



Critical regulation of follicular helper T cell differentiation and function by $G\alpha_{13}$ signaling

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GPCR- $G\alpha$ protein-mediated signal transduction contributes to spatiotemporal interactions between immune cells to fine-tune and facilitate the process of inflammation and host protection. Beyond this, however, how $G\alpha$ proteins contribute to the helper T cell subset differentiation and adaptive response have been underappreciated. Here, we found that $G\alpha_{13}$ signaling in T cells plays a crucial role in inducing follicular helper T (Tfh) cell differentiation in vivo. T cell-specific $G\alpha_{13}$ -deficient mice have diminished Tfh cell responses in a cell-intrinsic manner in response to immunization, lymphocytic choriomeningitis virus infection, and allergen challenges. Moreover, $G\alpha_{13}$ -deficient Tfh cells express reduced levels of Bcl-6 and CXCR5 and are functionally impaired in their ability to adhere to and stimulate B cells. Mechanistically, $G\alpha_{13}$ -deficient Tfh cells harbor defective Rho-ROCK2 activation, and Rho agonist treatment recuperates Tfh cell differentiation and expression of Bcl-6 and CXCR5 in Tfh cells of T cell-specific $G\alpha_{13}$ -deficient mice. Conversely, ROCK inhibitor treatment hampers Tfh cell differentiation in wild-type mice. These findings unveil a crucial regulatory role of $G\alpha_{13}$ -Rho-ROCK axis in optimal Tfh cell differentiation and function, which might be a promising target for pharmacologic intervention in vaccine development as well as antibody-mediated immune disorders.

$G\alpha_{13}$ | Tfh cell | germinal center reaction | ROCK

G α proteins, activated upon the binding of ligands to their respective G protein-coupled receptors (GPCRs), transduce outside signals to the cell's interior by binding to either GTP or GDP. $G\alpha$ proteins are classified into four families— $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13}$ —with each subfamily consisting of multiple members and many of its members being activated simultaneously by one GPCR to transmit signal. GPCRs are stimulated in response to a variety of ligands, from chemokines, nucleotides, and peptides to hormones and other lipids, and mediate a wide range of cellular function: adhesion, contraction, motility, and proliferation—to name a few. The role of GPCR is fundamental in dynamic biological processes, such as inflammation and immune responses, whereby the careful spatiotemporal orchestration of leukocytes is required. As such, the biological role of $G\alpha$ proteins in mediating adhesion, chemotaxis, migration, and the motility of immune cells through different tissue types and organ systems has been the interest of many for some time (1). For example, in the context of inflammation, chemokines, sphingosine-1-phosphate (S1P), lysophosphatidic acid, and thrombins signal via $G\alpha_{12/13}$ -coupled receptors to recruit immune cells to the site of inflammation and promote entry into lymphoid organs, thereby amplifying the inflammatory response (2).

In addition, several groups have provided insight with regard to $G\alpha$ protein's role in immune cell survival and retention in lymphoid organs (3–5). For example, the loss of $G\alpha_{12/13}$ -signaling cells has been shown to promote aberrant B cell survival and skewed retention in secondary lymphoid organs (SLOs), thereby

promoting B cell cancer (6–8). Similarly, $G\alpha_{12/13}$ -coupled receptors have been shown to be critical in T cell polarization, adhesiveness, and retention within the lymph nodes (LNs) (4, 9). Recent efforts have provided detailed descriptions of how motility and chemotactile gradients are fine-tuned by the interplay of various GPCRs (10, 11). In particular, follicular helper T (Tfh) cells seem to be one of the more sensitive subsets to GPCR-mediated signaling out of the helper T cell subsets, presumably because of its requirement to migrate to and from cellular regions within the SLOs to fully mature and accomplish its B cell help function (12). Antigen-specific Tfh cells access the B cell follicle by up-regulating CXCR5 (mediated via $G\alpha_i$ signaling) and by down-regulating CCR7, enabling B cell clonal expansion and subsequent germinal center (GC) response (13). GPR183 (or more commonly known as EBI2) is mediated by $G\alpha_i$ signaling and serves as another important receptor for Tfh cell commitment by guiding progenitor Tfh cells to interact with dendritic cells (DCs) (14). S1PR2, a GPCR mediated by $G\alpha_{12/13}$ signaling, regulates LN retention via chemorepulsion and has been shown to be also important

Significance

Optimal follicular helper T (Tfh) cell differentiation and function are required for effective humoral immunity against infection, while improper Tfh cell responses are associated with autoimmunity and allergy. We demonstrate that $G\alpha_{13}$ —a $G\alpha$ protein subunit known to be involved in mediating signals related to cytoskeletal integrity, chemotaxis, and migration—acts as an essential positive regulator in Tfh cell development and function. The deletion of $G\alpha_{13}$ in T cells results in dampened germinal center reactions in immunization and viral infection models. Mechanistically, $G\alpha_{13}$ -RhoA-ROCK2 axis is responsible for the Tfh cell differentiation from naïve precursors, and Rho agonists recuperate hampered Tfh cell function in $G\alpha_{13}$ -deficient mice. Such mechanistic insight underscores the possibility of targeting $G\alpha_{13}$ -mediated signaling to maneuver Tfh cell responses.

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in Tfh cell maturation by directing colocalization with B cell within the GCs (9).

Beyond this kinetic insight of G α protein's involvement on T cell function, there still exist a significant gap in our understanding of how exactly G α protein-mediated signaling can regulate the differentiation program of CD4 T cell subsets and the function of effector T cells in vivo. To address the knowledge gap, we employed a T cell-specific knockout (KO) mouse of a particular heterotrimeric G α protein subunit, G α_{13} , which in our results was shown to play nonredundant roles with its subfamily member protein. By illustrating how the deficiency of G α_{13} signaling can impact the development of a specific helper T cell subset and critically affect its function, our study highlights the importance of G α_{13} -RhoA-Rho-associated protein kinase 2 (ROCK2) signaling pathway in early Tfh cell differentiation and function in helping B cells.

Results

Contribution of G α_{13} Signaling in T Cells to Thymic Development and Distribution to Secondary Lymphoid Organs. As a first step to investigate the role of G α_{13} in T cells, we compared the expression of various members of the G protein α family in flow-sorted B220⁺ B cells, CD4⁺ T cells, and CD8⁺ T cells by Western blot. While the levels of G α_s and G α_{12} expression were comparable among the lymphocyte subsets, those of G α_{13} were slightly higher in CD4⁺ T cells compared to other lymphocytes (Fig. 1A and B and *SI Appendix, Fig. S1A*). T cell receptor (TCR) signaling triggered by anti-CD3/CD28 stimulation induced little changes in the level of G α_{13} expression in CD4⁺ T cells (Fig. 1C). The germline deletion of *Gna13* results in embryonic lethality (15). To investigate the role of G α_{13} in T cells, we established a T cell conditional G α_{13} KO (T Δ ^{Gna13}) mouse system by crossing *Gna13^{fl/fl}* strain with *CD4^{Cre}* mice. We

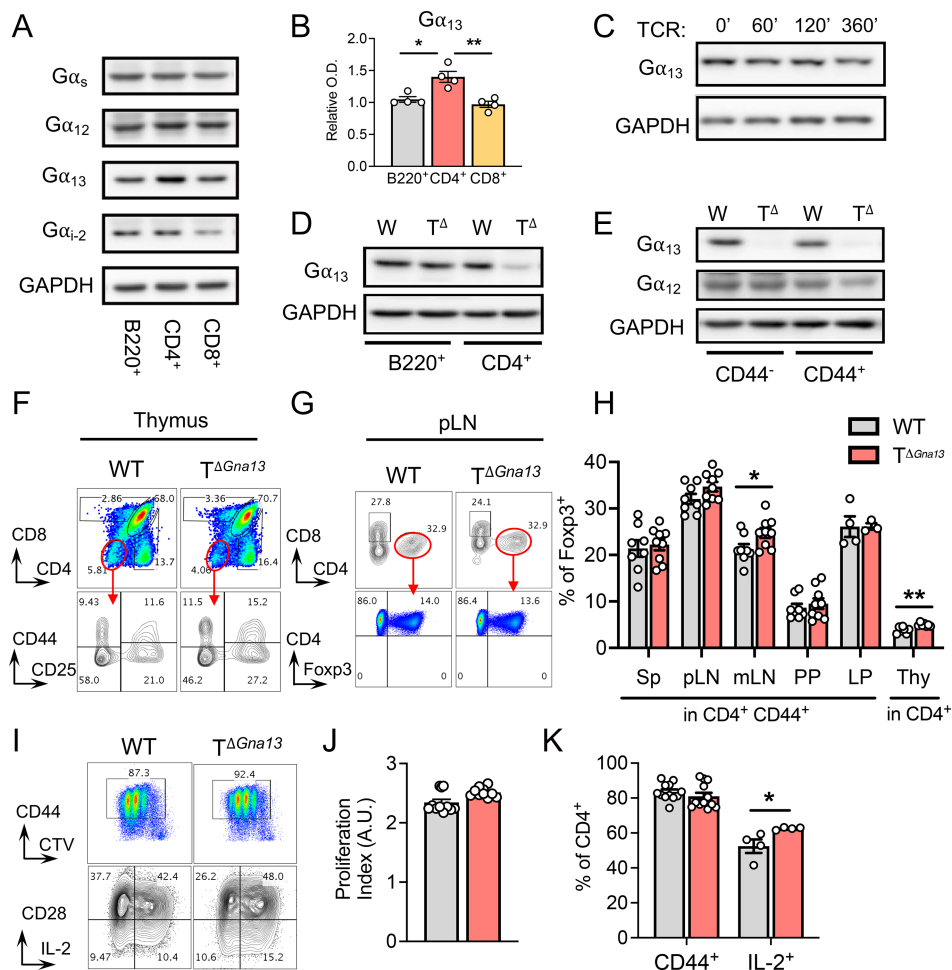


Fig. 1. G α_{13} signaling in T cells is dispensable for the development and activation of T cells. (A and B) WT lymphocytes were flow sorted and analyzed. (A) Western blot of WT B-, CD4⁺ and CD8⁺ T cells to detect G α subunit proteins, with GAPDH as the loading control and (B) corresponding, relative optical densitometry graphs ($n = 4$ per group). (C) Western blot of WT, naive CD4⁺ T cells that were unstimulated (0') or stimulated with anti-CD3/CD28 for the indicated times to detect G α_{13} . (D) Western blot of either WT or T Δ ^{Gna13} B and CD4⁺ T cells to detect G α_{13} protein. (E) Western blot of either naive or activated (CD44⁺) CD4⁺ T cells to detect G α_{12} and G α_{13} proteins. (F and G) Representative fluorescence-activated cell sorting (FACS) plots of T cell subset analysis in the thymus (F) and SLO pLN (G), peripheral LNs at steady state. (H) Frequencies of Foxp3⁺ Tregs in various organs from two independent experiments combined ($n = 4$ to 9 per group). Sp, spleen; mLN, mesenteric LN; LP, lamina propria; and Thy, thymus. (I–K) Naive T cells were stimulated with anti-CD3/CD28 for 2 d for proliferation analysis. (J) Representative FACS plots of CD44, CD28, IL-2 expression, and cell division. Calculated proliferation index denoted in arbitrary units (A.U.). (J) and frequencies (K) of CD44⁺ and IL-2⁺ CD4⁺ T cells ($n = 4$ to 13 per group). All data are representative of two independent experiments, unless stated otherwise. All data are mean \pm SEM; * $P < 0.05$; and ** $P < 0.01$.

confirmed the efficient deletion of $G\alpha_{13}$ in $CD4^+$ T cells but not in B cells (Fig. 1D). While the deletion of $G\alpha_{13}$ often leads to the up-regulation of $G\alpha_{12}$ as a complementary mechanism (15), the expression of $G\alpha_{12}$ was relatively unaltered in $G\alpha_{13}$ -deficient $CD4^+$ T cells, regardless of the activation status defined by CD44 expression (Fig. 1E).

$G\alpha_{13}$ signaling has been proposed to play a role in early thymocyte proliferation and survival in vitro (16). Additionally, $G\alpha_{12}$ and $G\alpha_{13}$ double-deficient mice have been shown to harbor increased cellularity in the thymus (4). When we analyzed the subsets of thymocytes between $T^{\Delta G\alpha_{13}}$ and littermate control ($Gna13^{fl/fl}$) mice, we found that the frequencies and the absolute number of all four double-negative stages were comparable between the two groups (Fig. 1F and SI Appendix, Fig. S1 B–D). Hence, $G\alpha_{13}$ signaling in T cells is dispensable for the development of T cells in the thymus. The analyses of SLOs demonstrated that both total cell numbers and frequencies of $CD4^+$ and $CD8^+$ T cells were comparable between the two groups regardless of CD44 expression level (Fig. 1G and SI Appendix, Fig. S1 E and F). In addition, Treg frequencies in the periphery and the thymus were either comparable or slightly

higher in the $T^{\Delta G\alpha_{13}}$ mice (Fig. 1H). To examine the involvement of $G\alpha_{13}$ in TCR-triggered activation, we compared the proliferation and expression of CD44, CD28, and IL-2 after stimulating naive $CD4^+$ T cells with plate-bound anti-CD3 and anti-CD28 and found similar degrees of activation, proliferation (Fig. 1 I–K), and apoptosis (SI Appendix, Fig. S1 H and I) overall, with an $\sim 10\%$ increase in IL-2 secretion in the $T^{\Delta G\alpha_{13}}$ group. These series of in vitro experiments, along with thymocyte analysis, indicate that the $G\alpha_{13}$ -mediated signaling in T cells is largely dispensable for the overall development of conventional T cells in the thymus, the distribution of mature T cells into SLOs in vivo, and the TCR-mediated activation and proliferation of $CD4^+$ T cells.

Lack of $G\alpha_{13}$ Signaling in T Cells Results in Diminished Tfh Cell Responses In Vivo. To define the role of $G\alpha_{13}$ -mediated signaling in peripheral T cell responses, we first investigated the differentiation capabilities of naive T cells under Th1-, Th2-, Th17-, and Treg-skewing conditions in vitro and found that the differentiated frequencies of Th1, Th2, and Treg cells were comparable, while Th17 cell frequencies were slightly increased in the

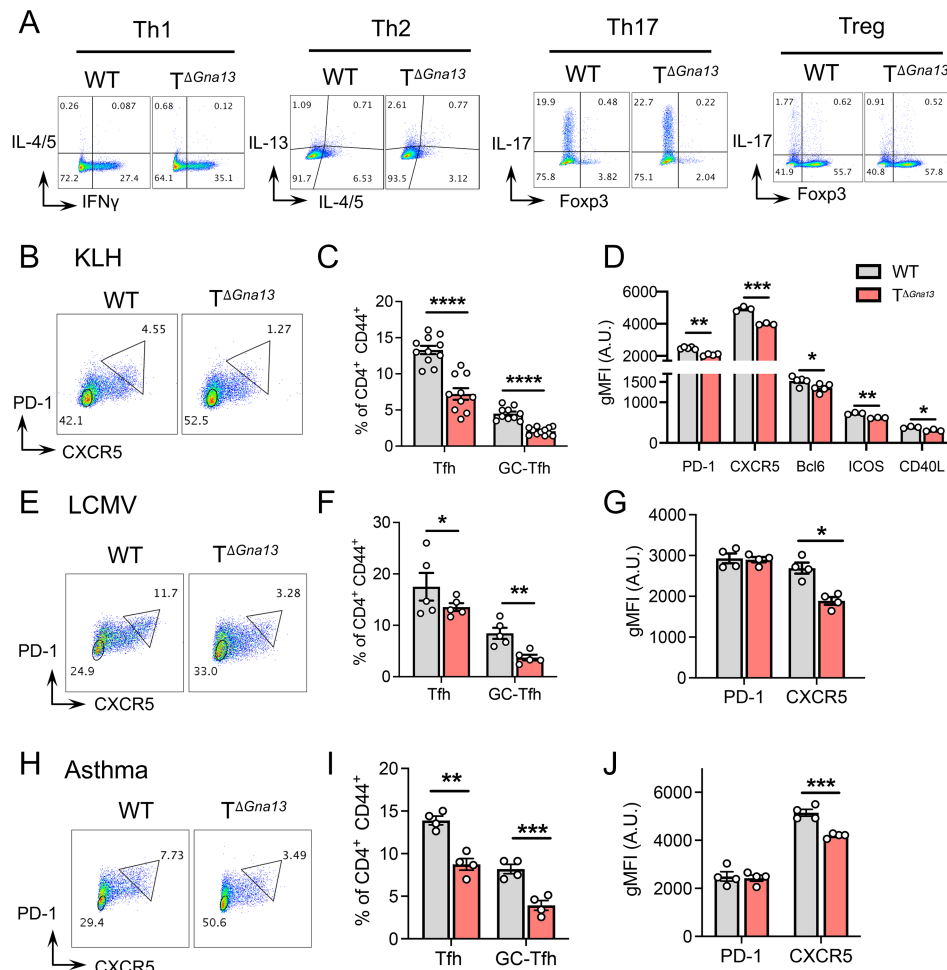


Fig. 2. $G\alpha_{13}$ deficiency leads to diminished Tfh cell responses in vivo. (A) Representative fluorescence-activated cell sorting (FACS) plots of naive T cells differentiated to Th1, Th2, Th17, and Treg conditions in vitro. (B–D) Mice were s.c. immunized with KLH emulsified in CFA at the side flanks, and inguinal LNs were analyzed on day 8. (B) Representative FACS plots of GC-Tfh, gated as CXCR5^{hi} PD-1^{hi}. The image representative of three independent experiments is shown. (C) Total and GC-Tfh frequencies ($n = 9$ to 10 per group). (D) Geometric mean fluorescence intensities (gMFI) of Tfh markers and molecules ($n = 3$ to 5 per group). (E–G) Mice were infected with 3×10^5 plaque-forming units (pfu) of LCMV Armstrong, and spleens were analyzed on day 6. (E) Representative FACS plots of GC-Tfh (CXCR5^{hi} PD-1^{hi}) analysis. Frequencies (F) and gMFI (G) of Tfh markers ($n = 4$ to 5 per group). (H–J) Mice were intranasally challenged with a mixture of OVA and *Aspergillus oryzae* (PAO), and mediastinal LNs were analyzed on day 9. (H) Representative FACS plots of GC-Tfh analysis. Frequencies (I) and gMFI (J) of Tfh markers denoted in arbitrary units (A.U.). ($n = 4$ per group). All data are representative of two independent experiments, unless stated otherwise. All data are mean \pm SEM; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; and **** $P < 0.0001$.

$T^{\Delta Gna13}$ group compared to littermate control (Fig. 2A and *SI Appendix, Fig. S2A*). We then analyzed CD4⁺ effector Th subsets in the SLOs of $T^{\Delta Gna13}$ mice at steady state and found that Th1 cell frequencies were slightly elevated in the gut-associated lymphoid tissues of $T^{\Delta Gna13}$ mice, while the opposite was true in the spleen (*SI Appendix, Fig. S2B*). The frequencies of Th17 cell and Treg cell were comparable in all examined secondary lymphoid tissues between the two groups overall, except for the slight increase in Treg cell frequencies in the mesenteric LNs (Fig. 1H and *SI Appendix, Fig. S2C*). Concomitantly, we observed a trend toward diminished Tfh cell frequencies (*SI Appendix, Fig. S2 D and E*) and absolute numbers (*SI Appendix, Fig. S2F*) in the Peyer's patches (PPs) of $T^{\Delta Gna13}$ mice, albeit no such differences in absolute cell numbers were observed for other Th subsets within the PPs (*SI Appendix, Fig. S2F*). Additionally, absolute cell counts of the above lymphoid organs were comparable between the two groups, with the exception of the spleen, which was slightly higher in the $T^{\Delta Gna13}$ group (*SI Appendix, Fig. S2G*).

To evaluate whether $G\alpha_{13}$ signaling regulates antigen-specific CD4⁺ T cell responses in vivo, we subcutaneously (s.c.) immunized $T^{\Delta Gna13}$ and littermate control mice with KLH in CFA and compared the CD4⁺ T cell subsets in the draining LNs. We observed that Th1 and Th17 cell frequencies were all comparable between the two groups, with a slight increase in Treg frequencies in the $T^{\Delta Gna13}$ group (*SI Appendix, Fig. S2H*). In accordance with these results, ex vivo KLH restimulation of lymphoid cells from the draining LNs showed no difference in IFN- γ and IL-17 production (*SI Appendix, Fig. S2I*). Total Tfh frequencies were defined to be CXCR5⁺PD-1⁺, among which GC-Tfh cells were defined as CXCR5^{hi}PD-1^{hi} (*SI Appendix, Fig. S3 A–C*). In sharp contrast to the other Th subsets, the frequencies, as well as absolute number, of total and GC-Tfh cells were significantly lower in the $T^{\Delta Gna13}$ mice than in the littermate control mice (Fig. 2B and C and *SI Appendix, Fig. S3D*). In addition, the signature surface markers of Tfh cells, such as CXCR5, PD-1, ICOS, and CD40L, showed significantly lower expression levels in $G\alpha_{13}$ -deficient Tfh cells than in $G\alpha_{13}$ -sufficient Tfh cells (Fig. 2D). The expression of Bcl-6, the master transcription factor that commits Tfh cells to the lineage, was also relatively diminished in the former (Fig. 2D). The Ki-67 staining of T cell subsets showed comparable proliferative capacity between the two groups (*SI Appendix, Fig. S3E*).

To determine further the importance of $G\alpha_{13}$ signaling in Tfh cell differentiation in vivo, we employed two additional animal models in which the development of Tfh cells upon antigenic encounter is well-established: lymphocytic choriomeningitis virus (LCMV) infection and allergic asthma (17, 18). In both animal models, we observed a significant reduction in the frequency of Tfh cells among CD4⁺ T cells in the $T^{\Delta Gna13}$ mice compared to that of control mice (Fig. 2E, F, H, and I, respectively, for each model). While frequencies of other helper T cell subsets remained comparable, those of Treg cells detected in the spleens of LCMV model were lower, while Treg cell frequencies in the mediastinal LNs of asthma model were significantly higher in $T^{\Delta Gna13}$ mice compared to those of control mice (*SI Appendix, Fig. S3 F and G*). Similar to the KLH model, the expression level of CXCR5 and Bcl-6 was consistently lower in the $G\alpha_{13}$ -deficient Tfh cells than $G\alpha_{13}$ -sufficient ones in both models (Fig. 2G and J and *SI Appendix, Fig. S3H*). Of note, the frequencies of the fully committed GC-Tfh cells, characterized by CXCR5^{hi} PD-1^{hi}, were all consistently lower in the $T^{\Delta Gna13}$ group in all three models, indicating that $G\alpha_{13}$ signal is integral for triggering Tfh cell commitment (Fig. 2E, F, H, and I). Altogether, these results indicate that the lack of $G\alpha_{13}$ -mediated signaling in T cells leads to specifically diminished Tfh cell responses in vivo, regardless of the antigen type or immunization method used.

$G\alpha_{13}$ Signaling in T Cell Is Required for Optimal GC Reactions and Humoral Responses. Tfh cells facilitate GC reactions within the SLOs by providing costimulation and cytokines to activated B cells. The diminished Tfh cell responses observed in the $T^{\Delta Gna13}$ mice prompted us to investigate whether GC reactions are affected in the $T^{\Delta Gna13}$ mice. While the frequencies of total B220⁺ B cells were comparable between the two groups at steady state (*SI Appendix, Fig. S4A, Left*), the frequencies of CD95⁺GL7⁺B220⁺ GC B cells were significantly lower in the PPs of the $T^{\Delta Gna13}$ mice compared to the littermate control mice (*SI Appendix, Fig. S4A, Left*). The comparison of absolute cell numbers between the two groups yielded similar trends (*SI Appendix, Fig. S4A, Right*). Such differences were not observed in other SLOs or were rather reversed in the case of mesenteric LNs (*SI Appendix, Fig. S4B*). No significant differences were observed in the levels of circulating total IgG at steady state (*SI Appendix, Fig. S4C*).

Unlike unimmunized mice, we observed a significant reduction in the frequencies of GC B cells and plasma cells (PCs) in the draining LNs of $T^{\Delta Gna13}$ mice upon s.c. immunization with KLH in CFA, compared to those of littermate control (Fig. 3A–C). The visualization of the GC showed the relatively poor colocalization of CD4⁺ T cells within each GC of the draining LNs in the $T^{\Delta Gna13}$ mice compared to that of control mice, while overall follicle formation seemed to be unaffected (Fig. 3D and *SI Appendix, Fig. S4D*). Moreover, serum levels of KLH-specific IgG—both IgG1 and IgG2 subclasses—were found to be significantly lower in the former, while those of IgM and IgG3 were comparable between two groups (Fig. 3E). Given that IgM and IgG3 production is largely independent of T cell help, the selective reduction in KLH-specific IgG1 and IgG2 subclasses strongly suggests that diminished Tfh cell responses likely led to reduced KLH-specific IgG in the $T^{\Delta Gna13}$ mice in this experimental setting.

$G\alpha_{13}$ deficiency not only led to diminished frequencies of Tfh cells, but also resulted in diminished expression of CXCR5, ICOS, and CD40L on Tfh cells, raising a possibility that $G\alpha_{13}$ -deficient Tfh cells are functionally impaired. To explore this possibility, we flow-sorted Tfh cells from the KLH-immunized draining LNs of the respective mice and cocultured them with naive B cells isolated from wild-type (WT) syngeneic mice (Fig. 3F, *Top*). The amount of IgG in the supernatant was significantly lower in the conditions in which $G\alpha_{13}$ -deficient Tfh were cocultured with B cells by approximately threefold (Fig. 3F, *Bottom*). Both IgG1 and IgG2c subclasses were significantly diminished in the coculture supernatant, similar to the in vivo serum analysis (Fig. 3F, *Bottom*). Trends of reduced humoral responses were also partially observed in LCMV and allergic asthma models, in which GC B cell or PC frequencies, as well as antigen-specific IgG levels, were lower in $T^{\Delta Gna13}$ mice (*SI Appendix, Fig. S4 E and F*). $G\alpha_{13}$ -sufficient, as well as -deficient, Tfh cells showed equivalent survival and proliferation (*SI Appendix, Fig. S4G*). These results collectively demonstrate that the $G\alpha_{13}$ -mediated signaling in T cells is integral not only for the differentiation of Tfh cells, but also for its function in facilitating optimal GC reactions and antigen-specific IgG production in vivo.

$G\alpha_{13}$ Signaling Promotes Tfh Cell Differentiation in a T Cell-intrinsic Manner. Next, we sought to determine the mechanism by which $G\alpha_{13}$ signaling contributes to Tfh cell responses in vivo. To test whether $G\alpha_{13}$ triggers Tfh cell differentiation in a cell-intrinsic manner, we employed two different in vivo models. First, we established a mixed bone chimeric mice system, in which congenically marked WT (CD45.1^{+/+}) bone marrow was mixed with $T^{\Delta Gna13}$ (CD45.2^{+/+}) bone marrow at 1:1 ratio before being adoptively transferred to bone marrow-ablated $Rag1^{-/-}$ mice (Fig. 4A). Following a 6-wk reconstitution period, mice

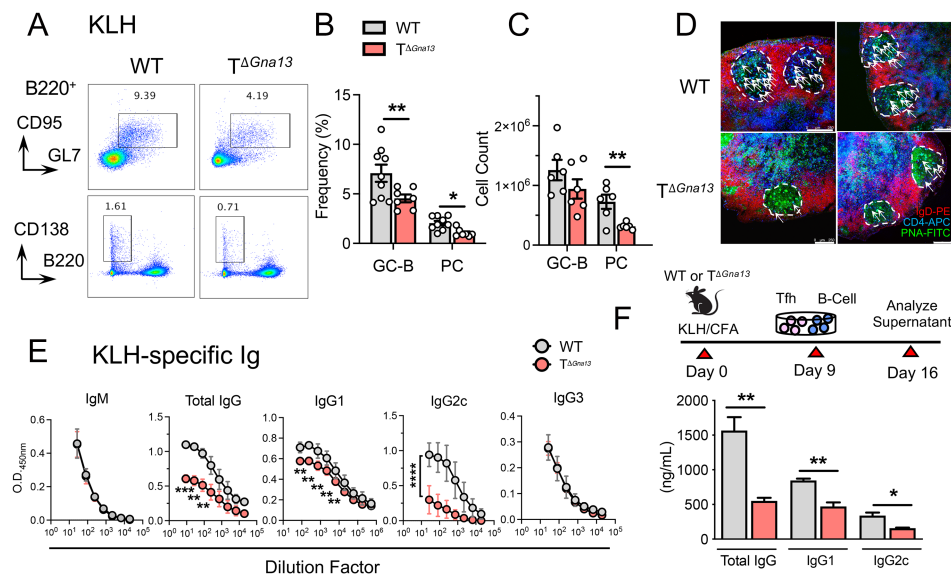


Fig. 3. $G\alpha_{13}$ signaling in T cells is required for optimal GC reactions and humoral responses. (A–E) Mice were s.c. immunized with KLH in CFA at the side flanks, and inguinal LNs were analyzed on day 8 for cell analysis or day 10 for serum analysis. (A) Representative fluorescence-activated cell sorting plots of GC B and PC analysis from three independent experiments. Frequencies (B) and absolute cell counts (C) of GC B cells and PC ($n = 6$ to 9 per group). (D) Representative immunofluorescence image of the GC within the draining LNs. White arrows indicate $CD4^+$ T and B cell colocalization within the B cell follicle. White bar on the lower right corner indicates the 250- μ m scale. (E) Antigen-specific serum Ig enzyme-linked immunosorbent assay (ELISA) analysis ($n = 3$ per group). Values are denoted as optical density (O.D.). (F) Tfh cells were flow sorted and cocultured with naive B cells ex vivo for 7 d prior to Ig ELISA analysis of the culture supernatant. Data are representative of three independent experiments ($n = 5$ per group). All data are representative of two independent experiments, unless stated otherwise. All data are mean \pm SEM; * $P < 0.05$; and ** $P < 0.01$.

were subjected to KLH immunization and subsequent analysis on day 9, adhering to the gating strategy as shown (SI Appendix, Fig. S5A). The frequencies of Tfh cells, and particularly CXCR5^{hi}PD-1^{hi} GC-Tfh cells, were significantly lower in the $T^{\Delta Gna13}$ compartment than WT (Fig. 4B). Moreover, the expression levels of Bcl-6, CXCR5, and PD-1 on $G\alpha_{13}$ -deficient Tfh cells were all significantly diminished compared to those of $G\alpha_{13}$ -sufficient Tfh cells in the same chimeric mice, although levels of ICOS were comparable (Fig. 4C). Unlike previous models, frequencies of Treg cell were slightly but significantly lower in the $T^{\Delta Gna13}$ group, while follicular regulatory T cell frequencies were relatively similar between the two donor populations (SI Appendix, Fig. S5 C and D), suggesting that the T cell-intrinsic differences in Tfh responses were not due to differences in Treg cells.

In the second model, we cotransferred WT (CD45.1⁺/CD45.2⁺) and $G\alpha_{13}$ -deficient (CD45.2^{+/+}) OT-II T cells at 1:1 ratio into congenic mice (CD45.1^{+/+}), followed by immunization with ovalbumin (OVA) in CFA (Fig. 4D and SI Appendix, Fig. S5B). Kinetic analysis revealed that $G\alpha_{13}$ -deficient OT-II T cells showed consistent impairment in Tfh cell differentiation, starting as early as day 3 postimmunization (Fig. 4E and SI Appendix, Fig. S5E), while the comparable expression of CD44 in both donor groups indicated that the overall activation of OT-II donor cells was comparable (SI Appendix, Fig. S5F). The impairment most affected CXCR5^{hi}PD-1^{hi} GC-Tfh cell population (SI Appendix, Fig. S5G) and led to significantly lower expression levels of Bcl-6, CXCR5, PD-1, and ICOS (Fig. 4F). To compare the efficiency of T-B cell conjugate formation between $G\alpha_{13}$ -sufficient and -deficient Tfh cells, OT-II Tfh cells were cocultured with either OT-II peptide-loaded DC or B cells (Fig. 4G). While demonstrating comparable T-DC conjugate formation, $G\alpha_{13}$ -deficient Tfh cells were significantly less efficient than $G\alpha_{13}$ -sufficient ones in forming conjugates with B cells (Fig. 4H). We observed similar results using in vitro-generated “Tfh-like” cells (SI Appendix, Fig. S5H). Thus, $G\alpha_{13}$ signaling plays a crucial, cell-intrinsic role to promote the

differentiation of Tfh cells that stably interact with cognate B cells.

$G\alpha_{13}$ Signaling Is Required for the Activation of RhoA-ROCK Pathway in Tfh Cell. We next compared the relative transcript levels of major transcription factors involved in the Tfh cell differentiation program and found that *Bcl6*, *Ascl2*, *Zbtb7b* (encoding Thpok), and *Lef1* expressions, but not *Bach2* and *Tcf7*, were significantly lower, while *Prdm1* (encoding Blimp1) expression was higher in $G\alpha_{13}$ -deficient Tfh cells than those in $G\alpha_{13}$ -sufficient Tfh cells (Fig. 5A and SI Appendix, Fig. S6A). Levels of messenger RNA-encoding cytokines important for Tfh function, such as *Ifng*, *Il4*, and *Il21*, were comparable or, rather, higher in the former (SI Appendix, Fig. S6B), ruling out the role of $G\alpha_{13}$ in regulating these cytokines in Tfh cells. Expression levels of the surface molecules important for Tfh cell function and maturation, including *Cd154* (encoding CD40L), *Cxcr4*, and *Slpr2*, were significantly down-regulated in $G\alpha_{13}$ -deficient Tfh cells (Fig. 5B).

To probe the mechanistic details by which $G\alpha_{13}$ signaling regulates Tfh cell differentiation in vivo, we profiled the transcriptome of flow-sorted Tfh cells isolated from KLH-immunized $T^{\Delta Gna13}$ mice and control mice via RNA sequencing (RNA-seq) analysis. At a setting of greater than twofold expression changes, with a raw P value < 0.05 and false discovery rate < 0.1 , 231 genes were up-regulated, and 765 genes were down-regulated in the $G\alpha_{13}$ -deficient Tfh cells, compared with the $G\alpha_{13}$ -sufficient Tfh cells (Dataset S1). In particular, we found that among the down-regulated differentially expressed genes (DEGs), genes previously known to be positively associated with the Tfh cell lineage program, such as *ApoE*, *Atf3*, *Mafb*, *Scd3*, *Spp1*, and *Zeb2*, were present (SI Appendix, Table S1) (19–24), indicating that Tfh transcriptome is selectively down-regulated in the absence of $G\alpha_{13}$. In fact, out of all the Tfh signature genes found within the DEGs, almost all genes were significantly down-regulated (SI Appendix, Table S1). As expected, nearly half of the top 20 terms of gene ontology enrichment analysis of DEGs using the

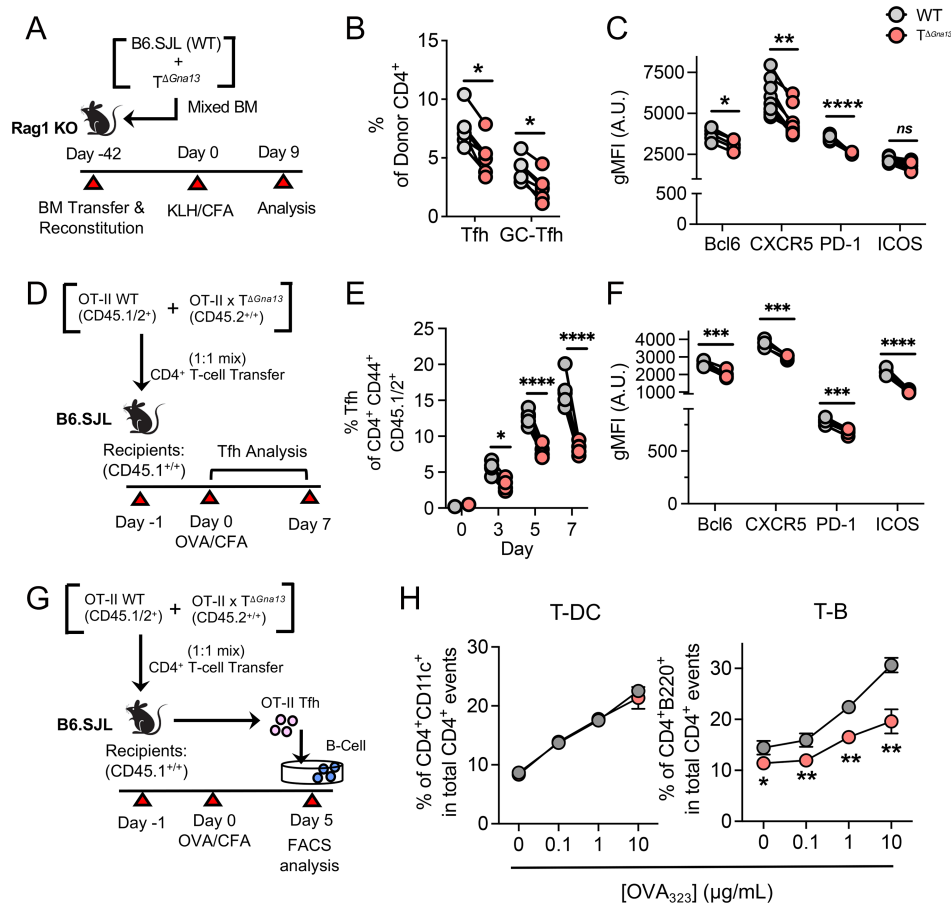


Fig. 4. $G\alpha_{13}$ signaling promotes Tfh cell differentiation in a cell-intrinsic manner. (A–C) Mixed bone marrow (BM) chimera experiments were conducted according to the scheme shown in A. Inguinal LNs were analyzed on day 9. (B) Summary of respective donor Tfh and GC-Tfh frequencies ($n = 5$ per group). (C) gMFI values of Tfh marker and molecules denoted as arbitrary units (A.U.) ($n = 6$ per group). (D–F) OT-II cotransfer experiment was conducted according to the scheme shown in D. Inguinal LNs were analyzed on days 0, 3, 5, and 7. (E) Summary of respective donor Tfh and GC-Tfh frequencies. (F) gMFI values of Tfh marker and molecules ($n = 5$ per group). (G) Experimental scheme of conjugate formation assay. (H) Antigen-specific T-DC and T-B conjugate efficiencies of WT or $G\alpha_{13}$ -deficient OT-II Tfh cells ($n = 3$ per group). All data are representative of two independent experiments. All data are mean \pm SEM; ns, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; and **** $P < 0.0001$.

gProfiler package were related to molecules involved in cell adhesion, chemotaxis, localization, and migration (SI Appendix, Fig. S6C). The gene ontology term analysis of DEGs using a web-based tool indicated that $G\alpha_{13}$ -deficient Tfh cells had impaired Rho signaling pathways, as demonstrated by the high, negative enrichment scores attributed to genes associated to signaling via Rho GTPase proteins (Fig. 5C). The $G\alpha_{12/13}$ signaling of the GPCRs involves the activation of Rho GTPase guanine exchange factors (RhoGEFs), and these link GPCRs to the activation of the small, monomeric GTPase RhoA and other downstream effectors (25). In hematopoietic cells, three RhoGEFs have been identified: PDZ-RhoGEF, leukemia-associated RhoGEF, and p115-RhoGEF (respective gene names: *Arhgef1*, *Argef12*, and *Arhgef11*) (26–28). Among the three RGS-RhoGEFs, the level of *Arhgef11* (encoding p115-RhoGEF) was significantly down-regulated in $G\alpha_{13}$ -deficient Tfh cells (Fig. 5D). The expression of *Rhoa* transcript and active GTP-bound RhoA levels were also significantly diminished in the $G\alpha_{13}$ -deficient Tfh cells (Fig. 5E). Since RhoA phosphorylates ROCK2 to regulate cellular proliferation (29) and movement-related factors such as cofilin (30), we examined the phosphorylation of ROCK2 and cofilin and found that the levels of phospho-ROCK2 and phosphocofilin were profoundly diminished in the $G\alpha_{13}$ -deficient Tfh cells (Fig. 5F and SI Appendix, Fig. S6D). Thus, in the absence of $G\alpha_{13}$ signaling, Tfh cells failed

to activate the RhoA-ROCK2 signaling pathway. As ROCK2 has been shown to modulate STAT3 signaling (31), we next questioned whether $G\alpha_{13}$ deficiency impacts STAT3 phosphorylation in $CD4^+$ T cells. Compared to WT, $G\alpha_{13}$ -deficient $CD4^+$ T cells displayed a slight but significant reduction in the level of phospho-STAT3 upon IL-6 stimulation (SI Appendix, Fig. S6E), suggesting a role for $G\alpha_{13}$ signaling on the optimal STAT3 activation in T cells.

Tfh cells are known to mediate various autoimmune diseases, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis, either via assistance with autoantibody production and/or the formation of tertiary lymphoid structures in nonlymphoid tissues (32, 33). In order to determine whether RhoA signaling is relatively enriched in Tfh cells compared to non-Tfh effector cells in humans, we analyzed available RNA-seq data from SLE patient blood samples (34, 35). Gene set enrichment analysis (GSEA) of Tfh cells compared to non-Tfh cells yielded a positive correlation with genes signatures associated with RhoA and RhoGTPase effectors, suggesting that RhoA activation is positively correlated with human Tfh cell lineage program (SI Appendix, Fig. S6F).

$G\alpha_{13}$ -RhoA-ROCK Axis Facilitates Tfh Cell Differentiation and Subsequent GC Reactions In Vivo. To determine whether the defective RhoA-pROCK2 pathway could account for the

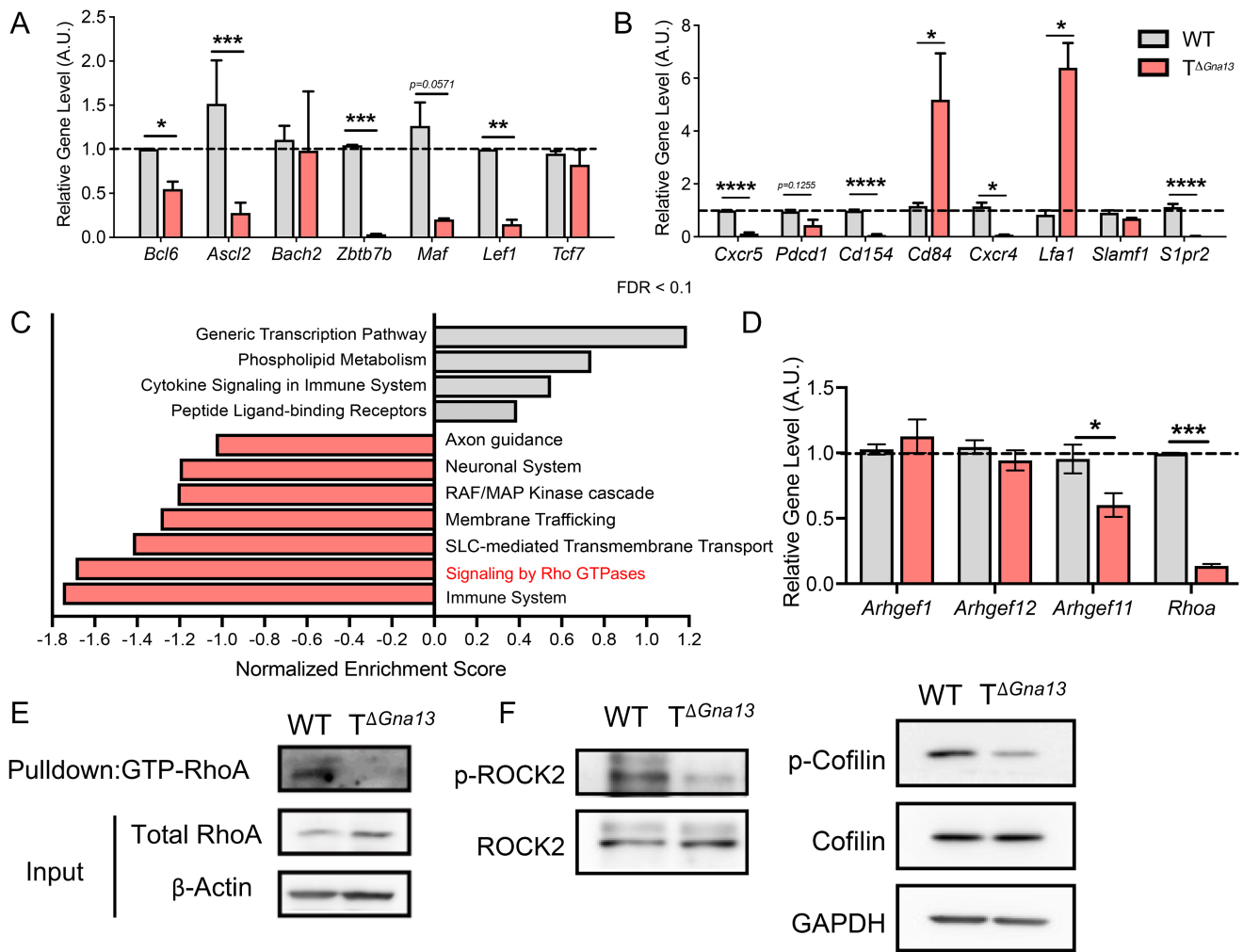


Fig. 5. $G\alpha_{13}$ signaling is required for the activation of RhoA-ROCK2 pathway in Tfh cells. (A–G) Tfh from the draining LNs of KLH-immunized mice were flow sorted on day 8 and analyzed. Relative transcript levels of signature transcription factors (A) and surface molecules (B) important in Tfh cell differentiation and function analyzed via qRT-PCR. Values are denoted as arbitrary units (A.U.). (C) DEG from bulk RNA-seq of Tfh cells were subjected to gene ontology analysis. False discovery rate (FDR) cutoff of 0.1 was applied. Solute carrier transporters (SLC). (D) Relative transcript levels of RhoGEFs proteins in Tfh cells. (E) Immunoblotting of active (GTP bound) RhoA. (F) Western blot of total and phosphorylated ROCK2 (Left) and cofilin (Right). All data are mean \pm SEM and *P* values are indicated where appropriate; **P* < 0.05; ***P* < 0.01; ****P* < 0.001; and *****P* < 0.0001.

observed, diminished Tfh cell responses in $T^{\Delta Gna13}$ mice in vivo, we examined if treatment with a RhoA agonist, narciolasine, could restore Tfh cell responses in $T^{\Delta Gna13}$ mice in a KLH immunization model (Fig. 6A). Narciclasine treatment showed little effect on the frequencies of Tfh cells in WT control mice; however, it significantly increased the frequencies of Tfh cells in $T^{\Delta Gna13}$ mice to the levels comparable to those of control mice (Fig. 6B and C). Concomitantly, the frequencies of GC B cells and PCs in the $T^{\Delta Gna13}$ mice were increased by the agonist treatment (Fig. 6D). While the agonist treatment had little effect on the frequencies of GC B cells, it increased that of PCs in control mice, suggesting that, besides regulating Tfh cell responses, the Rho agonist itself might be capable of directly promoting PC differentiation (Fig. 6D). The same observed enhancement in Tfh cell responses was shown in the LCMV infection model; Rho agonist treatment rescued Tfh cell frequencies in the $T^{\Delta Gna13}$ mice (SI Appendix, Fig. S7A–C), indicating that Rho activation positively regulates the Tfh cell differentiation program in vivo. The stimulation of naive $CD4^+$ T cells in the presence of IL-6 and neutralizing antibodies to IFN- γ , IL-4, and TGF- β 1 induces IL-21-expressing, Tfh-like cells (20). The increasing concentrations of narciolasine

treatment on naive T cells under Tfh-like-skewing conditions led to higher frequencies of CXCR5-expressing $CD4^+$ T cells in vitro (SI Appendix, Fig. S7D).

Since the direct in vivo treatment of narciolasine could introduce indirect effects and skew our interpretation, we first treated agonist on $G\alpha_{13}$ -deficient, naive OT-II T cells stimulated under Tfh-like-skewing conditions in vitro and then transferred them into congenically marked WT recipients, followed by immunization with OVA in CFA (Fig. 6E). This also allowed us to precisely determine whether such Rho agonist boosting in $CD4^+$ T cells can truly compensate the defective Tfh cell differentiation in $T^{\Delta Gna13}$ mice in vivo. Compared to those in the recipients of vehicle-treated, $G\alpha_{13}$ -deficient OT-II T cells, the frequencies of Tfh cells in the recipients of agonist-treated, $G\alpha_{13}$ -deficient OT-II T cells were significantly increased, although they were still lower than those in the recipient of WT OT-II T cells (Fig. 6F and G). While GC B cell frequencies in recipients of vehicle-treated, $G\alpha_{13}$ -deficient OT-II cells were significantly lower than those that received WT OT-II T cells, no differences were observed in the GC B cell frequencies between the recipients of WT OT-II T cells and agonist-treated OT-II T cells (Fig. 6H). While KLH-specific antibody levels of

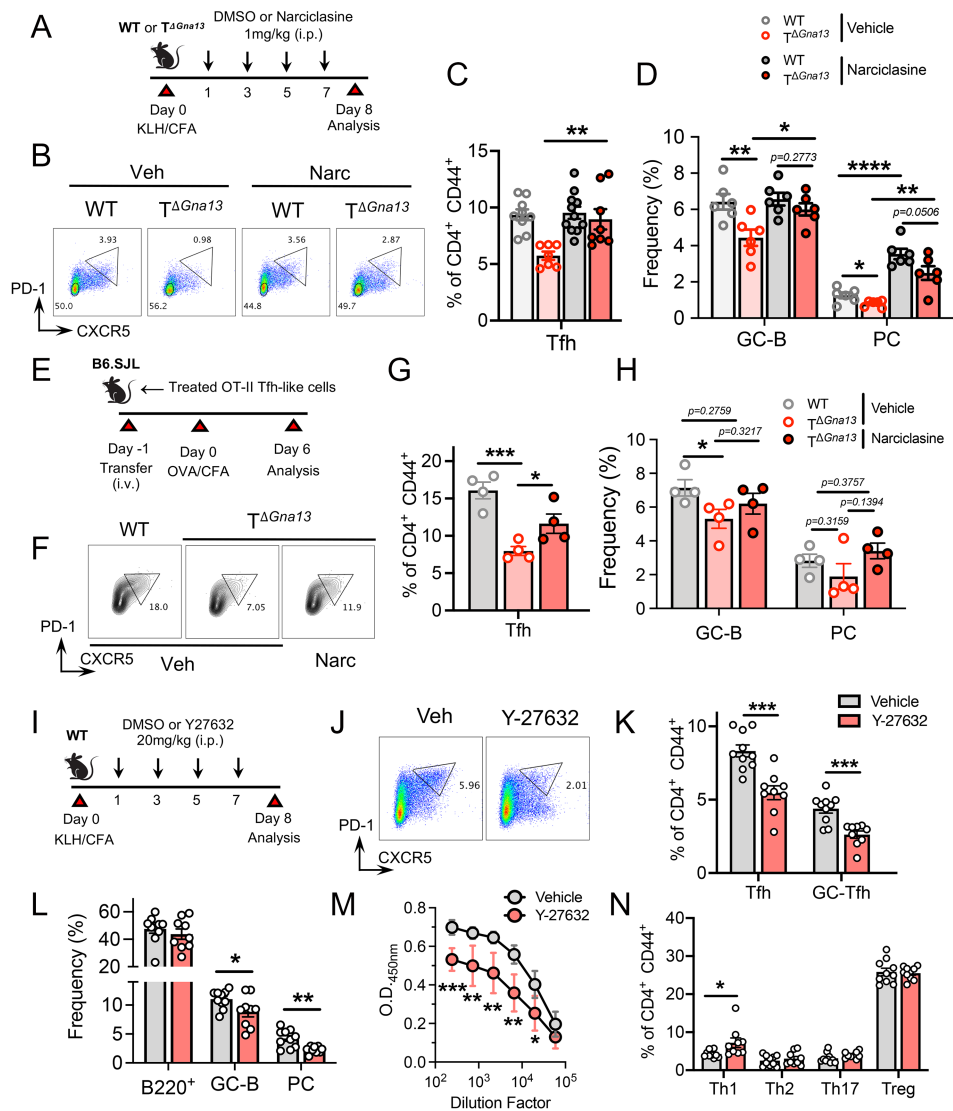


Fig. 6. Modulation of the RhoA-ROCK axis regulates Tfh and GC reactions in vivo. (A–D) WT and $T^{\Delta Gna13}$ mice were KLH immunized and treated with either DMSO vehicle (Veh) or narciclasine (Narc). Draining LNs were analyzed as indicated in A. (B) Representative fluorescence-activated cell sorting (FACS) plots of GC-Tfh cells. Tfh frequencies (C) combined from two independent experiments and B cell frequencies (D) ($n \geq 6$ per group). (E–H) Either DMSO or Narc was treated to WT and $G\alpha_{13}$ -deficient OT-II cells under Tfh-skewing conditions in vitro and were intravenously (i.v.) transferred to congenically marked WT recipients, which were then OVA immunized as shown in E. (F) Representative FACS plots of Tfh cells. Tfh (G) and B cell (H) frequencies ($n = 4$ per group). (I–M) KLH-immunized WT mice were subjected to ROCK inhibitor treatment as shown in I. (J) Representative FACS plots of Tfh cells. Tfh, GC-Tfh (K) and B cell (L) frequencies combined from two independent experiments ($n = 9$ per group). (M) KLH-specific serum total IgG levels. (N) Th subset frequencies in draining LNs. All data are representative of two independent experiments, unless stated otherwise. All data are mean \pm SEM and P values are indicated where appropriate; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; and **** $P < 0.0001$.

vehicle-treated T^{Δ} mice were significantly lower than those of vehicle-treated WT mice, narciclasine treatment tended to enhance antibody production in the T^{Δ} group, although not to the level of the WT group (SI Appendix, Fig. S7E). Both the frequency and absolute number of Treg cells did not change as a result of agonist treatment (SI Appendix, Fig. S7F). No differences were observed in endogenous Tfh cells of recipient origin, indicating that recipient Tfh cells did not contribute to the observed differences in B cell responses (SI Appendix, Fig. S7G). These data strongly suggest that the modulation of the Rho pathway can salvage the defective $G\alpha_{13}$ signaling in $T^{\Delta Gna13}$ mice.

Since treatment with a Rho agonist restored the frequencies of Tfh cells and GC B cells in $T^{\Delta Gna13}$ mice to the levels similar to WT mice in vivo, we hypothesized that the blockade of the RhoA-ROCK2 axis would lead to diminished Tfh cell

differentiation and subsequent GC reactions. In order to test this, we treated KLH-immunized C57BL/6 mice with Y27632, a selective ROCK inhibitor, or DMSO as vehicle before analyzing the draining LNs and serum (Fig. 6I). Tfh cell frequencies, as well as the expression levels of CXCR5 on Tfh cells, were significantly diminished in the inhibitor-treated group compared with vehicle-treated group (Fig. 6J and K and SI Appendix, Fig. S7H). A similar reduction in the frequencies of GC B cells and PC population were also observed (Fig. 6L). Consistently, the levels of KLH-specific IgG were also significantly diminished in the inhibitor-treated group in a IgG2c-dependent manner (Fig. 6M and SI Appendix, Fig. S7I). Frequencies of Th1 cells were slightly elevated in the inhibitor-treated group, which was somewhat in parallel with the results of steady-state analysis conducted previously (Fig. 6N and SI Appendix, Fig. S2 B and E). The frequencies of Th2, Th17, and Treg cells were all unaffected

as a result of inhibitor treatment (Fig. 6N and *SI Appendix, Fig. S7J*). Thus, the blockade of ROCK could suppress the differentiation of Tfh cells and subsequent GC reactions in vivo. Collectively, these results demonstrate that $G\alpha_{13}$ signaling in T cells promotes Tfh cell responses via activating the RhoA-ROCK2 cascade, subsequently contributing to the induction of transcription factors and surface molecules required for the Tfh cell lineage program (*SI Appendix, Fig. S7K*).

Discussion

It has been well documented that GPCRs mediate a wide array of cellular functions in T cells, most notably in cytoskeletal rearrangements and migration. In particular, the modulation of various chemokine receptors on chemotacting T cells has been shown to be mediated by a diverse range of $G\alpha$ proteins. Among $G\alpha$ proteins, the $G\alpha_{12/13}$ subfamily of proteins have been implicated to negatively regulate T cell trafficking via suppressing expressions of certain integrins. Although the role of $G\alpha$ proteins in T cell chemotaxis and motility is well documented, how $G\alpha$ -mediated signaling fine-tunes and modulates T cell subset differentiation and development has not been reported. Here, we demonstrate that the lack of $G\alpha_{13}$ signaling in T cells led to defective Tfh cell responses in vivo by using three different animal models: subcutaneous immunization, systemic LCMV infection, and intranasal allergen challenges. Thus, T cell-intrinsic $G\alpha_{13}$ signaling is crucially required for the Tfh cell lineage program in vivo, regardless of antigen types. Although differences in Treg frequencies were observed in certain cases at steady state and in KLH and asthma models, similar results were not observed in mixed bone marrow chimeras and cotransfer models, indicating that Treg cell deviations cannot account for the diminished Tfh cell responses in vivo. Many of the $G\alpha_{13}$ -mediated signals are also shared with $G\alpha_{12}$ proteins, and previous works have demonstrated $G\alpha_{12}$ activity can partly compensate for signals in the absence of $G\alpha_{13}$ (15). As such, we have confirmed that $G\alpha_{12}$ expression remains relatively unchanged in our $T^{\Delta Gna13}$ mouse system. These data indicate that $G\alpha_{13}$ protein has a nonredundant role in Tfh cell differentiation in vivo.

Work with other primary and cancer cells have revealed that $G\alpha_{13}$ proteins activate Rho GTPases via a direct interaction with RhoGEFs, which in turn activate intracellular mediators such as ROCK2 (36, 37). We have demonstrated that the same molecular pathway seems to be not only pertinent but also critical for naive T cell commitment to the Tfh cell lineage. In T cell lymphoma, a gain-of-function mutation in RhoA led to increased ICOS expression and a malignant transformation of Tfh cells in vivo (38). Previous work with human T cells has demonstrated that phosphorylated ROCK2 interacts with and binds to phosphorylated STAT3, thereby forming a complex to positively regulate *BCL6* (31, 39, 40). Given that the Bcl-6 expression levels of $G\alpha_{13}$ -deficient Tfh cells, as well as phospho-STAT3 levels, were diminished compared to $G\alpha_{13}$ -sufficient ones, we propose that $G\alpha_{13}$ -mediated RhoA-ROCK2-STAT3 signal cascade may account for the diminished Tfh cell responses in $T^{\Delta Gna13}$ mice in vivo. We have also shown that the failure to transduce optimal $G\alpha_{13}$ -Rho-ROCK2 pathway in T cells can lead to deterred, antigen-specific humoral responses in vivo, which seems to be somewhat recuperable by the agonistic modulation of the Rho pathway, although not completely. The partial rescue of humoral responses indicates that other intercellular regulations could also be at play besides the aforementioned mechanism. Active $G\alpha_{12/13}$ proteins have been shown to bind with and seize cadherins from catenins, which lead to up-regulated, β -catenin-mediated, and TCF/LEF-dependent transcriptional activation in cancer cells (41). TCF/LEF expression and regulation have been shown to be

important for Tfh cell polarization (42, 43), and such a pathway is certainly another plausible molecular mechanism. The convergence of multiple transcriptional pathways must produce a synergistic effect and lead to the ultimate cellular consequence to selectively deter commitment to the Tfh cell lineage.

$G\alpha_{13}$ -mediated signaling appears to be important not only for Tfh cell lineage commitment but also for Tfh cell stimulatory and adhesion function. Our analyses revealed that $G\alpha_{13}$ deficiency in T cells prompts multiple defects in Tfh cell function, including the significantly lower expression of receptors important for migration to and within B cell zones, such as CXCR5, S1PR2, and CXCR4, as well as costimulatory molecules important for directly activating B cells, such as ICOS and CD40L, while it seemed to up-regulate the expression of CD84 and LFA1. Histological analysis revealed that $G\alpha_{13}$ -deficient $CD4^+$ T cells failed to access the GC, presumably because of the reduced expression of CXCR5. Mixed bone marrow chimera studies, as well as OT-II cotransfer studies, indicate that the $G\alpha_{13}$ signaling in T cells is indispensable for the surface molecules important for Tfh commitment: CXCR5, CD40L, ICOS, and other cell surface molecules required for helping activated B cells. A similar reduction of CXCR5 expression on Tfh cells was observed in mice treated with ROCK inhibitor in vivo, suggesting that $G\alpha_{13}$ -Rho-ROCK signaling in T cells facilitates the expression of Tfh cell signature transcription factor and cell surface molecules. In addition to its role in Tfh cell differentiation, $G\alpha_{13}$ signaling was found to contribute to the “B cell help” function of Tfh cells, since $G\alpha_{13}$ -deficient Tfh cells were less efficient not only in forming conjugates with B cells but also in stimulating B cells to produce immunoglobulins.

Our findings raise the possibility that the blockade of Rho-ROCK pathway might be a promising approach to repress Tfh cell differentiation and subsequent GC reactions in antibody-mediated autoimmune diseases, such as SLE and pemphigus vulgaris, in humans. The GSEA of two separate human SLE patient datasets has demonstrated a positive correlation between active Rho gene signatures and DEGs in Tfh cells in comparison to non-Tfh cells. More thorough and comprehensive investigations are needed to determine whether such a relationship is based on cause and effect and whether other autoimmune datasets recapitulate the above correlations; our study presents preliminary grounds to believe that the selective manipulation of the RhoA pathway could salvage Tfh-mediated pathogenicity in humans. Several ROCK inhibitors have been approved for cerebral vasospasm and glaucoma (44, 45), and ROCK2-specific inhibitors are currently under phase II clinical trials for the treatment of multiple indications, including psoriasis, systemic sclerosis, graft-versus-host disease, idiopathic pulmonary fibrosis, and progressive supranuclear palsy (46, 47). It would be important to determine if pan-ROCK inhibitors or ROCK2-specific inhibitors can ameliorate pathogenic Tfh cell responses in humans and more rigorous investigations remain.

While the present study convincingly demonstrates a pivotal role of $G\alpha_{13}$ on Tfh cell responses in vivo, remaining questions include what kinds of external stimuli are involved and which of the receptor(s) coupling to $G\alpha_{13}$ have the important implications in early Tfh cell differentiation. Many have previously shed some light in an attempt to identify the membrane receptors that couple to $G\alpha_{13}$ in lymphocytes, including S1PR2, CXCR4, and LPARs, all of which are involved in cell chemotaxis and adhesion. Although CXCR5 is known to be primarily a $G\alpha_i$ -coupled receptor, there is evidence that it also capable of interacting with $G\alpha_{13}$ proteins upon CXCL13 binding (48), indicating that noncanonical signaling possibilities exist, as many of the GPCR signaling is mediated by more than one type of $G\alpha$ proteins (49). For example, integrins have been suggested to serve as noncanonical, $G\alpha_{13}$ -coupled receptors, and integrin activation can also lead to cross-talk with GPCRs via a

dynamic modulation of $G\alpha_{13}$ -RhoA activity (50). It is likely that all of the above-mentioned receptors and signals lead to the synergistic amplification of the $G\alpha_{13}$ -mediated signaling in early antigen exposure and Tfh cell development, rather than one dominant mechanism being at play.

In summary, our work provides a previously unappreciated insight into the mechanism by which $G\alpha$ proteins positively regulate Tfh cell differentiation and function, thereby fulfilling the need to understand the molecular requirements facilitating humoral responses in vivo. Targeting the $G\alpha_{13}$ -Rho-ROCK axis might be useful in developing pharmacologic interventions in vaccine development as well as antibody-mediated immune disorders.

Materials and Methods

Mice. C57BL/6, B6.SJL, and *Rag1*^{-/-} mice were purchased from Jackson Laboratory (Bar Harbor). *Cd4-cre* and OT-II Tg mice were kindly provided by Chen Dong, Tsinghua University. *Gna12*^{-/-} and *Gna13*^{fl/fl} mice were kindly provided by Melvin I. Simon, California Institute of Technology, Pasadena, CA, and Stefan Offermanns, Max Planck Institute, Bad Nauheim, Germany, respectively.

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Mice were crossed accordingly and either female or male mice of 6 to 12 wk, sex and age matched, were used for experiments, with *Gna13*^{fl/fl} littermate mice as WT controls. All mice were maintained in a specific, pathogen-free facility at the Seoul National University School of Pharmacy Animal Research Facility. For viral infection models, experiments were conducted at Biosafety level-2 research facility (SNUIBC-R190327-1–1 and SNU-190830-2-1). The rest is provided in *SI Appendix*.

Data Availability. RNA-seq were performed and analyzed in *SI Appendix* and *Dataset 1*.

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