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CD275-Independent IL-17–Producing T Follicular Helper–like Cells in Lymphopenic Autoimmune-Prone Mice

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Abstract

T cells undergo homeostatic expansion and acquire an activated phenotype in lymphopenic microenvironments. Restoration of normal lymphocyte numbers typically re-establishes normal homeostasis, and proinflammatory cytokine production returns to baseline. Mice deficient in guanine nucleotide exchange factor RasGRP1 exhibit dysregulated homeostatic expansion, which manifests as lymphoproliferative disease with autoantibody production. Our previous work revealed that autoreactive B cells lacking RasGRP1 break tolerance early during development, as well as during germinal center responses, suggesting that T cell–independent and T cell–dependent mechanisms are responsible. Examination of whether a particular T cell subset is involved in the breach of B cell tolerance revealed increased Th17 cells in *Rasgrp1*-deficient mice relative to control mice. *Rasgrp1*-deficient mice lacking IL-17R had fewer germinal centers, and germinal centers that formed contained fewer autoreactive B cells, suggesting that IL-17 signaling is required for a break in B cell tolerance in germinal centers. Interestingly, a fraction of Th17 cells from *Rasgrp1*-deficient mice were $CXCR5^{+}$ and upregulated levels of CD278 coordinate with their appearance in germinal centers, all attributes of T follicular helper cells (Tfh17). To determine whether CD278–CD275 interactions were required for the development of Tfh17 cells and for autoantibody, *Rasgrp1*-deficient mice were crossed with CD275-deficient mice. Surprisingly, mice deficient in RasGRP1 and CD275 formed Tfh17 cells and germinal centers and produced similar titers of autoantibodies as mice deficient in only RasGRP1. Therefore, these studies suggest that requirements for Tfh cell development change in lymphopenia-associated autoimmune settings.

As central regulators of immunity, Th cell subsets dictate immunity to foreign Ags and self-Ags. In autoimmunity, altered frequency and function of Th1, Th17, and T follicular helper (Tfh) subsets are linked to proinflammatory autoimmune conditions, such as multiple

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sclerosis (1), Crohn's disease (2), and systemic lupus erythematosus (SLE) (3). Targeted therapy disrupting T cell activation, costimulation and cytokines, or general T cell ablation generally ameliorates autoimmunity, thereby implicating particular Th subsets (4–10), although proof of their self-reactivity is often inferred rather than formally proven.

Tfh cells are defined by their location in germinal centers (GCs) resulting from upregulation of CXCR5 (11, 12), leading to migration toward ligand CXCL13 produced by follicular dendritic cells residing in B cell follicles (13, 14). In addition, Tfh cells exhibit increased levels of Bcl6 that are induced upon ICOS/CD278-ICOSL/CD275 interactions provided by both dendritic cells and B cells (15–17). CD275, CD278, and Bcl6 are each required for Tfh development, GCs, and Ab production (18, 19). Further, in autoimmune mice, such as the MRL-*lpr* strain, blockade or genetic deficiency in CD278 leads to amelioration of pathology, suggesting that this strategy may prevent development of autoreactive Tfh cells (20, 21).

In both humans and in mouse, deficiency in RasGRP1 predisposes to autoimmunity (22–25). For example, defective splicing of *Rasgrp1* transcripts and single nucleotide polymorphisms of *Rasgrp1* are associated with development of SLE and type I diabetes in humans, respectively (23, 26). *Rasgrp1*$^{-/-}$ mice develop a lymphoproliferative disorder that includes the development of spontaneous GCs and anti-nuclear Abs (ANAs) (27). Importantly, *Rasgrp1*-deficient nude mice do not exhibit splenomegaly and fail to produce ANAs, demonstrating that autoimmune features are T cell dependent (28). RasGRP1 is a guanine exchange factor (GEF) that is responsible, in part, for the activation of the Ras signal-transduction pathway downstream of AgRs in B and T cells (22, 29). In T cells, RasGRP1 is the predominant GEF mediating signals following AgR (TCR) engagement, and thymocytes deficient in *Rasgrp1* are less able to survive selection as the result of impaired TCR signaling (30). The resultant block in T cell development at the CD4^{+}CD8^{+} stage leads to T lymphopenia in the periphery (31, 32). *Rasgrp1*$^{-/-}$ CD4 cells that make it to the periphery are functionally impaired when strong signals are given via the TCR (33). Despite this functional impairment in vitro, *Rasgrp1*$^{-/-}$ CD4 T cells are activated in vivo as a consequence of homeostatic expansion (32). Paradoxically, the frequency and activity of regulatory T cells (Tregs) are reportedly increased in *Rasgrp1*$^{-/-}$ mice (34).

Ras pathway signaling operates upstream of ERK, and recent studies suggest that inhibiting ERK signaling skews Th cell differentiation toward the Th17 subset (35). Th17 cells are implicated in numerous autoimmune models as a pathogenic subset critical for disease. IL-17, the cytokine produced by Th17 cells, mediates class-switch recombination to all IgG subclasses, and Tfh-associated cytokine IL-21 can further enhance class-switch recombination to IgG1 and IgG2b (36). This contrasts with IFN-γ produced by Th1 cells that promotes IgG2a responses and suppresses IgG2b and IgG3 responses (37). Because *Rasgrp1*$^{-/-}$ mice show elevated serum Ab titers for all IgG subclasses, we speculated that Th17 cells have a role in regulating autoimmune responses.

Both the frequency and number of Th17 cells were increased in *Rasgrp1*$^{-/-}$ mice; further, these RORγt^{+} cells upregulated CXCR5 and CD278/ICOS and localized to GCs, consistent with a Tfh cell phenotype. To address whether Tfh17 cells in lymphopenic *Rasgrp1*-deficient mice were important for autoimmunity and, further, required CD278–CD275 interactions

similar to nonlymphopenic mice, we assessed Tfh17 cells in mice deficient in both RasGRP1 and IL-17RA, as well as in mice deficient in both RasGRP1 and CD275 (ICOSL). The frequencies of autoreactive B cells, spontaneous GCs, and autoreactive B cells within spontaneous GCs were significantly reduced in *Rasgrp1*-deficient mice also lacking IL-17RA compared with autoimmune-prone mice lacking only RasGRP1. However, although CD275–CD278 interactions are typically important for GCs and Tfh cells, *Rasgrp1*-deficient mice lacking CD275 developed frequent GCs, Tfh17 cells, and autoantibody. We conclude that lymphopenia-driven autoimmunity and the development of Tfh17 cells in *Rasgrp1*-deficient mice occur independently of CD275.

Materials and Methods

Mice

Mice were housed at the University of South Alabama in an AALAC International–accredited specific pathogen–free facility. *Rasgrp1*-deficient (*Rasgrp1*$^{-/-}$) (38) and littermate control *Rasgrp1*-sufficient heterozygous (*Rasgrp1*$^{+/-}$) mice were maintained on a C57BL/6 background (>10 generations backcrossed). Importantly, no phenotypic or functional differences were measured between mice with one or two functional *Rasgrp1* alleles. Mice lacking IL-17RA (C57BL/6 background) were crossed with *Rasgrp1* strains harboring the BCR knock-in transgene 564Igi (39). The 564Igi BCR recognizes multiple self-Ags (40, 41), and B cells expressing this transgene can be readily identified using anti-idiotypic Ab (39). CD275/B7-H2/ICOSL–deficient mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Maintenance of breeding colonies and all procedures involving mice were performed according to protocols approved by the University of South Alabama Institutional Animal Care and Use Committee.

Flow cytometric analysis and Abs

Single-cell suspensions of splenic mononuclear cells (MNCs) were isolated by density gradient centrifugation using Lympholyte M (Cedarlane Laboratories, Burlington, NC). For intracellular cytokine staining of T cells, total splenocytes were incubated with PMA and ionomycin for 2 h at 37°C with 5% CO_2, and GolgiStop and GolgiPlug (BD Biosciences, San Jose, CA) were added for an additional 3 h. Following staining with surface markers, splenocytes were permeabilized and fixed using the Foxp3 staining protocol (eBioscience, San Diego, CA). Intracellular staining for cytokines was subsequently performed. Abs used for the analysis of T cells included CD3ε (145-2C11), CD4 (GK1.5), CD8α (53-6.7), CD25 (PC61), CD44 (IM7), CD62L (MEL-14), CD69 (H1.2F3), CXCR5/CD185 (SPRCL5), CCR7/CD197 (4B12), ICOS/CD278 (7E.17G9), PD-1/CD279 (J43), Bcl6 (IG191E/A8, K112-91), IFN-γ (XMG1.2), IL-2 (JES6-5H4), IL-4 (11B11), IL-17A (eBio17B7), IL-21 (FFA21 or BL25168), and Foxp3 (FJK-16s). Combinations of these Abs conjugated to fluorophores FITC, PE, PE-Cy7, PE–Texas Red, PerCP-Cy5.5, allophycocyanin/eFluor 660, allophycocyanin-Cy7, and Pacific Blue/V450 were used (BD Biosciences, eBioscience, and BioLegend, San Diego, CA). Anti-idiotype Ab (B6.256) was used to identify 564Igi autoreactive B cells. Cells were analyzed by a FACSCanto II and sorted using a multilaser FACSAria II SORP housed in the University of South Alabama College of Medicine Flow Cytometry Laboratory. Data were analyzed with FlowJo software (TreeStar, Ashland, OR).

Immunofluorescent analysis of splenic sections

Five-micron cryosections of OCT-preserved (Tissue-Tek, Torrance, CA) spleens were prepared by placing trays onto a block of dry ice. Frozen tissues were stored at −80°C; 5-μm sections were placed onto Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA) using a Shandon FE/FSE Cryotome (Thermo Scientific, Waltham, MA). After rehydration with PBS, sections were incubated with anti-CD16/CD32 (2.4G2; Bio X Cell, West Lebanon, NH) before immunostaining to resolve T cells (using anti-CD4 Ab), B cells (anti-CD45R), and GCs (PNA-FITC) or to resolve Th17 cell (PE-conjugated anti-mouse IL-17A) localization counterstained with FITC-conjugated anti-mouse CD4, PE-Cy7–conjugated anti-mouse CD45R, and allophycocyanin-conjugated GL7. Images were acquired using a Nikon A1R confocal microscope (University of South Alabama Microscope Core Facility) and analyzed with Nikon Elements Software (Nikon Instruments, Melville, NY).

T cell isolation and stimulation assays

Splenic CD4 T cells were isolated using a MACS CD4 T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured using RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 IU penicillin, and 0.1 mg/ml streptomycin (Invitrogen, Grand Island, NY) and 2 mM 2-ME. Cells were activated using plate-bound anti-CD3 (2C11, 5 μg/ml) in the presence or absence of mitomycin C–treated T cell–depleted splenocytes. After 48–72 h of culture, cells and supernatants were collected and analyzed.

ELISAs

To measure cytokines, culture supernatants were analyzed for IFN-γ and IL-17 by sandwich ELISA using anti-cytokine Abs (R&D Systems, Minneapolis, MN). Biotinylated anti-cytokine Ab and streptavidin HRP were used for cytokine detection. HRP was visualized using 2, 2′-azino-bis-(3-benzthiazoline-6-sulfonic acid), and absorbance signals two times above background (C57BL/6 sera) were used as a threshold. Standard curves were generated using recombinant cytokines, and linear regression was applied to quantitate levels of IFN-γ and IL-17 produced by activated T cells.

For measurement of 564Ig-derived autoantibody titers, ELISA plates (Thermo Scientific) were coated with Abs to IgM or IgG2b/2c/3 (Southern Biotechnology Associates, Birmingham, AL). After blocking plates with 5% dry milk (Carnation) in PBS, serial dilutions of serum were incubated, and plates were washed in PBS containing 0.05% Tween 20. Sandwich was completed by addition of biotinylated anti-idiotypic Ab (clone B6-256), followed by streptavidin-HRP. Color detection was performed by addition of ABTS, and absorbance was measured. Absorbance signals two times above background (C57BL/6 sera) were used to determine 564Ig Ab titers.

ANA assays

ANA assays were performed, according to the manufacturer’s protocol, using mitotic HEp-2 cells stabilized on microscope slides (Antibodies, Davis, CA).

Statistical analysis

Statistical comparisons reported were generated using GraphPad Prism 6 software. One-way ANOVA was used to compare more than two means, with Tukey's multiple comparisons, as indicated in the figure legends. The unpaired Student *t* test was used to compare two means, and Welch's correction was applied when variances were unequal, as determined by the *F* test.

Results

IL-17–producing CD4$^+$ cells in Rasgrp1-deficient mice

Previous work demonstrated that *Rasgrp1*$^{-/-}$ mice produce increased autoantibodies of all IgG isotypes, as well as autoreactive B cells, within GCs (27). To examine T cell subsets that may contribute to autoantibody production and GCs, splenic CD4 T cells were isolated and stimulated in vitro with anti-CD3 alone and in the presence of APCs, and supernatants were analyzed for IFN-γ and IL-17A by ELISA (Fig. 1). Consistent with previous studies, CD4 T cells from *Rasgrp1*$^{-/-}$ mice and age-matched littermate control mice produced similar amounts of IFN-γ (Fig. 1A, 4.5 ± 2.2 ng/ml and 2.7 ± 1.0 ng/ml, respectively). In contrast to IFN-γ production, anti-CD3–stimulated CD4 T cells from *Rasgrp1*$^{-/-}$ mice produced >10-fold more IL-17A than did *Rasgrp1*-sufficient CD4 T cells (Fig. 1B, 8.0 ± 3.1 versus 0.5 ± 0.6 in the presence of APCs, $p \leq 0.02$). These data suggest that Th17 cells are present in *Rasgrp1*$^{-/-}$ mice and that they can be activated via TCR engagement.

To determine the relative frequency and cell numbers of Th cell subsets, splenocytes from *Rasgrp1*$^{-/-}$ and littermate control mice were stained for intracellular cytokines and analyzed by flow cytometry (Fig. 2). Consistent with previous work, the frequency and number of Th1 cells (CD4$^+$ IFNγ^+ IL-17A$^-$) was increased in *Rasgrp1*$^{-/-}$ mice relative to age-matched littermate control mice. The frequency of Th1 cells increased with age in spleens of *Rasgrp1*$^{-/-}$ and control mice, although the frequency and the number of Th1 cells remained significantly elevated in *Rasgrp1*$^{-/-}$ mice. Similarly, the frequency and number of Th17 cells (CD4$^+$ IFNγ^- IL-17A$^+$) were elevated in *Rasgrp1*$^{-/-}$ mice compared with age-matched littermate control mice. Importantly, although the frequency of Th17 cells in *Rasgrp1*-deficient mice varied with age, the number of Th17 cells increased with age (Fig. 2). Interestingly, a minor population of T cells producing both IFN-γ and IL-17A was also elevated in *Rasgrp1*$^{-/-}$ mice relative to littermate controls. No IL-4$^+$ staining was observed within splenic CD4$^+$ T cells from *Rasgrp1*$^{-/-}$ or control mice. In sum, these analyses reveal that *Rasgrp1*$^{-/-}$ mice develop Th17 cells, and this subset exhibits marked increases in the frequency and number compared with nonautoimmune littermate control mice.

Breach of GC B cell tolerance is IL-17R dependent

Autoreactive B cells break tolerance at multiple checkpoints in the absence of RasGRP1 (27). To measure the contribution of IL-17A production to the break in B cell tolerance within GCs, *Rasgrp1*-deficient mice containing the knock-in BCR transgene 564Igi were crossed with mice lacking IL-17RA. As previously reported (27), the frequency and number of autoreactive B cells were increased *Rasgrp1*-deficient mice compared with *Rasgrp1*-sufficient mice (Fig. 3A–C). Although the frequency and number of 564Igi B cells did not

differ in spleens of *Rasgrp1*-sufficient mice with and without IL-17RA, the frequency and number of 564Igi B cells in autoimmune-prone *Rasgrp1*-deficient mice was significantly increased compared with autoimmune-prone mice lacking IL-17RA. Thus, for example, although *Rasgrp1*-deficient mice had a mean frequency of 38.0% and 4.7×10^7 564Igi splenic B cells, mice lacking RasGRP1 and IL-17RA had means of 22.8% and 1.1×10^6 564Igi B cells. Therefore, the increase in 564Igi autoreactive B cells in *Rasgrp1*-deficient mice is partially dependent on IL-17RA.

IL-17RA signaling can promote B cell retention in GCs (42, 43). That Th17 cells are increased in *Rasgrp1*-deficient mice suggested that they may provide IL-17A to autoreactive B cells within GCs and, thereby, potentiate autoantibody production. Therefore, the frequency and number of 564Igi B cells within GCs was determined using flow cytometry (Fig. 3D, 3E). As previously reported (27), the frequency and number of autoreactive GC B cells were increased in *Rasgrp1*-deficient mice ($5.0 \pm 0.5\%$, $2.1 \pm 0.5 \times 10^6$, mean ± SEM) compared with *Rasgrp1*-sufficient mice ($3.5 \pm 0.3\%$, $3.6 \pm 0.7 \times 10^5$, Fig. 3D, 3E). Although the absence of IL-17RA did not significantly alter the frequency or number of auto-reactive GC B cells in the presence of RasGRP1, the frequency and number of 564Igi GC B cells were reduced in mice lacking RasGRP1 and IL-17RA ($2.8 \pm 0.3\%$, $4.4 \pm 1.7 \times 10^5$) compared with *Rasgrp1*-deficient mice. These data are consistent with a model whereby IL-17–producing CD4 T cells in GCs (i.e., Tfh17) provide IL-17 to autoreactive B cells within GCs, thereby facilitating their survival, selection, and differentiation.

Th17 cells localize to GCs in Rasgrp1$^{-/-}$ mice

Tfh17 cells characteristically display a CXCR5$^+$ PD1$^+$ ICOS/CD278$^+$ phenotype. To determine whether some Th17 cells observed in *Rasgrp1*$^{-/-}$ mice fulfill Tfh17 designation and localize to GCs, T cells were analyzed for Tfh markers and for their lo-calization in spleen. Compared with spleens from littermate control mice, 10-wk-old *Rasgrp1*$^{-/-}$ mice exhibited increases in the frequency and number of CXCR5$^+$ CD278$^+$ CD4 T cells (Fig. 4A–C). Notably, PD1 levels were increased on T cells from spleens of young *Rasgrp1*$^{-/-}$ mice that lacked GCs (presumably due to lymphopenia-associated activation; data not shown); therefore, PD1 was not used to distinguish Tfh cells. In contrast, CD278 levels on T cells from control and young *Rasgrp1*$^{-/-}$ mice were low. Although CD278 levels remained low on T cells from littermate control mice, CD278 levels increased markedly with age in *Rasgrp1*$^{-/-}$ mice (Fig. 4C, 4D). The increased levels of T cell CD278 correlated directly with the appearance of GCs in *Rasgrp1*$^{-/-}$ mice (27).

To directly examine localization of IL-17A$^+$ CD4$^+$ T cells, splenic sections were examined by immunofluorescent staining and confocal microscopy. GC staining in littermate control mice was infrequent, as was colocalized anti–IL-17A and anti-CD4 staining (Fig. 4E, *left panels*). In agreement with our earlier results, GCs were readily apparent in *Rasgrp1*$^{-/-}$ mice older than 2 mo of age. Further, IL-17A$^+$ CD4 cells frequently localized within regions staining with PNA (Fig. 4E, *right panels*), indicating that at least some Th17 cells reside within GCs of *Rasgrp1*$^{-/-}$ mice. Based on these analyses, we conclude that *Rasgrp1*-deficient have increased Tfh17 cells.

Bcl6 is essential for Tfh development, whereas RORγt is the canonical transcription factor for Th17 cells. To further explore the phenotype of Tfh17 cells that apparently develop spontane ously in *Rasgrp1*$^{-/-}$ mice, Bcl6 and RORγt levels were examined in IL17A^{+} and CXCR5^{+} IL17A^{+} CD4 T cells (Fig. 5). Consistent with the canonical RORγt defining Th17 cells, Th17 cells from *Rasgrp1*-sufficient and -deficient mice stained positive for RORγt, whereas a smaller fraction was positive for RORγt and CXCR5 (Fig. 5A). Compared with *Rasgrp1*-sufficient mice that generated a low frequency (0.7 ± 0.4 [mean ± SE]) and number (3,913 ± 3,017) of RORγt^{+} CXCR5^{+} Th17 cells, spleens from *Rasgrp1*-deficient mice had substantial increases (5.1 ± 0.9 and 55,648 ± 23,476, respectively, Fig. 5A–C). In general, although Th17 cells from all mice stained positive for Bcl6 relative to the isotype control (data not shown), the levels of Bcl6 were elevated in Th17 cells from mice lacking RasGRP1 (Fig. 5D–F). In addition, a small fraction of Th17 cells from autoimmune-prone *Rasgrp1*-deficient mice was positive for Bcl6 and CXCR5, although levels of Bcl6 in CXCR5^{+} Th17 cells were lower compared with the CXCR5^{neg} Th17 subset. Notably, few CXCR5^{+} Bcl6^{+} Th17 cells were observed in *Rasgrp1*-sufficient control mice. Taken together, these analyses suggest that CXCR5^{+} Th17 (i.e., Tfh17) cells are positive for canonical RORγt and Bcl6, although levels of Bcl6 were relatively low.

Role of CD278 in Tfh17 cells

CD278-CD275/B7-H2/ICOSL interactions are required for the development of Tfh cells, GCs, and Ab production in wild-type mice (17, 44, 45). Increased CD278 levels on CD4 T cells from *Rasgrp1*$^{-/-}$ mice, concomitant with the appearance of GCs previously shown to harbor autoreactive B cells, suggested that CD278-CD275 may also be important for regulating Tfh cell development in this lymphoproliferative mouse strain. To test this hypothesis, 12- to 16-wk-old mice deficient in RasGRP1 and CD275 (double knockout [DKO]) were compared with single *Rasgrp1*- and CD275-deficient and *Rasgrp1*-heterozygous litter-mate control mice. The frequency and number of Th1, Th17, and CD4 T cells producing IFN-γ and IL-17 were minimal in litter-mate control and CD275-deficient mice (Fig. 2B, 2C). In comparison, the frequency and number of Th17-expressing, as well as IL-17 and IFN-γ–coexpressing, CD4 populations were elevated in *Rasgrp1*$^{-/-}$ and DKO mice. Therefore, disrupting CD278–CD275 interactions had no effect on the development of T effector subset populations in the absence of RasGRP1.

ICOS/CD278 is induced upon stimulation of CD4 T cells, and further CD275–CD278 interactions mediate upregulation of CXCR5 and Bcl6. As expected, CD4 T cells from naive control mice and control mice deficient in CD275 had minimal levels of CXCR5 and CD278 (Fig. 4A, 4B). Surprisingly, CD4 T cells from *Rasgrp1*-deficient and DKO mice exhibited an increased frequency of CXCR5^{+} CD278^{+} cells. To further address whether Th17 cells from DKO mice were Tfh like, Bcl6 and RORγt levels were measured (Fig. 5). Although Th17 cells from all groups of mice were largely RORγt^{+}, few Th17 cells from control animals with and without CD275 stained positive for CXCR5. In addition, very few Th17 cells from control mice were positive for Bcl6. In contrast, a subpopulation of Th17 cells from *Rasgrp1*-deficient and DKO mice was double positive for Bcl6 and CXCR5, although, as noted previously, the levels of Bcl6 were lower in the CXCR5^{+} subset. Collectively, these

data suggest that Tfh17-like cells in *Rasgrp1*-deficient mice express RORγt and low levels of Bcl6 in a CD275-independent manner.

To determine whether the absence of CD275 affected the development of splenomegaly, a feature linked to autoimmunity in *Rasgrp1*-deficient mice, splenic mass was measured in 10- to 12-wk-old mice. Consistent with earlier work, mice lacking RasGRP1 developed marked splenomegaly compared with control mice (Fig. 6A). Interestingly, splenic mass was comparable between *Rasgrp1*-deficient mice in the presence and absence of CD275, indicating that splenomegaly was CD275 independent.

To understand whether the absence of CD275 affected the development of GCs, spleens of mice were examined using flow cytometry and immunohistology. As expected, the frequency of GC B cells, identified as PNA^{hi} $CD95^{+}$ B cells using flow cytometry, was low in *Rasgrp1* heterozygous and CD275-deficient mice (Fig. 6B). In contrast, the frequency of GC B cells was signifi-cantly elevated in $Rasgrp1^{-/-}$ and DKO mice. These results were consistent with immunohistological analysis of splenic sections showing an increased frequency of GCs in $Rasgrp1^{-/-}$ mice with and without CD275 (data not shown). In addition, $CD4^{+}$ $IL\text{-}17^{+}$ T cells were detectable in splenic GCs from $Rasgrp1^{-/-}$ and DKO mice (Fig. 4E), suggesting that GC and Tfh cell development are not impaired in the absence of CD275.

T cell–dependent Ab responses in wild-type mice require CD278–CD275 interactions (44). In addition, disrupting these interactions through administration of blocking Ab regulates autoantibody levels in certain autoimmune murine models (4). Therefore, autoantibody production was also measured in $Rasgrp1^{-/-}$ mice with and without CD275. As expected, littermate control and CD275-deficient mice produced minimal ANAs (Fig. 6C). In contrast, ANAs were readily detectable in $Rasgrp1^{-/-}$ mice and could be detected at high dilutions of serum, indicating that autoantibody production was elevated. DKO mice also produced ANA titers comparable with those from $Rasgrp1^{-/-}$ mice. To understand whether autoantibody production from a known population of autoreactive B cells participating in GC responses of $Rasgrp1^{-/-}$ mice was regulated by CD275, CD275-deficient mice were crossed with 564Igi-transgenic mice, and serum 564Ig autoantibody levels were measured. Similar to the ANA results, the level of 564Ig-derived IgG2b subclass Ab was elevated in serum from $Rasgrp1^{-/-}$ and DKO mice compared with controls (Fig. 6D). Similarly, total serum levels of several classes of Ab were elevated comparably in *Rasgrp1*-deficient mice with and without CD275 (Fig. 6E). These data suggest that autoantibody production in $Rasgrp1^{-/-}$ mice occurs independently of CD275.

In addition to IL-17A, production of IL-21 is implicated in Tfh cell–mediated autoimmunity (46, 47). To determine whether IL-21 production by Tfh17 cells may contribute to the autoimmune phenotypes exhibited in *Rasgrp1*-deficient and *Rasgrp1*- CD275–DKO animals, the frequency and number of IL-21–producing Tfh17 cells were measured by flow cytometry (Fig. 7). Consistent with a potential role in mediating autoimmunity, the frequency and number of $CD4^{+}$ $IL17A^{+}$ $CXCR5^{+}$ cells staining positive for IL-21 were increased in *Rasgrp1*-deficient mice relative to *Rasgrp1*-sufficient control mice (frequency, 0.8 ± 0.4 versus 4.1 ± 0.8; number, 427 ± 235 versus 21,987 ± 5,269 [mean ± SEM],

Rasgrp1-sufficient versus *Rasgrp1*-deficient). Interestingly, the frequency and number of Tfh17 cells making IL-21 in mice lacking RasGRP1 and CD275 did not differ statistically from those of control mice (0.8 ± 0.3 and 2404 ± 805). Therefore, although production of IL-21 by Tfh17 cells may contribute to increased autoantibody titers in *Rasgrp1*-deficient mice, it does not seem to explain the increased autoantibody titers in mice lacking RasGRP1 and CD275.

CD278-CD275 is reportedly important for Treg survival and expansion (48). To understand whether the absence of CD275 may indirectly affect Th17 frequency and number by altering the development of Tregs, Treg frequency and number were quantified by measuring $CD4^+$ $CD25^+$ $Foxp3^+$ cells (Fig. 8). Consistent with previous studies reporting that CD278-CD275 is required for Treg development and function, the frequency of Tregs was slightly reduced in CD275-deficient mice compared with CD275-sufficient mice. In contrast, the frequency and number of Tregs were similar between *Rasgrp1*$^{-/-}$ and DKO mice, suggesting that the absence of CD275 does not affect the development of Tregs in *Rasgrp1*$^{-/-}$ mice. The frequency and number of $Foxp3^+$ $CD25^+$ regulatory cells were also similar within gated Th1, Th17, and Tfh cell populations among all strains (data not shown), suggesting that differences in Tregs within GCs do not explain autoimmunity in *Rasgrp1*$^{-/-}$ and *Rasgrp1*$^{-/-}$ $CD275^{-/-}$ mice.

Discussion

Early evidence from *Rasgrp1*-deficient mice indicated impaired T cell development, with subsequent T cell lymphopenia in the periphery (38). This leads to homeostatic expansion that was found to be defective in the absence of RasGRP1, causing clonal exhaustion of primarily Th1 cells (32). This observation, in combination with the finding that the frequency and activity of Tregs are also increased, led to the suggestion that autoimmunity was self-limiting in *Rasgrp1*-deficient mice (34). In contrast, we found that autoantibody levels of all IgG subclasses were elevated in *Rasgrp1*$^{-/-}$ mice, concomitant with the appearance of GCs containing autoreactive B cells (26). This autoimmune phenotype appears to be substantiated in other *Rasgrp1* mutant strains of mice (33, 49). In this article, we demonstrate that the frequency and number of IL-17–producing CD4 T cells are increased in *Rasgrp1*$^{-/-}$ mice. These Th17 cells localize to GCs and resemble Tfh cells immunophenotypically. Importantly, autoreactive GC B cells are significantly reduced in mice lacking RasGRP1 and IL-17R, suggesting that Tfh17-like cells mediate a break in B cell tolerance within GCs. Surprisingly, the development and apparent function of Tfh17 cells, as well as the development of GCs and the production of autoantibody, occur independently of CD275 in *Rasgrp1*-deficient mice. We propose that this newly described Tfh17 cell population is uniquely regulated and mediates autoimmunity in lymphopenia-driven settings.

The frequency and number of Th17 cells were increased in *Rasgrp1*-deficient mice relative to *Rasgrp1*-sufficient littermate control animals, thereby accounting for increased IL-17A production by in vitro–stimulated splenic CD4 T cells from *Rasgrp1*-deficient mice (Fig. 1). In contrast, although the data for Th1 cells trend toward similar increases between *Rasgrp1*-deficient and *Rasgrp1*-sufficient animals, the differences were not statistically significant.

Consistent with the Th1 cell–frequency data, IFN-γ production by in vitro–stimulated CD4 T cells also did not differ statistically. Interestingly, CD4 T cells in *Rasgrp1*-deficient mice exhibit an impaired IFN-γ response to challenge with *Listeria monocytogenes*, likely due to their propensity for clonal exhaustion (32). We did not test whether exhaustion of stimulated *Rasgrp1*-deficient Th1 cells (34) or impaired activation in the absence of the predominant GEF necessary for Ras signaling (33, 38, 49) contributes to IFN-γ responses of anti-CD3–stimulated *Rasgrp1*-deficient Th1 cells. However, phenotypic differences, such as CD25 levels (50), between Th1 and Th17 cells could potentially mediate differential sensitivity of Th subsets to clonal exhaustion. Indeed, we confirm that CD25 levels are significantly reduced on Th17 cells compared with Th1 cells in *Rasgrp1*$^{-/-}$ mice (Fig. 8B, 8C). Because IL-2 is critical for T cell expansion (17, 50–52), it follows that reduced CD25 levels presumably would reduce the ability of GC T cells to proliferate. Signaling via CD25 inhibits Th17 differentiation by a mechanism involving modulation of Stat3 and Stat5 binding to regulatory regions of the IL-17 promoter (50, 53, 54). Importantly, in contrast to IL-17, *Rasgrp1*-deficient CD4 T cells produce little IL-2 after stimulation with anti-CD3 in vitro (33). Therefore, reduced IL-2 may influence Th17 cell and Tfh17-like cell development in *Rasgrp1*-deficient mice. With regard to increased production of IL-17 by CD4 T cells from *Rasgrp1*$^{-/-}$ mice in vitro, Tfh cells are potent producers of cytokines compared with conventional effector T cells; therefore, the increase in IL-17 production in *Rasgrp1*$^{-/-}$ CD4 T cells can be most easily explained by the increased frequency of Tfh17 cells in deficient mice.

Th17 and Tfh17 cells can produce IL-17A, as well as IL-21 (55), and both of these cytokines can influence GC B cell responses (56, 57). The importance of IL-17A in mediating autoimmunity in *Rasgrp1*-deficient mice was tested in mice lacking IL-17RA; a specific population of autoreactive B cells (564Igi transgenic) was reduced in GCs (Fig. 3). Notably, in nonautoimmune settings, IL-21 and CD275 are both required for the generation of Tfh cells (55). Tfh17 cells from *Rasgrp1*-deficient mice also produced IL-21, raising the possibility that IL-21 may contribute to anti-nuclear and 564Igi-derived autoantibody production. However, mice lacking RasGRP1 and CD275 generated similar titers of these autoantibodies, yet lacked the IL-21–producing Tfh17 population (Fig. 7). Although a more direct test of the importance of IL-21 is needed, we infer from our data that IL-21 production by Tfh17-like cells is not required for at least some autoantibodies produced in *Rasgrp1*-deficient mice. These results from *Rasgrp1*-deficient mice seemingly contrast with other autoimmune disease models, including MRL/lpr (20), sanroque (58, 59), and BXD2 (46) mice strains, in which CD275 and/or IL-21 potentiate Tfh cell development, GCs, and autoantibody production. Thus, Tfh17-like cell development in *Rasgrp1*-deficient mice may well involve a novel pathway.

Tfh17 cells are linked to several autoimmune disorders in mice and in humans. For example, an increase in Th17 cells was noted in peripheral blood from multiple cohorts of patients with SLE compared with healthy control samples (60–62). Th17 cells support class-switch recombination to multiple IgG subclasses in B cells (36); therefore, our observation that the frequency and number Tfh17 cells are increased in *Rasgrp1*$^{-/-}$ mice provides an explanation for increased autoantibodies of multiple IgG subclasses in deficient mice (Fig. 6) (27). Notably, Tfh17 cells in *Rasgrp1*$^{-/-}$ mice possess the conventional CD4^{+} CXCR5^{+} PD-1^{+}

CD278$^+$ immunophenotype, as well as express RORγt, although with lower than reported levels of Bcl6 (Figs. 4, 5). Previous work established that Bcl6 is necessary and sufficient for the development of Tfh cells (11, 18, 19). Bcl6 upregulation in T cells is potentiated by CD278–CD275 interactions, leading to upregulation of CXCR5 to potentiate migration of activated T cells into the B cell follicle. Therefore, it is surprising that the frequency and number of Tfh17 cells are comparable in *Rasgrp1*-deficient mice with and without CD275 (Figs. 4, 5). Under healthy physiologic settings, Tfh cell development is also dependent on Stat3 (63). Interestingly, Stat3 can be activated through IL-6 (64), and we observed elevated serum levels of IL-6 in *Rasgrp1*-deficient mice (~5 pg/ml and undetectable levels in *Rasgrp1*$^{-/-}$ and *Rasgrp1*$^{+/-}$ mice, respectively; data not shown). In addition, IL-6 and IL-21 were shown to be required for optimal Tfh development in lymphocytic choriomeningitis virus–infected mice (65). We propose that the proinflammatory state of lymphopenic *Rasgrp1*-deficient mice can compensate, in part, for CD278–CD275 interactions. If true, this could hold important implications for understanding and treating lymphopenia-coupled autoimmune diseases.

In addition to the increased frequency and number of Th17 cells, Th cells producing IFN-γ and IL-17 are increased in *Rasgrp1*$^{-/-}$ mice (Fig. 2). Similar double-positive subsets were observed in other models of autoimmunity (66), in which it was suggested that this population represented the most pathogenic of autoreactive Th subsets as a result of their ability to produce multiple proinflammatory cytokines. Another possibility is that this population represents an intermediate between Th1 and Th17 cells (67, 68). Regardless of their origin, the increase in *Rasgrp1*-deficient mice is at least consistent with a potential role in autoimmunity. However, CD4 T cells producing IFN-γ and IL-17 did not meet the immunophenotypic definition of Tfh cells (data not shown). The increased numbers of Tfh17 and IFNγ^+ IL-17$^+$ Th cells are likely caused by defective homeostatic expansion in *Rasgrp1*$^{-/-}$ mice and are grossly reflected by the development of splenomegaly. In nonautoimmune-prone mice, homeostatic expansion is regulated by IL-7 (69–71). In mice developing lymphoproliferative syndrome, additional mechanisms override IL-7 regulation, leading to accumulation of CD4 T cells (72–75), as well as other lymphocytes. The root cause of defective homeostatic expansion in *Rasgrp1*-deficient mice is unknown.

CD4 T cells from *Rasgrp1*-deficient mice upregulate several markers associated with homeostatic activation and expansion of CD4 T cells similar to what is reported for wild-type T cells placed in lymphopenic environments (OX-40, PD-1, PD-L1, CD69, and CD80; data not shown). Unlike CD4 T cells from wild-type mice (76), restitution of normal CD4 T cell numbers in *Rasgrp1*$^{-/-}$ mice does not result in reduction of the above markers to levels found in quiescent cells. Interestingly, CD4 T cells from *Rasgrp1*$^{-/-}$ mice do not upregulate CD278 during lymphopenia. Instead, CD278 levels only increase concomitantly with the appearance of spontaneous GCs (Fig. 4). This led us to test whether CD278–CD275 interactions are required for the development of Tfh cells and GCs. Notably, we observed that the development of Tfh17 cells and GCs is CD275 independent (Figs. 4, 5). We speculate that defective homeostatic expansion in mice lacking RasGRP1 contributes to a novel Tfh17 population that can arise independently of CD278–CD275 interactions.

Whether these novel Tfh17 cells in *Rasgrp1*$^{-/-}$ mice are auto-reactive is unknown. We showed previously that GCs developing spontaneously (i.e., without manipulation) in *Rasgrp1*-deficient mice contain autoreactive B cells (27); therefore, the presence of Tfh17 cells may directly affect autoantibody production. In this study, we observed that autoantibody production is elevated in *Rasgrp1*$^{-/-}$ mice independently of CD275. IL-17 potentiates B cell survival and promotes class switch to multiple IgG subclasses (36); therefore, it is noteworthy that 564Ig-derived autoantibody of multiple IgG subclasses (IgG1 could not be tested as a result of the anti-idiotypic detection Ab also being IgG1) was increased in *Rasgrp1*-deficient mice independently of CD275 (Fig. 6). Increased autoantibody of multiple IgG subclasses is consistent with at least some autoantibody being T cell dependent. CD278-CD275 is thought to enhance synapses between Ag-specific GC B cells and Tfh cells, leading to increased survival, as well as differentiation of GC B cells into memory and plasma cells. Surprisingly, our observations using DKO mice indicate that CD278–CD275 interactions are not required for autoantibody production. These results contrast with those derived from other autoimmune murine models (20, 58).

Tregs are key components for peripheral tolerance and are dependent on CD278/CD275 for their function. The frequency and function of Tregs are reportedly increased in *Rasgrp1*-deficient mice (34). Our own studies corroborate the increased frequency of Tregs, although we find this difference only within the first several weeks of life. Further, our studies indicate that Tregs lacking RasGRP1 are comparable functionally to Tregs from control mice (data not shown). In the current study, although the lack of CD275 appeared to affect the frequency of Tregs in *Rasgrp1*-sufficient mice, there was no statistical change in the frequency of Tregs in *Rasgrp1*-deficient mice (Fig. 8). Although we cannot rule out functional differences, it appears that CD275–CD278 interactions may not be required for Treg development in lymphopenia-associated autoimmune-prone *Rasgrp1*-deficient mice. A separate Treg subset, called T follicular regulatory (Tfr) cells, locate to GCs and share many markers of Tfh cells, including CXCR5, PD-1, and CD278, although they can be distinguished from Tfh cells based on Foxp3, CTLA-4, and higher levels of CD25 (77). Tfr cells inhibit GC responses by limiting numbers of Tfh and GC B cells, and altered Tfh/Tfr balance was recently implicated in autoimmunity in BXD2 mice (46). Interestingly, the imbalance in autoimmune BXD2 mice was potentiated by IL-21 production. Although we observed an increased frequency of IL-21^{+} Tfh17 cells in *Rasgrp1*-deficient mice relative to littermate control mice (Fig. 7), no differences in the frequency of Foxp3^{+} CXCR5^{+} CD4 T cells were observed (data not shown). Moreover, *Rasgrp1*-deficient mice remained autoimmune prone in the absence of CD275, despite the reduced frequency of IL-21–producing Tfh17 cells. Collectively, these data appear inconsistent with Tfr cells having a profound role in regulating autoimmunity in the absence of RasGRP1.

Blocking CD278–CD275 interactions shows efficacy in several autoimmune settings (78–80). Given that autoantibody production in *Rasgrp1*-deficient mice occurs independently of CD275, it is tempting to speculate that there may be subsets of patients with autoimmune disease who are unresponsive to such therapy. This may apply more specifically to autoimmunity developing in lymphopenic individuals, as appears in the *Rasgrp1*-deficient mouse model. More work is needed to further define the requirements and functions of

Tfh17 cells in *Rasgrp1*-deficient and other lymphopenia-associated autoimmune mouse models.

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Abbreviations used

ANA	anti-nuclear Ab
DKO	double knockout
GC	germinal center
GEF	guanine exchange factor
MNC	mononuclear cell
SLE	systemic lupus erythematosus
Tfh	T follicular helper
Tfr	T follicular regulatory
Treg	regulatory T cell

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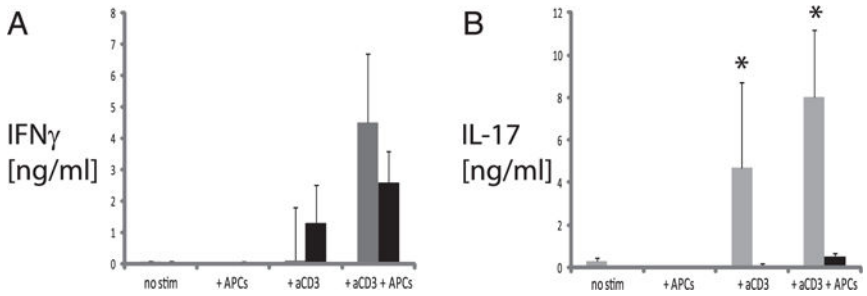

FIGURE 1.

IL-17 production from stimulated CD4$^+$ T cells from *Rasgrp1*-deficient mice. Isolated CD4$^+$ T cells were stimulated for 48–72 h with anti-CD3 in the presence or absence of autologous mitomycin C–treated APCs. Culture supernatants containing control (black bars) and *Rasgrp1*$^{-/-}$ (gray bars) CD4 T cells from 10-wk-old mice were collected and analyzed by ELISA for IFN-γ (**A**) and IL-17 (**B**). Cultures containing unstimulated CD4 T cells and mitomycin C–treated APCs alone were used as negative controls. Data are mean ± SE and summarize the results from four independent experiments. *$p \leq 0.02$, *t* test.

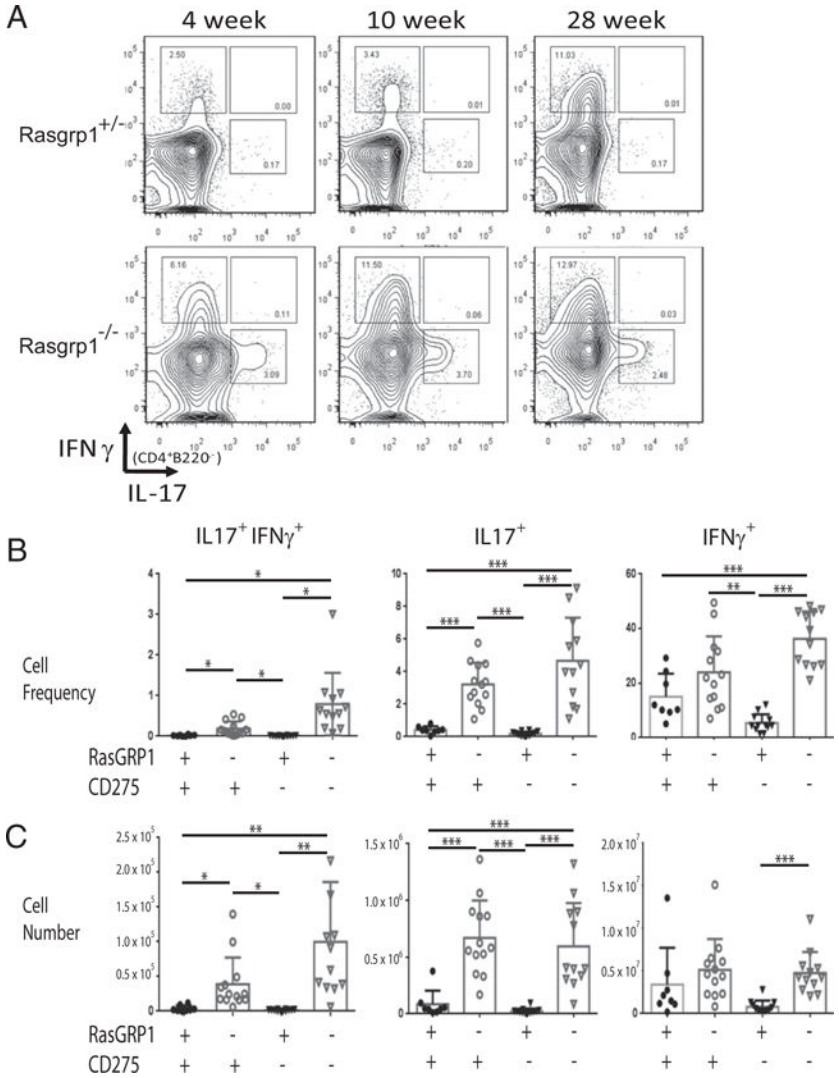

FIGURE 2.

Frequency and number of IFN-γ– and IL-17–producing CD4 T cells. (**A**) Splenic MNCs were gated on CD4 T cells, and the frequency of IFN-γ and IL-17 production was determined by intracellular staining. Shown are FACS plots for 4-week-old (*left panels*), 10-week-old (*middle panels*), and 28-wk-old (*right panels*) *Rasgrp1*$^{+/-}$ littermate control (*upper panels*) and *Rasgrp1*$^{-/-}$ (*lower panels*) mice. Data are a representative from one of five independent experiments. Summary of the frequencies (**B**) and numbers (**C**) of IFN-γ^{+} IL-17^{+} (*left panels*), Th17 (*middle panels*), and Th1 (*right panels*) CD4 T cell subsets from individual 10- to 12-wk-old *Rasgpr1*$^{+/-}$ (●), *Rasgrp1*$^{-/-}$ (○), *ICOSL/CD275*$^{-/-}$ (▼), and DKO (∇) mice. Each symbol represents an individual mouse (one-way ANOVA with Tukey's multiple-comparison test, *$p \leq 0.01$, **$p \leq 0.001$, ***$p \leq 0.0001$).

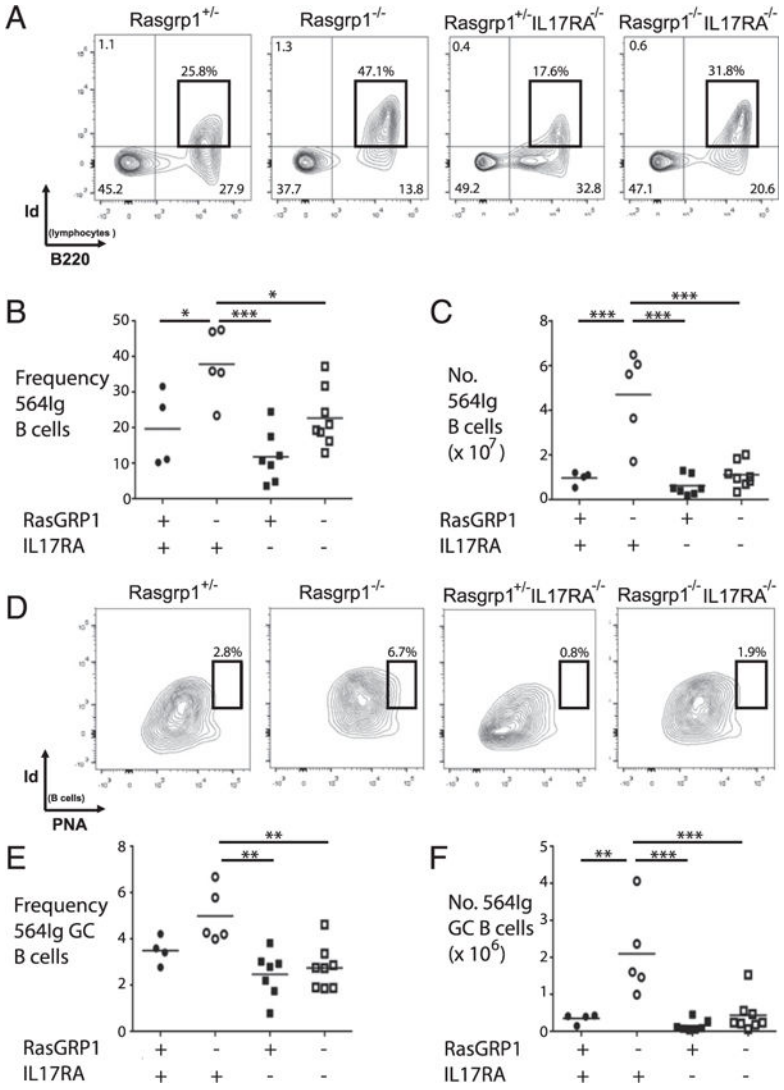

FIGURE 3.

Frequency and number of self-reactive 564Igi B cells in GCs of *Rasgrp1*-deficient mice are IL-17R dependent. (**A**) Representative FACS plots from spleens of 12- to 16-wk-old sufficient (*far left panel*) and *Rasgrp1*-deficient (*near left panel*) mice with and without IL-17R (*near right* and *far right panels*, respectively). Self-reactive 564Igi B cells were identified with an anti-idiotypic Ab, and gates were set by comparing to minus one controls (i.e., no anti-idiotypic Ab added, data not shown). Summary of the frequencies (**B**) and numbers (**C**) of 564Igi B cells from spleens of individual $Rasgpr1^{+/-}$ (●), $Rasgrp1^{-/-}$ (○), $IL17R^{-/-}$ (■), and $Rasgrp1^{-/-}$ $IL17R^{-/-}$ (□) mice. (**D**) Representative FACS plots to identify 564Igi-derived germinal center B cells in the indicated strains of mice. Frequency (**E**) and number (**F**) of 564Igi B cells localizing to GCs (one-way ANOVA with Tukey's multiple comparison test, $*p \leq 0.03$, $**p \leq 0.003$, $***p \leq 0.0003$).

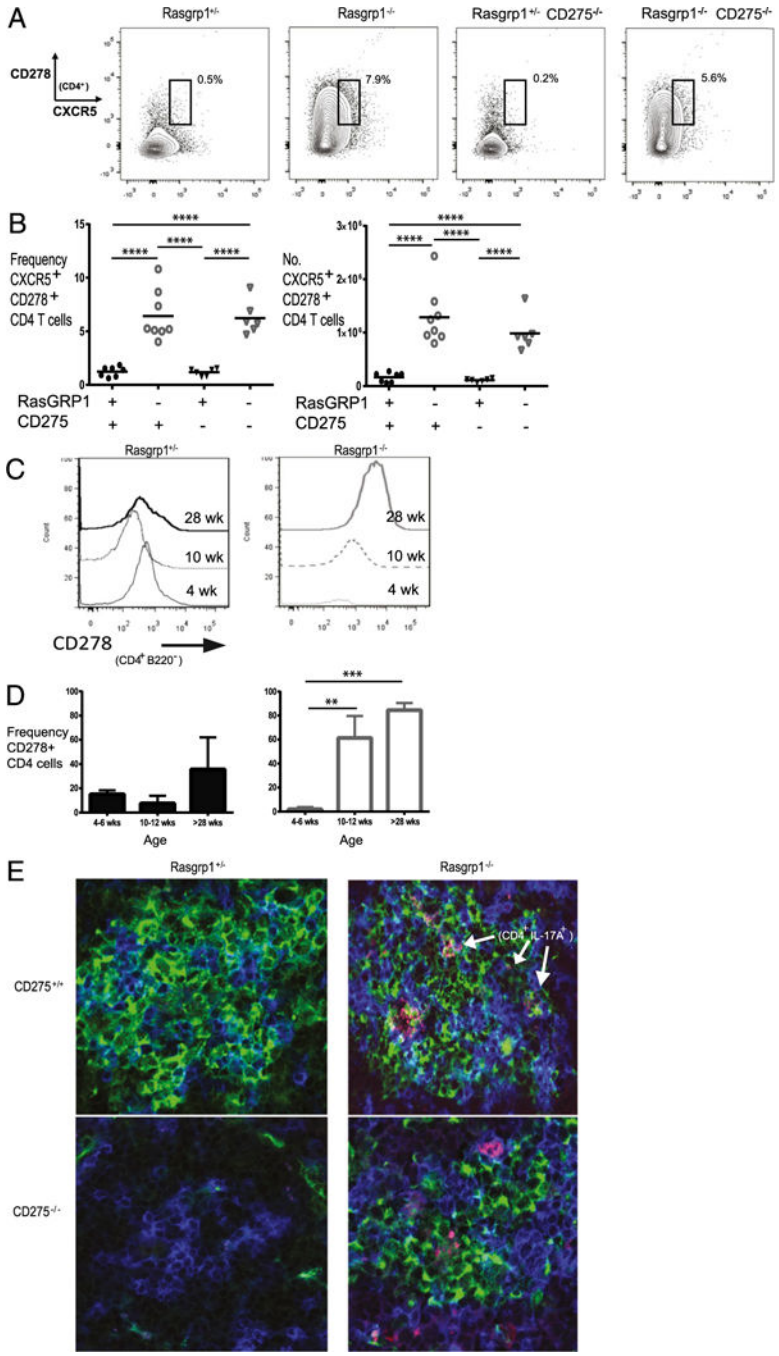

FIGURE 4.

CD278 levels and localization of IL-17–producing CD4 T cells in spleens of *Rasgrp1*$^{-/-}$ mice. (**A**) Frequency of CXCR5^{+} CD278^{+} CD4 T cells in representative *Rasgrp1*$^{+/-}$ littermate control and *Rasgrp1*$^{-/-}$ mice, as well as in both sets of mice lacking CD275. (**B**) Summary of the frequency (*left panel*) and number (*right panel*) of CXCR5^{+} CD278^{+} CD4 T cells for all groups of mice; each individual symbol represents the value for a single mouse (one-way ANOVA with Tukey's multiple comparison test, $p < 0.0001$). (**C**) Representative graphs of CD278 levels on CD4 T cells from spleens of 4-, 10-, and 28-wk-old *Rasgrp1*$^{+/-}$

littermate control mice (*left panel*) and *Rasgrp1*$^{-/-}$ mice (*right panel*). (**D**) Summary of age-related changes from multiple mice (**$p < 0.01$, ***$p < 0.001$, *t* test). (**E**) Representative splenic sections with GCs from 12-wk-old littermate control mice (*upper left panel*), *Rasgrp1*$^{-/-}$ mice (*upper right panel*), CD275$^{-/-}$ mice (*lower left panel*), and *Rasgrp1*$^{-/-}$ CD275$^{-/-}$ mice (DKO, lower right) were examined by confocal microscopy for GCs (PNA, green), CD4 (blue), and IL-17 (red) (original magnification ×600). Note that no GC was detected in the section from the CD275$^{-/-}$ mouse.

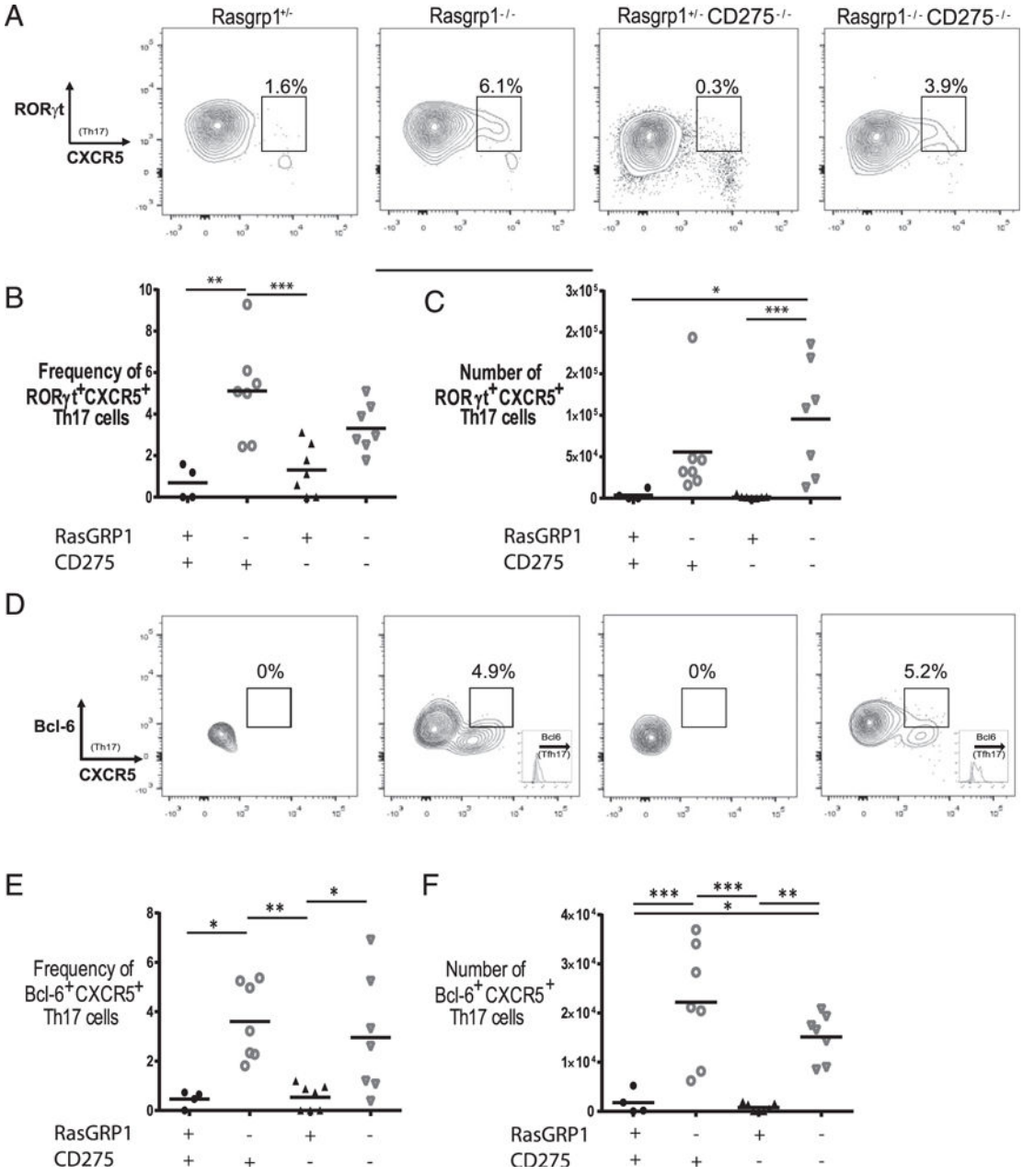

FIGURE 5.

ROR γt and Bcl6 levels in Th17 cells. Splenic MNCs were gated on Th17 cells, and the levels of ROR γt (**A**) and Bcl6 (**D**) were plotted against CXCR5. Scatter plots of the cell frequency and number of ROR γt^+ CXCR5$^+$ (**B** and **C**) or Bcl6$^+$ CXCR5$^+$ (**E** and **F**) Th17 cells from individual 12-to 16-wk-old *Rasgpr1*$^{+/-}$ (●), *Rasgrp1*$^{-/-}$ (○), *ICOSL/CD275*$^{-/-}$ (▲), and DKO (∇) mice (one-way ANOVA for all, except ROR γt number [$p = 0.007$] with Tukey's multiple-comparison test, *$p \leq 0.01$, **$p \leq 0.001$, ***$p \leq 0.0001$).

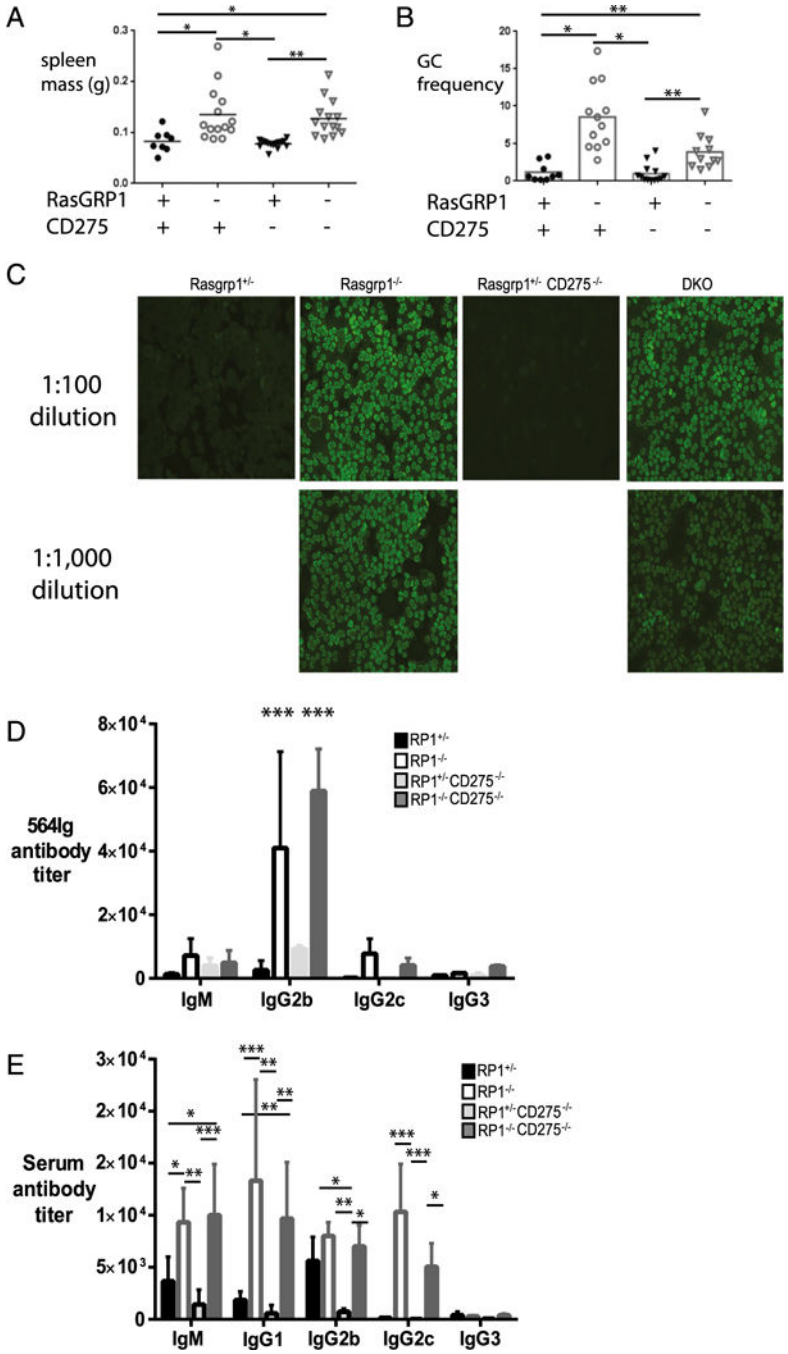

FIGURE 6.

Autoimmunity in *Rasgrp1*-deficient mice is CD275 independent. (**A**) Splenic mass was measured in individual 12-to 16-wk-old *Rasgpr1*$^{+/-}$ (●), *Rasgrp1*$^{-/-}$ (○), *ICOSL/CD275*$^{-/-}$ (▼), and DKO (∇) mice (one-way ANOVA with Tukey's multiple-comparison test, *$p \leq 0.03$, **$p \leq 0.007$, ***$p = 0.0003$). (**B**) Summary of the frequency of PNA^{+} CD95^{+} GC B cells in individual mice (one-way ANOVA, **$p < 0.001$, ***$p < 0.0001$). (**C**) Hep-2 ANA staining from representative *Rasgpr1*$^{+/-}$ mice (*far left panel*), *Rasgrp1*$^{-/-}$ mice (*near left panels*), *ICOSL/CD275*$^{-/-}$ mice (*near right panel*), and DKO mice (*far right panels*) mice at

1:100 (*upper panels*) and 1:1000 (*lower panels*) dilutions of serum (original magnification ×200). (**D**) Titers of 564Ig-derived serum autoantibodies were measured in *Rasgpr1*$^{+/-}$ (black bars), *Rasgrp1*$^{-/-}$ (white bars), *ICOSL/CD275*$^{-/-}$ (light gray bars), and DKO (dark gray bars) 564Igi-transgenic mice, as previously described (27). Data are mean ± SEM from five or six mice per genotype (two-way ANOVA with Tukey's multiple-comparison test, ***$p < 0.0001$). (**E**) Total serum Ig titers in *Rasgrp1*-deficient mice. Titers of serum Abs in *Rasgpr1*$^{+/-}$, *Rasgrp1*$^{-/-}$, *ICOSL/CD275*$^{-/-}$, and DKO mice were measured using ELISA. Data are mean ± SEM from five or six mice per genotype (two-way ANOVA with Tukey's multiple-comparison test, *$p < 0.04$, **$p < 0.001$, ***$p < 0.0003$).

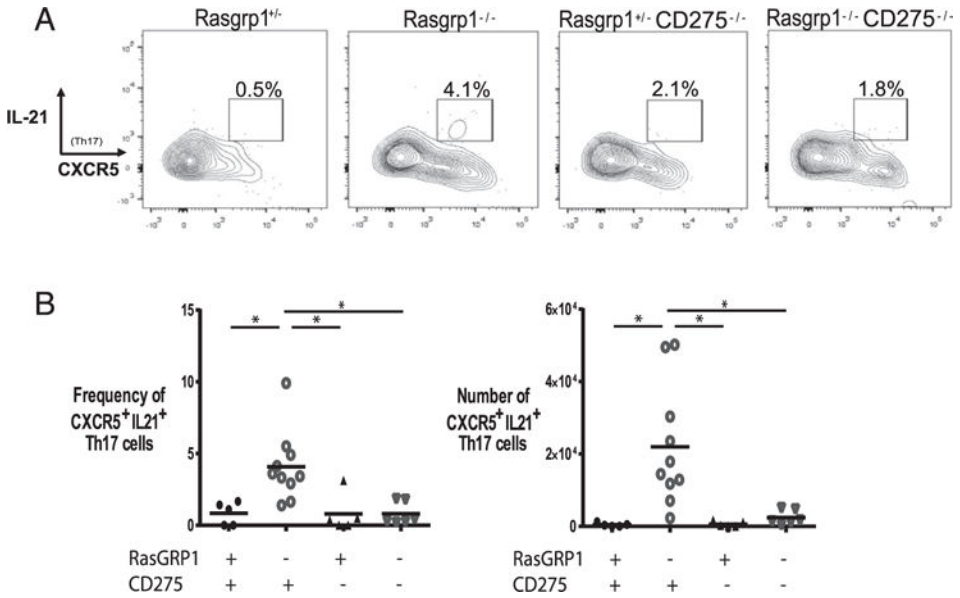

FIGURE 7.

Generation of IL-21–producing Tfh17 cells is CD275 dependent. (**A**) Splenic MNCs were gated on Th17 cells, and levels of IL-21 were plotted against CXCR5. (**B**) Scatter plots of cell frequency (*left panel*) and number (*right panel*) of IL-21$^+$ CXCR5$^+$ Th17 cells from individual 12- to 16-wk-old *Rasgpr1*$^{+/-}$ (●), *Rasgrp1*$^{-/-}$ (○), *ICOSL/CD275*$^{-/-}$ (▲), and DKO (∇) mice (one-way ANOVA with Tukey's multiple-comparison test, *$p < 0.01$).

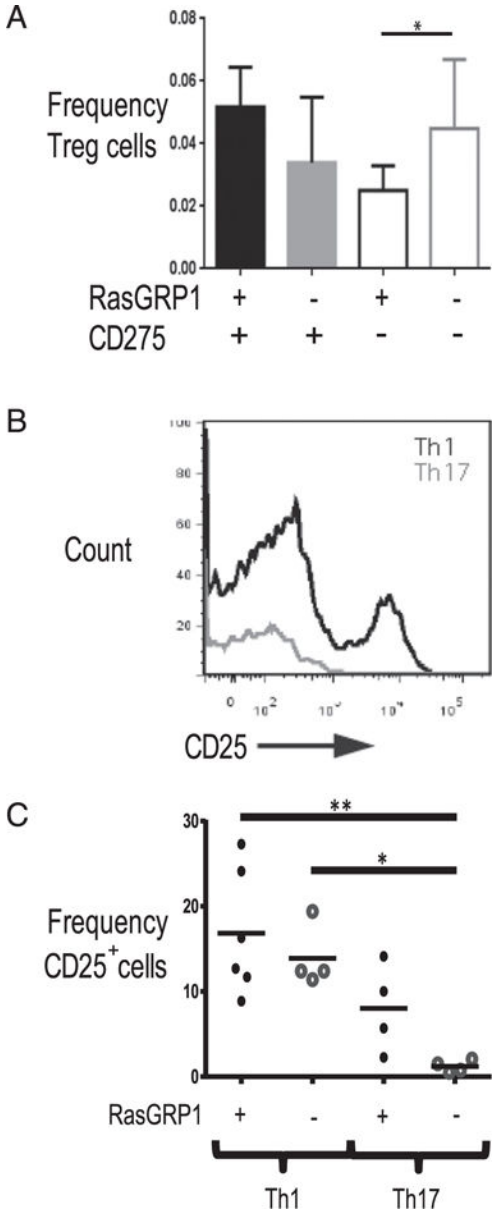

FIGURE 8.

Frequency of Tregs in *Rasgrp1*-deficient mice with and without CD275 and CD25 levels on Th1 and Th17 cells. Tregs were identified as $CD4^+$ $CD25^+$ $Foxp3^+$ cells. (**A**) Frequency of Tregs in 12- to 16-wk-old $Rasgpr1^{+/-}$ (filled black bar), $Rasgrp1^{-/-}$ (filled gray), $ICOSL/CD275^{-/-}$ (open black), and DKO (open gray) mice. Data are mean 6 SD from 5–10 mice per group (one-way ANOVA with Tukey's multiple-comparison test, $*p < 0.01$). (**B**) CD25 levels on Th1 and Th17 cells from $Rasgrp1^{-/-}$ mice. (**C**) Summary of CD25-positive Th1 and Th17 cells from spleens of 12- to 16-wk-old control and $Rasgrp1^{-/-}$ mice (one-way ANOVA with Tukey's multiple-comparison test, $*p < 0.01$, $**p < 0.001$).