IL-17A Controls IL-17F Production and Maintains Blood Neutrophil Counts in Mice[1]

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G-CSF, its receptor, and IL-17 receptor A (IL-17RA) are all required to maintain baseline neutrophil counts in mice. In this study, we tested whether IL-17F could compensate and maintain baseline neutrophil counts in the absence of IL-17A. Unlike the reduced neutrophil counts found in IL-17RA-deficient mice, neutrophil counts were mildly increased in IL-17A-deficient (*Il17a*$^{-/-}$) animals. There was no evidence for infection or altered neutrophil function. Plasma G-CSF and IL-17F levels were elevated in *Il17a*$^{-/-}$ compared with wild-type mice. IL-17F was mainly produced in the spleen and mesenteric lymph nodes, but IL-23 was unaltered in *Il17a*$^{-/-}$ mice. Instead, *Il17a*$^{-/-}$ splenocytes differentiated with IL-6, TGF-β, and IL-23 ex vivo produced significantly more IL-17F in response to IL-23 than wild-type cells. Adding rIL-17A to *Il17a*$^{-/-}$ splenocyte cultures reduced IL-17F mRNA and protein secretion. These effects were also observed in wild-type but not IL-17RA-deficient cells. We conclude that IL-17A mediated suppression of IL-17F production and secretion requires IL-17RA and is relevant to maintain the normal set point of blood neutrophil counts in vivo. ***The Journal of Immunology,*** **2009, 183: 865–873.**

Neutrophilic granulocytes (neutrophils) rapidly localize to sites of infection and are crucial for primary antimicrobial host defense (1). Their short life span requires continuous replacement and robust mechanisms to control their numbers in circulating blood. Beyond transient elevations during acute inflammatory responses, increased baseline circulating neutrophil numbers are a risk factor for all-cause mortality and progression of chronic disease such as atherosclerosis and chronic renal failure (2–4).

The proinflammatory cytokine IL-17A, the signature cytokine of T_H17 cells (5, 6), has been shown to increase neutrophil counts via induction of G-CSF (7, 8). IL-17A is increased in different strains of constitutively neutrophilic, neutrophil adhesion molecule-deficient mice (8). The IL-17RA plays an important role in host defense and reactive neutrophilia (9–12) and is required for bone marrow regeneration (13). In healthy mice lacking IL-17RA (*Il17ra*$^{-/-}$), blood neutrophil counts are ~40% decreased (14, 15). Short-term Ab-mediated IL-17A blockade has been reported to reduce (15) or have no effect (16) on circulating neutrophil counts.

Of the six known members of the IL-17-family, IL-17A and IL-17F share most sequence and structural homology. Both IL-17A and IL-17F require IL-17RA to signal (17–20). IL-17A binds to IL-17RA with higher affinity than IL-17F (18), but the receptor complex contains additional molecules (21). Although IL-17A and F and their heterodimer (22) induce similar cytokines and chemokines, IL-17F induced weaker G-CSF, but similar GM-CSF secretion as IL-17A (19). IL-17F instillation induced less airway neutrophilia than either IL-17A or the IL-17A/F heterodimer (22). In contrast, in vivo adenoviral overexpression of IL-17A and IL-17F resulted in near equal induction of peripheral blood neutrophilia and spleen and bone marrow hematopoietic CFU (23).

Targeted deletion of IL-17A in mice (*Il17a*$^{-/-}$) had differential effects on inflammation and host defense. Contact and airway hypersensitivity as well as collagen-induced arthritis were ameliorated (24, 25), but chemical colitis and graft-versus-host disease were more severe (26, 27). Host defense against *Klebsiella pneumoniae* but not *Listeria monocytogenes* was preserved in *Il17a*$^{-/-}$ mice (12, 28). It is not known whether IL-17A and F have differential roles in maintaining baseline blood neutrophil counts.

Both IL-17A and F can be induced in $CD4^+$ T cells by a combination of TGF-β and IL-6 alone or together with IL-23 (22, 29, 30). Similar histone acetylation patterns were associated with IL-17A and F transcription during T_H17 polarization of $CD4^+$ T cells (31), but IL-17F mRNA appears to be expressed more broadly beyond lymphocytes (18, 32). The nuclear orphan receptor retinoic acid receptor-related orphan receptor (ROR)[3] α has recently be shown to be essential for expression of IL-17A but not F, whereas RORγt is required for both (33).

Our initial screening of *Il17a*$^{-/-}$ mice revealed elevated blood neutrophil counts. Since this is opposite to the reported phenotype of *Il17ra*$^{-/-}$ mice (14, 15) and since IL-17F, like IL-17A requires IL-17RA, we suspected that IL-17F might compensate for the absence of IL-17A. To explore the role of IL-17F in neutrophil homeostasis, we studied blood neutrophils, G-CSF, and IL-17F in mice lacking IL-17A and wild-type controls.

Materials and Methods

Animals

Mice lacking IL-17A (*Il17a*$^{-/-}$) (24), IL-17 receptor (*Il17ra*$^{-/-}$) (11), and wild-type C57BL/6 mice (The Jackson Laboratory) were used between 8 and 16 wk of age. They were kept in specific pathogen-free conditions in barrier facilities. All mice were on a C57BL/6 background for at least 10 generations, genotyped by PCR, and animal experiments were approved by the Institutional Animal Care and Use Committee of the University of

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[3] Abbreviations used in this paper: ROR, retinoic acid receptor-related orphan receptor; for, forward; rev, reverse; MLN, mesenteric lymph node.

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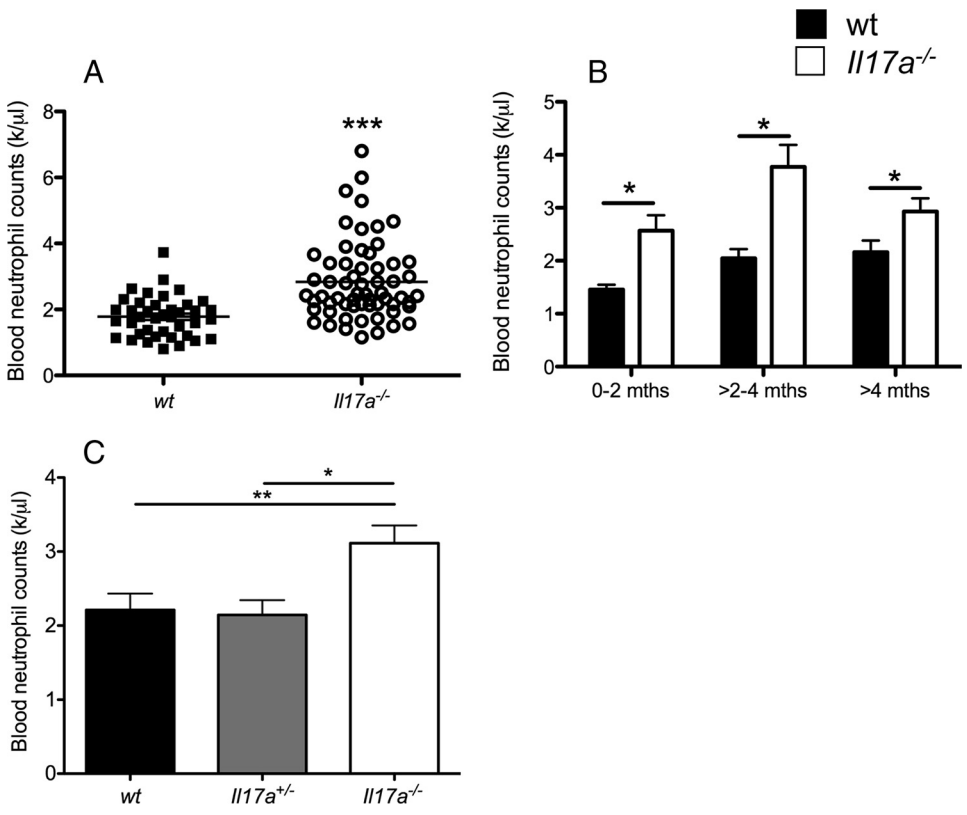

FIGURE 1. Increased peripheral blood neutrophil counts in *Il17a*$^{-/-}$ mice. Neutrophil counts were assessed by an automated counter in mice from 3 wk to 7 mo of age. *Il17a*$^{-/-}$ mice on the C57BL/6 background ($n = 59$) showed significantly increased counts compared with wild-type (wt) C57BL/6 mice ($n = 38$; *A*). *Il17a*$^{-/-}$ neutrophil counts were significantly increased in all age groups (*B*). In a separate experiment, neutrophil counts in *Il17a*$^{+/-}$ heterozygous mice were compared with *Il17a*$^{+/+}$ and *Il17a*$^{-/-}$ mice (*C*, $n = 5$–8 mice at 4–6 wk of age) (∗, $p < 0.05$; ∗∗, $p < 0.01$; and ∗∗∗, $p < 0.001$).

Virginia and the Animal Care Committee at La Jolla Institute for Allergy and Immunology, respectively. Blood was taken via tail bleed into EDTA-coated capillary tubes, analyzed by an automatic analyzer (Hemavet 950FS; DREW Scientific), and differential counts were confirmed by manual counts after Wright-Giemsa stain (Sure stain; Fisher) with a ×100 oil objective on a Nikon eclipse 80i microscope.

Neutrophil function assays

Bone marrow neutrophils (5×10^6 cells/ml) were incubated with PMA or buffer control for 15 min at 37°C and stained with PE-conjugated L-selectin Ab (MEL-14; BD Pharmingen). For assessment of respiratory burst, stimulation was preceded by a 5-min loading step in 1 μM dihydrorhodamine (Invitrogen) and generation of rhodamine was analyzed by flow cytometry. Spontaneous apoptosis was assessed by an annexin V/7-aminoactinomycin D staining kit (BD Pharmingen) according to the manufacturer's instructions after a 20-h culture in complete RPMI 1640 supplemented with 10% FCS at 37°C and 5% CO_2.

Lymphocyte stimulation, staining, and flow cytometry

Cells were stimulated for 5 h with 10 ng/ml PMA, 500 ng/ml ionomycin (both Sigma-Aldrich), and 2 μM monensin (eBioscience). PerCP-Cy5.5-conjugated anti-CD3ε (145-2C11), PE-conjugated anti-δ TCR (GL3), allophycocyanin-conjugated anti-TCR β-chain (H57-597; BioLegend), FITC-conjugated anti-IL-17F (IC2057F; R&D Systems), Pacific Blue-conjugated anti-CD4 (RM 4-5), PE-Cy7-conjugated anti-CD8 (53-6.7), and a LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen) were used. Cells were permeabilized using BD Pharmingen Fix-Perm. Flow cytometry was performed on a BD Biosciences LSRII and analyzed using FlowJo software (Tree Star).

Lymphocyte culture

Splenocytes were stimulated in complete medium (RPMI 1640 containing 10% FCS, nonessential amino acids, 2 mM L-glutamine (all from Invitrogen), and 1% penicillin/streptomycin) by plate-bound purified anti-CD28 and anti-CD3 (BioLegend) in the presence of IL-6 (50 ng/ml), TGF-β (1 ng/ml; PeproTech), and IL-23 (20 ng/ml unless otherwise indicated; eBioscience) in the presence or absence of goat anti-mouse IL-17RA (R&D Systems), IL-17A, and IL-17F (PeproTech, dissolved in dH_2O from lyophilized powder as recommended by the manufacturer, stored at −20°C, and further diluted in RPMI 1640 with all additives, full RPMI 1640 addition served as control) as indicated and replated on untreated plates on day 3. Addition of rIL-17A and F did not significantly alter cell number or viability as assessed by hemocytometer and live-dead stain.

ELISA

Mouse IL-17A and F and G-CSF were assayed using Duo-Set ELISA development kits (R&D Systems) and IL-23 using ELISA Ready-SET-Go (eBioscience) according to the manufacturers' instructions. The manufacturer's claim of specificity for either form of IL-17 was verified by addition of rIL-17A and F. Detection of the IL-17A/F heterodimer was studied using recombinant insect cell IL-17A/F heterodimer (eBioscience).

mRNA quantification

RNA was isolated using TRIzol (Invitrogen) and reverse transcription was conducted with an Omniscript RT-kit (Qiagen) after DNase digest (RNase-free DNase; Promega) according to the manufacturer's instructions. Real-time PCR was performed with a QuantiTect SYBR Green RT-PCR Kit (Qiagen) on a LightCycler 480 (Roche). Primers were selected using primer bank (34) as follows: *Il17f* forward (for), TGCTACTGTTGATG TTGGGAC and reverse (rev), AATGCCCTGGTTTTGGTTGAA; *Il23p19* for, ATGCTGGATTGCAGAGCAGTA and rev, ACGGGGCACATTAT TTTTAGTCT; *Il23p40* for, TGGTTTGCCATCGTTTTGCTG and rev, AC AGGTGAGGTTCACTGTTTCT; *Il23r* for, TTCAGATGGGCATGAATG TTTCT and rev, CCAAATCCGAGCTGTTGTTCTAT; *Il21* for, GGAC CCTTGTCTGTCTGGTAG and rev, TGTGGAGCTGATAGAAGTTCA GG; and *Il22* for, ATGAGTTTTTCCCTTATGGGGAC and rev, GCTG GAAGTTGGACACCTCAA; RORγt primers were as is in Ref. 35. Products were confirmed by melting temperature analysis and gel electrophoresis. Transcript level was normalized to β-actin according to the ΔC_t method.

Statistical analysis

The two-tailed Student *t* test was used after one- or two-way ANOVA if appropriate as indicated. Values of $p < 0.05$ were considered significant. Data are expressed as mean ± SEM.

Results

Increased peripheral blood neutrophil counts in Il17a$^{-/-}$ *mice*

Unlike *Il17ra*$^{-/-}$ mice (14, 15), blood neutrophil counts were significantly elevated in *Il17a*$^{-/-}$ mice (Fig. 1*A*). This was true in all

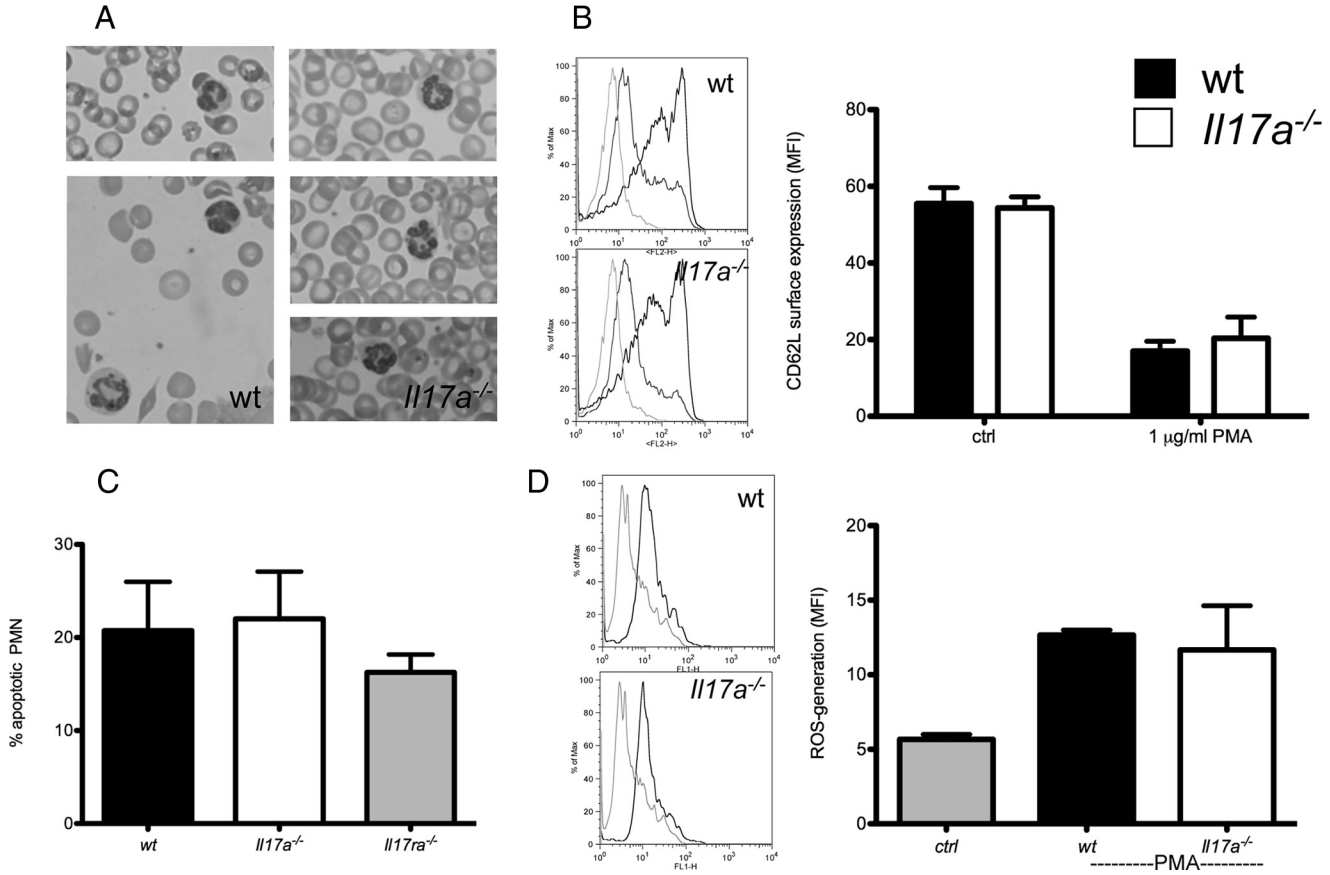

FIGURE 2. Neutrophil characterization in *Il17a*$^{-/-}$ mice. Neutrophils from peripheral blood were morphologically normal as assessed by Wright-Giemsa stain (*A*, representative images from 100 neutrophils of three mice per genotype; ×100 oil objective). There was no evidence for mobilization of immature progenitors. Neutrophil activation was assessed by L-selectin surface expression (*B*, $n = 3$ and representative image; black line, unstimulated cells; gray line, stimulation with 1 μg/ml PMA; light gray line, isotype control (ctrl)). Spontaneous apoptosis of bone marrow neutrophils from wild-type (wt), *Il17a*$^{-/-}$, and *Il17ra*$^{-/-}$ mice was measured by annexin V-PE and 7-aminoactinomycin D staining after a 20-h culture in RPMI 1640 supplemented with 10% FCS (*C*, $n = 4$). Respiratory burst of bone marrow neutrophils (*D*; gray line, unstimulated cells; black line, stimulation with 1 μg/ml PMA for 15 min at 37°C) was assessed by dihydrorhodamine ($n = 3$).

age groups (Fig. 1*B*) and in both male and female animals (data not shown). Very similar results were obtained in two animal facilities (University of Virginia and La Jolla Institute for Allergy and Immunology). Blood neutrophil counts were normal in *Il17a*$^{+/-}$ heterozygous animals (Fig. 1*C*). Counts were unchanged after 4 wk of oral antibiotic treatment with trimethoprim/sulfomethoxazole ($n = 6$ mice; data not shown), making bacterial infection unlikely and suggesting an endogenous cause of increased neutrophil counts.

Neutrophils are functionally normal in Il17a$^{-/-}$ *mice*

Since IL-17A is an important proinflammatory cytokine, we assessed neutrophil function in *Il17a*$^{-/-}$ mice. Blood smears revealed morphologically normal neutrophils and no evidence for immature progenitor mobilization (Fig. 2*A*). L-selectin was equally shed from wild-type and *Il17a*$^{-/-}$ neutrophils (Fig. 2*B*). There was no evidence of altered spontaneous apoptosis of bone marrow neutrophils from *Il17a*$^{-/-}$ or *Il17ra*$^{-/-}$ mice (Fig. 2*C*). IL-17A and F in concentrations up to 100 ng/ml did not alter apoptosis of BM neutrophil progenitors (data not shown). Activation-dependent generation of reactive oxygen species in *Il17a*$^{-/-}$ neutrophils was very similar to that in wild-type neutrophils (Fig. 2*D*).

Plasma G-CSF and IL-17F are significantly increased in Il17a$^{-/-}$ *mice*

Neutrophil numbers correlate positively with plasma G-CSF levels (7, 8). We found a significant elevation of G-CSF in *Il17a*$^{-/-}$ mouse plasma (Fig. 3*A*) and in stimulated bone marrow supernatants (Fig. 3*B*). These data suggest that in the absence of IL-17A, more functionally normal neutrophils are generated via elevation of circulating G-CSF.

To test the role of the other known IL17-RA agonist, IL-17F, its production was studied at the mRNA and protein levels. IL-17F plasma concentration was significantly increased in *Il17a*$^{-/-}$ mice compared with nondetectable levels in wild-type animals (Fig. 3*C*). IL-17F mRNA expression was significantly up-regulated in mesenteric lymph nodes (MLN) of *Il17a*$^{-/-}$ but not *Il17a*$^{+/-}$ mice under baseline conditions (Fig. 3*D*). To further assess the organs responsible for elevated IL-17F levels, cell suspensions were stimulated ex vivo and supernatants were assayed for IL-17F (Fig. 3*E*). Elevated IL-17F levels were found in *Il17a*$^{-/-}$ but not *Il17a*$^{+/-}$-stimulated supernatants from spleen, MLN, and thymus. Since the IL-17F ELISA was ~10-fold less sensitive for detecting IL-17A/F heterodimer, the measured levels effectively represent IL-17F/F homodimer. No significant differences between wild-type and *Il17a*$^{-/-}$ mice was found in small intestine or bone marrow IL-17F mRNA expression or IL-17F protein secretion into the supernatants (data not shown). The number of IL-17F-producing cells as assessed by flow cytometry after intracellular IL-17F staining was significantly increased in spleen and MLN of *Il17a*$^{-/-}$ mice (Fig. 3*F*). Most IL-17F producers expressed the γδ TCR and were CD4, CD8 negative (Fig. 3*G*). Similar to previously

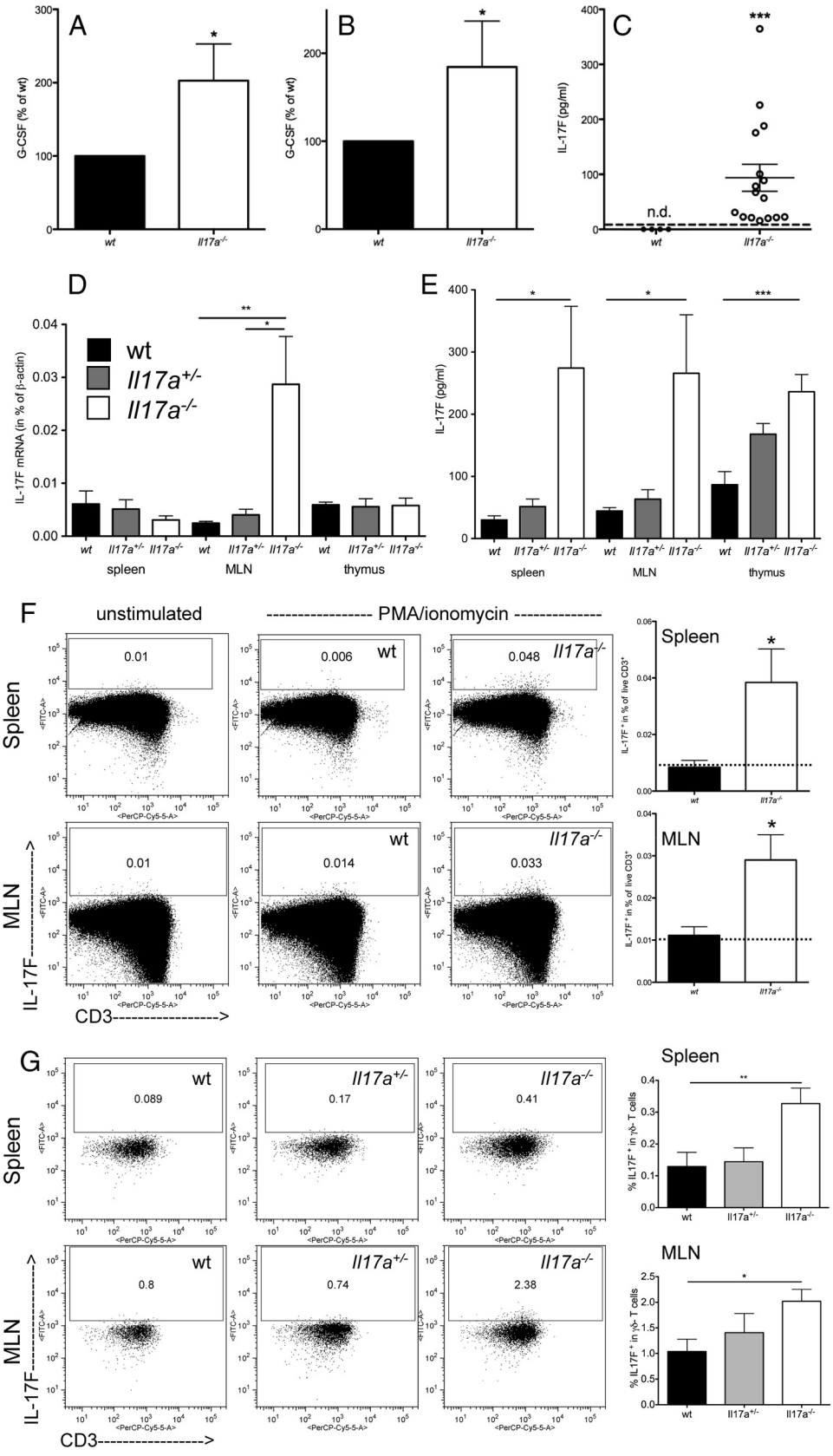

FIGURE 3. Increased G-CSF and IL-17F in $Il17a^{-/-}$ mice. G-CSF was measured in plasma (*A*, $n = 4$) and in PMA/ionomycin-stimulated bone marrow supernatants (*B*, $n = 13$) from $Il17a^{-/-}$ and wild-type (wt) mice. IL-17F in plasma from wild-type and $Il17a^{-/-}$ animals is shown in *C* ($n = 4$ for wild type and $n = 16$ for $Il17a^{-/-}$). IL-17F mRNA was significantly increased in MLN of $Il17a^{-/-}$, but not in $Il17a^{+/-}$ mice (*D*, $n = 5$ per genotype). IL-17F protein was measured in supernatants of wild-type, $Il17a^{+/-}$, and $Il17a^{-/-}$ splenocytes, MLN, and thymocytes after a 5-h PMA/ionomycin stimulation (*E*, $n = 6$). IL-17F-expressing cells were analyzed by flow cytometry after intracellular staining, (gated for live, CD3-positive cells). Their proportion was significantly increased in $Il17a^{-/-}$ compared with wild-type mice in spleen and MLN but not in thymus (*F*, $n = 5$ each and data not shown). IL-17 expression was analyzed as in *F* and gated for the γδ-TCR- positive compartment (*G*, $n = 10$ (wild type), $n = 5$ ($Il17a^{+/-}$) and 17 ($Il17a^{-/-}$)). Percentages of IL-17F-positive cells are given with the gate set to include 0.01% of unstimulated splenocytes/MLN cell controls (dotted line in *right panel*) (*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$).

reported findings (27), we did not find significant differences in the proportion of $CD8^+$, $CD4^+$, αβ, or γδ T cells in $Il17a^{-/-}$ mice (data no shown). These data show that IL-17F production is increased in the absence of IL-17A in vivo and suggest that IL-17F induces G-CSF in the absence of IL-17A, thereby driving granulopoiesis.

No evidence for increased spontaneous IL-23 expression in the absence of IL-17A

IL-23 is a major factor in propagating IL-17A- and F-producing T cells (5, 29, 36). Since IL-17A deficiency might result in

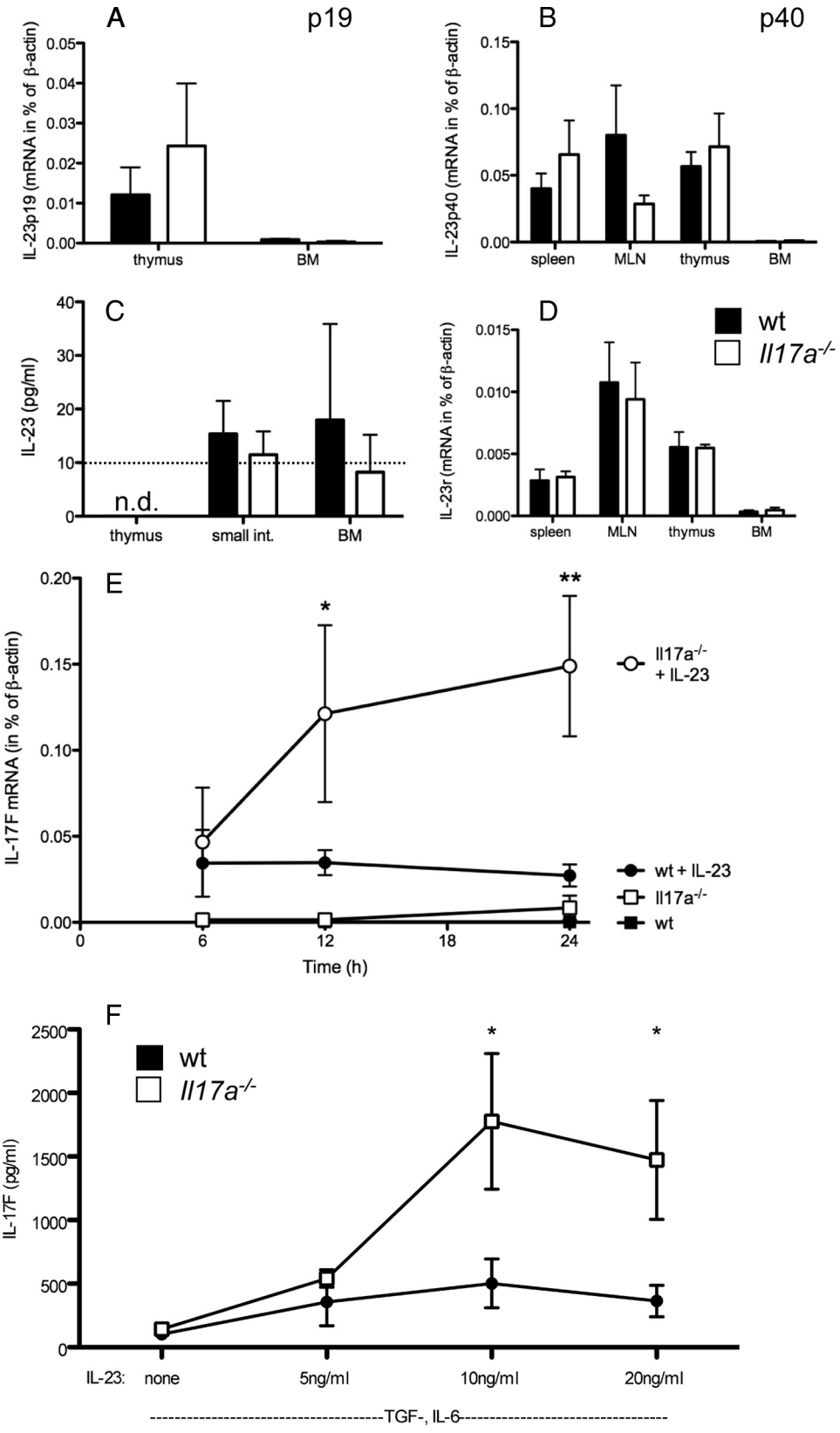

FIGURE 4. Increased IL-17F response to IL-23 in *Il17a*$^{-/-}$ mice. IL-23 mRNA was measured by quantitative real-time PCR for the p19 (*A*, $n = 4$–7) and p40 subunits (*B*, $n = 4$–9) in the indicated organs from wild-type (wt; ■) and *Il17a*$^{-/-}$ (□) mice. IL-23p19 levels in spleen and MLN were not detectable. IL-23 secretion was assessed by ELISA in PMA/ionomycin-stimulated organ supernatants (*C*, $n = 3$–5) revealing no significant differences (dotted line, detection limit). IL-23 receptor mRNA expression was also similar in *Il17a*$^{-/-}$ and wild-type spleen, MLN, thymus, and bone marrow (BM; *D*, $n = 5$). Total splenocytes were differentiated toward IL-17 production by IL-6 (50 ng/ml) and TGF-β (1 ng/ml) with and without IL-23 (20 ng/ml) (black: wild-type mice; white *Il17a*$^{-/-}$, squares without IL-23; circles, IL-23). IL-17F mRNA expression was assessed after 6, 12, and 24 h (*E*, $n = 4$). IL-17F production as assessed by ELISA after 3-day rest and restimulation with PMA/ionomycin was increased in *Il17a*$^{-/-}$ cells compared with wild-type cells at 10 and 20 ng/ml IL-23 (*F*, $n = 4$; $*$, $p < 0.05$ for difference between wild type and *Il17a*$^{-/-}$ after two-way ANOVA).

reactively increased IL-23 production (37), we tested whether IL-23 production was altered in *Il17a*$^{-/-}$ mice. We found no difference in mRNA expression of either the IL-23-specific p19 subunit or the p40 subunit shared with IL-12 (Fig. 4, *A* and *B*). There was no significant difference in stimulated IL-23 protein secretion between wild-type and *Il17a*$^{-/-}$ tissues (Fig. 4*C*). The IL-23 receptor is strongly induced by IL-23 (36, 38, 39). Consistent with unaltered IL-23 concentrations, mRNA for the IL-23 receptor was not altered in the *Il17a*$^{-/-}$ mice (Fig. 4*D*).

Spontaneous mRNA expression of the nuclear receptor RORγt, which has been shown to be essential in IL-17A, but less so in

IL-17F expression (33) was very similar in wild-type and *Il17a*$^{-/-}$ spleen, MLN, and thymus (supplemental Fig. 1[4]).

Increased secretion of IL-17F in response to IL-23 in the absence of IL-17A

To test the response of IL-17F production to IL-23 in *Il17a*$^{-/-}$ and wild-type splenocytes, IL-17-producing T cells were induced according to established protocols (29, 35) by culturing mouse splenocytes with IL-6, TGF-β, and IL-23 on plate-bound anti-CD28 and anti-CD3ε. In splenocytes from 6-wk-old *Il17a*$^{-/-}$ and wild-type mice, IL-17F mRNA expression after 12 and 24 h of differentiation was increased in *Il17a*$^{-/-}$ compared with wild-type splenocytes (Fig. 4*E*). With IL-6 and TGF-β, but without IL-23, *Il17a*$^{-/-}$ splenocytes showed a trend toward more IL-17F production at 24 h (Fig. 4*E*). No difference was seen when no cytokines were added (data not shown). On day 3, cells were washed, transferred to untreated plates, and cultured for an additional 3 days. IL-17F secretion into the supernatant after stimulation with PMA/ionomycin for 5 h was significantly elevated in *Il17a*$^{-/-}$ splenocytes treated with either 10 or 20 ng/ml IL-23 (Fig. 4*F*). Collectively, these data suggest that removing IL-17A from the mouse genome induces systemic IL-17F overproduction.

IL-17A reduces IL-17F-producing T cells via IL-17RA

Since the lack of IL-17A resulted in an increase in IL-17F production, we tested whether exogenous IL-17A would suppress IL-17F production. IL-17A was added during days 1–3 of splenocyte culture on anti-CD28 and anti-CD3ε. After a 3-day rest period, IL-17F secretion into the supernatant was assessed (Fig. 5*A*). IL-17F production from *Il17a*$^{-/-}$ splenocytes was significantly blunted by the addition of rIL-17A. In contrast, addition of IL-17F did not alter IL-17F secretion. Concomitant with reduced IL-17F protein levels, IL-17F mRNA after 24 h of differentiation was dose-dependently decreased by IL-17A (Fig. 5*B*). The same effect was found for IL-22, but not IL-21, other signature cytokines of the T_H17 lineage (Fig. 5*C*). Expression of the IL-23 receptor and the transcription factors RORα and RORγt were unaffected (data not shown). These results show that exogenous IL-17A reduces IL-17F mRNA and protein production in *Il17a*$^{-/-}$ splenocytes. The lower levels of IL-17F secreted by wild-type splenocytes suggest that endogenous levels of IL-17A curb IL-17F secretion.

To test whether IL-17A also regulates IL-17F production in wild-type cells, we exposed splenocytes from wild-type mice to IL-17A during the early phase of polarization. IL-17F mRNA (Fig. 6*A*) after 24 h of IL-6, TGF-β, and IL-23 treatment was significantly decreased in the presence of IL-17A (10 ng/ml; Fig. 6*A*). This effect was not seen in the presence of anti-IL-17RA Ab. Spontaneous IL-17F secretion from wild-type splenocytes after 24 h was decreased by the addition of IL-17A (Fig. 6*B*). Adding IL-17A during the first 3 days of differentiation dose-dependently decreased the number of IL-17F-producing splenocytes as detected by flow cytometry (Fig. 6*C*). No decrease in IL-17F producers was observed in *Il17ra*$^{-/-}$ splenocytes, confirming that IL-17A-mediated inhibition of IL-17F required IL-17RA (Fig. 6*D*). To test whether IL-17A itself or another soluble factor secreted in response to IL-17A from other splenocytes was responsible for IL-17F inhibition, IL-17F mRNA expression in *Il17ra*$^{-/-}$ splenocytes was assessed after a 24-h culture. It was neither inhibited by direct addition of IL-17A (data not shown) nor by incubation with tissue culture supernatant from IL-17A-treated *Il17a*$^{-/-}$ splenocytes (Fig. 6*E*), suggesting that the inhibitory action is directly on the IL-17 producers and not mediated via another soluble agent. These data establish that IL-17A negatively regulates IL-17F production in normal mice. This feedback inhibition is unavailable in *Il17a*$^{-/-}$ mice, explaining elevated levels of IL-17F and G-CSF and elevated neutrophil counts.

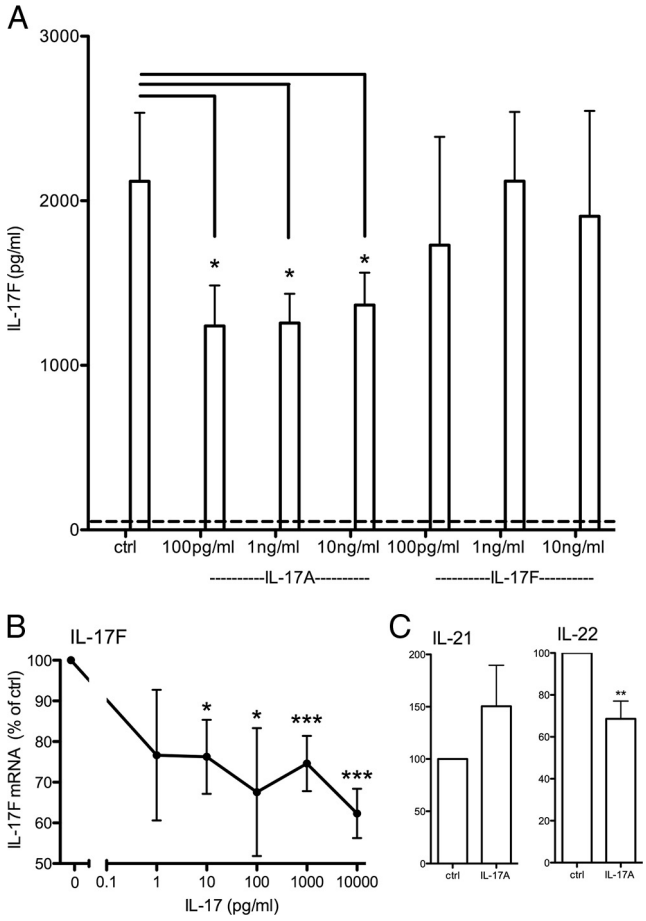

FIGURE 5. Effect of IL-17A on IL-17F producer differentiation. Total splenocytes were differentiated toward IL-17 production by IL-6, TGF-β, and IL-23 (20 ng/ml) in the presence or absence of rIL-17A or IL-17F. After 72 h, cells were washed, transferred to an untreated plate, rested for 72 h, and subsequently restimulated with PMA/ionomycin. IL-17F secretion into the supernatant was assessed by ELISA. IL-17A significantly inhibited IL-17F production from *Il17a*$^{-/-}$ cells (*A*, $n = 4$). IL-17F mRNA expression after 24 h of differentiation in the presence or absence of different doses of IL-17A in *Il17a*$^{-/-}$ splenocytes was assessed by real-time RT-PCR (*B*, $n = 6–8$). The expression of IL-21 and IL-22 in *Il17a*$^{-/-}$ splenocytes as further signature cytokines of IL-17 producers was assessed after 24 h with and without 10 ng/ml IL-17A (*C*) (*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$).

Discussion

Il17a$^{-/-}$ mice have elevated neutrophil counts and G-CSF and IL-17F levels. IL-17F production was most elevated in spleen and MLN. We did not find evidence for infection as a trigger of increased neutrophil counts, nor did neutrophils in *Il17a*$^{-/-}$ mice show signs of activation such as spontaneous L-selectin shedding or an altered life span. Together with the reduction of IL-17F production by IL-17A, these data show that a physiologically relevant feedback loop is nonfunctional in *Il17a*$^{-/-}$ mice, leading to IL-17F overproduction. Similar to our results, elevated IL-17F protein in the absence of IL-17A was shown in a colitis model in a different strain of *Il17a*$^{-/-}$ mice (40) while this manuscript was

[4] The online version of this article contains supplemental material.

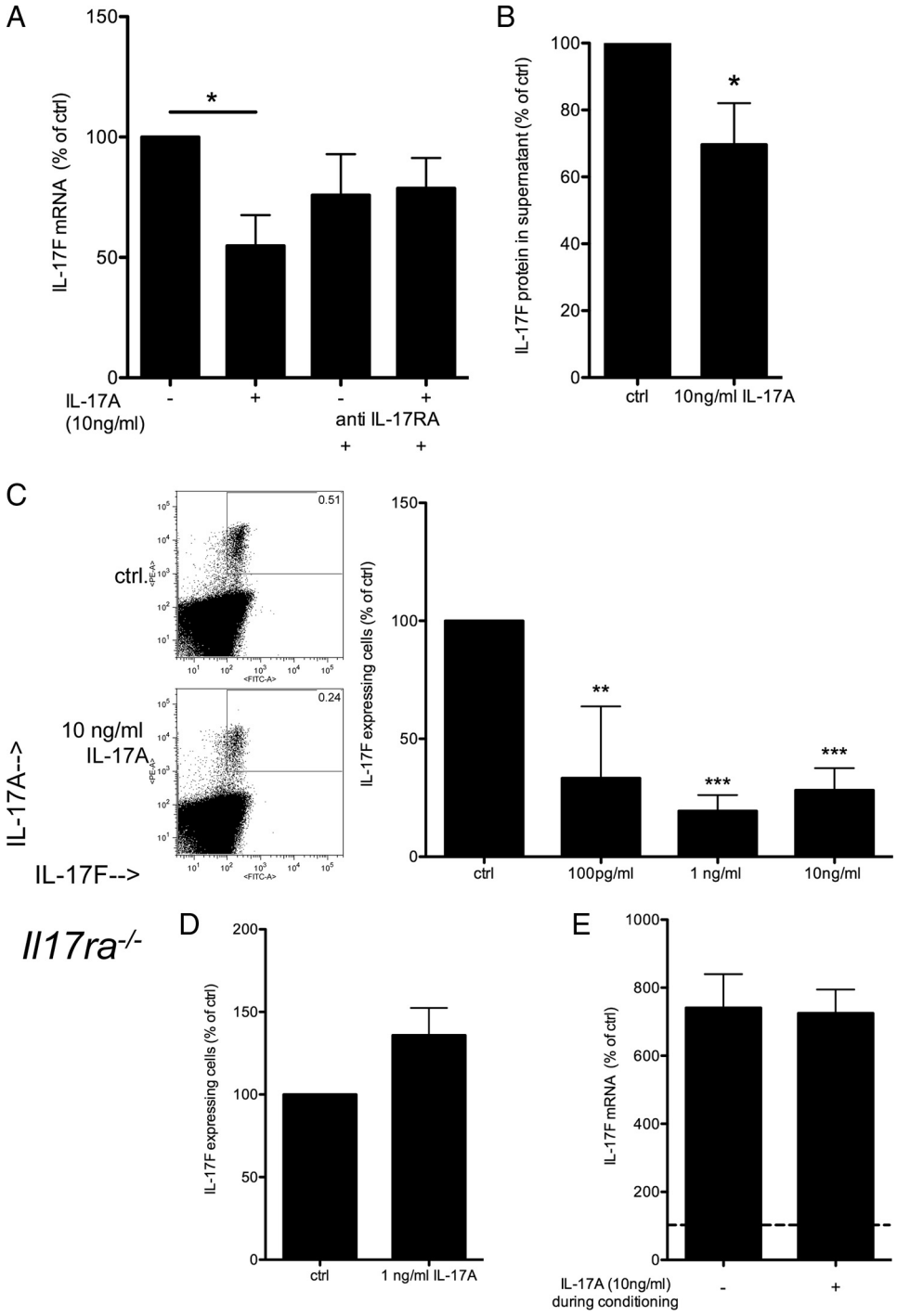

FIGURE 6. IL-17A suppresses IL-17F-production in wild-type splenocytes. IL-17F mRNA expression with and without exogenous IL-17A (10 ng/ml) during 24 h of differentiation in wild-type splenocytes with and without polyclonal anti-IL-17RA Ab (10 μg/ml; *A*, $n = 6$). Spontaneous IL-17F secretion into the supernatant during the first 24 h of differentiation was also significantly decreased by exogenous IL-17A (*B*, $n = 3$). To test whether this early difference translated into an effect on the number of IL-17F producers, their percentage of CD3-positive, live cells was determined by flow cytometry on day 6 (*C*, representative plots and compared with splenocytes without exogenous IL-17A, $n = 3$–7). No change was seen in *Il17ra*$^{-/-}$ splenocytes stimulated with IL-17A, confirming that IL-17RA was required for IL-17A-mediated inhibition of IL-17F (*D*, $n = 4$). To test whether IL-17A acts directly on the IL-17F producers or induces secretion of another soluble factor from other splenocytes, *Il17ra*$^{-/-}$ splenocytes were incubated with 50% conditioned medium (from *Il17a*$^{-/-}$ splenocytes stimulated with IL-6/TGF-β/IL-23 for 24 h with and without IL-17A (10 ng/ml)) and 50% fresh RPMI 1640 for 24 h on anti-CD3/anti-CD28. IL-17F mRNA expression was determined (*E*, $n = 8$, data expressed as induction over unconditioned RPMI 1640) (*, $p < 0.05$ and **, $p < 0.01$ after ANOVA if more than two conditions were compared). ctrl, Control.

under review. Also, recent work on IL-17A and IL-17F in a number of infection and autoimmune models (41) contains flow cytometry data that, in the absence of statistical analysis, may be suggestive of higher IL-17F expression in ex vivo-polarized and restimulated *Il17a*$^{-/-}$ lymphocytes.

IL-17A and F are both known to affect G-CSF production and release (19, 23), but IL-17A has higher affinity for the IL-17RA receptor (18). Our observations extend previous evidence of in vivo IL-17F granulopoietic activity from adenoviral overexpression (23) to endogenous cytokine production. These results, along with recent data on more pronounced neutrophil recruitment in response to IL-17F than IL-17A (42), establish IL-17F as an effective in vivo neutrophil regulatory agonist.

IL-23 is a key cytokine for IL-17A and F production and is involved in maintaining baseline and increased neutrophil counts (29, 43). Its expression in macrophages and dendritic cells can be suppressed by phagocytosis of apoptotic neutrophils and thereby act as a neutrophil regulatory feedback loop (37). In this study, in *Il17a*$^{-/-}$ mice with moderately increased neutrophil counts, IL-23 production was normal, arguing that IL-17F overexpression was unlikely to result from up-regulated IL-23. Instead, we found that IL-17F production from *Il17a*$^{-/-}$ splenocytes was markedly more responsive to IL-23 than wild-type cells.

Our finding that this phenotype could at least partially be reversed by exogenous IL-17A proposes IL-17A as an autocrine inhibitor of IL-17F production. Evidence for an IL-17-autocrine

feedback loop was first suggested by IL-17A and F mRNA inhibition in ex vivo-stimulated splenocytes from β_2 integrin-deficient mice that have an expanded population of IL-17 producers (15). The initial work on IL-23-induced IL-17 production in $CD4^+$ T cells (29) found no effect of anti-IL-17A Abs on IL-17A producer differentiation, but the investigators did not measure IL-17F protein and did not add exogenous IL-17A. The present work is the first to show that IL-17F can be inhibited by IL-17A in wild-type cells at the protein level and that absence of IL-17A leads to elevated blood neutrophil counts. Similar to findings in splenocytes from β_2 integrin-deficient mice (15), we did not find a significant down-regulation of IL-17F protein by up to 50 ng/ml IL-17F (Figs. 5 and 6 and data not shown) that exceed by far concentrations found in plasma. IL-17F mRNA appears to be down-regulated by IL-17F at very high doses in $CD4^+$ T cells (44); however, IL-17F protein was not reported in that study. In healthy wild-type mice, most IL-17 producers are $\gamma\delta^+$- or $CD4^-\alpha\beta^{low}$ cells rather than $CD4^+$ T cells (15, 37, 45, 46). We found most splenic IL-17F producers in the $\gamma\delta$-TCR^+ compartment. Whether differential IL-17 receptor subunit expression in the IL-17- producing T cell subtypes accounts for differential responses to IL-17A and F remains to be investigated.

Inhibition of excess IL-17F production in *Il17a*$^{-/-}$ splenocytes by IL-17A was incomplete. We applied exogenous IL-17A and IL-17F during the first 3 days of IL-6, IL-23, and TGF-β treatment on anti-CD28 and anti-CD3 and some IL-17F production may have recovered during the subsequent rest period. Also, some of the IL-17F protein made by wild-type cells may have been secreted as A/F heterodimer (22). IL-17A/F heterodimer is recognized by the ELISA used in this study but with 10-fold lower sensitivity. Thus, IL-17A/F heterodimer, if present, would have been underestimated.

The *Il17a* and *Il17f* genes reside in tail-to-tail orientation in relative proximity on mouse chromosome 6 (47) with most promoter activity at least for IL-17A located upstream of the gene (48). Our findings of normal IL-17F mRNA expression and neutrophil counts in *Il17a*$^{+/-}$ heterozygous mice exclude that elevated IL-17F levels are a side effect of homologous recombination. Together with our finding that IL-17A inhibited IL-17F in splenocytes from wild-type mice, this suggests that the observed inhibition is not an artifact of the *Il17a*$^{-/-}$ mice and IL-17A suppresses IL-17F in normal mice.

The feedback by IL-17A on IL-17F appears to dampen IL-17F secretion in vivo. This may explain why IL-17A deficiency is not beneficial in all forms of pathological inflammation. In a model of reactive chemical colitis, mice lacking IL-17A were more affected than wild-type mice (49). Conversely, mice without IL-17F had less weight loss than wild-type mice and less intestinal chemokine expression in a similar model (26). Also, a recent publication showed aggravation of graft-versus-host disease in the absence of IL-17A (27). IL-17F expression was not investigated in this model, but increased IL-17F production due to lack of feedback inhibition could possibly have played a role in the more severe disease. In models of pulmonary inflammation, although IL-17F instillation induced less neutrophil airway infiltration than either IL-17A or the IL-17A/F heterodimer (22), IL-17A-deficient mice had more neutrophil airway infiltration after OVA challenge than either IL-17F- or IL-17RA-deficient mice (26). Since IL-17A and IL-17F are induced at the same time in various forms of inflammation (5, 29, 50, 51), a lack of IL-17A-mediated IL-17F suppression would provide one pathway to explain these findings.

Under physiological conditions, while IL-17A and F act as peripheral IL-17 effectors, our data suggest that IL-17A curbs IL-17F production, resulting in limited secretion despite the presence of IL-17-stimulating factors such as IL-23. Elevated IL-17F explains elevated G-CSF and blood neutrophil levels in *Il17a*$^{-/-}$ mice, which are likely caused by the lack of this short circuit "brake" resulting in an altered IL-17F production set point.

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Disclosures

The authors have no financial conflict of interest.

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