS16, modulates Gαq (31) and Gαi (32) pathways, results in impaired T lymphocyte proliferation and IL-2 production in response to TCR or PMA stimulation (33). rgs2-/- mice also exhibit delayed and reduced responses to lymphocytic choriomeningitis virus infection, which is a model of Th1-dependent inflammation.

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Received for publication December 20, 2002. Accepted for publication May 27, 2003.

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2 Abbreviations used in this paper: PTX, pertussis toxin; RGS, regulator of G protein signaling; GAP, GTPase-activating protein; GPCR, G-protein-coupled receptor; AHR, airway hyperreactivity; RT-QPCR, reverse transcriptase-quantitative PCR; C57, threshold cycle; ORF, open reading frame; MAP, mitogen-activated protein; HA, hemagglutinin; CCL, CC chemokine ligand; CXCL, CXC chemokine ligand; PECAM, platelet endothelial cell adhesion molecule; BAL, bronchoalveolar lavage; BALF, BAL fluid; rag, recombinase-activating gene; AC, adenylyl cyclase; WT, wild type; Tg, transgenic.

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0022-1767/03/$02.00
Although RGS16 and RGS2 display similar GAP activity in vitro, it is unclear whether RGS16 plays an equivalent immunoregulatory role in T cells. Few in vivo models of RGS biology exist, and a primary physiological function has been established only for one RGS protein, RGS9-1, which regulates phototransduction in the retina (34). To explore the role of RGS16 in T cell-mediated immune responses, we generated transgenic (Tg) mice expressing RGS16 in CD4+ and CD8+ lymphocytes. Although lymphocyte distribution in thymus and lymphoid organs in the absence of inflammation was normal in Tg mice, T lymphocytes migrated aberrantly to a site of i.p. injection of select chemokines. In addition, while Th2 recruitment to the lung in response to allergen challenge was impaired, rgs16 Tg T cells displayed an enhanced activation phenotype, including increased proliferation and cytokine production, which correlated with increased serum eotaxin levels and airway hyperreactivity (AHR). These results suggest that RGS16 modulates T cell activation and migration to an inflammatory stimulus in vivo by regulating signaling through specific chemokine receptors.

Materials and Methods

RGS16 expression analysis and Abs

Th1 and Th2 cells were differentiated as described elsewhere (35). In brief, human peripheral blood CD4+ CD45RA- cells were cultured in plates containing immobilized anti-CD3 (10 ng/ml), JY cells, and exogenous cytokines. For Th1 cells, cultures were grown in the presence of IL-18 (5 ng/ml), IL-6 (5 ng/ml), IL-12 (20 ng/ml), and anti-IL-4 (5 μg/ml). Th2 cells were differentiated by culturing T cells in the presence of IL-18 (5 ng/ml), IL-6 (5 ng/ml), IL-4 (20 ng/ml), and anti-IL-12 (5 μg/ml). At weekly intervals, cultures were centrifuged in Ficoll-Hypaque (Amersham Biosciences, Piscataway, NJ) to remove dead accessory cells and subsequently cultured with the above cytokines and Abs. Activated Th1 and Th2 cells were maintained in this manner for three cycles and then reactivated using the cytokine combinations described above for the desired period of time. ELISA (Endogen, Woburn, MA; R&D Systems, Minneapolis, MN) was used to quantify cytokine (IFN-γ, IL-5, and IL-13) production by Th1 and Th2 cultures.

Real-time reverse transcriptase-quantitative PCR (RT-PCR) was performed using the TaqMan method (PerkinElmer, Wellelsley, MA). Total RNA was isolated using RNA STAT-60 (Tel-Test, Friendswood, TX), DNase I treatment, and phenol extraction. cDNA was synthesized from 5 μg of total RNA using the Superscript kit and oligo(dT) (Life Technologies, Rockville, MD). A no-amplification control containing RNA without reverse transcriptase was prepared to ensure the DNase I treatment was complete. No-tissue controls that contained buffer and enzyme only were also included. The expression level of rgs16 and the internal reference β2-microglobulin was measured by real-time PCR using primers labeled with FAM or VIC (PerkinElmer), respectively. The primers (forward, TT TGCCTGTGCTGCTATTGTAGCT, and reverse, CCGCTTGGCA GAA) and FAM probe (TGGTTGATCCTGGTTTCTAAATCCCGA) were designed using Primer Express software (PerkinElmer). The simultaneous measurement of rgs16-FAM and β2-microglobulin-VIC allowed for normalization of the amount of cDNA added per sample. Duplicate PCRs were performed using the TaqMan Universal PCR Master Mix and the ABI PRISM 7700 sequence detection system (PerkinElmer) using the following thermal cycle routine: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s, followed by 60°C for 1 min. A comparative threshold cycle (CT) method (User Bulletin no. 2; PerkinElmer) was used to determine gene expression relative to the no-tissue control (calibrator). To determine relative expression levels, we used the following formula:

\[ \Delta \Delta CT = \frac{\Delta CT_{\text{sample}}}{\Delta CT_{\text{calibrator}}} \]

For immunoblotting, cells were lysed in radioimmuno precipitation analysis buffer, subjected to SDS-PAGE (12% Tris-glycine gels), and transferred to polyvinylidene difluoride membranes. RGS16 was detected using polyclonal antiserum raised against His6RGS16, purified by protein A affinity chromatography, or with an anti-RGS16 antisera that has been described previously (CT265) (36). Signal was revealed using goat anti-rabbit IgG-HRP and ECL according to standard methods (Pierce, Rockford, IL). Antiserum against Gαq (AS7) was the kind gift of A. Spiegel (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health).

Generation of rgs16 Tg mice and genotyping

A SalI/XhoI fragment of the murine rgs16 cDNA was inserted into a Tg expression vector containing the murine CD4 enhancer and promoter lacking the CD8 silencer (p37.1, the kind gift of D. Littman (New York University School of Medicine, New York, NY) (37). Tg mice were generated by pronuclear injection of linearized plasmid into FVB (Taconic Farms, Germantown, NY) or BALB/CByJ (The Jackson Laboratory, Bar Harbor, ME) oocytes using standard protocols. Transgene-carrying mice were identified by Southern blotting of genomic DNA purified from mouse tails using a SalI/EcoRI fragment of the rgs16 cDNA (covering the first 300 bp of the rgs16 open reading frame (ORF), which is highly specific to rgs16 as opposed to other RGS family members) or by PCR using the following primers specific for the transgene: Tg16-1, GGGTTTTGTCGACCCA CCGGTCGCGCCACGG; and Tg16-2, TTCCCCACCTCGAGTTCCAC CTCTCTTCCCCGTTGCG. The following primers specific for endogenous rgs16 were used as a control for the PCR: En16-1, GAAGCTTTCTCA GAAGATGTAC; and En16-2, ACCTCTTTAGGGGTTCCG. Double Tg mice were obtained by crossing rgs16 Tgs with mice carrying the transgene for the DO11.10 TCR αβ, which recognizes residues 323–339 of chicken OVA in association with I-Ad (Taconic Farms). All research involving the use of mice was performed in accordance with protocols approved by Millennium Pharmaceuticals and/or the National Institutes of Health Institutional Animal Care and Use Committees. Male or female mice (6–20 wk of age) were used for all experiments.

For Northern blotting, total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA), and 20 μg per sample was fractionated on a 1% formaldehyde denaturing gel. RNA was transferred to nitrocellulose and hybridized with the aforementioned rgs16-specific probe at 60°C using Rapid-Hyb buffer (Amersham Pharmacia Biotech, Piscataway, NJ).

Mitogen-activated protein (MAP) kinase assays and measurement of adenyl cyclase (AC) activity

These assays were conducted exactly as described previously (38). Abs against p-Erk, hemagglutinin (HA), and Erk1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Jurkat cells were transfected with either pcDNA3 or pcDNA3-HA-RGS16 (29) using Superfect (Qiagen), and stable transfectants were selected with G418.

In situ hybridization

Frozen 6-μm sections of normal and Tg mouse lymph nodes, spleens, and thymuses were fixed in 4% paraformaldehyde, washed in PBS, acetylated in 0.1 M triethanolamine-HCl and 0.25% acetic anhydride (Sigma-Aldrich, St. Louis, MO) for 10 min, washed in 2× SSC, dehydrated with ethanol, and air dried. Slides were hybridized overnight at 55°C with 5′S-labeled human aminotransferase riboprobes at 2.5 × 10⁶ cpm/slide. Slides were subsequently washed and dipped in photographic emulsion (Kodak, Rochester, NY) and exposed for 6 wk at 4°C before autoradiography. Slides were then counterstained with H&E to assess histology. The following primers were used to generate the rgs16 riboprobe: T3, AAATACCCCTACTAAA GGGTTGCGCAGTAAGATACAA, and T7, TAATAGCCTCAC TATAGGCTCAATTGGGCTCC. The platelet endothelial cell adhesion molecule (PECAM) probe consisted of a PCR fragment comprising 1246–1983 (GenBank accession number M37780).

In vivo chemotaxis assays

Peritoneal recruitment assays were performed as previously described (39). Briefly, mice were injected i.p. with CXC chemokine ligand (CXCL)12 or CC chemokine ligand (CCL)12 (R&D Systems) (200 μl of a 5 μg/ml solution) or PBS. At different time points after injection, peritoneal leukocytes were collected by lavage and enumerated. To determine the percentage of each leukocyte subtype, we pelleted cells (5 × 10⁶) onto glass slides by cytospin at 600 rpm and stained with Wright-Giemsa. The number of each leukocyte subtype was counted in eight high-power fields (magnification, ×40; total area, 0.5 mm²) per area and divided by the total number of cells per high-power field. To obtain the absolute number of each leukocyte subtype in lavage, these percentages were multiplied by the total number of cells recovered from the peritoneal fluid. Immunophenotyping of migrated cells was determined by flow cytometry using fluorochrome-conjugated anti-CD4, -CD8, and -CXCR3 (BD Pharmingen, San Diego, CA).
OVA sensitization and challenge and measurement of AHR

Mice were sensitized by an i.p. injection of 100 µg of OVA (Sigma-Aldrich) in 200 µl of 50% Injekt Alum (Pierce). After 7–10 days, mice were subjected to a 30-min nebulization of 1% OVA in PBS daily for three consecutive days. Seventy-two hours after the last challenge, airway resistance was evaluated using an intubated plethysmography system (Flexivent; SCIREQ, Montreal, Quebec, Canada). Bronchoalveolar lavage (BAL) was performed by injecting 500 µl of PBS containing 1% BSA, and a 50-µl aliquot was removed and subjected to cytocentrifugation for cell quantitation. After centrifugation at 500 x g, supernatants were stored at −80°C for measurement of cytokines.

Immunophenotyping

Lungs from sensitized and challenged mice were dissected en bloc and ground into single-cell suspensions using a Medimachine (BD Biosciences, Bedford, MA) were then added either in medium alone (AIM V; Life Technologies), in medium containing Con A (2.5 µg/ml; Sigma-Aldrich) as a positive control or in medium plus OVA at 2 or 20 µg/ml. After a 48-h incubation for IL-4 or 72-h incubation at 37°C, plates were washed three times with PBS and four times with PBS containing 0.025% Tween 20 (PBS-T). Secondary biotinylated Ab was then added (all Abs were diluted 1/250 in PBS containing 1% BSA and 0.025% Tween 20 (PBS-BT)). The plates were incubated overnight at 4°C, washed three times in PBS-T, and incubated for 90 min at room temperature with a 1/250 dilution of HRP-streptavidin in PBS-BT. After four more washes in PBS, peroxidase activity was revealed by 3-amino 9-ethyl-carbazol in the presence of H2O2. Cytokines in supernatants were stored at −1544 RGS16 REGULATES T LYMPHOCYTE FUNCTION

Generation of rgs16 T cell Tg mice

Because of its expression in activated T lymphocytes and regulation of chemokine signaling, we hypothesized that RGS16 could play a role in T cell migration and activation in vivo. To explore this hypothesis in a whole organism model, we generated rgs16 Tg mice in which rgs16 transcription is driven by the CD4 enhancer/promoter lacking the CD8 intronic silencer, allowing expression in CD4+ and CD8+ lymphocytes, but not in B cells, macrophages, NK cells, or monocytes (Fig. 2A) (37). Southern blotting confirmed the presence of the transgene (Fig. 2B). Consistent with T lineage expression, we observed the Tg RNA species in thymus, spleen, and lymph nodes from Tg mice, but not WT mice (Fig. 2C). To examine RGS16 protein expression in Tg T lymphocytes, we sorted CD4+ and CD8+ populations from freshly isolated splenocytes by flow cytometry and immunoblotted cell lysates with anti-RGS16 Abs. In Tg mice, RGS16 was expressed constitutively in both CD4+ and CD8+ populations, but not in resting CD4+ or CD8+ cells from WT mice nor in CD4-CD8- cells from either WT or Tg mice (Fig. 2D). In contrast, levels of Gr1, in CD4+ cell membranes were similar in WT and Tg mice. Two Tg founder lines were generated in either the FVB or BALB/c strains, which expressed broadly comparable levels of RGS16 and virtually indistinguishable phenotypes (our unpublished data).

To confirm that RGS16 overexpression modulates chemokine signaling in primary murine T cells, we isolated splenocytes from WT or Tg mice and measured MAP kinase activation evoked by CXCR4 stimulation. We observed reduced phosphorylation of Erk1 in Tg splenocytes stimulated with CXCL12, indicating that constitutive RGS16 expression inhibits MAP kinase activation mediated by CXCR4 in murine splenocytes (Fig. 2E).
RGS16 expression does not impair T cell development or homeostatic trafficking to secondary lymphoid organs

**rgs16** Tg mice were fertile, and no overall difference in mortality compared with that of WT mice was observed. No significant changes in the number of circulating CD3+CD4+, CD4+, CD8+, or B cells were observed in mice carrying the transgene (our unpublished data). To ascertain whether RGS16 affected basal lymphocyte trafficking, we performed histological examination of spleen, thymus, and lymph nodes from Tg mice, which revealed no abnormalities (Fig. 3). In situ hybridization revealed normal expression of PECAM-1 (CD31) in vascular endothelium (Fig. 3A, thymus) and in high endothelial venules (B, lymph nodes; C, spleen), suggesting normal organ architecture. Furthermore, analysis of rgs16 mRNA expression in these tissues demonstrated a normal distribution of T lymphocytes. These data indicate that compartmental homing of naive lymphocytes to secondary lymphoid organs in the absence of inflammatory or pathogenic stimuli was normal in Tg mice. Finally, no colon pathology was observed in Tg animals, in contrast to the inflammatory colitis seen in **rgs16**−/−deficient mice (our unpublished data).

Selectively impaired lymphocyte chemotaxis/homing in **rgs16** Tg mice

Our previous studies showed that RGS16 interacts with AlF4-activated G0i and G0q in Jurkat cells and inhibits signaling induced by transfected CXCR1 receptor stimulation of these G proteins in HEK 293 cells (29). Because of RGS16 G0i GAP activity, we hypothesized that **rgs16** Tg lymphocytes would migrate abnormally to chemokine stimuli in vivo. To assess chemokine-induced homing of naive, circulating lymphocytes in the absence of immune provocation, we injected chemokines directly into the peritoneum of WT or Tg mice, harvested cells by peritoneal lavage, and quantitated the number of recruited cells. Although there was no difference in the quantity of WT or Tg lymphocytes recruited to the peritoneum by sham (PBS) injection, the number of lymphocytes migrating from 1–6 h after CXCL12 injection was dramatically reduced in Tg mice (Fig. 4, A and B). There was no significant difference between Tg and WT mice in numbers of eosinophils, macrophages, or neutrophils in the peritoneum after CXCL12 injection (Fig. 4A). To characterize the phenotype of migrated cells, we measured cell surface marker expression by flow cytometry. In the lymphocyte-gated population, the percentage of CD4+ cells in peritoneal lavage fluid was reduced from 23.6 ± 2.4% in WT mice to 15.2 ± 2.7% in Tg animals (mean ± SEM of two independent experiments of three to five mice in each group), and the percentage of CD8+ cells was 9.6 ± 4% in WT compared with 3.2 ± 1.3% in Tg mice. In addition, the number of CD4+CXCR4+ and CD8+CXCR4+ cells was reduced in peritoneal fluid obtained from Tg mice in comparison with those of WT (Fig. 4C).
RGS16 REGULATES T LYMPHOCYTE FUNCTION

To determine whether the impaired migration of Tg lymphocytes was specific to CXCL12, we injected CCL12, which acts on CCR2. Neutralizing Abs to CCL12 have been shown to reduce AHR in mouse asthma models by affecting eosinophil trafficking within the lung. There was no change in CCR2-mediated recruitment of peritoneal lymphocytes/monocytes or eosinophils in Tg mice compared with WT (Fig. 4D). These results suggest that RGS16 expression in T lymphocytes attenuates migration selectively evoked by the chemokine ligand for CXCR4, but not CCR2.

Tg lymphocyte migration in acute T cell-dependent inflammation

To determine whether RGS16 expression affected T cell migration and/or activation in response to an inflammatory stimulus, we characterized pulmonary responses induced by OVA challenge. In this model, OVA-specific CD4+ Th2 cells are recruited to the lung, where their secreted cytokines act directly on airways to attract and activate primary effector cells in the allergic response such as epithelial cells, mast cells, basophils, and eosinophils. These cells, in turn, secrete proinflammatory mediators such as chemokines, histamine, leukotrienes, and TNF-α, which cause bronchoconstriction, submucosal edema, epithelial sloughing, and increased mucus production (4, 5, 13, 41, 42). Histological lung changes result in AHR to inhaled stimuli such as methacholine, an agonist of airway smooth muscle m3 muscarinic receptors. Mice were sensitized once with an i.p. injection of OVA, followed by three challenges with nebulized OVA solution (Fig. 5A). We assessed AHR by determining airway resistance in response to methacholine and lung cellularity by counting cells in BALF. However, in this model, we observed essentially no difference between WT and Tg mice in the number of lymphocytes, neutrophils, eosinophils, or macrophages recruited to the lung (Fig. 5B).

We hypothesized that, although overall BALF lymphocyte numbers were similar in WT and Tg mice, the migration of discrete chemokine receptor-bearing T cell populations could be affected specifically by RGS16 expression. To explore this possibility further, we analyzed cell surface expression of CD4, CD8, CCR3 (expressed on Th2 but not Th1 cells), CCR5 (preferentially expressed on Th1 cells), and CXCR4 (expressed mainly on Th2 cells) in T cell-enriched preparations derived from lung single-cell suspensions. There was no significant decrease in the percentage of CD4+ or CD8+ cells in T lymphocyte suspensions from Tg lungs compared with WT (Table I). Because CD4+ cells are thought to be the predominant effector T cells mediating allergic inflammation in the lung (42), we characterized chemokine receptor expression on this subpopulation. Strikingly, the percentages of CD4+CCR3+, CD4+CCR5+, and CD4+CXCR4+ cells were reduced in lungs of Tg mice (Table I), as was the level of CCR3 and CCR5 expression on CD4+ lymphocytes (Fig. 5C). These results suggest that RGS16 expression affects CCR3-, CCR5-, and CXCR4-dependent trafficking patterns of lymphocytes in response to OVA challenge.

To assess chemokine receptor expression under basal conditions and to determine whether OVA-induced lymphocyte trafficking to other lymphoid compartments might be altered, we measured CCR3 expression on splenic T cells before and after OVA challenge. Naive spleens from Tg mice contained similar (or slightly decreased) percentages of CD4+, CD8+, and CCR3+ T lymphocytes compared with those of WT (Fig. 5D). In OVA-challenged and -sensitized Tg mice, the overall number of CD4+ and CD8+ lymphocytes was either unchanged or decreased compared with those of WT. However, percentages of CD4+CCR3+ and CD8+CCR3+ cells were increased 2- to 3-fold (Fig. 5E). These data suggest that sensitized and challenged Tg spleens contain...
more Th2 lymphocytes than do WT controls, in contrast to the makeup of lung lymphocyte populations.

Because this analysis implied that differentiated Tg Th2 cells are restricted from infiltrating the lung parenchyma in response to allergen, we expected decreased AHR in Tg mice. Surprisingly, however, although there were no significant differences in baseline airway reactivity between the two groups, Tg mice exhibited a significant increase in airway resistance to methacholine after OVA challenge compared with WT mice (Fig. 5F). Thus, the decrease in numbers of relevant proinflammatory T lymphocytes in the lung is offset by increased airway reactivity in rgs16 Tg mice.

RGS16 expression is associated with enhanced T cell cytokine secretion

Although adoptive transfer of PTX-treated Th2 lymphocytes impairs their migration to OVA-challenged lungs, the lymphocytes

FIGURE 3. Normal homeostatic lymphoid organ compartmentation in rgs16 Tg mice. In situ hybridization was performed on sections from WT (i, iii, and v) or Tg (ii, iv, and vi) thymuses (A), lymph nodes (B), and spleens (C). Dark-field images (×100) are shown for sections hybridized with rgs16 antisense (iii and iv), rgs16 sense (vi), or PECAM antisense (v) riboprobes. Bright-field images (i and ii) are shown for each organ after H&E staining.
FIGURE 4. RGS16 inhibits lymphocyte chemotaxis to the peritoneum in response to select chemokines. A, WT or Tg mice were injected with PBS or CXCL12. Two hours later, cells were harvested by peritoneal lavage, and lymphocytes/monocytes were enumerated by Giemsa staining. Left graph represents mean ± SEM from six mice in each group stimulated with PBS and nine WT or Tg mice injected with CXCL12. (**, $p = 0.001$, repeated measures ANOVA with Tukey-Kramer posthoc test). Right graph represents numbers of each cell type in a count of 400 cells from peritoneal lavage fluid after cytocentrifugation and H&E staining (mean ± SEM of two independent experiments of three to five mice in each group; ***, $p = 0.009$ between WT and Tg lymphocytes, standard t test). C, Experiment as in A, except that cells were harvested at 1, 2, and 6 h after chemokine injection and quantitated as above. The zero time point represents mice injected with PBS. Values are mean ± SEM from two to three mice in each group. D, Immunophenotype of peritoneal cells. Cells from peritoneal lavage after CXCL12 stimulation were fixed and analyzed for CD4, CD8, and CXCR4 expression by flow cytometry. Numbers represent percentages of double-positive cells (CD4 or CD8 and CXCR4) from two pooled mice representative of six mice in each group. E, Mice were injected with CCL12, and after 2 h, peritoneal lymphocytes/monocytes and eosinophils were quantitated as above.
are still functional and able to induce AHR when instilled directly into the airway (18). Thus, the apparent discrepancy between AHR and T cell lung infiltration in rgs16 Tg mice led us to examine the activation status of Tg T cells directly. We hypothesized that, because loss of Gαi2 function results in accentuated T cell responses (19, 20), RGS16 overexpression could augment activation, because RGS16 inhibits Gαi2 function. In turn, enhanced cytokine secretion might cause direct lung injury and AHR independent of cellular infiltration, which has been demonstrated in previous studies (8, 44–48). Surprisingly, the number of CD4+CD25+ cells in lung was decreased in Tg mice compared with that of WT (Table I). These cells could represent either activated cells or regulatory T cells with a suppressive phenotype (49). To analyze T cell activation further, we measured cytokine production by splenocytes in recall assays after in vivo OVA sensitization. Although we detected no differences in IL-2 production, rgs16 Tg lymphocytes secreted significantly more IL-4 and IL-5 in response to OVA (Fig. 6A). Alternatively, elevated cytokine levels could reflect the increased population of Th2-differentiated cells in the spleen of OVA-exposed Tg animals (see Fig. 5E).

To further clarify the role of RGS16 in T cell activation, we generated mice Tg for both rgs16 and a TCR specific for the OVA peptide D011.10. We cultured naïve splenocytes and measured cytokine production in response to anti-CD3 plus anti-CD28 or OVA peptide. In contrast to primed T lymphocytes, naïve Tg T cells produced similar amounts of IL-2, IL-5, and IFN-γ in response to either of these stimuli (Fig. 6B). However, when double Tg mice were sensitized with OVA before splenocyte recall with OVA peptide ex vivo, lymphocytes produced slightly less IFN-γ but increased IL-5. There was a significant difference in the ratio of IL-5/IFN-γ between WT and Tg mice (Fig. 6C). Production of IL-13 by Tg lymphocytes was also increased ~2-fold compared with WT in response to anti-CD3 plus anti-CD28. Collectively, these data suggest that RGS16 expression is associated with increased production of Th2 cytokines in CD4+ cells that have been primed with allergen in vivo. Whether these findings represent increased general activation or skewing toward a Th2 response will require assessment of lymphocyte responses to a pathogen that evokes a Th1 response.

Another cytokine implicated in allergic responses is eotaxin, which enhances recruitment and activation of eosinophils. Serum eotaxin levels correlate with impairment of lung function in human asthmatics (13). Although eotaxin (as well as IL-4 and -13) levels in BALF or supernatants from activated splenocytes were too low to measure in our model, serum eotaxin was increased ~2-fold in challenged Tg mice compared with WT. Taken together, the increased IL-4, -5, -13, and eotaxin levels in Tg mice indicate a more pronounced Th2 microenvironment, which could contribute to enhanced airway responses.

Abnormal allergen-induced migration and cytokine secretion by adoptively transferred Tg lymphocytes

To confirm that rgs16 Tg lymphocytes traffic aberrantly in response to allergen, we passively transferred sensitized Tg lymphocytes into an OVA-challenged recipient. We sensitized donor WT or Tg mice and recipient mice with OVA. Spleens were harvested from donors, and whole splenocytes were fluorescently labeled with CFSE. Equal numbers of labeled cells were then systemically injected into recipient WT mice. After three subsequent OVA challenges, we collected BALF, followed by harvest of lungs, spleen, and cervical lymph nodes of recipient mice. We then quantified numbers of CFSE+CD3+ cells in each organ by flow cytometry (Fig. 7A). To measure the effect of the rgs16 transgene on cell recruitment, we determined the ratio of CFSE+CD3+ cells to CFSE+CD3− cells, which corrects for inequities in total numbers of labeled cells injected into recipient mice. Although there was no difference in the quantity of Tg and WT lymphocytes in the spleen, there were ~20% fewer CD3+ cells in lungs and 35% fewer T lymphocytes in draining lymph nodes of recipients of rgs16 Tg splenocytes after allergen challenge (Fig. 7B). Similarly, numbers of injected CD4+ cells were reduced in both lung (by 25%) and lymph nodes (40%) in recipients of Tg splenocytes compared with WT.

To assess the activation of injected, sensitized T lymphocytes after injection into recipient mice, we first measured cytokine levels in BALF after OVA challenge. Although BALF IL-2 levels were comparable in recipients injected with either WT or Tg splenocytes, IL-5 levels were substantially increased (~2.5-fold) in BALF from recipients of rgs16 Tg cells (Fig. 7C). These data support the hypothesis that adoptively transferred rgs16 Tg lymphocytes display both impaired migration to a site of inflammation as well as increased cytokine secretion in response to allergen exposure.

Despite these abnormalities, we observed no significant differences in AHR between recipients injected with lymphocytes from WT or Tg mice. We hypothesized that, because the majority of the inflammation in recipients was likely mediated by endogenous host lymphocytes, differences in AHR would be discounted. To minimize the host response, we injected sensitized, CFSE-labeled WT or rgs16 Tg splenocytes into rag2−/− mice, which lack endogenous lymphocytes. We then quantitated lymphocytes in spleen and lung after OVA challenge. Similar to the previous transfer experiments, there were nearly identical numbers of T cells in the spleens of recipients. In contrast, lungs from rgs16 Tg lymphocyte recipients contained ~40% less CD3+ lymphocytes after allergen exposure than lungs from recipients of WT lymphocytes (Fig. 7D). This result supports the idea that homing of rgs16 Tg lymphocytes to a site of OVA-induced inflammation is impaired. To determine whether the reduction in lymphocyte infiltration in the lung correlated with AHR, we measured airway responses to methacholine. Recipient mice injected with rgs16 Tg splenocytes exhibited enhanced responses to methacholine, indicating increased AHR (Fig. 7E). To assess whether altered lymphocyte activation contributed to this abnormality, we measured BALF cytokine levels. Although there was a trend toward increased IL-5 in recipients of rgs16 Tg lymphocytes, this result was not significant (our unpublished data). We attribute this result to the fact that overall BALF cytokine levels at this time point were very low or undetectable, most likely due to the low overall numbers of lymphocytes recruited to the lung in recipient mice. As an alternative measure of activation, we assessed cell proliferation by measuring CFSE content. Because of the lag between allergen challenge and cell harvest, divided cells will contain less CFSE fluorescence than when first injected. Thus, the ratio of CFSEhigh to CFSELow CD3+ cells provides an estimate of cell division. This analysis revealed a significantly higher ratio of divided/nondivided Tg lymphocytes compared with WT controls in spleen and pulmonary lymph nodes from recipient mice (Fig. 7F). Collectively, these experiments demonstrate that, despite the fact that injected Tg lymphocytes proliferate more and produce more cytokines compared with WT, they are prevented from migrating to the lung after allergen challenge.

Discussion

Go signaling is critical for T cell function from the time the lymphocyte leaves the thymus to the point at which it migrates to a site of inflammation as evidenced by the dramatic blockade of these
FIGURE 5. Lymphocyte migration and airway responsiveness after acute allergen challenge in Tg mice. A, Protocol for OVA sensitization and challenge. Mice were subjected to an i.p. injection of OVA followed by three daily nebulizations of 1% OVA beginning 7–10 days after sensitization. On the fourth day after the initial challenge, airway reactivity was assessed by Flexivent plethysmography. B, BALF lymphocyte counts from sensitized and challenged mice. BAL was performed, and cell differential counts were determined by counting 400 total cells in a cytocentrifugation preparation stained with H&E. Bar graph represents mean ± SEM of two independent experiments of six WT and six Tg mice sensitized and challenged with OVA. There was no difference in BALF cell numbers in sham (PBS)-challenged Tg mice in comparison with WT (not shown). C, Immunophenotype of lung lymphocytes after OVA challenge. T cells were enriched from lung single-cell suspensions by negative Ab selection and magnetic bead sorting. Cells were fixed and stained with FITC-conjugated Abs to CD4 and PE-conjugated CCR3 or CCR5 before analysis by flow cytometry. (Figure legend continues)
The regulation of specific chemokine receptors by RGS16 can also be inferred by the phenotype of Tg mice under normal and pathological conditions. That RGS16 expression had no effect on T cell development, thymic architecture, and homeostatic lymphoid organ compartmentation suggests that RGS16 might be an ineffective regulator of CCR7 signaling (53, 54). In support of this concept, preliminary studies indicate that adoptively transferred *rgs16* Tg T lymphocytes traffic normally to lymph nodes injected with CCL21 (the principal ligand of CCR7) in *pbb* mice, which lack endogenous CCL21 (secondary lymphoid chemokine) (our unpublished data). Similarly, although we were unable to comprehensively measure chemokine receptor expression on lung lymphocytes due to a lack of sufficient cells, the reduced migration of RGS16-expressing, sensitized T lymphocytes to the lung after adoptive transfer and allergen challenge also implies a role for RGS16 in the regulation of CCR4- and CCR8-mediated trafficking. Both of these receptors are expressed on Th2 lymphocytes, and deficiencies in either receptor are associated with impaired T cell recruitment to the lung in mouse asthma models (13, 50).

Our results suggest that RGS16, like RGS2, may play a role in T cell activation. *rgs16* Tg T cells produced more Th2 cytokines (IL-4, -5, and -13) in response to allergen or TCR stimulation, whereas naive cells exhibited normal cytokine secretion. Several studies have indicated a positive role for chemokines in T cell responses, such as CXCL8 (55) and CXCL12 (56), which augment expression of anti-CD3-induced T cell activation markers and cytokine production. Because these responses are inhibited by PTX, one might predict that RGS expression would similarly attenuate T cell responsiveness, because RGS proteins block Gαi signaling. However, *rgs2−/−* T cells exhibited reduced cytokine production and proliferation evoked by anti-CD3 (33), suggesting that RGS16 overexpression could result in the opposite phenotype. Our studies also imply that in vivo sensitization is a prerequisite for the effect of RGS16 on cytokine production. Differential chemokine receptor expression in naive vs polarized, sensitized cells might partially explain these results, and indeed, we observed a striking increase in the number of Th2-like, CCR3+ T cells in spleens of Tg mice after allergen exposure.

It is unclear whether the overall increases in cytokine secretion are due to enhanced activation and/or differentiation of Tg T cells or are a result of increased numbers of cells in lung or spleen that are dividing rather than migrating. Indeed, cell trafficking has been linked to the cell cycle. Cells in G0/G1 migrate more efficiently than do naive cells, despite equal expression of receptors. AHR in challenged mice. Airway resistance was measured in anesthetized, mechanically ventilated mice after nebulization of the indicated concentrations of methacholine intratracheally. Graph represents the percentage increase over baseline resistance (mean ± SEM of six WT and seven Tg mice).

Table 1: Receptor expression on lung T lymphocytes in OVA-challenged mice

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Lung</th>
<th>WT</th>
<th>Tg</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+</td>
<td>62.3 ± 2.9</td>
<td>67.9 ± 1.3</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>CD8+</td>
<td>7.8 ± 4</td>
<td>6.6 ± 1</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>CD4+CCR3</td>
<td>20.8 ± 7.2</td>
<td>5 ± 3.1</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>CD4+CCR7</td>
<td>11.3 ± 1</td>
<td>3.6 ± 1.3</td>
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</tr>
<tr>
<td>CD4+CCR8</td>
<td>93.3 ± 1.3</td>
<td>67.9 ± 6.2</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>CD4+CXCR4</td>
<td>80.9 ± 3.6</td>
<td>65.5 ± 4</td>
<td>0.01</td>
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</tr>
<tr>
<td>CD4+CD25</td>
<td>6.8 ± 0.1</td>
<td>5.2 ± 0.3</td>
<td>0.009</td>
<td></td>
</tr>
</tbody>
</table>

a Percentage of total cells in lymphocyte-gated population positive for both markers.

b Values represent percentage of CD4+ or CD8+ cells that express CCR3, CCR5, or CXCR4 (mean ± SEM of six mice in each group). The p values were derived by the Student’s t test.

c Values represent percentage of CD4+ or CD8+ cells expressing specific chemokine receptors, as determined by flow cytometry. Values represent percentage of CD4+ or CD8+ cells expressing specific chemokine receptors, as determined by flow cytometry.

d Values represent percentage of CD4+ or CD8+ cells expressing specific chemokine receptors, as determined by flow cytometry.
RGS16 may have a primary effect on T cell activation pathways. Further biochemical studies may clarify whether TCR-induced signaling is directly affected by RGS16 expression.

Despite reduced lymphocyte numbers in lungs of Tg mice, AHR was increased after allergen challenge in Tg mice and in rag2−/− recipients of sensitized Tg T cells. Elevated levels of systemic cytokines might partially explain this phenotype, because Th2 cytokines may cause direct pathological airway changes in asthma. Adoptive transfer of Stat6−/−-sensitized Th2 cells results in AHR independent of airway eosinophilia (48). Similarly, elevated IL-4, -5, and -13 may have direct effects on airway pathology that enhance AHR. Both IL-5 and IL-13 induce changes in the asthmatic airway independently of inflammatory cells (41, 44–48). Instillation of rIL-13 directly into the airways induces increased mucus production, airway smooth muscle contraction, and AHR without a concurrent influx of eosinophils (44, 45).

Indeed, we found increased serum eotaxin in Tg mice, which might also partially explain the normal numbers of eosinophils in lungs of Tg mice despite the decrease in Th2 cell numbers. Other cytokines, such as IL-4, -5, and -13, could be produced by Tg lymphocytes retained in the spleen, or could be elaborated by either T lymphocytes or resident lung cells such as mast cells, basophils, or epithelial cells. In particular, CD4+CD8−NKT cells, which produce IL-4 and -13, have recently been shown to be required for eosinophilia and AHR in a mouse asthma model (60). Conceivably, these cells could migrate to the lung in increased numbers in Tg mice to compensate for the missing T cell population.

In summary, RGS16 expression in T lymphocytes, in contrast to PTX (18), results in selective blockade of T cell migration to certain chemokines and enhancement of T cell cytokine synthesis in response to a complex inflammatory stimulus. Whether these findings reflect true specificity of RGS16 toward particular GPCRs or G proteins will require examination of the biochemical responses of T cells lacking RGS16 to chemokine and other GPCR ligand stimulation. To better define the molecular GPCR and G protein targets of RGS16 and their role in T cell immune responses, we are generating conditional knockout mice lacking RGS16 in T lymphocytes. Nonetheless, the current study suggests that therapeutic
FIGURE 7. Reduced allergen-induced lung recruitment of Tg lymphocytes after adoptive transfer. A, Donor WT or Tg mice and WT BALB/c recipient mice were sensitized with 100 μg of OVA. One week later, whole splenocytes were extracted from donor mice and labeled with CFSE before tail vein injection into recipients. Mice were then subjected to three daily challenges of nebulized OVA solution before measurement of AHR and quantitation of cell numbers in lung, spleen, and lymph nodes by flow cytometry. B, Cell counts from organs of mice receiving either WT (□) or Tg (●) splenocytes after allergen challenge. Spleen, lymph nodes, and lungs were dissected from recipient mice and ground into single-cell suspensions. CFSE− T lymphocytes were identified by flow cytometry after staining with anti-CD3. Bar graph represents the ratio of CD3+ lymphocytes identified by dual fluorescence normalized to the number of CFSE−CD3− cells (●, p = 0.02, two-factor ANOVA). C, BAL was performed on mechanically ventilated mice after challenge and assessment of AHR. IL-2 and IL-5 in BALF were measured by ELISA (mean ± SEM from five mice in each group; ●, p < 0.05, two-factor ANOVA). D, Sensitized rag2−/− recipients were injected with donor WT (□) or Tg (●) lymphocytes and challenged with OVA as in A. (Figure legend continues) Organs were harvested and analyzed as in B. Bar graph represents mean ± SEM of four mice in each group. (●, p = 0.04, two-factor ANOVA). E, AHR was assessed in OVA-sensitized and -challenged rag2−/− recipients by measuring airway resistance by plethysmography after exposure to the indicated concentrations of methacholine. Graph represents percentage increase over baseline resistance (mean ± SEM of seven mice in each group). F, Cell proliferation was assessed by determining the ratio of CD3+ lymphocytes from spleens or draining lymph nodes with high vs low CFSE fluorescence by flow cytometry. Bar graph represents the mean ± SEM of seven mice in each group. (●, p = 0.04, two-factor ANOVA).
agents that mimic or antagonize specific RGS proteins such as RGS16 might selectively affect distinct T cell trafficking and T cell-dependent immune responses.

Acknowledgments
We thank Dr. Virgilio Bundoc for expert technical assistance and Dean Metcalfe for continued support.

References