Divergent requirement for Gαs and cAMP in the differentiation and inflammatory profile of distinct mouse Th subsets

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Introduction
The stimulatory Gtα subunit (Gtαs) of the trimeric G protein Gs plays a central role in GPCR-mediated signal transduction by coupling the receptors to the activation of adenylyl cyclase (AC) and increased synthesis of cAMP (1). Increases in cAMP can inhibit T cell function, e.g., blunting CD4+ T cell activation, proliferation, and production of certain cytokines, such as IFN-γ and TNF-α (2, 3). In contrast to these data, cAMP has also been shown to stimulate inflammation by promoting Th17 cell expansion (4–6). For example, the mucosal adjuvant cholera toxin (CT), via a cAMP-dependent mechanism, provokes Th17 immune response at mucosal sites (7).

To help resolve such discrepant findings regarding the role of cAMP in CD4+ T cell differentiation and function, we generated conditional knockout mice whose CD4+ T cells lack the gene for Gtαs (GnasΔCD4 mice) and thus have decreased production of cAMP. We found that CD4+ T cells isolated from GnasΔCD4 mice had reduced cAMP levels, decreased Ca2+ influx, and weak Th17 and Th1 responses but normal Th2 and Treg responses both in vitro and in vivo. Our data thus suggest that cAMP in CD4+ T cells is proinflammatory by altering the differentiation and activation of Th subsets.

Results
GnasΔCD4 CD4+ T cell mice have reduced IL-17 and IFN-γ production. GPCR-mediated increase in intracellular cAMP requires the activation of AC by Gtαs (3). We used the Cre-loxP system to generate mice with a deletion of Gnas (the gene that encodes Gtαs) targeted to T cells (8). The targeted genetic deletion of Gtαs with CD4-Cre occurs at the CD4+CD8+ stage. Consequently, we found that both peripheral CD4+ and CD8+ T cells from GnasΔCD4 mice lacked Gtαs expression (Figure 1A). GnasΔCD4 and WT mice had similar numbers of CD4+ and CD8+ T cells and a similar percentage of effector memory (CD44hiCD62Llo) and naive (CD44loCD62Lhi) CD4+ and CD8+ T cells (Supplemental Figure 1, A–E; supplemental material available online with this article; doi:10.1172/JCI59097DS1), indicating that the loss of Gtαs did not affect T cell development overall.

Figure 1B shows that compared with WT CD4+ cells, CD4+ T cells from GnasΔCD4 mice, have blunted cAMP accumulation in response to the AC activator forskolin and the agonists of Gtαs-coupled GPCRs PGE2 and isoproterenol in the presence of the phosphodiesterase 4 (PDE4) inhibitor rolipram. Rolipram was used based on the high expression of PDE4B and the greater ability of PDE4 inhibitors to increase cAMP compared with other family-specific PDE inhibitors in CD4+ T cells (Supplemental Figure 2). Reduced cAMP accumulation in GnasΔCD4 CD4+ T cells in response to AC activation by forskolin is consistent with the observation that Gtαs is necessary for maximal forskolin-stimulated AC activation (9). GPCR-stimulated cAMP levels were similar in CD11c+ bone marrow–derived dendritic cells (BMDCs) from GnasΔCD4 and WT mice (Figure 1C), consistent with a T cell–specific deletion of Gtαs (Figure 1A).

Assessment of the cytokine profile of CD4+ T cells isolated from the GnasΔCD4 mice revealed lower levels of IL-17A, IL-22, and IFN-γ production upon stimulation with anti-CD3/CD28 Abs compared with the responses of WT CD4+ T cells (Figure 1D). Despite the change in the cytokine profile, we observed no significant differ-
ences in the mRNA levels of the Th17- and Th1-lineage commitment transcription factors Rorc and Tbet (Figure 1E), suggesting that lineage commitment is not altered in GnasΔCD4 mice. Functionally GnasΔCD4 mice also had reduced ability to clear Citrobacter rodentium compared with WT mice despite similar bacterial loads at 1 week after infection (Supplemental Figure 1F). Also, the CD4+ T lymphocytes from the colon lamina propria (LPLs) of GnasΔCD4 infected mice had a lower expression of IL-17A+ cells and IFN-γ+ cells (Supplemental Figure 1G).

We also evaluated the possible contribution of CD8+ T cells and BMDCs from GnasΔCD4 mice to CD4+ T cell function. As shown in Supplemental Figure 3, deletion of Gts in these cells did not affect OVA-specific CD4+ T cells responses.

GnasΔCD4 mice do not mount an antigen-specific Th17 response. The data generated with anti-CD3/CD28 Ab stimulation (Figure 1D) suggest a defect in Th17 cytokine production by GnasΔCD4 CD4+ T cells. To determine antigen-specific Th17 immune responses in GnasΔCD4 CD4+ T cells, we used in vitro and in vivo immunization protocols with OVA and CT (7). Figure 2A shows that compared with control OT-2 cells, OT-2/GnasΔCD4 CD4+ T cells differentiated in vitro had reduced production of Th17 cytokines (IL-17A and IL-22). Furthermore, CD4+ T cells isolated from the spleens of in vivo OVA/CT-immunized WT mice, but not GnasΔCD4 mice, produced greater amounts of IL-17A+ cells and IFN-γ+ cells (Supplemental Figure 1G).

Reduced cAMP, IL-17, and IFN-γ production in GnasΔCD4 CD4+ T cells. (A) Deficient expression of Gnas mRNA in GnasΔCD4 CD4+ and CD8+ T cells. Messenger RNA was prepared from sorted splenic CD4 T cells (TCRβ+CD4+), CD8+ T cells (TCRβ+CD8+), B cells (TCRβ+CD19+), and DCs (CD11c+TCRβ+CD19+). ND, not detected. (B) Reduced cAMP accumulation in CD4+ T cells of GnasΔCD4 mice. cAMP accumulation was assessed in CD4+ T cells from WT and GnasΔCD4 mice treated with vehicle, forskolin (Fsk), isoproterenol (Iso), or PGE2 in the presence of the PDE4 inhibitor rolipram (Rol). Data are mean ± SEM, n = 3, from one representative experiment of 3 with similar results; *P < 0.05, GnasΔCD4 compared with WT cells under the same conditions. (C) cAMP accumulation in CD11c+ BMDCs from WT and GnasΔCD4 mice treated with vehicle or isoproterenol in the presence of rolipram. (D) Reduced Th17 and Th1 cytokine in GnasΔCD4 CD4+ T cells. Splenic CD4+ T cells from WT or GnasΔCD4 mice were stimulated with anti-CD3/CD28 Abs for 72 hours, and the levels of cytokines were determined (ELISA). n = 7–8 for each group from 2 experiments with similar results; *P < 0.05. (E) mRNA levels of transcription factors in GnasΔCD4 CD4+ T cells. Messenger RNA was prepared from cells used in D to assess transcription factors by quantitative PCR. The expression levels (A and E) were normalized to the expression of the Rplp0 housekeeping gene.
Detailed studies. To an IL-6/IL-23/IL-1 differentiation protocol (13) compared with cultured the cells for 4 days under Th17, Th1, Th2, and Treg polarization and TNF-α expression in Tregs between WT and ΔCD4+ T cells (Figure 3E). These results provide further evidence for the contribution of Gαs to Th1 and Th17 differentiation but do not exclude a defect in the cells’ ability to migrate to the colon.

GnasACD4 CD4+ T cells display reduced Th17 and Th1 cell differentiation in vitro. Figures 1–3 indicate a role for Gαs in the acquisition of Th17 and Th1 effector functions. To further elucidate the role of Gαs in Th subset differentiation of CD4+ T cells, we used an in vitro differentiation system for each Th subset: after FACS sorting of naive CD4+ T cells from the spleens of WT and GnasACD4 mice, we cultured the cells for 4 days under Th17, Th1, Th2, and Treg polarization conditions; stimulated the cells with PMA/ionomycin; and assessed (by flow cytometry) the intracellular levels of IL-17, IFN-γ, IL-4, and Foxp3 (Figure 4). WT CD4+ T cells had a 2.8-fold greater increase (i.e., 41.6% vs. 14.9%) in IL-17+ cells in response to an IL-6/TGF-β differentiation protocol (12) and a 22-fold increase in IL-17+ cells in response to an IL-6/IL-23/IL-1β differentiation protocol (13) compared with GnasACD4 mice (Figure 4A), thus demonstrating a role for Gαs in Th17 differentiation. Furthermore, WT CD4+ T cells had an 81-fold increase in IFN-γ-producing cells (Th1, Figure 4B) but a similar number of IL-4-producing cells (Th2, Figure 4C) compared with GnasACD4 CD4+ T cells. We also observed no significant differences in Tregs between WT and GnasACD4 mice: Foxp3+ expression (Figure 4D) and stability (Supplemental Figure 4A), IL-10 production (Supplemental Figure 4B), and the suppressive effect on WT CD4+ T cell proliferation (Supplemental Figure 4C) were similar in Tregs from WT and GnasACD4 mice. Taken together, these data suggest that Gαs in CD4+ T cells regulates Th17 and Th1, but not Th2 or Treg, cell differentiation.

cAMP restores the ability of GnasACD4 CD4+ T cells to promote Th17 and Th1 differentiation. Since GnasACD4 CD4+ T cells have blunted cAMP accumulation, we hypothesized that addition of exogenous cAMP would reverse the effects of the genetic deletion. We thus treated GnasACD4 FACS-sorted naïve CD4+ T cells cultured under Th17 and Th1 polarization conditions with 8-bromo-cAMP (8Br-cAMP), a cell-permeable cAMP analog, and found (Figure 5A) a 2.1-fold increase in IL-17+ cells in response to the IL-6/Th17 differentia
tion and a 3.3-fold increase in response to the IL-6/IL-23/IL-1β differentiation protocol. The increase in IL-17+ cells by 8Br-cAMP was dose dependent (Supplemental Figure 5A). 8Br-cAMP treatment of WT CD4+ T cells increased Th17 cells by 50%–200% in both WT and GnasACD4 CD4+ T cells (Figure 5B). 8Br-cAMP increased GnasACD4 CD4+IFN-γ- Th1 cells by 5.8-fold (Figure 5C) and of GnasACD4 IFN-γ-TNFα cells by 5.2-fold (Figure 5D) but did not alter either cell population in WT CD4+ T cells. 8Br-cAMP did not increase IL-4+ cells (Th2) or Foxp3+ cells (Tregs) in either WT or GnasACD4 CD4+ T cells, further implying that cAMP has divergent effects on Th subset differentiation (Supplemental Figure 5, B and C). Collectively, these data indicate that GnasACD4 CD4+ T cells lack normal Th17 and Th1 differentiation as a result of reduced cAMP accumulation.

Transcriptional regulation of Th17 differentiation in GnasACD4 CD4+ T cells. The reduced production of IL-17+ cells from GnasACD4 CD4+ T cells (Figure 4) and the restoration of IL-17 production by 8Br-cAMP (Figure 5) might imply that cAMP regulates the expression of transcription factors involved in Th17 differentiation (14). However, the presence of 8Br-cAMP produced no significant changes in the mRNA levels of Rorc (15), Rora, or aryl-hydrocarbon receptor (Ahr) under either IL-6/TGF-β or IL-6/IL-23/IL-1β polarization conditions (Supplemental Figure 6, A–C). The expression of Rorc mRNA in WT and GnasACD4 CD4+ T cells was comparable during Th17 differentiation (Supplemental Figure 7A), even though the mRNA level of Il17a was reduced in GnasACD4 CD4+ T cells (Supplemental Figure 7B).

Since the phosphorylation of Stat3 by IL-6 or IL-23 is mandatory for Th17 differentiation, we investigated its phosphorylation in WT and GnasACD4 CD4+ T cells (16, 17). As shown in Supplemental Figure 8, we found elevated levels of Stat3 phosphorylation in GnasACD4 CD4+ T cells under each Th17 differentiation condition. We conclude that the lower number of IL-17+ cells among GnasACD4...
CD4⁺ T cells that was observed with Th17 polarization conditions was not due to an inhibition of RORγt expression or Stat3 activation during the Th17 differentiation program.

CAMP, via PKA, increases Ca²⁺ influx and promotes Th17 differentiation. Since Ca²⁺ influx is mandatory for TCR signaling and is regulated by cAMP in a number of cell types, we investigated whether Ca²⁺ had a role in the phenotype of GnasΔCD4 CD4⁺ T cells (18). TCR stimulation increased cAMP levels in CD4⁺ T cells, implying a physiological role for cAMP in T cell activation (Supplemental Figure 9). Ca²⁺ influx was reduced in GnasΔCD4 CD4⁺ T cells compared with WT CD4⁺ T cells (Figure 6A); this reduction could be reversed by addition of 8Br-cAMP (Figure 6, B and C). The Ca²⁺ transport ATPase of intracellular store-operated Ca²⁺ channels (SERCA) is inhibited by thapsigargin (Tg) (12). Treatment with Tg did not alter intracellular Ca²⁺ levels in CD4⁺ T cells incubated in Ca²⁺-free medium (Figure 6D); however, upon Ca²⁺ addition, Ca²⁺ influx increased to a greater extent in WT CD4⁺ T cells than in GnasΔCD4 CD4⁺ T cells. These data imply that cAMP mediates Ca²⁺ influx in CD4⁺ T cells, especially because we observed no differences in the mRNA expression of Orai13, Stim1 and Stim2, or L-type Ca²⁺ channels between WT and GnasΔCD4 CD4⁺ T cells (Supplemental Figure 10 and refs. 12, 19).

To determine whether the cAMP-mediated increase in Ca²⁺ influx is required for Th17 differentiation, we incubated FACS-sorted naive CD4⁺ T cells from WT and GnasΔCD4 mice under Th17 polarization conditions (IL-6/TGF-β) with 8Br-cAMP in the presence or absence of the L-type Ca²⁺ channel blocker diltiazem (20). Diltiazem inhibited the increase in the number of Th17 cells stimulated by 8Br-cAMP in both WT and GnasΔCD4 CD4⁺ T cells (Figure 6E). The increase in Ca²⁺ influx by cAMP is PKA dependent (21): Figure 7, A and B, shows that the PKA inhibitor H-89 reduced Ca²⁺ influx and the number of IL-17⁺ cells induced by 8Br-cAMP in CD4⁺ T cells under Th17 polarization conditions (IL-6/TGF-β). These data thus highlight a role for cAMP/PKA/Ca²⁺ in Th17 subset differentiation that is independent of lineage commitment factors, such as RORγt.
We then assessed the recipients over a 4-week period. Rag1−/− mice that received Th17 cells that had been differentiated in the presence of 8Br-cAMP developed more severe colitis compared with those that received Th17 cells differentiated without 8Br-cAMP (Figure 8, B and D, and Supplemental Figure 11, B and D). Consistent with previous reports of Th17 plasticity (22), CD4+ T cells isolated from the mesenteric lymph nodes and spleens of recipients displayed a phenotype different from that observed prior to their transfer. The in vitro differentiation of CD4+ T cells in the presence of 8Br-cAMP resulted (after transfer) in a lower percentage of IL-17+ cells, but a higher percentage of IFN-γ+ cells and IL-17+IFN-γ+ double-positive cells (Figure 8C and Supplemental Figure 11, B and D). These results highlight the proinflammatory role of cAMP in CD4+ T cells and its ability to increase the colitogenic properties of Th17 cells.

Discussion

Gαs mediates the activation of AC and generation of cAMP in response to GPCR agonists (1). Global Gαs deficiency is embryonically lethal; therefore, we generated mice with a T cell–selective deletion of Gαs (8, 23, 24). GnasΔCD4CD4-Cre mice developed normally, but their CD4+ T cells did not accumulate cAMP; had reduced Ca2+ influx; secreted lower levels of IL-17, IL-22, and IFN-γ (but normal IL-4) compared with WT CD4+ T cells; and did not mount an antigen-specific Th17 response upon CT/OVA immunization (Figure 2). The adoptive transfer of naive GnasΔCD4 CD4+ T cells into Rag1−/− recipients provoked minimal colonic inflammation compared with that induced by the transfer of naive WT CD4+ T cells. Consistent with this phenotype, GnasΔCD4 CD4+ T cells isolated from recipients’ spleens displayed a lower frequency of IFN-γ+, IFN-γ+IL-17+, and TNF-α+ cells. To better elucidate the role of Gαs in Th cell differentiation, we used an in vitro differentiation system. CD4+ T cells from GnasΔCD4 mice displayed fewer Th17 and Th1 cells but comparable expression of Th2 or Tregs relative to WT CD4+ T cells (Figure 4). Collectively, these in vitro and in vivo data indicate a divergent role for Gαs in Th subset differentiation and function.

Figure 4

Reduced Th17 and Th1 cell differentiation of GnasΔCD4 naive CD4+ T cells in vitro. FACS-sorted naive CD4+ T cells from WT or GnasΔCD4 spleens were cultured for 4 days under Th polarization conditions for (A) Th17, (B) Th1, (C) Th2, and (D) Tregs as described in Methods. The CD4+ T cells were then stimulated with PMA/ionomycin for 4 hours. IL-17A, IFN-γ, IL-4, and Foxp3 levels were determined by flow cytometry. The number in each quadrant indicates the frequency of cells. Data are representative of 3 experiments with similar results.

cAMP enhances the colitogenicity of in vitro differentiated Th17 cells.

To assess the in vivo effect of cAMP on Th17 cells, we transferred WT Th17 cells that were differentiated in vitro under IL-6/TGF-β and IL-6/IL-23/IL-1β conditions in the absence or presence of 8Br-cAMP into Rag1−/− mice (Figure 8A and Supplemental Figure 11A). To determine whether the phenotype of GnasΔCD4 CD4+ T cells was the result of reduced cAMP accumulation, we used 8Br-cAMP to restore cAMP levels and assessed in vitro Th differentiation. 8Br-cAMP restored the expression of GnasΔCD4 IL-17+ and IFN-γ+ but did not alter IL-4+ or Foxp3+ CD4+ T cells, implying that reduced
cAMP accumulation in Gnas<sup>ACD4</sup> CD4<sup>+</sup> T cells selectively modulates Th17 and Th1 differentiation. Furthermore, restoring cAMP increased the expression of IL-17<sup>+</sup>TNF-α<sup>+</sup> and IFN-γ<sup>+</sup>TNF-α<sup>+</sup> cells, demonstrating that cAMP enhances the inflammatory phenotype of Th17 and Th1 subsets (Figure 5). The 8Br-cAMP–promoted Th17 phenotype observed in CD4<sup>+</sup> T cells from Gnas<sup>ACD4</sup> mice was not associated with increased levels of Th17 lineage-specific transcription factors (Supplemental Figures 7 and 8), thus indicating that a transcriptional defect does not account for the impairment in Th17 differentiation.

Figure 5

cAMP restores Th17 and Th1 differentiation of Gnas<sup>ACD4</sup> CD4<sup>+</sup> T cells. (A and B) FACS-sorted naive CD4<sup>+</sup> T cells from WT or Gnas<sup>ACD4</sup> spleens were cultured for 4 days under two Th17 polarization conditions: IL-6/TGF-β or IL-6/IL-23/IL-1β with or without 8Br-cAMP (25 μM). (C and D) FACS-sorted naive CD4<sup>+</sup> T cells were cultured for 4 days under Th1 polarization conditions with or without 8Br-cAMP (25 μM). The CD4<sup>+</sup> T cells were stimulated with PMA/ionomycin for 4 hours, and the intracellular levels of IL-17A, IFN-γ, and TNF-α were determined by FACS analysis. The number in each quadrant indicates the frequency of cells. The data are representative of 3 independent experiments with similar results.
In addition to the expression of lineage-specific transcription factors, differentiation of Th17 or Th1 cells is regulated by Ca\(^{2+}\) signaling, with Ca\(^{2+}\) influx primarily mediated by calcium release-activated channels (CRACs), inositol-1,4,5-trisphosphate receptors (IP3Rs), and L-type calcium channels (25, 26). Defective CRACs or L-type calcium channels in CD4\(^+\) T cells can inhibit Th17 and Th1 cell differentiation without inhibiting the expression of RORγt or T-bet (12). Since cAMP can activate L-type calcium channels (27), we postulated that Gnas\(^{ACD4}\) CD4\(^+\) T cells have reduced Ca\(^{2+}\) influx that contributes to their Th phenotype. Indeed, we found that cAMP, via increasing Ca\(^{2+}\) influx, restores Th17 and Th1 differentiation of Gnas\(^{ACD4}\) CD4\(^+\) T cells. (A–C) Ca\(^{2+}\) influx in enriched WT or Gnas\(^{ACD4}\) splenic CD4\(^+\) T cells. CD4\(^+\) T cells stained with anti-CD3 Ab were cross-linked with goat anti-hamster Abs (αHam) in Ca\(^{2+}\)-free HBSS medium, followed by the addition of CaCl\(_2\) (2 mM); WT (CFSE-labeled) or Gnas\(^{ACD4}\) CD4\(^+\) T cells (no label) were mixed, incubated with the calcium indicator Indo-1 AM, and assayed in one tube at the same time in the same environment. Ca\(^{2+}\) influx was measured by changes in the mean fluorescence ratio of Indo-1 AM at violet (405 nm) to blue laser (510 nm). 8Br, 8Br-cAMP, 25 μM. The data are representative of 1 of 3 independent experiments. (D) Reduced Ca\(^{2+}\) influx in Gnas\(^{ACD4}\) splenic CD4\(^+\) T cells stimulated by Tg. WT or Gnas\(^{ACD4}\) splenic CD4\(^+\) T cells in Ca\(^{2+}\)-free HBSS medium (EGTA, 0.5 mM) were stimulated by Tg (1 μM), followed by the addition of CaCl\(_2\) (2 mM). (E) Diltiazem inhibits the 8Br-cAMP increase in Th17 cells. FACS-sorted naive CD4\(^+\) T cells were cultured for 4 days under Th17 differentiation conditions (IL-6/TGF-β) as indicated. 8Br, 25 μM; diltiazem, 20 μM. The CD4\(^+\) T cells were stimulated with PMA/ionomycin for 4 hours. Intracellular cytokine levels were determined by flow cytometry. The number in each quadrant indicates the frequency of cells. Data are representative of 2 independent experiments with similar results.
upregulation of their inflammatory responses. Importantly, these effects were not observed for Th2 cells, thus implying that their differentiation is independent of cAMP and cAMP/PKA-induced Ca\(^{2+}\) influx. This observation is consistent with evidence that the activation of Th1 and Th2 under the same conditions results in different patterns of Ca\(^{2+}\) signaling and cytokine production (33).

Our results thus indicate that cAMP has a proinflammatory and not, as previously proposed (3), an immunosuppressive effect, in terms of regulation of the function of Th cells. A possible explanation for differences in such results is that we assessed the role of cAMP during Th cell differentiation while others evaluated such a role after differentiation of Th cells. The Th-specific effects by cAMP suggest that it may be exploited for therapeutic immunomodulation. Such therapeutic effects might be achieved by targeting: (a) cAMP formation through the activation of G\(_\alpha_s\)-coupled or inhibition of G\(_\alpha_i\)-coupled GPCRs, (b) cAMP degradation by inhibiting PDE isoforms, or (c) L-type Ca\(^{2+}\) channels through their blockade in CD4\(^+\) T cells. It will be of interest to test such approaches in future studies.

**Methods**

*Mice.* C57BL/6 (B6) mice were purchased from Harlan, CD4-Cre transgenic mice from Taconic, and OT-2 (B6) mice expressing a transgenic TCR that recognize class II–derived OVA peptide and Rag1\(^{-/-}\) mice (B6) from The Jackson Laboratory. To generate G\(_{nas}\)−/− mice, C57BL/6 (B6) mice were used as control (WT). To generate OVA-specific G\(_{nas}\)ΔCD4 mice, we crossed G\(_{nas}\)ΔCD4 to OT-2 mice. Seven- to 12-week-old mice were used in all the experiments and were maintained under specific pathogen–free conditions.

*Reagents.* Reagents were obtained as follows: 8Br-cAMP and PKA inhibitor H-89 from Calbiochem; TGF-β from PeproTech; IL-2 from Sigma-Aldrich; anti–mouse CD3e (clone 145-2C11), anti–mouse CD28 (clone 37.51) Ab from BioXcell; rolipram and diltiazem from Tocris Bioscience. Tg, forskolin, PGE\(_2\), and isoproterenol were purchased from Sigma-Aldrich.

**CD4\(^+\) T-cell isolation and differentiation in vitro.** CD4\(^+\) T cells were isolated by immunomagnetic selection (EasySep CD4-negative selection kit, StemCell Technologies) from a single-cell suspension of splenocytes or peripheral lymph node cells. Naive CD4\(^+\) T cells (CD62L\(^{hi}\)CD44\(^{lo}\)CD45R\(^{lo}\)) were isolated from CD4\(^+\) T cells by FACS sorting (Aria, BD Biosciences). Cells were incubated in complete IMDM medium (Invitrogen) supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin, 50 \(\mu\)M β-mercaptoethanol, and 10% FCS. Culture plates (48-well) were coated with goat anti-hamster Ab (0.1 mg/ml, USBiological). Naive CD4\(^+\) T cells (1 \(\times\) 10\(^{5}\)/ml) were cultured in medium with soluble anti-CD3 Ab (0.25 \(\mu\)g/ml), anti-CD28 Ab (1 \(\mu\)g/ml), and neutralizing antibodies for IL-4 (10 \(\mu\)g/ml) and IFN-γ (10 \(\mu\)g/ml).

Naive CD4\(^+\) T cells were differentiated into Th17 cells according to two protocols: (a) TGF-β-dependent Th17 cells were obtained by addition of IL-6 (20 ng/ml) and TGF-β (4 ng/ml) to the Th0 cultures (34); (b) IL-23–mediated Th17 differentiation by addition of IL-6 (20 ng/ml), IL-23 (10 ng/ml), and IL-1β (10 ng/ml) (13). Naive CD4\(^+\) T cells were cultured in goat anti-hamster Ab–coated culture plates in complete IMDM medium with soluble anti-CD3 Ab (5 \(\mu\)g/ml) and anti-CD28 Ab (1 \(\mu\)g/ml), added with IL-12 (20 ng/ml) and neutralizing anti–IL-4 Ab (10 \(\mu\)g/ml) for Th1 differentiation; or IL-4 (10 ng/ml) and neutralizing anti–IFN-γ Ab (10 \(\mu\)g/ml) for Th2 differentiation into Th17 cells. Th1 and Th2 cell differentiation were assessed by FACS analysis using anti-CD3 Ab (eBioscience) and anti–CD4 Ab (eBioscience). Naive CD4\(^+\) T cells were transfected with either a scrambled or an antisense G\(_{nas}\)ΔCD4 plasmid (pSpZ Respect, Open Biosystems) using the Amaxa Nucleofector system (Amaxa). G\(_{nas}\)ΔCD4 plasmid (35) was previously shown to efficiently block G\(_{nas}\) signaling in T cells, whereas the scrambled plasmid did not alter G\(_{nas}\) activity. G\(_{nas}\)ΔCD4 plasmid is expressed from a retroviral vector in T cells, thereby allowing the evaluation of the role of G\(_{nas}\)ΔCD4 in either naive or differentiated T cells. G\(_{nas}\)ΔCD4 and scrambled plasmids were transduced into naive CD4\(^+\) T cells by retroviral transduction. Two days after transduction, retrovirus–infected cells were sorted using fluorescence-activated cell sorting (FACS) analysis (Aria, BD Biosciences).

**Analysis of intracellular cytokine production.** Naive CD4\(^+\) T cells were stimulated in vitro with anti-CD3 (1 \(\mu\)g/ml) and anti–CD28 (1 \(\mu\)g/ml) to induce Th17 cell differentiation. After 4 days, cells were stimulated with anti-CD3 (1 \(\mu\)g/ml) and IL-2 (20 ng/ml) (13) and IL-1β (10 ng/ml) (34) and stained with anti–intracellular IFN-γ Ab (clone XMG1.2, BD Biosciences) and anti–intracellular IL-17A Ab (clone TBF2, Serotec). Stained cells were analyzed by flow cytometry (FACS) analysis (FACSCaliber, BD Biosciences) and the percentage of IFN-γ– and IL-17A–positive cells was determined. The results were expressed as the percentage of cytokine–positive cells in the total CD4\(^+\) T-cell population. Nonstimulated cells served as controls.

**GFP expression.** GFP expression was assessed by flow cytometry (FACS) analysis (FACSCaliber, BD Biosciences). GFP–positive cells were determined by the percentage of GFP–positive cells in the total CD4\(^+\) T-cell population.
differentiation. At day 2, recombinant human IL-2 (20 U/ml) was added into the Th1 or Th2 culture. For Treg differentiation, TGF-β (10 ng/ml) and IL-2 (100 U/ml) were added into IMDM culture medium. After 4 days, cells were collected for analysis.

ELISA measurement of cytokines. Splenic CD4+ T cells were enriched by immunomagnetic cell selection (EasySep CD4 negative selection kit, StemCell Technologies) to greater than 95% purity by negative selection. CD4+ T cells (1 × 10^6 cells) were stimulated with plate-bound anti-CD3 Ab (10 μg/ml) and anti-CD28 Ab (1 μg/ml) for 72 hours in complete RPMI medium (Mediatech Inc.) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM β-mercaptoethanol, and 10% FCS. Cytokine levels in the supernatant were determined using ELISA kits for IL-17A, IL-5, IL-10, IFN-γ (eBioscience), and IL-22 (Antigenix America Inc.) following the manufacturers’ instructions.

Flow cytometry and intracellular staining. All antibodies used for cell labeling were purchased from BD Biosciences — Pharmingen and eBiosciences. The data were acquired by an LSR II, FACSCalibur, and C6 Accuri flow cytometer (BD Biosciences) and analyzed by FlowJo software (Tree Star Inc.). For measurements of intracellular cytokines, CD4+ T cells were stimulated with PMA (50 ng/ml) and ionomycin in the presence of GolgiStop (BD Biosciences — Pharmingen) for 4 hours. Cytokines were analyzed using antibodies to IL-17A, TNF-α, IL-4, Foxp3, or IFN-γ (eBioscience) according to the manufacturer’s instructions. For measurements of p-Stat3 levels, we incubated CD4+ T cells with anti-CD3 (10 μg/ml) and anti-CD28 (1 μg/ml) Abs with the indicated cytokines for 15 minutes. Cells were fixed by 1.5% formaldehyde, 100% methanol, and stained by anti–p-Stat3–PE Ab (pY705, BD Biosciences — Pharmingen).

cAMP assay. cAMP accumulation was measured as previously described (35). CD4+ T cells or CD11c+ BMDCs were enriched by magnetic selection and equilibrated in RPMI-1640 medium containing 10% FCS for 4 hours at 37°C and then incubated with stimulatory agonists for 10 minutes in the absence and presence of PDE inhibitors (added 30 minutes before the addition of agonists). Reactions were terminated by aspiration of the
medium and addition of 50 μl of cold 7.5% (wt/vol) trichloroacetic acid (TCA) per million cells. cAMP content in TCA extracts was determined by radioimmunoassay and normalized to the amount of cells per well.

OVA-specific immune responses upon in vitro immunization. Naïve OT-2 CD4+ T cells from spleens of WT or GnasΔCD4 mice were enriched by immunomagnetic cell selection (EasySep CD4 negative selection kit, StemCell Technologies) to greater than 95% purity and were further depleted of CD25+ cells (anti-CD25 Biotin Ab and Biotin selection kit, StemCell Technologies) by negative selection. The WT splenic DCs were isolated by use of a CD11c positive selection kit (StemCell Technologies). DCs (0.5 × 10^6) were then cocultured with WT or OT-2/GnasΔCD4 naïve T cells (1 × 10^6) in RPMI-1640 medium in the presence of class II OVA peptide (aa 323–339) (10 μg/ml) for 5 days. OT-2 cells (1 × 10^6) were then collected and stimulated by plate-bound anti-CD3 Ab (10 μg/ml) and soluble anti-CD28 Ab (1 μg/ml) for 24 hours. Supernatants were collected and cytokine levels were determined (ELISA).

Adoptive transfer of naïve CD45RBlo or OT-2/ΔCD45RBhi CD4+ T cells induced colitis. Colitis was induced by adoptive transfer of naïve CD45RBlo CD4+ T cells: sorted naïve CD4+ T cells (CD4+CD45RBloCD25−, 1 × 10^6 cells/mouse) from WT or GnasΔCD4 mice were adoptively transferred into 12-week-old sex- and age-matched Rag1−/− mice as described previously (37). A group of Rag1−/− mice also received 0.5 × 10^6 CD45RBhiCD25− (Tregs) from WT mice; naïve CD4+ T cells (1 × 10^6 cells) were used controls. After transfer, mice were monitored for weight loss and signs of intestinal inflammation. Diseased animals were sacrificed for analysis around 4 and 6 weeks after transfer. Spleen and mesenteric lymph node (MLN) cells were cultured in RPMI-1640 medium and stimulated by PMA/ionomycin for 5 hours in the presence of Golgistop (BD Biosciences—Pharmingen) for intracellular staining. The colon explants were isolated and cultured as described previously (37), and cytokines levels in the supernatant were measured (ELISA).

Histological evaluation of colitis. The colon was excised, opened longitudinally, rolled onto a wooden stick, fixed with 10% neutral buffered formaldehyde solution, and embedded in paraffin. Tissue sections (5 μm) were prepared, deparaffinized, and stained with H&E. Sections were analyzed in a blinded fashion as described in previous studies (37).

Quantitative PCR analysis. Isolation of RNA was carried out using an RNeasy Mini Kit (QIAGEN) according to the manufacturer’s instructions. The cDNA was synthesized using Superscript III First-Strand system (Invitrogen). Quantitative PCR analysis was performed as described previously (37). SYBR Green PCR Master Mix was used for real-time PCR (7300 system, Applied Biosystems). Samples were run in duplicate or triplicate and normalized to a housekeeping gene (mouse RpL32 and mouse 18s rRNA). The primer sequences are provided in Supplemental Table 1.

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