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Interleukin-21 Controls ILC3 Cytokine Production and Promotes a Protective Phenotype in a Mouse Model of Colitis

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Abstract

Group 3 innate lymphoid cells (ILC3s) have dual roles in intestinal health, acting in both protective and pathogenic capacities, and importantly, modulations in this population of ILCs have been implicated in inflammatory bowel disease (IBD). Further, subpopulations of ILC3s have been described as serving specific functions in maintaining homeostasis or responding to infection, and aberrant activation of one or more of these subpopulations could exacerbate IBD. However, the signals that enforce the protective and pathogenic features of ILC3s are not fully elucidated. Here we show that IL-21, a cytokine primarily produced by CD4 T cells, acts on a subpopulation of intestinal ILC3s to promote a protective phenotype. IL-21 signaling does not affect the MHCII expressing ILC3 subset but promotes ILC3s that express Tbet and are poised to produce IL-22. Consistent with a protective phenotype, IL-21-deficiency dampens cytokine-induced IL-17A production. We show that exacerbated colitis develops in mice lacking the IL-21 receptor, in agreement with a protective role for IL-21 signaling on ILC3s. These data reveal a novel role for IL-21 in shaping ILC responses in the intestine and provide one mechanism by which effector CD4 T cells can influence innate immunity.

INTRODUCTION

Innate lymphoid cells (ILCs) are a diverse group of innate immune cells that lack an antigen receptor and are especially populous in the body's barrier organs, such as the lungs, skin, and intestine (1–4). Because these cells lack an antigen receptor, ILCs are triggered to respond by stimulation with cytokines. ILCs can be divided into groups that parallel the effector CD4 T cell subsets based on the expression of transcription factors and production of cytokines (5, 6). Group 1 ILCs (ILC1s), which include NK cells as well as a non-NK ILC1 subset, express T-bet and/or Eomes and produce IFNγ and/or cytotoxic granules. Group 2 ILCs (ILC2s) express GATA-3 and produce IL-13 and IL-5 in response to IL-25 and IL-33. Finally, Group 3 ILCs (ILC3s) express RORγt and produce IL-22, IL-17A, GM-

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CSF, and/or IFNγ in response to IL-23 and IL-1β. Importantly, functionally distinct subpopulations of ILC3s have been identified using single cell sequencing, and it is yet undetermined how these discrete populations are differentially regulated to effect both protective and pathogenic immune responses(7).

The common γ-chain (γc) cytokine family consists of six members, IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, and is so named because all utilize the γc receptor, CD132, in combination with specific receptor chains for signaling (8). This family of cytokines is known to impact multiple cell types within the immune system, including helper CD4 T cells and ILCs (9). For example, classic NK cells are reliant on IL-15 for their development, and specific ILC subsets require IL-7 for homeostasis (5). All ILC populations express the γc chain, and, notably, these cells are absent in γc-deficient mice (5, 10). This indicates an important role for this family of cytokines in ILC biology, however it is unclear if all γc chain cytokines influence ILC development and function.

Given the similarity of ILC3s to Th17 CD4 T cells, we posited that these subsets may share additional cytokine networks besides IL-23, IL-17A, and IL-22. IL-21 acts to solidify the Th17 lineage and drives their function via upregulation of RORγt, Ahr, IL-23R, IL-17A, and IL-22 (11–14); however, the influence of IL-21 signaling on ILC3s has not yet been investigated. To investigate the effect of IL-21 on the regulation of ILC3 phenotype and function, we examined these cells in mice lacking either IL-21 or the IL-21 receptor. In the absence of IL-21 signaling, the bulk ILC3 population in the intestine is not compromised but the proportion of plastic ILC3s is reduced, with fewer NKp46+ ILC3s and decreased Tbet expression. Additionally, IL-21 is necessary for optimal IL-22 production by ILC3s and this is consistent with exacerbated colitis in mice lacking the IL-21 receptor on ILCs. Together, these data highlight an important role for IL-21 in calibrating ILC3 subsets in the intestine and emphasize the complex network on signals that regulate ILC3 homeostasis.

MATERIALS AND METHODS

Mice.

Wild-type C57BL/6 (WT) and B6.129S7-Rag1tm1Mom/J (Rag1−/−) mice were purchased from The Jackson Laboratory. IL-21R−/− and IL-21−/− mice were generated as described (15). Rag1−/−IL-21R−/− and Rag1−/−IL-21R+/− mice were generated at University of Alabama at Birmingham. Mice were used at 6–12 weeks of age and all experiments were performed with sex and age-matched groups. For colitis experiments, littermate controls were used as recipient mice. All animals were bred and maintained according to Institutional Animal Care and Use Committee regulations.

Isolation of CD4 T cells and B cells.

Freshly explanted spleens were mechanically disrupted into single-cell suspensions using mesh screens. Erythrocytes were subsequently removed by lysis using 0.83% (w/v) NH4Cl. CD4 T cells were isolated using Dynabeads FlowComp Mouse CD4 kit and B cells were isolated using Dynabeads Mouse CD43 (Untouched B Cells) kit (Invitrogen) according to the manufacturer’s instructions.

Isolation of lamina propria cells.

Lamina propria lymphocytes were isolated using the Lamina Propria Dissociation Kit (Miltenyi Biotec) according to the manufacturer's protocol. Cells were filtered through 100μm cell strainers in PBS with 0.5%FBS. Cells were resuspended in 5ml of 30% Percoll (GE Healthcare) and centrifuged at 25°C at 800 RCF for 20 minutes. Cells were collected at the bottom of the Percoll gradient, washed, filtered through 70μm filters, and resuspended in complete media (RPMI 1640 with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 1× nonessential amino acids, 1 μM sodium pyruvate, and 2.5 μM 2-ME).

ILC identification.

Except where otherwise indicated ILC3s were identified with the following markers: Lineage- (CD4, TCRβ, TCRγδ, CD8α, CD8β, CD19, CD11b, CD11c, DX5, Gr-1, Ter119, NK1.1) CD45+ Thy1.2+ RORγt+ GATA-3lo.

Antibodies and flow cytometry.

Isolated cells were stained in PBS for 20–30 minutes on ice. Fc-receptor blockade was performed with anti-CD16/32 mAbs prior to cell surface staining. For the detection of transcription factors and cytokines, cells were stimulated for 4 hours with 10 ng/ml rIL-23 (eBioscience) and 10 ng/ml rIL-1β (Peprotech). Brefeldin A (10 μg/ml; BD Biosciences) was added for the last 3 hours of stimulation. Cells were fixed and permeabilized for intracellular staining using the Foxp3-staining kit (eBioscience) according to the manufacturer's protocol. Dead cells were excluded from analysis using LIVE/DEAD Fixable Aqua Dead cell stain (Invitrogen). Doublets were excluded prior to analysis. Samples were acquired using an LSRII flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (Tree Star).

Biotinylated mAbs from Biolegend were used as lineage markers: CD4 (GK1.5), TCRβ (H57–597), TCRγδ (eBioscience UC7–13D5), CD8α (53–6.7), CD8β (YTS156.7.7), CD19 (6D5), CD11b (M1/70), CD11c (N418), DX5, Gr-1 (RB6–8C5), Ter119, NK1.1 (PK136). Streptavidin-APC-eFluor780 (eBioscience) was used to stain lineage positive cells.

Additional mAbs used: CD45 (eBioscience 30-F11), Thy1.2 (eBioscience 53–2.1), KLRG1 (eBioscience 2F1), IL-7Rα (eBioscience A7R34), NKp46 (eBioscience 29A1.4), MHC-II (eBioscience M5/114.15.2), Tbet (eBioscience eBio4B10), RORγt (eBioscience B2D), GATA-3 (BD L50–823), IL-17A (eBioscience eBio17B7), IL-22 (Biolegend Poly5164).

For FACS sorting, pooled lamina propria cells were stained and sorted using a FACSAria II cell sorter (BD Biosciences) in the UAB Comprehensive Arthritis, Musculoskeletal and Autoimmunity Center flow cytometry facility. ILC2s were identified as Lineage-CD45+Thy1.2intKLRG1+; ILC3s were identified as Lineage-CD45+Thy1.2hiKLRG1-IL-7Rα+.

pSTAT3 staining.

For the detection of pSTAT3, cells were stained for lineage markers in PBS for 30 minutes, then stimulated for 30 minutes with 10 ng/ml rIL-21 (eBioscience). Surface marker and pSTAT3 staining was performed following fixation with 4% paraformaldehyde and permeabilization with 100% methanol. pSTAT3 staining was achieved with Phospho-Stat3 (Y705) antibody (Cell Signaling)..

ILC culture.

ILC3s were FACS sorted from SI lamina propria and 1×104 cells/well were plated in 100 μl complete media supplemented with 10 ng/ml rIL-7 (Biolegend), 10 ng/ml stem cell factor (Biolegend), and +/− 50 ng/ml rIL-21 (eBioscience). Cells were cultured overnight, and RNA was extracted for real-time PCR as described below.

RNA isolation, cDNA synthesis, and real-time PCR.

RNA was extracted from cells using the RNeasy Micro Plus Kit (Qiagen) and cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. Real-time PCR was performed using the iQ SYBR-Green Supermix (Bio-Rad) and the primer pairs listed below with β2-microglobulin as the housekeeping gene. Reactions were run in triplicate on the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). Relative gene expression was calculated according to the ΔΔ threshold cycle (Ct) and data was normalized to WT splenic CD4 T cells.

B2m FWD: CCTGCAGAGTTAAGCATGCCAG

B2m REV: TGCTTGATCACATGTCTCGATCC

Rorγt FWD: CCGCTGAGAGGGCTTCAC

Rorγt REV: TGCAGGAGTAGGCCACATTACA

Gata3 FWD: CAGAACCGGCCCCTTATCA

Gata3 REV: CATTAGCGTTCCTCCTCCAGA

Il21r FWD: GTGACCCCGTCATCTTTCAGA

Il21r REV: CCGCTGTGCTCCCTGTACA

Tbx21 FWD: ACCAGAGCGGCAAGTGGG

Tbx21 REV: TGGACATATAAGCGGTTCCCTG

Colitis induction.

Lymphocytes were isolated from spleens and lymph nodes of WT mice and stained for CD4, CD25, and CD45RB. CD4+CD45RBhiCD25- cells were FACS sorted and 5 × 105 cells per mouse were injected intraperitoneally. Mice were monitored for weight loss and sacrificed after four weeks. Large intestines were excised, opened longitudinally, and fixed in formalin.

Tissue was embedded in paraffin, sectioned, and stained with Haematoxylin and Eosin (H&E) by the UAB Comparative Histology Laboratory. Slides were scored in a blinded fashion by a veterinary pathologist for signs of disease.

Statistics.

Statistical significance was calculated as indicated in figure legends using Prism software (GraphPad). All t-tests performed are two-tailed and p values < 0.05 were considered significant unless specifically indicated otherwise in the text.

RESULTS

IL-21 influences the composition of the intestinal ILC3 population by regulating Tbet levels.

To determine how IL-21 signaling impacts ILC3s, we compared several characteristics of ILC3s from the lamina propria of wild-type (WT) and IL-21 deficient (IL-21−/−) mice. Throughout this study, ILC3s are identified as lineage- CD45+ Thy1.2+ RORγt+ GATA-3lo cells (Fig. S1A; see Materials and Methods for the description of the lineage panel). The frequency of Tbet+ ILC3s is decreased in both the small intestine (SI) and large intestine (LI) of IL-21−/− mice, with the per-cell expression level of Tbet also reduced in the SI (Fig. 1A–B), suggesting that IL-21 promotes Tbet expression. To corroborate this finding, we sorted ILC3s from the SI lamina propria of WT mice, confirmed their identity as RORγt+ ILC3s with quantitative realtime PCR (Fig. S1B–C), and cultured them overnight in the presence or absence of recombinant IL-21. ILC3s cultured in the presence of IL-21 express higher levels of *Tbx21* mRNA, which encodes Tbet, than ILC3s cultured in the absence of IL-21 (Fig. 1C). This confirms that IL-21 induces Tbet expression in ILC3s. Since Tbet has been shown to control the expression of NKp46 on ILC3s (16, 17), we next investigated whether this subset of ILC3s is impacted by IL-21 deficiency. Importantly, no differences in the total number of ILC3s was observed (Fig. 1E). In the absence of IL-21 signaling, the frequency of Tbet+NKp46-ILC3s is decreased, with no impact on the frequency of Tbet +NKp46+ ILC3s. Unsurprisingly, we observed a reciprocal increase in Tbet-NKp46- ILC3s in the absence of IL-21 (Fig. 1D–E), indicating that IL-21 regulates ILC3 plasticity via Tbet expression but not NKp46 expression.

A subset of ILC3s that are Tbet-NKp46- has been shown to express class II major histocompatibility complex (MHC II) and is remarkable for its ability to dampen CD4 T cell-driven microbiota-dependent intestinal inflammation (18). Importantly, TCR-MHC II binding is one mechanism by which CD4 T cells engage in crosstalk with ILCs(18–20); therefore, we investigated whether CD4 T cell-derived IL-21 modulates MHC-II expression. Despite our finding of an increased frequency of the Tbet-NKp46- subset of ILC3s in the absence of IL-21, we did not observe an appreciable difference in the frequency of MHC II+ ILC3s in IL-21−/− mice, implying that MHC II expression marks a unique subset of cells (Fig. 2). This is consistent with a recent publication showing that MHCII+ ILC3s are a distinct subset based on transcriptional and functional profiling (7). We do observe a slight decrease in the level of MHC II in the SI and a trend in the LI indicating that ILC3s from IL-21−/− mice express lower levels of MHC II on a per-cell basis. However, it is unlikely

that this slight change in MHC II expression would affect the ability of ILC3s to successfully present antigen to CD4 T cells. This demonstrates that IL-21 does not impact the development of MHC II+ ILC3s.

IL-21 promotes a protective ILC3 phenotype by preferentially augmenting an IL-22 producing ILC3 subset.

A salient feature of ILC3s is the ability to produce IL-22, which can act on intestinal epithelial cells to induce production of anti-microbial peptides such as RegIIIβ and RegIIIγ in order to protect against bacterial infections and commensal-induced inflammation (4). In addition, IL-22 has been linked to the promotion of epithelial survival and repair in the intestine as well as amelioration of intestinal inflammation and induction of healing in certain models of inflammatory bowel disease (IBD) (1, 21–23). Further, IL-22 producing ILC3s are shown to be a distinct subset from those that express MHC-II(7). Since we saw that the NKp46-Tbet- population was affected by IL-21 signaling, but the MHC-II+ ILC3s showed no difference in population size or MHC-II expression, we hypothesized that IL-21 is specifically influencing the IL-22 producing subset of ILC3s. IL-22 producing ILC3s are capable of producing other cytokines, such as GM-CSF (24, 25), IFNγ (26), and IL-17A (27–30) but the regulation of this heterogeneity has yet to be established. To ascertain the role of IL-21 signaling on ILC3s, cells were isolated from the LI lamina propria of WT and IL-21−/− mice, cultured with media alone or IL-23 and IL-1β, and ILC3s were evaluated for cytokine production. Following stimulation with IL-23/IL-1β, the frequency of IL-22+IL-17A- ILC3s, which is the most abundant fraction during homeostasis, is reduced in IL-21−/− mice (Fig. 3A–B). In contrast, the small fraction of IL-22-IL-17A+ ILC3s is increased in the absence of IL-21 following cytokine stimulation (Fig. 3A–B). Finally, not only is the frequency of IL-22+IL-17A- ILC3s decreased in the absence of IL-21, but we also note a reduction in the amount of IL-22 produced on a per-cell basis following IL-23/IL-1β stimulation (Fig. 3C–D). This indicates that IL-21 regulates ILC3 production of IL-22 and IL-17A.

Group 3 ILCs express the receptor for and respond to IL-21.

Our data show that distinct features of ILC3s are modulated by the presence of IL-21, leading us to hypothesize that IL-21 acts directly on a specific subset of ILC3s. IL-21 signals via its heterodimeric receptor consisting of the IL-21R chain and γc chain (8). Since it is known that all ILCs express the common γc chain, we examined the expression of IL-21R on subsets of ILCs. ILC2s and ILC3s were sorted from the SI and LI lamina propria of WT mice (Fig. S1B). Subset identity was confirmed via real-time PCR with ILC2s expressing low levels of RORγt and high levels of GATA-3 and ILC3s expressing high levels of RORγt and low levels of GATA-3 (Fig. S1C). Strikingly, ILC3s, but not ILC2s, express *Il21r* mRNA at levels similar to that of B cells (Fig. 4A), which have been shown to harbor elevated levels of the IL-21R (31). This data indicates that ILC3s are capable of responding to IL-21.

It has been shown that STAT3 signaling is critical for the production of IL-22 by ILC3s and thus, for innate defense against *Citrobacter rodentium* infection (32). Since IL-21 has been shown to be a potent inducer of STAT3 activation(14), we tested whether ILCs phosphorylate STAT3 in response to IL-21. Cells were isolated from the mLNs, and LI

lamina propria of WT mice, cultured in the presence or absence of IL-21 for 30 minutes, and then ILCs were evaluated for expression of phosphorylated STAT3 (pSTAT3). Stimulation with IL-21 induced a 2-fold and a 4.5-fold increase in the frequency of pSTAT3+ cells in mLNs and LI, respectively, compared to cells cultured with media alone (Fig. 4B–C). This is also reflected in the total number of pSTAT3+ ILC3s. This effect is specific to IL-21 signaling, as pSTAT3 is not induced by IL-21 in ILCs that lack IL-21R (data not shown). Collectively, these data indicate that IL-21 can induce STAT3 activation in ILCs via IL-21R and are consistent with our previous observations that discrete subsets of ILC3s are modulated by IL-21.

Loss of IL-21R by innate immune cells is associated with exacerbated CD4 T cell-mediated colitis.

IBD is characterized by chronic intestinal inflammation, the causes of which are still largely unknown. There is much evidence that the IL-23/IL-17A axis plays a role in driving intestinal inflammation, as genome-wide association studies have identified polymorphisms in IL-23R and STAT3 that are associated with IBD (33–35). Recent studies have revealed that ILCs are innate sources of IL-17A that drive intestinal inflammation (27, 29). In contrast, several groups have shown that IL-22-producing ILC3s are protective against CD4 T cell-driven intestinal inflammation (23, 36, 37). Since we found that ILC3s have enhanced IL-22 yet reduced IL-17A production in the presence of CD4 T cell-derived IL-21, we posited that IL-21 signaling on ILCs would be protective in the CD45RBhi transfer model of colitis. To test this, we transferred naïve CD4 T cells (CD4+ CD45RBhi CD25-) from WT mice into Rag1−/−IL-21R−/− recipients or Rag1−/−IL-21R+/− littermate controls and evaluated disease severity by weight loss and intestinal pathology. Recipient mice deficient in IL-21R expression lost weight more rapidly than their littermate controls (Fig. 5A). These mice also had more severe intestinal pathology in both the cecum and colon as evidenced by epithelial hyperplasia, goblet cell loss, crypt exudate, and inflammatory cell accumulation (Fig. 5B–C). These data support a protective role for IL-21 signaling in non-adaptive cells, including ILC3s, in CD4 T cell-mediated colitis.

DISCUSSION

It is critical to understand the cytokine networks active in regulating ILC3 subsets to fully elucidate their function and role in the complex intestinal niche. Here, we have revealed IL-21 as a new player in ILC3 regulation; IL-21 signaling on ILC3s results in reduced IL-17A production and augmented IL-22 and Tbet expression, culminating in a protective variety of ILCs that can dampen pathogenesis. Further, our data suggests a novel mechanism by which IL-21 production by activated CD4 T cells direct the phenotype and function of ILCs, implying a unique function for this innate cell subset beyond the recruitment and activation of the adaptive immune response.

IL-21 is associated with chronic inflammation in the intestine and single-nucleotide polymorphisms in the *IL2/IL21* gene locus are linked with the development of IBD(38), yet how IL-21 contributes to disease is still unknown. IL-21 is known to impact numerous cell types, including many that reside in the intestine(14, 39), and it has been shown that during

Th17 differentiation IL-21 supports the upregulation of IL-23R and promoting IL-17A expression(13). Herein, we show that IL-21 actually decreases IL-17A and augments IL-22 production by ILC3s, illustrating an additional mechanism by which IL-21 may contribute to intestinal homeostasis and disease. Moreover, these data reveal both parallel and divergent regulatory networks that exist between ILC3s and Th17 cells. Interestingly, we find that stimulation of ILC3s with IL-21 does not directly enhance IL-22 production (data not shown); however, ILC3s from mice with intact IL-21 respond to stimulation with IL-23 and IL-1 with higher IL-22 production. Therefore, IL-21 possibly gives a priming signal that enhances the responsiveness of ILC3s, perhaps through upregulation of IL-23R or IL-1R1. Further investigation will be necessary to delineate the effector molecules influencing IL-21-mediated ILC3 activation.

The influence of tissue microenvironments, especially the commensal microbes of the intestine, on the phenotype and function of ILC populations is becoming increasingly evident(40). Recent transcriptome analysis reported little to no IL-21R expression by ILC3s isolated from the small intestine(41), which is in stark contrast to our data demonstrating striking IL-21R expression by ILC3s in the intestine. Since commensal bacteria and their metabolic products play a role in many aspects of ILC development, function, and phenotype (40, 42, 43), it is possible that this environmental factor influences the proportion of specific ILC3 subsets. Importantly, single cell analysis of ILCs was recently used to track transcriptome responses to changes in microbial colonization and demonstrated that the abundance and composition of the microbiota has an impact on the diversity and phenotype of intestinal ILC3s (7). It will be important to evaluate the influence of the microbiota on the ability of the ILC population to detect pro-inflammatory signaling molecules such as IL-21 as this could influence treatment plans for ILC-mediated autoinflammatory conditions.

In this study, we show that IL-21 can regulate the phenotype and function of the ILC3 population. Interestingly, recent reports reveal that IL-21R is expressed by ILC1s in the intestine, salivary glands, and, to a lesser extent, in the spleen and liver(7, 41), yet the effect of IL-21 on this ILC subset is unknown. ILC1s have been found to be associated with the inflamed lesions in IBD patients and these cells can produce the pro-inflammatory cytokines IFNγ and TNFα(44, 45). Future work investigating whether IL-21 is required for the development, maintenance, or function of ILC1s and how this affects the progression of inflammatory disease will enhance understanding of the overall impact of IL-21 on ILC responses.

Finally, our data highlight the heterogeneity and diversity of the ILC3 population. In line with recent evidence that there is functional compartmentalization under the ILC3 umbrella (7, 46), we show that cytokine signaling specifically directs the activity of IL-22-producing ILC3s without influencing MHCII+ ILC3s. Further, IL-21 signaling on this subset of ILC3s is necessary to protect against T cell-mediated intestinal inflammation. The demonstration that IL-21 acts on this small but crucial subset of ILCs may be of critical importance to understanding the mechanisms by which chronic intestinal inflammation is mediated and sustained.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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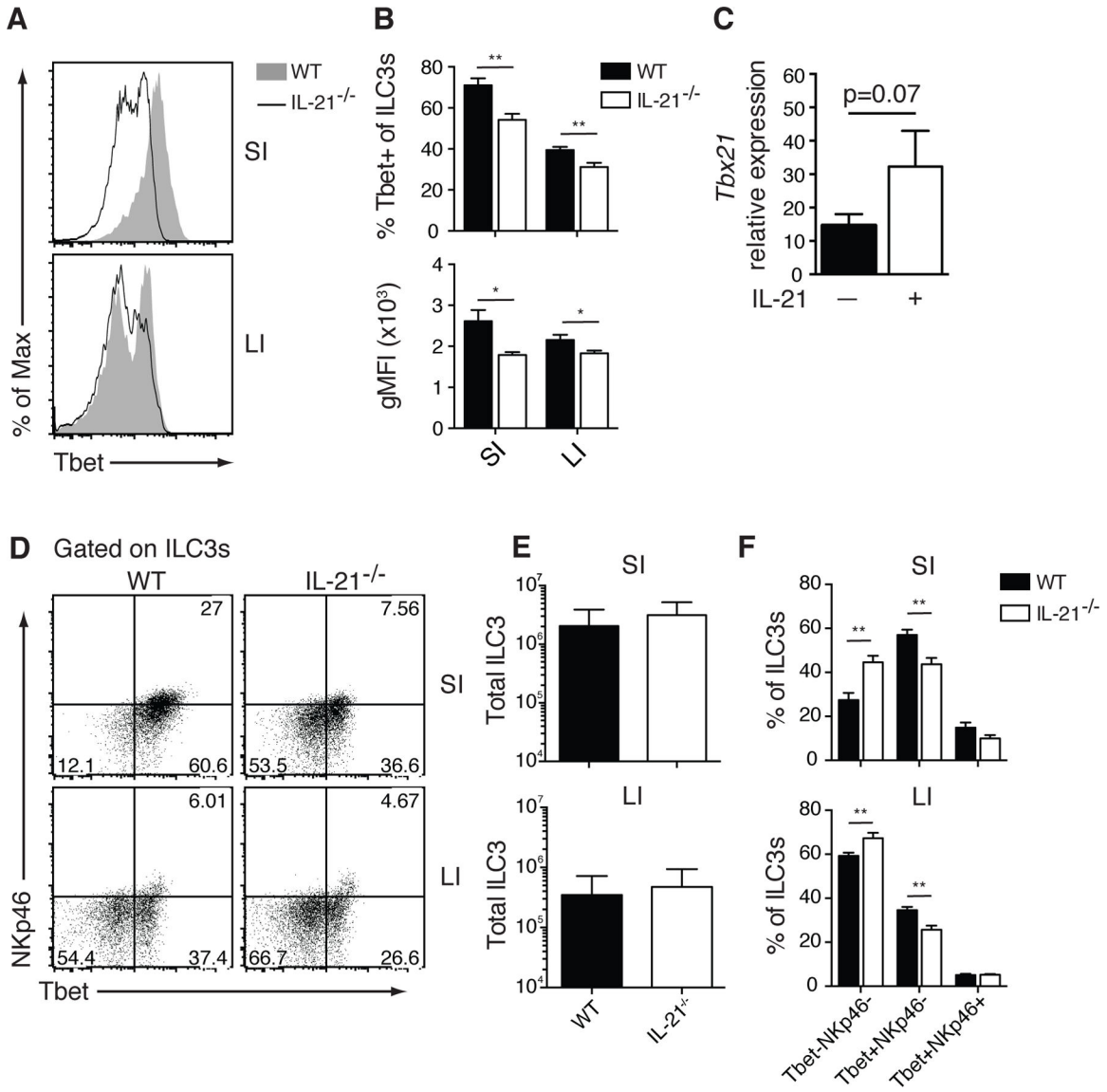

Figure 1. IL-21 induces Tbet expression in ILC3s.

(**A-B**) SI and LI lamina propria ILC3s from WT and IL-21−/− mice were stained for Tbet expression. ILC3s were identified as lineage- CD45+ Thy1.2+ RORγt+ GATA-3lo lymphocytes. (A) Representative histograms gated on intestinal ILC3s show Tbet staining. (B) The graphs show the frequency of Tbet+ ILC3s combined from 8 independent experiments (n=6–12) and the geometric MFI of Tbet staining combined from 4 independent experiments (n=6). t-test with Holm-Sidak multiple comparisons test. (**C**) WT SI lamina propria ILC3s (FACS sorting panel = lineage-CD45+Thy1.2hiKLRG1-IL-7Rα+) were FACS sorted and stimulated with IL-21. Expression of *Tbx21* mRNA was determined by real-time PCR. Data combined from 3 independent experiments (2 mice pooled per experiment). One-tailed t-test. (**D-E**) SI and LI lamina propria ILC3s from WT and IL-21−/− mice were analyzed for NKp46 and Tbet expression. (D) Representative plots gated on ILC3s. (E) The frequency of ILC3 subsets combined from 3 independent experiments (n=6–

12). t-test with Holm-Sidak multiple comparisons test. Error bars show SEM. ** $p<0.01$; *$p<0.05$.

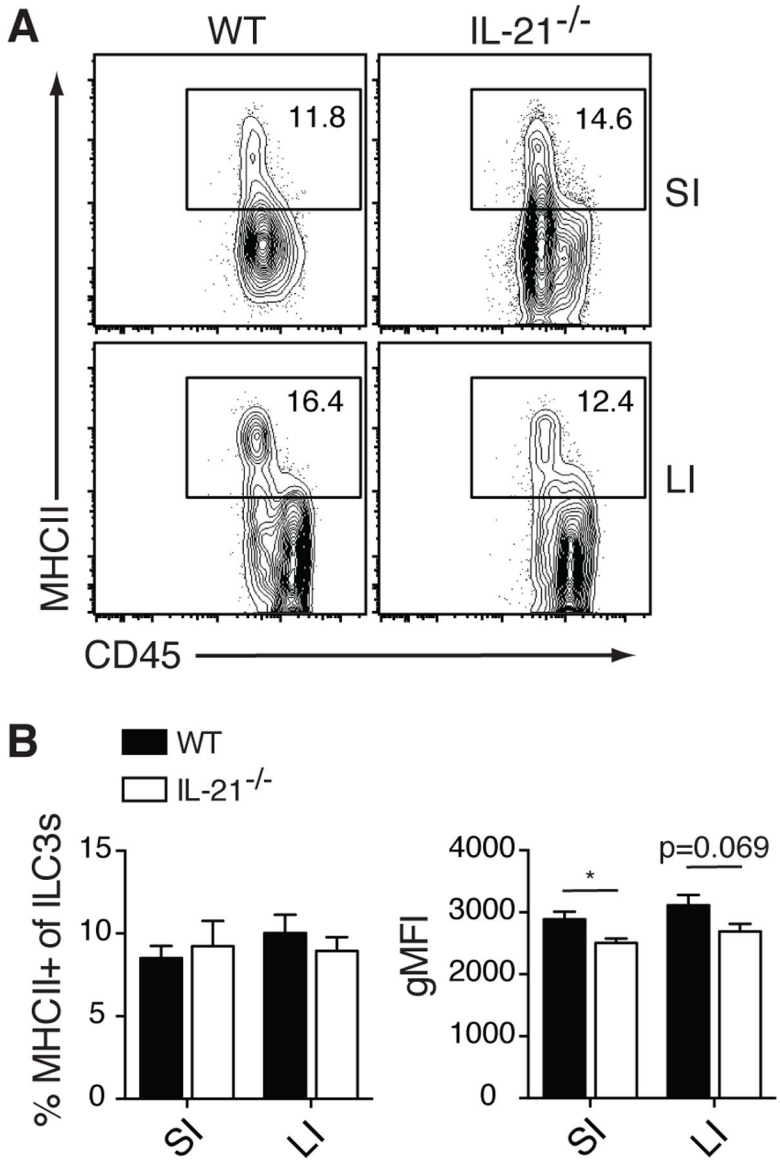

Figure 2. IL-21 does not impact MHC-II expression on ILC3s.

(**A**) Representative plots show MHC II expression by ILC3s isolated from the SI and LI lamina propria of WT and IL-21−/− mice. ILC3s were identified as lineage- CD45+ Thy1.2+ RORγt+ GATA-3lo lymphocytes. (**B**) Graphs show the (*left*) frequency of MHC-II + ILC3s combined from 8 independent experiments (n=6–12), and (*right*) geometric MFI of MHC II staining combined from 4 independent experiments (n=6). Error bars indicate SEM; t-test with Holm-Sidak multiple comparisons test. *p<0.05.

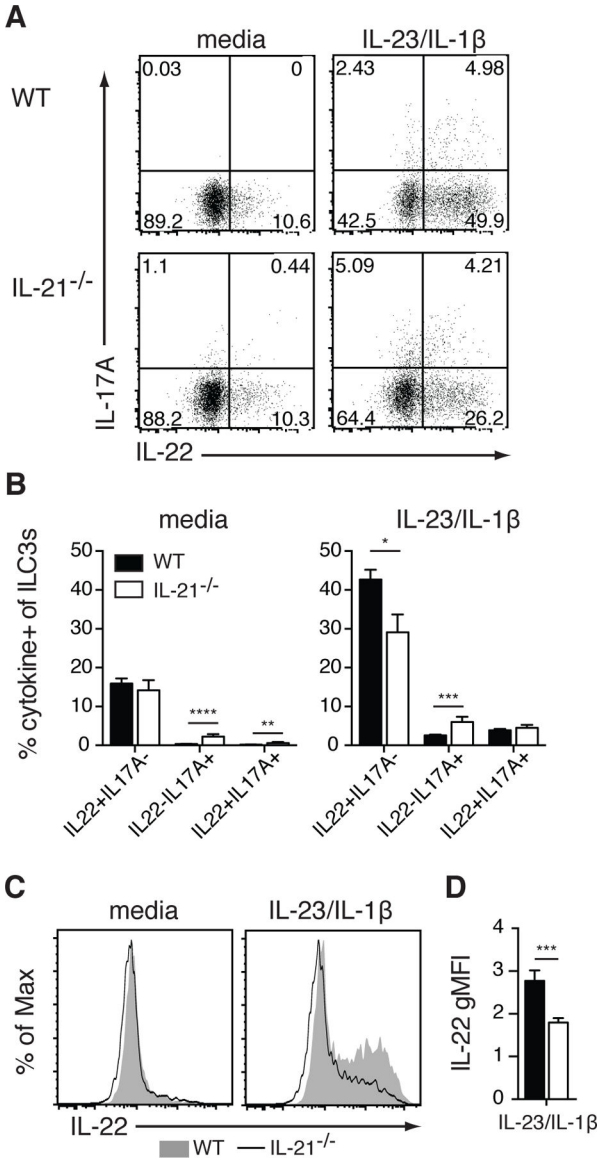

Figure 3. IL-21 promotes a protective ILC3 phenotype by preferentially augmenting an IL-22 producing ILC3 subset.

Mononuclear cells were isolated from LI lamina propria from WT and IL-21−/− mice and cultured for 4 hours with media or IL-23 and IL-1β. ILC3s were identified as lineage- CD45+ Thy1.2+ RORγt+ GATA-3lo lymphocytes. (**A**) Representative plots gated on ILC3s show intracellular staining for IL-17A and IL-22. (**B**) The frequencies of cytokine-producing ILC3 populations combined from 10 independent experiments (n=6–15) are shown. (**C**) Representative histograms of IL-22 expression in LI ILC3s after the indicated stimulations. (**D**) The ratio of IL-22 geometric MFI from stimulated versus non-stimulated ILC3s; data combined from 6 independent experiments (n=6–9). Error bars show SEM; t-test with Holm-Sidak multiple comparisons test. ****p<0.0001; ***p<0.001; ** p<0.01; *p<0.05.

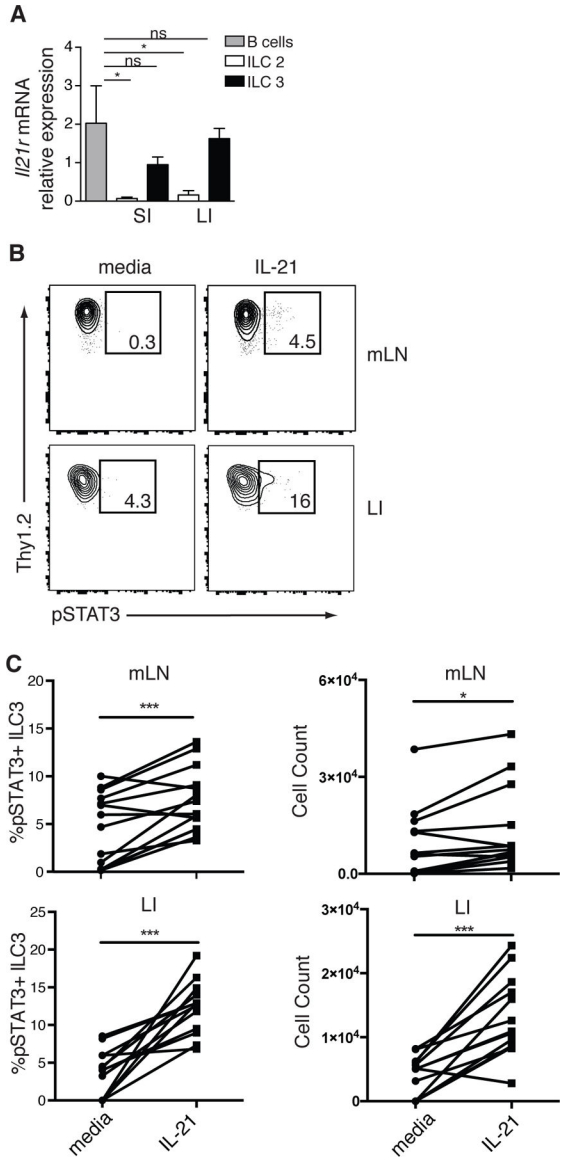

Figure 4. Group 3 intestinal ILCs selectively express the IL-21 receptor and respond to IL-21.

(**A**) IL-21R expression was determined by real-time PCR on FACS sorted ILC2s and ILC3s from the SI and LI lamina propria of WT mice; splenic B cells used as a positive control. ILC2s were identified as lineage-CD45+Thy1.2intKLRG1+; ILC3s were identified as lineage-CD45+Thy1.2hiKLRG1-IL-7Rα+. Data combined from 3 experiments. Error bars show SEM; one-way ANOVA with Dunnett's multiple comparisons test. (**B**-**C**) Intracellular pSTAT3 staining in ILC3s from WT mice after IL-21 stimulation. (B) Representative plots gated on ILC3s (lineage- Thy1.2hiKLRG1-IL-7Rα+) from mLN and LI lamina propria. (C) Graphs indicate the paired frequencies of pSTAT3+ ILCs from mLN LI lamina propria in media versus IL-23/IL-1β conditions. Data from 4–6 independent experiments (n=12–14). paired samples t-test compares pSTAT3 levels in media versus cytokine stimulation conditions. ****p<0.0001; *p<0.05; ns, not significant.

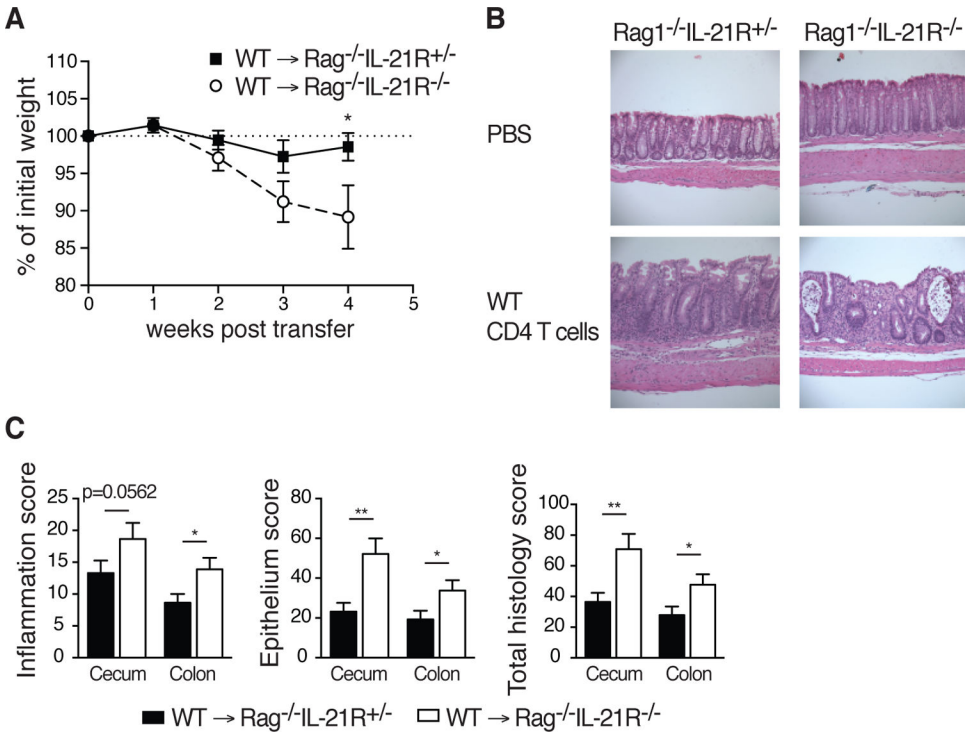

Figure 5. Loss of IL-21R by innate immune cells is associated with exacerbated CD4 T cell-mediated colitis.

Colitis was induced by transfer of WT CD4 T cells (CD4+CD45RBhiCD25-) into Rag1−/−IL-21R−/− mice or Rag1−/−IL-21R+/− littermate controls. (**A**) Weight loss combined from 2 independent experiments (n=13–18). Error bars show SEM; t-test with Holm-Sidak multiple comparisons test. (**B**) Representative H&E stained colon sections of Rag1−/−IL-21R−/− mice or Rag1−/−IL-21R+/− littermate controls that received WT CD45RBhi CD25-CD4 T cells. (**C**) Histology scores of recipient mice. Data from 2 independent experiments (n=12–17). Mann-Whitney U test. **p<0.01; *p<0.05.