IL-17A Inhibits the Expansion of IL-17A-Producing T Cells in Mice through "Short-Loop" Inhibition via IL-17 Receptor[1]

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IL-23 and IL-17A regulate granulopoiesis through G-CSF, the main granulopoietic cytokine. IL-23 is secreted by activated macrophages and dendritic cells and promotes the expansion of three subsets of IL-17A-expressing neutrophil-regulatory T (Tn) cells; $CD4^-CD8^-\alpha\beta^{low}$, $CD4^+CD8^-\alpha\beta^+$ (Th17), and $\gamma\delta^+$ T cells. In this study, we investigate the effects of IL-17A on circulating neutrophil levels using IL-17R-deficient (*Il17ra*$^{-/-}$) mice and *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ mice that lack both IL-17R and all four β_2 integrins. IL-17R deficiency conferred a reduction in neutrophil numbers and G-CSF levels, as did Ab blockade against IL-17A in wild-type mice. Bone marrow transplantation revealed that IL-17R expression on nonhemopoietic cells had the greatest effects on regulating blood neutrophil counts. Although circulating neutrophil numbers were reduced, IL-17A expression, secretion, and the number of IL-17A-producing Tn cells were elevated in *Il17ra*$^{-/-}$ and *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ mice, suggesting a negative feedback effect through IL-17R. The negative regulation of IL-17A-producing T cells and IL-17A and IL-17F gene expression through the interactions of IL-17A or IL-17F with IL-17R was confirmed in splenocyte cultures in vitro. We conclude that IL-17A regulates blood neutrophil counts by inducing G-CSF production mainly in nonhemopoietic cells. IL-17A controls the expansion of IL-17A-producing Tn cell populations through IL-17R. *The Journal of Immunology,* **2008, 181: 1357–1364.**

Interleukin-17A has biological roles at the interface between the adaptive and innate immune systems (1, 2). IL-17A contributes to the pathogenesis of multiple autoimmune diseases including rheumatoid arthritis (3–6), experimental autoimmune encephalitis (7, 8), and inflammatory bowel disease (9, 10) through the actions of $CD4^+$ Th17 cells. IL-17A also controls host responses against extracellular pathogens by regulating the expression of many inflammatory mediators including IL-6, CCL2, CXCL1, CXCL6, and CXCL8, as well as G-CSF, GM-CSF, and matrix metalloproteinases (1, 8, 11).

IL-17A is secreted primarily by three subsets of activated memory T cells, which we have collectively named neutrophil-regulatory Tn cells; $CD4^+CD8^-\alpha\beta^+$ (also known as Th17 cells), $CD4^-CD8^-\alpha\beta^{low}$ T cells, and $\gamma\delta^+$ T cells (12, 13). Although most studies have focused on Th17 cells (14, 15), $\gamma\delta^+$ T cells are the most abundant IL-17A-producing cells in normal wild-type (WT)[3] mice (12, 13). The nuclear receptor RORγt and the transcription factor Stat3 have been identified as key regulators involved in the polarization of naive $CD4^+$ T cells into Th17 cells following stimulation with IL-6, IL-21, and TGF-β1 (8, 15–22). Replacing the coding region of the RORγt gene with that of GFP locus revealed three GFP^+ cell populations in these mice (19), analogous to the three populations characterized by Stark et al. (12). The polarization of Th17 cells can be suppressed by the cytokines IFN-γ and IL-4, as well as IL-27 and IL-2 (8, 23–25).

In mice, IL-17A and IL-17F have a single known homodimeric receptor, IL-17R, which is ubiquitously expressed (26). In humans, IL-17A and IL-17F can bind to IL-17R alone and to a heterodimeric complex of IL-17R and IL-17RC (27). IL-17R-deficient mice have much higher mortality rates following infection with *Klebsiella pneumoniae* or *Toxoplasma gondii* due to defective neutrophil recruitment to the lung, although the cellular source of IL-17A in these murine models was not determined (28–30).

In addition to its role in host defense against infections, our group (12, 31) and others (28–30, 32, 33) have shown that IL-17A is a potent regulator of granulopoiesis and neutrophil recruitment under normal and inflammatory conditions. Organ-specific overexpression of IL-17A increases circulating neutrophil numbers and recruitment into the organs by induction of CXCL2, IL-1β, and G-CSF (32–34). We have previously shown that blood neutrophil numbers correlate with serum IL-17A and G-CSF levels in normal and adhesion molecule-deficient mice and that G-CSF acts downstream of IL-17A (31). Neutrophilia is induced by bacterial infection but is also commonly found in adhesion molecule-deficient mice with defective neutrophil trafficking (31, 35). One such mouse with a severe neutrophil trafficking defect is the *Itgb2*$^{-/-}$ mouse (36), which lacks all four β_2 integrins and mimics many aspects of the human leukocyte adhesion deficiency-I (37). *Itgb2*$^{-/-}$ mice have 10- to 20-fold elevated neutrophil counts as neutrophils are unable to traffic into many tissues (31, 36).

In this study, we investigate the role of IL-17R in regulating blood neutrophil numbers using IL-17R-deficient mice with and without neutrophilia. Specifically, we focus on the effects of defective IL-17R signaling on hemopoietic and nonhemopoietic

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[3] Abbreviations used in this paper: WT, wild type; MLN, mesenteric lymph node; LP, lamina propria; BM, bone marrow; PMN, polymorphonuclear neutrophil.

cells, and identify a "short-loop" negative regulatory pathway by which IL-17A regulates the polarization of IL-17A-producing Tn cells through its cognate receptor.

Materials and Methods

Animals

Mice lacking the β_2 integrin subunit (*Itgb2*$^{-/-}$) (36), IL-17R (*Il17ra*$^{-/-}$) (29), and WT C57BL/6 mice (The Jackson Laboratory) were used between 8 and 16 wk of age and kept in specific pathogen-free conditions in a barrier facility. Mice deficient in both CD18 and IL-17R were generated by breeding *Itgb2*$^{+/-}$ with *Il17ra*$^{-/-}$ mice using a standard four generation scheme. All mice were on a C57BL/6 background for at least ten generations, and animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Virginia. For Ab blockade of IL-17A (TC11-18H10.1; BioLegend), 100 μg of Ab or rat IgG1 isotype control (eBioscience) per mouse was injected i.v. into WT mice and blood neutrophil counts assessed after 24 h. Blood counts were taken via tail bleed into EDTA-coated capillary tubes, analyzed by automatic analyzer (Hemavet 850; CDC Technologies), and confirmed by Kimura-stained manual counts using a hemocytometer.

Bone marrow (BM) transplantation

BM was isolated from *Itgb2*$^{-/-}$ and *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ mice as previously described (38). In brief, 10^7 BM cells resuspended in 500 μl sterile PBS were injected i.v. into lethally irradiated WT or *Il17ra*$^{-/-}$ recipients under sterile conditions. Mice were given autoclaved water supplemented with antibiotics (5 mM sulfamethoxazole, 0.86 mM trimethoprim (Sigma-Aldrich). All experiments were conducted 8–9 wk post BM transplantation. Completeness of reconstitution was monitored by amplifying splenocyte DNA of female mice reconstituted with BM from male mice by real-time PCR using primers targeted against the Y6 gene located on the Y chromosome. Reconstitution was ~95% (data not shown).

Abs and recombinant proteins

mAbs were from BD Pharmingen: purified or allophycocyanin-conjugated anti-CD3ε (145-2C11), allophycocyanin-CY7- or PerCP-conjugated anti-CD4 (RM4-5), anti-CD16/CD32 (2.4G2; The Lymphocyte Culture Center, University of Virginia), purified anti-CD28 (37.51), FITC- or allophycocyanin-conjugated anti-TCR β-chain (H57-597), FITC- or biotin-conjugated anti-$\gamma\delta$ TCR (GL3), purified or PE-conjugated anti-IL-17A (TC11-18H10.1), and PerCP-conjugated streptavidin used for the detection of the biotin-conjugated primary Ab. IL-6 (50 ng/ml; R&D Systems), IL-23 (20 ng/ml; R&D Systems), TGF-β1 (1 ng/ml; PeproTech), and IL-17A (10 ng/ml; R&D Systems) were used at the concentrations indicated.

Flow cytometry

Animals were anesthetized with an i.p. injection of a mixture containing ketamine hydrochloride (Sanofi Winthrop Pharmaceuticals; 125 mg/kg), xylazine (TranquiVed, Phoenix Scientific; 12.5 mg/kg), and atropine sulfate (Fujisawa USA; 0.025 mg/kg) then killed by cervical dislocation. Single-cell suspensions of the spleen, mesenteric lymph nodes (MLN), and Peyer's patches were prepared by straining tissues through a 70-μm screen (BD Falcon). RBC were lysed and white blood cells washed twice with PBS-FBS. Lamina propria (LP) lymphocytes were prepared as previously described (39). For flow cytometry, cells were cultured in RPMI 1640 containing 10% FBS, 1× nonessential amino acids (Life Technologies), 10 mM HEPES, 2 mM L-glutamine (Life Technologies), 1 mM sodium pyruvate (Life Technologies) and 1% penicillin/streptomycin for 5 h with 10 ng/ml PMA (Sigma-Aldrich), 500 ng/ml calcium ionophore (Sigma-Aldrich), and GolgiStop (BD Pharmingen).

Following stimulation, cells were re-suspended at 10^7/ml. Fcγ III/IIRs were blocked with 0.5 μg anti-CD16/CD32 (2.G42) and the cell suspension was incubated with an optimal concentration of mAbs for 20 min at 4°C in staining buffer (5% FBS in PBS) and washed. When biotinylated mAbs were used, streptavidin-PerCP was added for 20 min at 4°C and cells washed twice in staining buffer. Intracellular staining was performed using Fix & Perm cell permeablization reagents (Caltag Laboratories) according to the manufacturer's instructions. Flow cytometry analysis was performed on a Becton Dickinson FACSCalibur dual laser, and data were analyzed using FlowJo software (Tree Star). Gates were set by isotype controls. Alternatively, cells were visualized using Amnis Imagestream100 (Amnis).

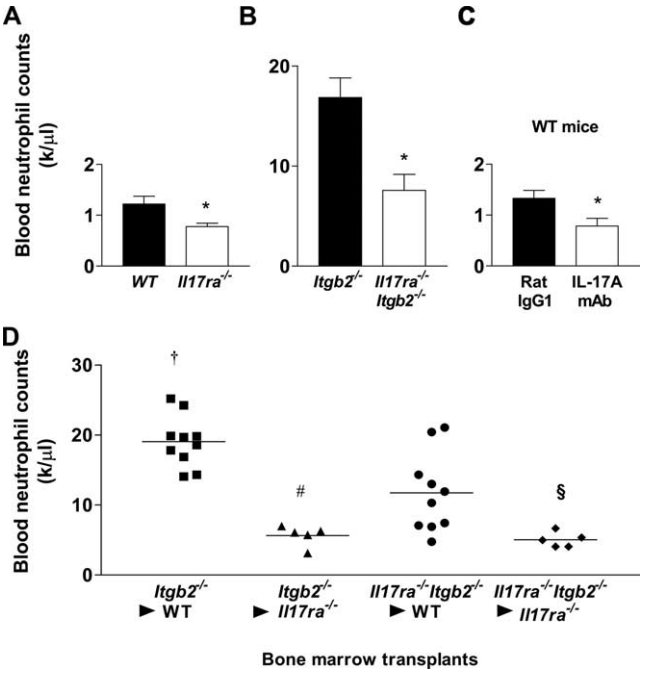

FIGURE 1. Blood neutrophil counts are reduced in IL-17R-deficient mice. Blood neutrophil counts were measured in *A* IL-17R-deficient mice ($n = 14$) compared with WT mice ($n = 15$), in *B* *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ mice ($n = 7$) compared with *Itgb2*$^{-/-}$ mice ($n = 22$), and in *C* WT mice following Ab treatment against IL-17A for 24 h ($n = 4–8$; *, $p < 0.05$ by nonparametric Mann-Whitney *U* test). BM transplantations (*D*) from *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ or *Itgb2*$^{-/-}$ donors into irradiated WT or *Il17ra*$^{-/-}$ recipients ($n = 5–10$) were performed and blood counts assessed after 6 wk. †, $p < 0.05$ from all other groups; #, $p < 0.05$ compared with *Itgb2*$^{-/-}$ BM into WT; §, $p < 0.05$ compared with *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ BM into WT mice.

Lymphocyte culture

Splenocytes from WT or *Il17ra*$^{-/-}$ mice were isolated under sterile conditions and cultured on plate-adsorbed purified anti-CD3ε (10 μg/ml) and soluble anti-CD28 (1 μg/ml), in the presence or absence of IL-23 (20 ng/ml), IL-6 (50 ng/ml), and TGF-β1 (1 ng/ml) for 3 days (17). The cell

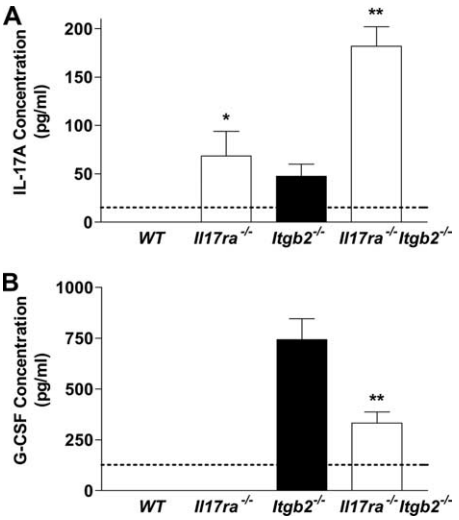

FIGURE 2. Effects of IL-17R deficiency on serum cytokine levels. Analysis of IL-17A ($n = 3–6$) (*A*) and G-CSF ($n = 6$) (*B*) in the serum of WT, *Il17ra*$^{-/-}$, *Itgb2*$^{-/-}$, and *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ mice. Detection limit of each ELISA indicated by dotted line. *, $p < 0.05$; **, $p < 0.01$ by nonparametric Mann-Whitney *U* test.

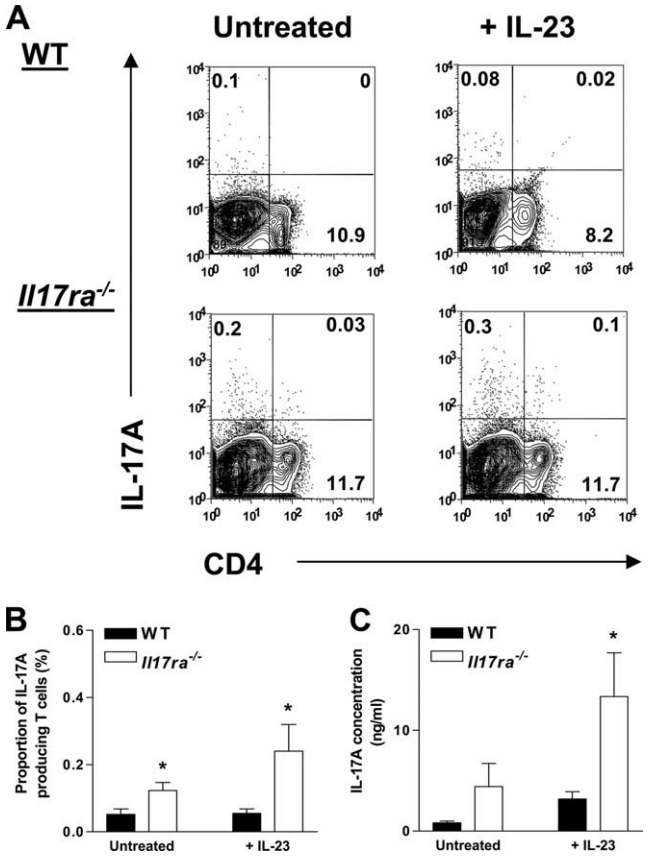

FIGURE 3. Effects of IL-17R deficiency on the number of IL-17A-producing Tn cells and IL-17A secretion. Splenocytes from *Il17ra*$^{-/-}$ and WT mice were stimulated for 3 days on plate-adsorbed anti-CD3ε and soluble anti-CD28 Abs in the presence or absence of IL-23 (20 ng/ml), followed by a 3 day rest. Cells were reactivated by PMA/ionomycin in the presence of GolgiStop for 5 h before intracellular staining for IL-17A. *A*, Representative plots from WT and *Il17ra*$^{-/-}$ mice. *B*, IL-17A-producing Tn cells (n = 3–4) as a proportion (%) of the gated lymphocyte population in *Il17ra*$^{-/-}$ splenocytes (□) compared with WT splenocytes (■). *C*, IL-17A protein in the supernatants (n = 3–5). *, $p < 0.05$ by unpaired t test.

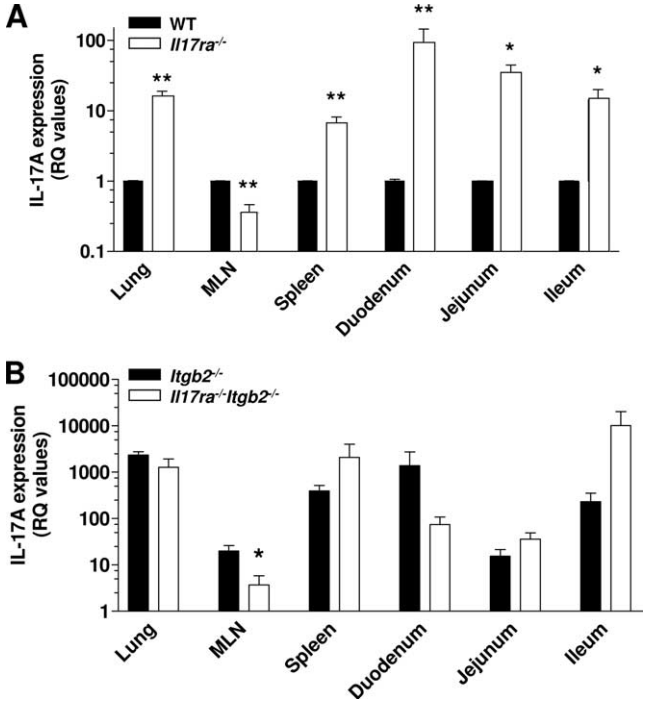

FIGURE 4. IL-17A is up-regulated in the tissue of IL-17R-deficient mice. Basal expression of IL-17A was examined in organs of (*A*) IL-17R-deficient mice (□) compared with WT (■) (n = 3–4) and (*B*) *Il17ra*$^{-/-}$ *Itgb2*$^{-/-}$ mice (□) compared with *Itgb2*$^{-/-}$ mice (■) (n = 3–5). For the *Il17ra*$^{-/-}$, *Itgb2*$^{-/-}$, and *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ mice, the relative quantification (RQ) values were expressed as a fold-increase compared with IL-17A expression in WT mice (the calibrator control which is equal to 1). *, $p < 0.05$; **, $p < 0.01$ by unpaired students t test.

suspensions were then transferred to fresh, nontreated plates for 3 days. On day 6, the splenocytes were reactivated for 5 h with 10 ng/ml PMA (Sigma-Aldrich), 500 ng/ml calcium ionophore (Sigma-Aldrich), and GolgiStop (BD Pharmingen). Alternatively, splenocytes from *Itgb2*$^{-/-}$ mice were treated with 10 ng/ml IL-17A or IL-17F before being prepared for RNA isolation. IL-17A and G-CSF protein in serum or cell culture supernatants were measured by Quantikine M mouse ELISA kits (R&D Systems) according to the manufacturer's directions.

mRNA quantification

RNA was extracted from isolated splenocytes or tissue following homogenization in Trizol (Invitrogen), according to manufacturer's instructions. Reverse transcription and PCR steps were performed using QuantiTect SYBR Green RT-PCR kit (Qiagen) as previously described (12). IL-17A primer sequences were as follows:

Sense: ATCCCTCAAAGCTCAGCGTGTC

Anti-Sense: GGGTCTTCATTGCGGTGGAGAG

FAM-labeled mouse IL-17F and IL-22 Assay on Demand probes and Vic-labeled TaqMan Ribosomal RNA control reagent (Applied Biosystems) were performed using the Quantitech Probe One-Step RT-PCR kit (Qiagen). Then, 1 μg of total RNA was used for all tissues. Values were determined using iCycler iQ Real-Time Detection System Software v3.0 (Qiagen). The corresponding values were normalized to 18s RNA and then normalized to individual WT mouse organs as the calibrator control (always equal to 1), thereby expressing the values as relative quantification (RQ) values.

Statistical analysis

Data were expressed as mean ± SEM. Statistical significance between groups was set at $p < 0.05$ using unpaired t tests, nonparametric Mann-Whitney U test, or One-Way ANOVA with Bonferroni's posthoc tests.

Results

IL-17R in nonhemopoietic cells regulates peripheral blood neutrophil counts

Circulating neutrophil numbers were reduced by ~37% in *Il17ra*$^{-/-}$ mice compared with WT mice, confirming previous studies (Fig. 1*A*) (12). To assess the effects of IL-17R deficiency in cases of severe neutrophilia, *Il17ra*$^{-/-}$ mice were crossed with neutrophilic, adhesion molecule-deficient (*Itgb2*$^{-/-}$) mice. Neutrophil counts were significantly attenuated in *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ mice by ~56% compared with *Itgb2*$^{-/-}$ mice, but absence of IL-17R signaling did not completely rectify the neutrophilia (Fig. 1*B*). Under barrier vivarium conditions, *Il17ra*$^{-/-}$ mice did not show any overt phenotype, as previously reported (29). However, *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ mice were consistently smaller in size than *Itgb2*$^{-/-}$ mice and weighed significantly less (10.5 ± 0.5 g vs 14.7 ± 1.2 g; $p < 0.05$). *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ mice exhibited splenomegaly and extramedullary hematopoiesis causing an expansion in splenic red pulp and subsequent decrease in white pulp. A large infiltration of neutrophils was also found in the lung tissue (data not shown). Furthermore, *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ mice failed to thrive, and many had to be euthanized between 12 and 14 wk of age. Ab blockade against IL-17A in WT mice caused a significant reduction in neutrophil numbers, by ~39%, confirming that IL-17A signaling via IL-17R regulates blood neutrophil numbers in WT mice (Fig. 1*C*).

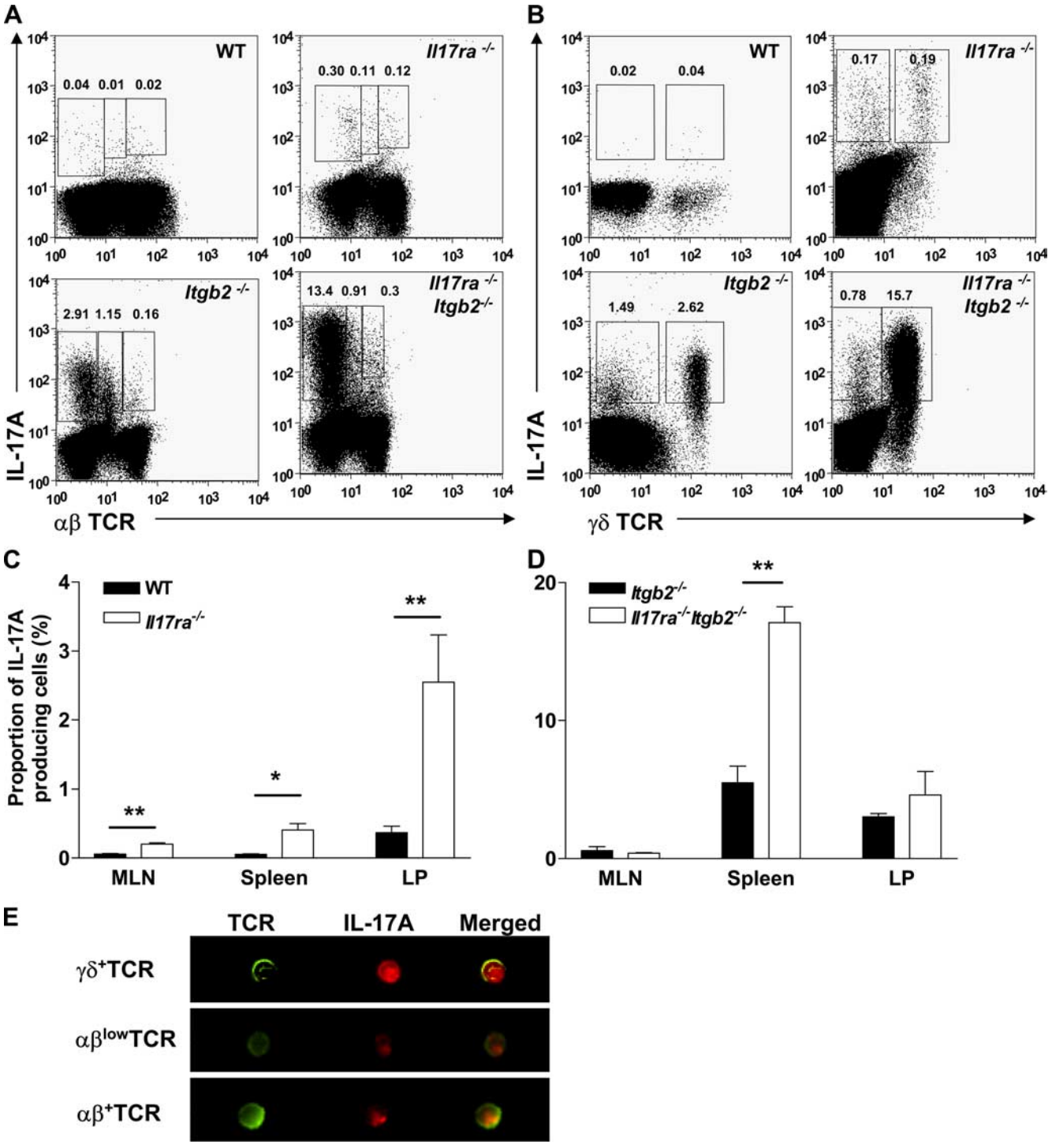

FIGURE 5. IL-17A-producing Tn cell populations are expanded in IL-17R-deficient mice. $\alpha\beta^{high}$ and $\alpha\beta^{low}$ T cells (*A*) and $\gamma\delta^+$ T cells (*B*) in the spleen of WT, *Il17ra*$^{-/-}$, *Itgb2*$^{-/-}$, and *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ mice. IL-17A-producing Tn cell populations are elevated in the MLN, spleen, and LP of *Il17ra*$^{-/-}$ mice compared with WT mice ($n = 4$–12) (*C*), and in the spleen of *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ mice compared with *Itgb2*$^{-/-}$ mice ($n = 3$–13) (*D*). Representative images of IL-17A-producing Tn cell subsets taken by Amnis (*E*). *, $p < 0.05$; **, $p < 0.01$ by unpaired student's *t* test.

Because β_2 integrins are only expressed on BM-derived cells (except glia cells in the brain), blood cells of lethally irradiated mice reconstituted with *Itgb2*$^{-/-}$ BM are completely devoid of all four β_2 integrins (40). To investigate whether the expression of IL-17R on hemopoietic or nonhemopoietic cells was important in regulating circulating neutrophil numbers, BM cells were isolated from *Itgb2*$^{-/-}$ or *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ mice and injected i.v. into lethally irradiated WT or *Il17ra*$^{-/-}$ mice. Mice were analyzed 6–7 wk after BM transfer. *Il17ra*$^{-/-}$ mice reconstituted with *Itgb2*$^{-/-}$ BM had much lower neutrophil counts than WT recipients of *Itgb2*$^{-/-}$ BM (Fig. 1*C*). Their blood neutrophil counts were not significantly different from those of *Il17ra*$^{-/-}$ mice reconstituted with *Itgb2*$^{-/-}$*Il17ra*$^{-/-}$ BM. These findings demonstrate that IL-17R on nonhemopoietic cells has a dominant effect on neutrophil counts. WT mice reconstituted with *Itgb2*$^{-/-}$*Il17ra*$^{-/-}$ BM have high neutrophil counts but not as high as WT mice reconstituted with *Itgb2*$^{-/-}$ BM (Fig. 1*C*). This

suggests that IL-17R on hemopoietic cells also contributes to neutrophil homeostasis, but less than IL-17R on nonhemopoietic cells.

Systemic levels of IL-17A and tissue expression of IL-17A are elevated in IL-17R-deficient mice

Blood sera from *Il17ra*$^{-/-}$ and *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ mice and their respective control groups were analyzed for IL-17A protein by ELISA. IL-17A was not detectable in WT mice, but was elevated in the sera of *Il17ra*$^{-/-}$ mice (70 ± 22 pg/ml). A significant further elevation in systemic IL-17A levels was seen in *Il17ra*$^{-/-}$ *Itgb2*$^{-/-}$ mice compared with the *Itgb2*$^{-/-}$ control group (Fig. 2*A*). This suggests that IL-17A secretion may be controlled by an IL-17R-dependent process and this mechanism is defective in *Il17ra*$^{-/-}$ mice.

G-CSF levels were not detectable in WT or *Il17ra*$^{-/-}$ mice but were elevated in *Itgb2*$^{-/-}$ mice. G-CSF levels were significantly reduced in *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ mice, suggesting that G-CSF is regulated by IL-17A in vivo through IL-17R (Fig. 2*B*). IL-17A has also been reported to induce CCL2, CXCL1, and IL-6 release from stromal cells (11). However, circulating levels of CCL2, CXCL1, and IL-6 were not significantly affected in *Il17ra*$^{-/-}$ mice on either a WT or *Itgb2*$^{-/-}$ background (data not shown). This excludes a role for these cytokines in the regulation of blood neutrophil numbers and suggests that G-CSF is the major cytokine controlling neutrophil production.

Elevated levels of IL-17A and IL-17A-producing Tn cells in IL-17R-deficient mice

To investigate the sources of the elevated systemic levels of IL-17A seen in *Il17ra*$^{-/-}$ mice, we cultured splenocytes from WT and *Il17ra*$^{-/-}$ mice on plate-absorbed anti-CD3ε and soluble anti-CD28 for 3 days in the presence or absence of IL-23, followed by a rest period of 3 days. On day 6, splenocyte cultures were reactivated with a PMA/ionomycin and GolgiStop mixture. In mixed splenocytes, only a small percentage of all cells polarized into IL-17A producers, less than the percentage previously reported for purified naive CD4$^+$ cell cultures (17, 18, 41). The number of IL-17A-producing Tn cells in *Il17ra*$^{-/-}$ splenocyte cultures was significantly increased compared with WT splenocytes cultures. This difference was further enhanced by treatment with IL-23 (Fig. 3, *A* and *B*). Concomitantly, IL-17A secretion into the cell culture supernatants was also significantly increased in *Il17ra*$^{-/-}$ splenocyte cultures (Fig. 3*C*).

In vitro, IL-17A-producing T cells can only be detected following stimulation using PMA/ionomycin. However, detectable serum levels of IL-17A in *Il17ra*$^{-/-}$, *Itgb2*$^{-/-}$, and *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ mice suggests that some T cells actively secrete IL-17A in vivo. To investigate whether basal levels of IL-17A mRNA expression matched the increase in IL-17A protein seen in sera of IL-17R-deficient mice, we monitored IL-17A mRNA expression in various organs (Fig. 4*A*). In IL-17R-deficient mice, an increase in IL-17A mRNA expression was found in the lung, spleen, and small intestines, but not the MLN (Fig. 4*A*). As reported previously, IL-17A message was elevated in the tissue of *Itgb2*$^{-/-}$ mice (12). The levels of IL-17A mRNA were also high in all organs tested (excepting the MLN) of *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ mice (Fig. 4*B*).

IL-17A-producing Tn cell populations are expanded in IL-17R-deficient mice

To investigate the source of elevated IL-17A found in IL-17R-deficient mice, the numbers of IL-17A-producing T cells were assessed in various organs. IL-17A-secreting T cells were significantly expanded in the spleen, MLN, and LP of *Il17ra*$^{-/-}$ mice compared with WT mice, with the majority of IL-17A-producing Tn cells found in the LP (Fig. 5, *A–C*). The proportion (%) of Tn cell subsets remained similar between *Il17ra*$^{-/-}$ and WT mice, with γδ$^+$ T cells being the major Tn cell population, followed by Th17 cells and then CD4$^-$CD8$^-$αβ^{low} T cells (Fig. 5*B* and Table I). Representative Th17, CD4$^-$CD8$^-$αβ^{low}, and γδ$^+$ T cells were analyzed using Amnis technology (Fig. 5*E*).

On the neutrophilic (*Itgb2*$^{-/-}$) background, most Tn cells were found in the spleen and LP (Fig. 5*D*). The expansion of IL-17A-producing T cells found in the spleen of *Itgb2*$^{-/-}$ mice was further exacerbated in *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ mice. γδ$^+$ T cells were the major Tn cell population in both *Itgb2*$^{-/-}$ and *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ mice in all organs assessed. The population of CD4$^-$CD8$^-$αβ^{low} T cells was also expanded above those of Th17 cells (Table II).

Table I. *IL-17A-producing Tn cells in IL-17R-deficient mice*[a]

	Percentage of All IL-17A-Producing Cells					
	γδ$^+$		CD4$^-$αβ^{low}		Th17	
Organs	WT	Il17ra$^{-/-}$	WT	Il17ra$^{-/-}$	WT	Il17ra$^{-/-}$
MLN	54 ± 17	33 ± 7	17 ± 5	28 ± 4	29 ± 7	39 ± 5
Spleen	59 ± 10	54 ± 2	25 ± 17	22 ± 7	16 ± 4	24 ± 9
LP	73 ± 16	65 ± 14	10 ± 6	13 ± 6	17 ± 9	22 ± 11

[a] Cells were gated by forward-side scatter for lymphocytes.

IL-17R regulates the polarization of IL-17A-producing Tn cells

The expansion of IL-17A-producing Tn cells in IL-17R-deficient mice compared with the control groups led us to hypothesize that IL-17A may negatively regulate the expansion of these cells through IL-17R signaling in an autocrine or paracrine manner. To test this hypothesis, splenocytes were isolated from *Itgb2*$^{-/-}$ or *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ mice and cultured as before. IL-17A-producing Tn cells were expanded in *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ splenocyte cultures compared with *Itgb2*$^{-/-}$ cultures by 48% (Fig. 6*A*). IL-17A secretion in the cell culture supernatants was also elevated following cytokine treatment (data not shown).

Treatment of unfractionated *Itgb2*$^{-/-}$ but not *Il17ra*$^{-/-}$ *Itgb2*$^{-/-}$ splenocytes with rIL-17A or IL-17F for 0–6 h caused a significant reduction in IL-17A gene expression compared with untreated cells (Fig. 6, *B* and *C*). Furthermore, IL-17A and IL-17F treatments caused a reduction in IL-17F gene expression in *Itgb2*$^{-/-}$ but not *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ splenocytes (Fig. 6, *D* and *E*). IL-22 mRNA was also down-regulated by 90–99% in response to IL-17A or F (data not shown). Taken together, these data show that both IL-17A and IL-17F can inhibit their own or each other's production through a short feedback loop that requires IL-17R. Whether this is a T cell autonomous or paracrine process remains to be determined.

Table II. *IL-17A-producing Tn cells in* Itgb2$^{-/-}$ *mice*

	Percentage of All IL-17A-Producing Cells					
	γδ$^+$		CD4$^-$αβ^{low}		Th17	
Organs	Itgb2$^{-/-}$	Il17ra$^{-/-}$ Itgb2$^{-/-}$	Itgb2$^{-/-}$	Il17ra$^{-/-}$ Itgb2$^{-/-}$	Itgb2$^{-/-}$	Il17ra$^{-/-}$ Itgb2$^{-/-}$
MLN	57 ± 5	43 ± 11	24 ± 10	17 ± 1	19 ± 6	40 ± 5[a]
Spleen	79 ± 15	95 ± 10	17 ± 6	3 ± 1	4 ± 1	2 ± 1
LP	74 ± 9	70 ± 32	17 ± 7	12 ± 4	9 ± 3	18 ± 11

[a] $p < 0.05$ by nonparametric Mann-Whitney test.

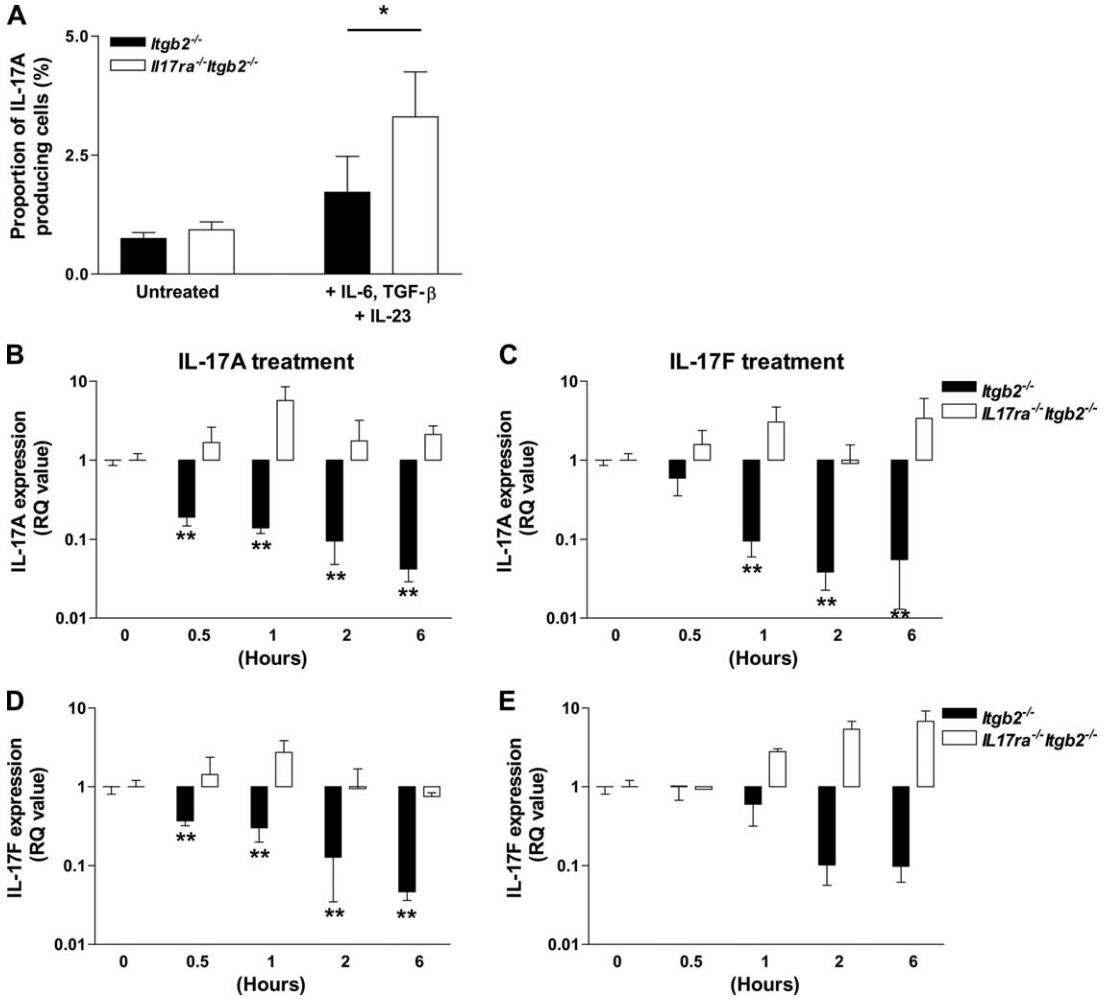

FIGURE 6. IL-17A and F negatively regulate their own expression and expansion of IL-17A-producing Tn cells. Unfractionated splenocytes from *Itgb2*$^{-/-}$ or *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ mice were cultured on anti-CD3ε and soluble anti-CD28, in the presence or absence of IL-6, TGF-β, and IL-23 for 3 days followed by a 3 day rest. Cells were reactivated by PMA/ionomycin in the presence of GolgiStop for 5 h before intracellular staining for IL-17A. Proportion of IL-17A-producing Tn cells (*A*) in cytokine-stimulated *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ cultures (□) compared with *Itgb2*$^{-/-}$ cultures (■) ($n = 3$–4). ∗, $p < 0.05$ by unpaired student's *t* test. IL-17A (*B*: $p < 0.0001$; and *C*: $p < 0.001$ by one-way ANOVA) and IL-17F (*D*: $p < 0.0001$; and *E*: $p < 0.01$ by one-way ANOVA) gene expression was determined in unfractionated *Itgb2*$^{-/-}$ ($n = 3$–4) or *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ ($n = 2$) splenocyte cultures treated with IL-17A (10 ng/ml) or IL-17F (10 ng/ml) from 0 to 6 h. ∗, $p < 0.05$; ∗∗, $p < 0.01$ compared with untreated samples (0 h) by Bonferroni's post hoc test.

Discussion

Mice lacking IL-17R have reduced neutrophil numbers and G-CSF levels. Most of this effect is due to IL-17R expression on nonhemopoietic cells. IL-17A expression, secretion, and the percentage of IL-17A-producing Tn cells were expanded in IL-17R-deficient mice and IL-17A or IL-17F treatment inhibited IL-17A gene expression. This suggests a short feedback loop by which IL-17A (and probably IL-17F) blunts the expansion of IL-17A-producing T cells in vivo and in vitro. Taken together, our data show a key role for IL-17R signaling in neutrophil homeostasis and in controlling the expansion of IL-17A-producing Tn cells (Fig. 7).

The IL-17R-deficient mouse was first generated by Ye et al. (29) to investigate the role of IL-17R signaling in response to infection by *K. pneumoniae*. These mice failed to recruit neutrophils into the lung, resulting in 100% mortality. Circulating neutrophil counts in *Il17ra*$^{-/-}$ mice and the cellular source of IL-17A were not reported (29). As part of its protective role against bacteria, IL-17A has been shown to regulate circulating blood neutrophil counts, with neutrophil numbers positively correlating with IL-17A serum levels in neutrophilic mice (31). Previous work has shown that irradiated *Il17ra*$^{-/-}$ mice show increased mortality and a dose-dependent decrease in blood neutrophil counts and BM and spleen CFU compared with WT mice (42). These findings suggest that IL-17R on stromal cells is required for hemopoietic recovery. Previous studies have demonstrated that various BM stromal cell lines respond to IL-17A by secreting G-CSF and up-regulating the expression of stem cell factor and, thus, must express IL-17R (34). IL-17R expression has also been demonstrated on fibroblast-like cells, mononuclear cells, polymorphonuclear cells, endothelial cells, BM cells, and bone lining cells in the joints of mice (6). Lubberts et al. (6) used BM chimeric mice with IL-17R expression on either hemopoietic or nonhemopoietic cells to demonstrate that IL-17R signaling on nonhemopoietic cells was an essential component of the destructive synovitis found in adjuvant induce arthritis models. This fits well with the present data showing that IL-17R on nonhemopoietic cells is important in regulating the granulopoietic process.

We have previously demonstrated that IL-23 regulates both IL-17A and G-CSF secretion in neutrophilic mice (43). IL-17A regulates G-CSF release from BM-derived stromal cell lines and increases surface expression of stem cell factor, the ligand for c-Kit, in vitro (34). In this study, we demonstrate that G-CSF secretion

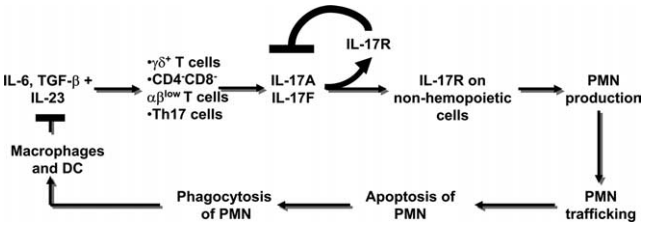

FIGURE 7. Neutrostat feedback loop. Cytokines released by macrophages and dendritic cells (DC) induce the release of IL-17A from three Tn cell subsets. IL-17A binds to IL-17R on nonhemopoietic cells in the BM inducing G-CSF release and elevating blood neutrophil (polymorphonuclear neutrophil; PMN) counts. PMN traffic into the tissue, undergo apoptosis, and become engulfed by tissue resident macrophages and dendritic cells (DC). Phagocytosis of apoptotic PMN curbs IL-23 release from these cells, closing the "long" feedback loop (12, 13). The present findings add a "short" feedback loop (bold lines), which negatively regulates IL-17A and IL-17F expression via IL-17R.

was significantly reduced in IL-17R-deficient mice, which became apparent on a neutrophilic background. Mice lacking G-CSF have chronic neutropenia (44), suggesting that the reduction in neutrophil numbers in *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ mice probably reflects the lack of G-CSF secretion by BM stromal cells in these mice. Even though large amounts of IL-17A are made, IL-17A is ineffective because the receptor is absent on the relevant cells. However, removing IL-17A signaling in *Il17ra*$^{-/-}$*Itgb2*$^{-/-}$ mice did not reduce neutrophil counts to normal WT levels, indicating that other, IL-17R-independent mechanisms can regulate neutrophil numbers in these mice. Such mechanisms might include alternative cytokine pathways.

The increased expression and systemic secretion of IL-17A in IL-17R-deficient mice suggests a possible compensatory mechanism in these mice involving IL-17A. Consistent with our data, Ye et al. (29) found elevated IL-17A levels in the bronchoalveolar lavage fluid of *Il17ra*$^{-/-}$ mice in response to *K. pneumoniae* infection compared with the control group (29). Furthermore, the expansion of IL-17A-producing Tn cell numbers in IL-17R-deficient mice (between 3- and 20-fold) suggests that IL-17A may be able to negatively regulate the polarization of IL-17A-producing cells through IL-17R. The cytokines IFN-γ, IL-2, IL-4, and IL-27 have previously been described to limit the polarization of Th17 cells in vitro and in vivo (8, 23–25). In this study, we demonstrate the expansion of IL-17A-producing Tn cells in vivo using IL-17R-deficient mice. IL-17R also binds two other ligands, IL-17F and IL-17A/F heterodimers. All three ligands can induce downstream signaling events and the release of inflammatory mediators in vitro and in vivo (26, 27, 45–48). Therefore, it is plausible that these ligands may also inhibit the expansion of IL-17A-producing T cells via IL-17R. Indeed, treatment of *Itgb2*$^{-/-}$ but not *Il17ra*$^{-/-}$ *Itgb2*$^{-/-}$ splenocytes with rIL-17A or IL-17F significantly inhibited IL-17A and IL-17F mRNA expression as well as IL-22, but the effects of IL-17A treatment was stronger and more sustained. Taken together, our results suggest that IL-17A is able to moderate its own secretion by inhibiting the expansion of IL-17A-producing Tn cells.

It is clear that the IL-23/IL-17A/G-CSF axis is responsible for most of the neutrophilia seen in mice lacking β_2 integrins (12, 31, 43). This pathway may, therefore, also be of key importance in the neutrophilia seen in human leukocyte adhesion deficiency syndromes (37). The ability of IL-17A to negatively regulate the expansion of IL-17A-producing Tn cells leads us to question the usefulness of anti-IL-17R therapies for autoimmune diseases because they may lead to unwanted expansion of IL-17A-producing T cells. Additionally, if such therapies reduce the circulating neutrophil counts, they can significantly compromise host defense in the patients receiving therapies. It is likely that alternative ways of curbing the proinflammatory effects of IL-17A can be found that circumvent these problems.

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Disclosures

The authors have no financial conflict of interest.

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