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RGS16 Attenuates Pulmonary Th2/Th17 Inflammatory Responses

Sucharita P. Shankar,* Mark S. Wilson,† Jeffrey A. DiVietro,* Margaret M. Mentink-Kane,† Zhihui Xie,* Thomas A. Wynn,† and Kirk M. Druey*

The regulators of G protein signaling (RGS) protein superfamily negatively controls G protein-coupled receptor signal transduction pathways. RGS16 is enriched in activated/effector T lymphocytes. In this paper, we show that RGS16 constrains pulmonary inflammation by regulating chemokine-induced T cell trafficking in response to challenge with Schistosoma mansoni. Naive Rgs16−/− mice were “primed” for inflammation by accumulation of CCR10+ T cells in the lung. Upon pathogen exposure, these mice developed more robust granulomatous lung fibrosis than wild-type counterparts. Distinct Th2 or putative Th17 subsets expressing pulmonary inflammation.

S. mansoni with factors mediating Th2 recruitment to lungs acutely challenged secreted cytokines, particularly IL-13 (5). Although chemokine T lymphocytes were partially excluded from lung granulomas in Rgs16−/− lungs following challenge and produced proinflammatory cytokines IL-13 and IL-17B. CCR4+Rgs16−/− Th2 cells migrated excessively to CCL17 and localized aberrantly in challenged lungs. T lymphocytes were partially excluded from lung granulomas in Rgs16−/− mice, instead forming peribronchial/perivascular aggregates. Thus, RGS16-mediated confinement of T cells to Schistosome granulomas mitigates widespread cytokine-mediated pulmonary inflammation. *The Journal of Immunology, 2012, 188: 6347–6356.

Chemokines dictate coordinated movement of leukocytes through lymphoid organs and sites of inflammation. Naive and activated leukocyte populations express unique chemokine receptors, and gene-targeting studies have implicated specific chemokines and receptors in leukocyte activation, differentiation, and lymphoid organ development (1). Schistosomiasis, induced by infection with the helminth Schistosoma mansoni (among other species), represents a global disease burden because of resultant hepatic fibrosis and portal hypertension (2). S. mansoni cercariae breach host skin and develop into adult male–female worm pairs that generate hundreds of eggs per day (3, 4). Eggs then enter circulation or embolize in host tissues such as liver, intestine, and lung, where a granulomatous reaction and fibrosis develop in an effort to sequester the foreign eggs Ags (3). In published mouse models of schistosomiasis, the pulmonary granulomatous reaction is initiated by CD4+ Th2 cells and their secreted cytokines, particularly IL-13 (5). Although chemokine factors mediating Th2 recruitment to lungs acutely challenged with S. mansoni eggs have been suggested (e.g., CCL17/CCL22 and CCR4/8), signaling pathways involved in pulmonary inflammation have not been fully defined (6).

Chemokine receptors are G protein-coupled receptors (GPCRs) linked to Gαi and possibly Gαq to induce chemotaxis (7). The primary signal transducer of GPCRs, the heterotrimeric G protein complex of α, β, and γ subunits, induces pathway activation through GDP–GTP exchange on Gα and stimulation of numerous effectors including kinases, phospholipases, and ion channels (8, 9). The intrinsic GTPase activity of the α subunit, which promotes Gα reassociation with βγ to form an inactive heterotrimer, terminates ligand-induced signaling. The regulators of G protein signaling (RGS) superfamily, which has >30 members in mammalian cells, negatively regulates G protein activity (10). All RGS proteins contain a characteristic 120-aa “RGS box,” which facilitates binding to Gα subunits and GTPase-accelerating protein (GAP) activity (11). RGS GAP activity hastens GPCR pathway inactivation by catalyzing the GTPase reaction. Although molecular determinants of RGS activity have been elucidated over the past decade, most physiological functions of RGS proteins in mammals remain unknown. Gαi inactivation by pertussis toxin disrupts physiological hematopoietic cell trafficking including thymic emigration, transendothelial leukocyte migration into lymph nodes (LN), and Ag-induced recruitment of cells to inflamed tissue (7). Because RGS proteins are physiologically relevant inhibitors of Gαi, they are poised to regulate chemokine-mediated responses in vivo (7).

RGS16 was initially discovered as an IL-2–dependent activation gene in human T lymphocytes (12). RGS16 may control Th2 lymphocyte migration in vivo because it is upregulated in activated human Th1 and Th2 cells relative to naive T cells, and RGS16 overexpression inhibits Th lymphocyte chemotaxis in vitro (13). To explore intracellular regulation of chemokine pathways in pulmonary inflammation, we generated Rgs16−/− mice and studied their response to sensitization with S. mansoni egg Ag followed by an i.v. bolus of live S. mansoni eggs (14). These studies revealed that RGS16 inhibits Th2 chemotaxis to chemokines including CCL17, which constrains T cell localization to Schistosome egg granulomas, thereby reducing the tissue-damaging effects of Th2-induced pulmonary inflammation by confining cytokines to specific regions(s) of the lung.

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Materials and Methods

**Generation of Rgs16−/− mice**

C57BL/6 wild-type (WT) mice were purchased from The Jackson Laboratory. Rgs16−/− mice were generated directly onto a C57BL/6 background as outlined in Fig. 1. All mice were housed in pathogen-free conditions and research performed in accordance with protocols (LAD3e and LP1616e) approved by the National Institutes of Health Institutional Animal Care and Use Committee. Male or female mice between 6 and 12 wk of age were used for all experiments.

**S. mansoni lung challenge model**

Mice were sensitized i.p. with 5000 S. mansoni eggs that were derived previously from sterile, LPS-free BALB/c mice harboring a liver infection (3). Fourteen days later, mice were challenged i.v. with 5000 live eggs of a Puerto Rican strain of S. mansoni (NMRI) as described elsewhere (3). The challenged mice were euthanized at 4 or 7 d post i.v. injection by CO2 inhalation. Lungs, spleens, and mediastinal LNs (MLNs) were harvested from both groups of challenged mice for analysis.

**Histopathology and immunohistochemistry**

Organs were fixed in 10% neutral-buffered formalin (EMD Chemicals) and embedded in paraffin. Tissue sections (5 μm) were evaluated using hematoxylin and eosin staining, and IHC was performed using previously described methods with primary antibodies from Biogenex (Fremont, CA) and anti–GFP antibody (Abcam). Slides were deparaffinized and stained as outlined in Materials and Methods. Images were obtained using a Leica DMi 4000B microscope and quantified using Image Pro Plus software (Media Cybertek).

**Real-time PCR and gene expression arrays**

Total RNA was isolated using the RNeasy mini kit (Qiagen), followed by DNase I treatment (Invitrogen). cDNA was synthesized from 0.25 to 1 μg RNA using the Supernscript III First-Strand Synthesis kit (Invitrogen). Real-time RT-quantitative PCR (qPCR) was performed using the TaqMan strategy (Applied Biosystems). No reverse transcription controls were included to verify cDNA digestion. Gene expression (Rgs16 [NM_011267.3], suppressor of cytokine signaling 2 [Socs2] [NM_007076.3], Ccl2 [NM_020729.5], Il13 [NM_016971.2], Il17a [NM_019508.1], or the internal reference control β-actin [NM_007393.3]) was measured by multiplex PCR using probes labeled with FAM. The simultaneous measurement of target gene-FAM and β-actin-FAM 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 7500 Standard or Step One Plus sequence detection systems. PCRs were performed using the TaqMan Universal PCR Master Mix and the ABI 7500 Standard or Step One Plus sequence detection systems.

**Cytokine analysis**

Single-cell suspensions were prepared from MLNs harvested 7 d following i.p. challenge; 0.5 × 10^6 cells/well were plated in triplicate wells of 96-well round polystyrene plates (Corning) in RPMI 1640 medium plus 10% FBS (Invitrogen). Cells were left untreated or stimulated with Schistosome egg Ag (SEA) from S. mansoni in PBS (10 μg/ml final concentration) for 3 d at 37°C. Cell supernatants were collected, and cytokine levels were measured with a fluorescence-based Bio-Plex Pro mouse cytokine Th1/Th2 assay (Bio-Rad), according to the manufacturer’s protocols. Cells were fixed by the addition of PBS containing 2% BSA and 4% paraformaldehyde (Electron Microscopy Sciences). ELISAs were performed to quantify secretion of mouse cytokines IL-4, IL-13, IFN-γ, IL-17A, or IL-17B (R&D Systems) in accordance with the manufacturer’s protocols.

**Flow cytometry and intracellular staining**

Organs were harvested and fixed at 7 d post challenge and converted to single-cell suspensions followed by fixation in PBS containing 2% BSA and 4% paraformaldehyde. Surface markers were analyzed with the following Abs: CD3 (17A2), B220 (RA3-6B2), CD11c (N418) (E Bioscience), CD4 (RM4-5), CD8 (53-6.7), CCR3 (83103), CCR4 (2B11) CXCR3 (CXCR3-173), CCR6 (C34-3448) (BD Biosciences), CCR4 (2G12) (Biolegend), or CCR10 (28H18) (R&D Systems), and cytokines were detected with anti–IL-5 (TRFK5) (BD Biosciences), anti–IL-13 (ebio13A), anti–IFN-γ (XMGI2) (E Bioscience), or anti–IL-17B (R&D Systems). For intracellular cytokine staining, cells were cultured with PMA (50 ng/ml) and ionomycin (0.5 μM) (Sigma–Aldrich) for 6 h at 37°C in the presence of brefeldin A (BD Biosciences). Cells were permeabilized with PBS/0.1% saponin (Sigma–Aldrich) and blocked with 5% PBS/milk (Santa Cruz Biotechnol– ogy) for 15 min, 4°C and then stained with Fluo-488 (BD Biosciences). Fixed permeabilized cells were stained in the dark with fluorescence-conjugated Abs (1 h, 4°C) and washed twice with FACS buffer. Samples were analyzed by flow cytometry using an LSR Fortessa (BD Biosciences), and data were analyzed using FlowJo (Tree Star).

**In vitro Th1, Th2, or Th17 culture**

Single-cell suspensions were generated from peripheral LNs from naive mice: naive T cells (CD4+ or CD8+ T cells) were isolated using the naive T cell isolation kit II (Miltenyi Biotec). A total of 5 × 10^5 cells/well were plated in 6-well plates precoated with anti–CD3 (1 μg/ml) and anti–CD28 (3 μg/ml) containing RPMI 1640 medium plus 10% FBS, recombinant mouse IL-4 (10 ng/ml) (PeproTech), mouse–anti–IFN-γ (10 μg/ml) (BD Biosciences), and 50 μM 2-ME for 3 d at 37°C. For differentiation into a Th1 phenotype, naive T cells were cultured in media containing IL-2 (10 ng/ml), IL-12 (10 ng/ml) (R&D Systems), and anti–IL-4 (10 μg/ml) (BD Biosciences), referred to as the Th1 mixture. For differentiation into a Th17 phenotype, naive T cells were cultured in media containing IL-23 (5 ng/ml), IL-1β (10 ng/ml), human TGF-β (5 ng/ml), anti–IL-4 (10 μg/ml) (BD Biosciences), and anti–IL-12 (10 ng/ml) (R&D Biosciences), referred to as the Th17 mixture. The differentiated activated Th1 or Th2 cells were then expanded in medium containing recombinant mouse IL-2 (10 ng/ml) and IL-7 (5 ng/ml) (PeproTech) or in IL-2 alone for Th17 cells.

**Generation and purification of TAT fusion proteins**

The coding region of human RGS16 was amplified by PCR using pC DNA3.1-RGS16 as a template and subcloned in-frame into the plasmid vector pTat-H6HA-GFP, which results in an N-terminally tagged GFP. TAT fusion proteins (TAT-GFP-RGS16 or TAT-GFP control) were expressed and purified by nickel-affinity chromatography as described previously (17). TAT proteins (TAT-GFP-RGS16 or TAT-GFP control) were added directly to T cell cultures for 2 h prior to stimulus addition.

**Chemotaxis assay**

Differentiated Th2 cells (0.5 × 10^6 cells/well) were plated in upper wells of 5-μm-pore polycarbonate membrane Transwell migration chambers containing RPMI 1640 medium plus 0.5% BSA (Corning). The bottom wells contained chemokines (CXCL12, CCL21, CCL17, CXCL9, or CCL20) (R&D Systems). Control wells, in which upper and lower chambers had equivalent chemokine concentrations, were used to determine chemokinesis. Cells migrated to the lower chamber were counted after 3 h by flow cytometry using a FACSCalibur (BD Biosciences).

**CFSE assay for analysis of cell proliferation**

Cells were labeled using the CellTrace CFSE cell proliferation kit (Invitrogen) plated in 96-well plates left untreated or precoated with anti–CD3/
Rgs16 was targeted by flanking exons 2–4 with a floxed-Neor cassette (PGKNeo) by two homologous arms of the endogenous Rgs16 gene. Shown are restriction maps and exons (solid vertical bars) of the endogenous and targeted Rgs16 loci (blue bars). WT and floxed alleles were identified by Southern blotting of BglII-digested genomic DNA with a 5′ probe (red), yielding 10.6- or 5.5-kb fragments, respectively (middle). Mice homozygous for the floxed allele were crossed with Rosa-Cre strain, resulting in deletion of PGKNeo and exons 2–4, which was identified as an 8.3-kb fragment.

Architecture of spleens, lungs, and peripheral LNs in naive WT or Rgs16−/− mice was evaluated by H&E staining. Original magnification ×5.

**Results**

**Generation of Rgs16−/− mice**

Rgs16 was targeted by flanking exons 2–4 with loxP sites (Fig. 1A). We generated knockouts by crossing mice with floxed Rgs16 allele with Rosa-Cre mice, which have the gene encoding Cre recombinase inserted into the ubiquitous Rosa26 locus. Correct targeting of the Rgs16 allele was confirmed by Southern blot analysis (Fig. 1B). Homozygous mice were viable and fertile and exhibited no gross phenotypic abnormalities. Architecture of the spleens, lungs, and peripheral LNs in naive Rgs16−/− mice was comparable to WT mice (Table I).

**RGS16 inhibits Th1 and Th2 chemotaxis**

To investigate function(s) of RGS16 in T cell-dependent inflammation, we examined its expression pattern in mouse effector T cells and their chemotactic responses. Compared with naive (CD4+CD62L+) T cells isolated from peripheral LNs, activated T cells were polarized to a Th2 phenotype by IL-4, and anti–IFN-γ Ab expressed 5-fold more Rgs16 mRNA, whereas those polarized to Th1 phenotype by a Th1 mixture or Th17 phenotype by a Th17 mixture expressed 2-fold more Rgs16 mRNA (Fig. 2A).

We measured chemotaxis of naive CD4 T cells to gradients of CCL21 and CXCL12 because these chemokines have an important function in homeostatic lymphocyte trafficking in lymphoid organs (18). Consistent with relatively low Rgs16 expression in naive CD4 T cells, CXCL12 and CCL21 induced equivalent chemotaxis of naive splenocytes or naive splenic CD4 T cells from unchallenged WT and Rgs16−/− mice (Fig. 2B). Th2 cells up-regulate several “inflammatory” chemokine receptors not typically expressed by naive cells including CCR4 and CCR8, which mediate trafficking to inflamed tissues containing CCL17 (19). According to in vitro-differentiated Th2 cells from mice lacking RGS16 migrated much more toward CCL17 gradients than WT counterparts (Fig. 2C). At the CCL17 concentration inducing a peak response (50 nM), nearly double the number of RGS16-deficient Th2 cells than WT cells migrated to the lower chamber (∼80 versus 40%).

In contrast to Th2 cells, Th1 and Th17 cells express a distinct set of chemokine receptors including CXCRL3 and CCR5 (Th1) or...
Similar to the behavior of RGS16-deficient Th2 cells, Th1 cells differentiated from Rgs16−/− mice migrated significantly more toward a gradient of CXCL9 (CXCR3 ligand) than did WT counterparts (Fig. 2C). For unclear reasons, we did not observe significant chemotaxis of murine Th17 cells toward CCL20 gradients in Transwell assays, independent of genotype (Supplemental Fig. 1). WT and Rgs16−/− T cells migrated to a similar extent in the presence of equivalent chemokine concentrations in the upper and lower chambers, indicating that the loss of RGS16 affected chemotaxis rather than chemokinesis (Fig. 2D). Rgs16 expression correlated with chemokine resistance. WT T cells retained in the upper chamber in the presence of a CCL17 gradient for 3 h (“nonmigratory”) expressed significantly more Rgs16 than cells migrating to the lower chamber during that time period (“migratory”) (Fig. 2E). Finally, because WT and Rgs16−/− Th-polarized subsets expressed similar levels of surface chemokine receptors including CXCR3 for Th1 cells, CXCR4, CCR10, or CCR4 for Th2 cells, and CCR6 for Th17 cells, these
results indicated that RGS16 inhibits chemotaxis downstream of receptors (Supplemental Fig. 1). Consistent with this interpretation, reconstitution of RGS16-deficient Th2 cells with TAT-RGS16 reduced CCL17-evoked chemotaxis compared with untreated cells or cells incubated with a control TAT protein (GFP) (Fig. 2F). Collectively, these results suggest that RGS16 directs trafficking of Th1 or Th2 lymphocytes by curbing their response to chemokine.

Rgs16−/− mice have increased granulomatous lung fibrosis after challenge with S. mansoni

To evaluate Th2 trafficking in vivo in Rgs16−/− mice and its impact on an acute inflammatory response, we sensitized mice with S. mansoni eggs i.p., followed by i.v. injection of eggs 14 d later (Fig. 3A). Mice were sacrificed 4 and 7 d after the i.v. inoculation, at which time S. mansoni eggs embolize in the lungs, resulting in synchronous pulmonary inflammation characterized by granulomas, infiltration of neutrophils, eosinophils, and Th2 lymphocytes, and collagen deposition/fibrosis (23). Consistent with a role for RGS16 in the regulation of host responses to S. mansoni, Rgs16 mRNA expression was increased in the lungs of sensitized challenged mice 7 d following the i.v. Schistosoma challenge (Fig. 3B). We also detected RGS16 in the lungs of challenged mice by immunohistochemistry (Fig. 3C). RGS16+ cells localized predominantly in granulomas surrounding lodged S. mansoni eggs. The lungs of Rgs16−/− mice developed significantly more inflammation and fibrosis than those of WT mice (Fig. 3D). Fibrosis scores (Fig. 3E), granuloma volumes (Fig. 3F), and eosinophil scores (Fig. 3G) were all higher in lungs of knockout mice compared with WT C57BL/6 mice. These results indicated that RGS16 constrains acute granulomatous pulmonary fibrosis induced by S. mansoni infection.

RGS16 deficiency induces anomalous lymphocyte trafficking in vivo

To determine which lymphocyte subsets mediated the atypical fibrotic response of Rgs16−/− mice lungs to S. mansoni, we immunophenotyped pulmonary inflammatory infiltrates by means of flow cytometry. Although we detected no substantial differences in the percentages or total numbers of B and T cells in the lungs of challenged WT and Rgs16−/− mice, there was a skewed Th2 response in knockout mice as evidenced by increased percentages of IL-13+ T cells in the lungs 4 d following helminth challenge. The ratio of IL-13+ (Th2)/IFN-γ (Th1) frequencies was significantly increased in Rgs16−/− lungs relative to WT (Fig. 4A, 4B). Because Th cells from WT and Rgs16−/− mice exhibited comparable rates of differentiation, proliferation, and survival in vitro (Supplemental Fig. 2; data not shown), aberrant cell growth and/or death probably did not account for the differences in the cellular composition observed.

Splenic CD4 T cells isolated from challenged Rgs16−/− mice migrated more toward CCL17 gradients than cells from WT mice (Fig. 4C). Thus, irregular trafficking patterns of fibrosis-inducing IL-13+ Th2 cells might contribute to the enhanced inflammation in Rgs16−/− mice following helminth challenge. Consistent with this hypothesis, we observed starkly atypical T lymphocyte localization patterns in the lungs of RGS16-deficient mice following S. mansoni inoculation compared with WT. Whereas T cells were distributed uniformly within fibrotic granulomas surrounding embolized eggs in WT lungs, they were restricted to the periphery of granulomas in the lungs of Rgs16−/− mice (Fig. 5A). In addition, we detected dense perivascular/peribronchial T cell aggregates that were largely absent in WT mice (Fig. 5B, 5C). These data...
suggest that RGS16 attenuates inflammation in Schistosome egg-challenged lungs by retaining T lymphocytes within granulomas.

**RGS16 regulates lymphocyte trafficking in vivo mediated by CCR4 and CCR10**

Because Th2 cells express CCR4, and RGS16-deficient T cells migrated excessively toward CCL17, we hypothesized that the CCL17/22–CCR4 pathway mediated uncharacteristic migration of a subset of Th2 cells (CCR4+IL-13+) in the challenged lungs of Rgs16<sup>2/2</sup> mice. Consistent with this hypothesis, we detected CCR4+IL-13+ T cells in the lungs of Rgs16<sup>2/2</sup> mice much earlier than in WT mice (4 d after challenge with *S. mansoni*) (Fig. 6A). To determine whether other chemokine/receptor pairs contributed to T lymphocyte mislocalization in the lungs of helminth-challenged Rgs16<sup>2/2</sup> mice, we analyzed differential gene expression by qPCR array (full gene list in Supplemental Table I). Notably, we found selectively increased expression of *Il17b* and *Ccr10* mRNA in the lungs of Rgs16<sup>2/2</sup> mice compared with WT. CCR10 is expressed by effector/memory T cells, Langerhans cells, and plasma cells, among others (24–26), and its ligands CCL27/28 are produced by epidermal keratinocytes (27) and displayed on the surface of dermal endothelial cells (28). CCR10 expression on skin-homing Th2 cells (29) has been implicated in the pathogenesis of T cell-mediated inflammatory skin diseases including atopic dermatitis and contact hypersensitivity (26, 27). We detected CCR10 in the lungs of challenged mice by immunohistochemistry, and its staining pattern mirrored T cell localization in WT and Rgs16<sup>2/2</sup> mice (predominantly in granulomas or peribronchial/perivascular areas, respectively) (Fig. 6B). This result suggests a role for CCR10 in *S. mansoni*-evoked inflammation and in the aberrant trafficking of cytokine-producing T cells we observed in Rgs16<sup>2/2</sup> mice.

Although we found decreased or comparable expression of Ccl17 and CCR10 ligands Ccl27 and Ccl28 in the lungs of WT and Rgs16<sup>2/2</sup> mice (in the presence or absence of *S. mansoni* challenge) (Fig. 6C, Supplemental Table I; data not shown), the naive lungs of knockout mice had significantly increased Ccr10 expression (Fig. 6D). We detected CCR10<sup>+</sup> T lymphocytes in the naive lungs of Rgs16<sup>2/2</sup> mice but not in WT mice (Fig. 6E), suggesting that anomalous migration of RGS16-deficient CCR10<sup>+</sup> T cells to the lungs accounted for the increased Ccr10 gene expression. Moreover, lungs from Rgs16<sup>2/2</sup> mice, but not from WT

**FIGURE 4.** Aberrant Th2 migration in Rgs16<sup>2/2</sup> lungs following *S. mansoni* challenge. (A and B) Frequencies of IL-13<sup>+</sup> or IFN-γ<sup>+</sup> T cells (CD3<sup>+</sup>) (A) or ratio IL-13<sup>+</sup>/IFN-γ<sup>+</sup> T cell percentages (B) in the lungs 4 d following *S. mansoni* challenge were quantified by flow cytometry (*p = 0.04; unpaired t test). The numbers in each quadrant represent a percentage of total T cells. (C) Chemotaxis of splenic CD4<sup>+</sup> T cells collected from the spleens of WT or Rgs16<sup>2/2</sup> mice 4 d following helminth challenge (mean ± SEM of four to five mice per group; *p = 0.03; unpaired t test).

**FIGURE 5.** Anomalous lymphocyte localization in helminth-challenged Rgs16<sup>2/2</sup> lungs. (A and B) T cell localization in helminth-challenged lungs evaluated by immunohistochemistry with a CD3 Ab. Images in (A) show parenchymal T cell accumulation in granulomas, whereas those in (B) demonstrate perivascular/peribronchial cell aggregates (arrowheads). Original magnification ×5. Total area containing cellular aggregates around airways and vessels was quantified using Image Pro Plus software (*p = 0.04; unpaired t test) as indicated in (C). Images represent eight mice per group evaluated in two independent experiments.
mice, contained cytokine-producing CCR4+ or CCR10+ T cells (IL-13+ or IL-17B) 4–7 d following S. mansoni challenge (Fig. 6F–H). Collectively, these results indicate that ectopic trafficking of CCR4+ and CCR10+ T effector cells to the lungs of Rgs16−/− mice may exacerbate fibrogenesis in response to pulmonary helminth challenge through enhanced cytokine production.

Cytokine abnormalities in S. mansoni-challenged Rgs16−/− mice

In addition to increased Ccr10 expression, array analysis also revealed upregulation of cytokines in helminth-challenged Rgs16−/− lungs compared with WT—specifically, Il13 and Il17b. We confirmed these results by real-time PCR (Fig. 7A, 7B) and investigated the source(s) of cytokines by restimulating MLN cells from WT or Rgs16−/− mice with SEA. LN supernatants from Rgs16−/− mice had significantly more IL-5, IL-10, and IL-13 than WT (Fig. 7C). Because effector T cells are generally considered to be the most common sources of these cytokines, we analyzed quantities of LN cytokine-producing CD4 T cells in WT and knockout mice. In contrast to the increased numbers of cytokine+ T cells present in the lungs of S. mansoni-challenged Rgs16−/− mice relative to WT at the earlier time point (4 d postchallenge), frequencies of IL-13+ or IL-17B+ T cells and the intensity of IL-13 staining were similar in WT and Rgs16−/− MLNs at the 7-d time point (Fig. 7D, 7E). These results suggested that further characterization of these cell populations is required to determine the source of increased cytokines in MLN supernatants of Rgs16−/− mice relative to WT following re-exposure to S. mansoni.

Discussion

We have elucidated a function for a modifier of GPCR signaling, RGS16, in a Th2-mediated murine pulmonary inflammatory response to helminth challenge—specifically, through regulation of lung T cell trafficking and cytokine production. The loss of RGS16 in mice triggered an enhanced granulomatous reaction in the lung following challenge with S. mansoni eggs, resulting in more fibrosis and eosinophil influx, anomalous localization of T cells, and increased cytokine production. This study also highlights an unanticipated function of the CCR10–CCL27/8 chemokine axis and IL-17B in the pathogenesis of S. mansoni-associated inflammation.

Several lines of evidence suggest that RGS16 directly controls differentiated Th/effector T cell migration patterns but does not regulate trafficking of quiescent, naive T lymphocytes. RGS16 expression is highly upregulated in differentiated mouse and human Th1, Th2, and Th17 cells compared with naive CD4 T cells (Fig. 2A) (13). Chemokines involved prominently in the maintenance of lymphoid compartments through induction of lymphocyte ex-
travasation through high endothelial venules of the spleen and LNs (CCL21 and CXCL12) induced comparable chemotaxis of naive WT and RGS16-deficient T cells. In contrast, Rgs16<sup>2/2</sup> effector Th2 lymphocytes differentiated in vitro or cells extracted from <i>S. mansoni</i>-challenged lungs had exaggerated chemotaxis toward a Th2-associated chemokine (CCL17). The degree of RGS16 expression correlated inversely with the extent of migration, and chemotaxis of Rgs16<sup>2/2</sup> T cells was reduced by reconstitution with RGS16. Spleen and LN lymphocyte populations and organ architecture in the absence of immune challenge were unchanged in either RGS16-Tg or Rgs16<sup>2/2</sup> mice (Ref. 13 and this study), whereas acute inoculation with <i>S. mansoni</i> accentuated trafficking of differentiated Th2 cells to these sites.

A surprising finding of this study is the anomalous collection of CCR10<sup>+</sup> Th2 cells in the lungs of Rgs16<sup>2/2</sup> mice. Although the CCR10–CCL27 axis has been previously associated with Th2-mediated inflammation in the skin, its role in pulmonary pathology induced by <i>S. mansoni</i> challenge was unknown (20). CCL27 is produced in skin epidermal keratinocytes (27) and presented by dermal endothelial cells (28). CCL17 also promotes CCL27 induction by keratinocytes in the presence of TNF-α (30). CCL28, another CCR10 ligand, has been implicated in CCR10-mediated leukocyte homing to the respiratory tract in a murine asthma model (31). The presence of similar or reduced amounts of CCL27/28 ligands in the lungs of Rgs16<sup>2/2</sup> mice compared with WT suggests that aberrant chemotactic responses of RGS16-deficient T cells to these ligands underlies their accumulation in lungs in the absence of immune challenge. However, for unknown reasons, we did not observe chemotaxis of Th2 cells (WT or knockout) toward these chemokines in Transwell assays in vitro despite expression of CCR10.

We also found increased II13 expression in the challenged lungs of Rgs16<sup>2/2</sup> mice relative to WT, consistent with its central contribution to fibrosis induced by helminth infection (32–33). Although previous work has also defined a role for IL-17A in murine lung inflammation (34), we observed increased II17b expression in the lungs of <i>S. mansoni</i>-challenged Rgs16<sup>2/2</sup> mice relative to WT but did not detect II17a. These results suggest an unexpected function of IL-17B in helminth immunity. Among other IL-17 family members, neutralization of IL-17B in a collagen-induced

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**FIGURE 7.** Cytokine abnormalities in Rgs16<sup>2/2</sup> mice inoculated with <i>S. mansoni</i>. (A and B) Expression of II13 (A) or II17b (B) was evaluated in naive or helminth-challenged lungs by real-time PCR (*p = 0.03; **p = 0.005; unpaired t test). (C) Cells were harvested from lung-draining MLNs 7 d following inoculation with <i>S. mansoni</i>. Cells were left untreated or restimulated with SEA for 3 d, followed by measurement of the indicated cytokine levels in supernatants (*p = 0.005; ***p = 0.0001; unpaired t test). (D) Frequencies of IL-13<sup>+</sup> or IFN-γ<sup>+</sup> T cells (CD3<sup>+</sup>) in LNs 7 d following <i>S. mansoni</i> challenge were quantified by means of flow cytometry Numbers in each quadrant represent percentage of total T cells. The graph on the right shows intensity of IL-13 staining (geometric mean fluorescence intensity, MFI) in the CD4 T cell population. (E) Frequencies of IL-17B<sup>+</sup> T cells (CD3<sup>+</sup>) in LNs 4 or 7 d following <i>S. mansoni</i> challenge were quantified by means of flow cytometry. All data were generated using three naive mice per group, three to five challenged WT mice, or four to five challenged Rgs16<sup>2/2</sup> mice. For (C), data are representative of seven to eight mice per group evaluated in two independent experiments.
model of murine arthritis suppressed disease progression by reducing cell infiltration and production of proinflammatory cytokine such as IL-1β and TNF-α (35), factors also known to induce CCL28 in airway epithelial cells (36, 37). Although we detected IL-17B+ T cells in the challenged lungs, it is unclear whether these cells are conventional Th17 cells. Indeed, in vitro-differentiated Th17 cells produced IL-17A but not IL-17B (Supplemental Fig. 3). Taken together, these findings suggest the presence of a unique proinflammatory environment downstream of IL-17B in the lungs of Rgs16−/− mice challenged with S. mansoni. The absence of RGS16 may promote T cell chemotaxis to CCL27/28 displayed on the surface of endothelial cells, which could account for the peribronchial/perivascular accumulation of T cells we observed in the challenged lungs of Rgs16−/− mice.

How RGS16 regulates Ag-induced cytokine production requires further study. We detected unique populations of IL-13 or IL-17B+ mice with increased IL-17A but not IL-17B (Supplemental Fig. 4; data not shown). These data suggest that lung T cells in Rgs16−/− mice are primed for increased Th2 cytokine production as a result of reduced Socs2 expression (38).

In contrast, although levels of immunoreactive cytokines in the supernatants of Ag-restimulated MLN cells extracted from Rgs16−/− mice 7 d post-S. mansoni challenge were significantly higher than those from WT mice, flow cytometric analysis demonstrated equivalent cytokine+ T cell frequencies and intensity of cytokine staining in these same LN T cell populations. Polarized Th cells from WT and Rgs16−/− mice secreted roughly equivalent cytokine amounts (IFN-γ for Th1, IL-4 or IL-13 for Th2, and IL-17A for Th17) in response to TCR stimulation with anti-CD3 and anti-CD28 in vitro (Supplemental Fig. 3; data not shown). Increased accumulation of specific populations of cytokine-producing Th cells in MLNs of Rgs16−/− mice compared with WT (presumably because of altered trafficking patterns) could also account for the overall increases in secreted cytokines observed. Thus, on the basis of these data alone, we cannot yet determine whether Ag-stimulated, RGS16-deficient T lymphocytes generate more cytokine than WT cells on a per cell basis.

Although our work and that of others have shown that RGS proteins inhibit chemokine-mediated lymphocyte chemotaxis and adhesive responses in vitro (39, 40), function(s) of RGS proteins in T cell-mediated immunity have not been explored in detail. Surprisingly, Rgs2−/− mice had reduced footpad swelling following local inoculation with lymphocytic choriomeningitis virus compared with WT, which correlated with impaired proliferation of, and IL-2 production by, RGS2-deficient T cells. Taken together, these results and our studies suggest unique, context-dependent functions of individual RGS proteins in immune cells that may or may not be predictable, based on their shared biochemical (GAP) activity. Further exploration of RGS16 in specific T cell populations and in the setting of immune challenges will be needed to fully clarify its function(s) in adaptive immunity. Although we present abundant evidence that anomalous Th2 trafficking and increased T cell-derived cytokines contribute to the enhanced pulmonary inflammation in S. mansoni-challenged, RGS16-deficient mice relative to WT (Figs. 4–7), non–Th17-dependent factors may also mediate fibrosis in this setting.

Additional models of inflammation and approaches such as adoptive T cell transfer and/or generation of bone marrow chimeras will be needed to determine the relative importance of T cell-intrinsic and T cell-independent factors to the immune responses of mice lacking RGS16.

Disclosures
The authors have no financial conflicts of interest.

References


