T-Lineage Cells Require the Thymus but Not V(D)J Recombination to Produce IL-17A and Regulate Granulopoiesis In Vivo[1]

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IL-17A and IL-17F regulate granulopoiesis and are produced by memory T cells. *Rag1*$^{-/-}$ recombinase-activating gene-deficient mice cannot produce mature T cells but maintain normal neutrophil counts. Athymic nude mice are neutropenic or have near-normal neutrophil counts, depending on the prevailing intestinal flora, and do not produce IL-17A. By contrast, thymi from *Rag1*$^{-/-}$ mice contain as much IL-17A as those from wild-type (WT) mice. IL-17A-producing cells are found in the double negative DN1 compartment of the *Rag1*$^{-/-}$ thymus and express intracellular CD3. These cells colonize the spleen and mesenteric lymph node and secrete IL-17A in vitro following stimulation with IL-23 at a level similar to that of WT splenocytes. Adoptively transferred *Rag1*$^{-/-}$ or WT thymocytes correct neutrophil counts in neutropenic nude mice. We conclude that the development of IL-17A-producing T-lineage cells requires an intact thymic epithelium, but not V(D)J recombination. ***The Journal of Immunology,*** **2009, 183: 5685–5693.**

Interleukin-17A has pleiotropic effects and is important in the pathology of many disease processes, including rheumatoid arthritis (1–3), experimental autoimmune encephalitis (4, 5), and inflammatory bowel disease (6, 7). IL-17A also controls neutrophil homeostasis (8–11) and elevated granulopoiesis induced by infections (12–16). IL-17A is produced by activated T cells with a memory phenotype (CD44^{high}CD62L^{low}) (17). Three major subpopulations of IL-17A-producing T cells have been identified, which we have collectively termed neutrophil regulatory T cells: CD4$^-$CD8$^-$ $\alpha\beta^{low}$, CD4$^+$CD8$^-$ $\alpha\beta^+$ (Th17 cells), and $\gamma\delta^+$ T cells (10, 18). $\gamma\delta^+$cells are the most common IL-17A-producing T cells in C57BL/6 wild-type (WT)[4] mice, and CD4$^-$CD8$^-$ $\alpha\beta^{low}$ cells are the most common in severely neutrophilic mice (15, 18). Naive CD4$^+$ T cells develop into Th17 cells in the presence of IL-6, IL-23, TGF-β_1, and TCR stimulation if the Th1 and Th2 cytokines IFN-γ and IL-4 are neutralized (5, 19–23). Some IL-17A-producing T cells express CD8 (24). Beyond T cells, IL-17A has been shown to be produced by lymphoid tissue inducer (LTi) cells (25). The baseline serum concentration of IL-17A in WT mice, in the absence of inflammation, is below or near the detection limit of existing ELISAs (26, 27).

Much less is known about IL-17F, which has been reported to be involved in inflammatory diseases of the airways (28). In the initial description of the IL-17F knockout mouse (*Il17f*$^{-/-}$), it appeared that absence of IL-17F alone did not produce much of a spontaneous phenotype, but neutrophil counts and granulopoiesis were not investigated (30). When challenged, T cells from *Il17f*$^{-/-}$ mice showed reduced secretion of IL-17A in response to immunization with keyhole limpet hemocyanin and increased IgG2a production (29). *Il17f*$^{-/-}$ mice showed a striking lack of CCL2, CCL5, and CCL7 expression in a model of dextran sulfate sodium-induced colitis (29). In a separate study, Ishigame et al. found that IL-17F was also produced by intestinal epithelial cells (30). In many situations, IL-17A and F are produced by the same cells, but both IL-17A and IL-17F single-positive lymphocyte populations exist (23, 31).

T cell development occurs in phases of increasing lineage commitment (32). Some murine T cell deficiency models are neutropenic and have perturbed granulopoiesis, suggesting that basal granulopoiesis is regulated by mature T cells (33, 34). One neutropenic model is the nude mouse, which has a mutation at the nude locus in the *whn* gene, also called *Foxn1*, resulting in an undeveloped thymic epithelium and almost no thymus-derived T cells (35). In nude and athymic mice, neutropenia is thought to occur due to an accumulation of immature myeloid and band cells in the bone marrow (BM), as neutrophils are unable to differentiate effectively (33, 36). In other reports, athymic mice have been shown to have normal or even elevated blood neutrophil counts (37–39). The reason for this discrepancy is unknown. In contrast to nude mice, the number of blood neutrophils is normal in RAG-1-deficient mice, although these mice do not produce mature T cells owing to an absolute block during T cell development at the double negative (DN)3 stage due to defective V(D)J rearrangement (40, 41). Similar to *Rag1*$^{-/-}$ mice, *Myb*$^{f/f}$ cwLckCre mice (where "f" is "floxed") also have a block at the DN3 to DN4 stage of

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[4] Abbreviations used in this paper: WT, wild type; BM, bone marrow; DN, double negative; f, floxed; LP, lamina propria; LTi, lymphoid tissue inducer; MLN, mesenteric lymph node.

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thymocyte development (42) but no B cell defect. These mice have a normal phenotype, although their total thymus cellularity is reduced by 70% compared with littermate controls due to a lack of double-positive cells (42). Blood neutrophil numbers were not previously reported in *Myb*$^{f/f}$ cwLckCre mice.

Previous work has demonstrated that adoptive transfer of WT thymocytes can correct neutropenia found in nude mice (33, 43). These studies predated the discovery of the roles of IL-17A and IL-17F in neutrophil homeostasis (10), so IL-17A and IL-17F-producing cells were not investigated. To test whether a lack of IL-17A- and/or IL-17F-producing lymphocytes may be responsible for the neutropenia found in nude mice, we measured IL-17A and IL-17 F at the mRNA and protein levels in nude mice kept in two different vivaria at the University of Virginia (Charlottesville, VA) and the La Jolla Institute of Allergy and Immunology (La Jolla, CA), respectively. In both environments, nude mice were unable to produce IL-17A. Although nude mice were neutropenic at the Virginia facility, they were not neutropenic at the La Jolla Institute of Allergy and Immunology. IL-17F can compensate for a deficiency in IL-17A with respect to controlling blood neutrophil counts (27). Recent work also suggests that the intestinal flora controls the production of IL-17A (44). Th17 cell differentiation in the lamina propria (LP) of the small intestine was found to require specific commensal bacteria, and Th17 polarization was inhibited by treating mice with selective antibiotics. Furthermore, mice from different sources had marked differences in their Th17 cell numbers, and animals lacking Th17 cells acquired them after the introduction of bacteria from Th17 cell-sufficient mice (44).

To test the role IL-17A-producing T cells and their precursors in neutrophil homeostasis, we transferred unfractionated or DN1 cell-sorted thymocytes from WT or *Rag1*$^{-/-}$ mice into neutropenic nude mice. Following the adoptive transfer of WT thymocytes, IL-17A-producing T cells predominantly homed to the LP and were largely CD4^{+} Th17 cells. IL-17A was efficiently produced by cells lacking a functional TCR and without prior TCR engagement. IL-17A-producing cells were found in the thymus and periphery of WT, *Rag1*$^{-/-}$, and *Myb*$^{f/f}$ cwLckCre mice but not nude mice with a neutropenic phenotype. Collectively, our data suggest that the development of IL-17A-producing T-lineage cells requires input from the thymic epithelium, but not mature TCR, to regulate granulopoiesis.

Materials and Methods

Animals

Rag1$^{-/-}$ (41), β_2 integrin (*Itgb2*$^{-/-}$; Ref. 45), C57BL/6$^{nude/nude}$ (nude; 35), *Myb*$^{f/f}$ cwLckCre and littermate controls (42), and WT C57BL/6 (CD45.2) or congenic C57BL/6 CD45.1 (The Jackson Laboratory) mice were used between 6 and 16 wk of age. Myb$^{f/f}$ cwLckCre mice and littermate controls were on a mixed 129/SVJ and C57BL/6 background as previously described (42). All other mice were on a C57BL/6 background for at least 10 generations. All animal experiments were approved by the Animal Care and Use Committee of the University of Virginia or the La Jolla Institute of Allergy and Immunology. Both animal facilities are certified specific pathogen free, and a list of pathogens regularly tested for can be found in supplemental Table I.[5]

Recombinant proteins and Abs

The following mAbs (all reagents from BD-Pharmingen unless otherwise indicated) were used: biotin-conjugated lineage negative panel, PE-conjugated anti-IL-17A (TC11-18H10.1); purified or allophycocyanin-conjugated anti-CD3ε (145-2C11); allophycocyanin-CY7- or PerCP-conjugated anti-CD4 (RM4-5); allophycocyanin-conjugated anti-CD8a (53-6.7); FITC-, Pacific Blue-, or PE-conjugated anti-CD11b (M1/70); allophycocyanin-conjugated anti-CD19 (1D3); FITC-conjugated anti-Pan NK Cells (DX5); anti-CD16/CD32 (2.4G2; Lymphocyte Culture Center, University of Virginia); FITC-conjugated anti-CD24; PE-CY7-conjugated CD25 (PC61); purified anti-CD28 (37.51); allophycocyanin-CY7-conjugated anti-CD44 (IM7); PE-conjugated anti-CD45.1 (A20); FITC-conjugated anti-CD45.2 (104); PerCp-CY5.5-conjugated anti-CD69 (H1.2F3); FITC- or allophycocyanin-conjugated anti-GR-1 (RB6-8C5); allophycocyanin-conjugated anti-NK1.1 (PK136); FITC- or allophycocyanin-conjugated anti-TCR β-chain (H57-597); FITC-conjugated anti-$\gamma\delta$ TCR (GL3); allophycocyanin-conjugated anti-CD117 (2B8; eBioscience); allophycocyanin-conjugated anti-CD127 (A7R34; eBioscience); PE-CY7-conjugated anti *Sca*I (D7) (Biolegend); FITC-conjugated CD90.2 (Thy-1) (53-2.1); FITC-conjugated anti-integrin β_7 chain (M293); allophycocyanin-conjugated anti-CCR7 (4B12) and allophycocyanin-conjugated anti-CCR9 (242503; R&D Systems); allophycocyanin-conjugated anti-L-selectin (MEL-14). PerCP-conjugated streptavidin was used for the detection of the biotin-conjugated primary Ab.

Lymphocyte cell culture

Splenocytes were isolated as previously described (10) and cultured in RPMI 1640 containing 10% FBS, 1× nonessential amino acids (Invitrogen), 10 mM HEPES, 2 mM L-glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen), and 1% penicillin/streptomycin in the presence or absence of plate-absorbed purified anti-CD3ε (10 μg/ml) and soluble anti-CD28 (10 μg/ml), 10 ng/ml PMA (Sigma-Aldrich) and 500 ng/ml calcium ionophore (Sigma-Aldrich), IL-23 (20 ng/ml; R&D systems), IL-6 (100 ng/ml; R&D systems), and/or TGF-β_1 (1 ng/ml; PeproTech) for 3 days.

Flow cytometry

Single-cell suspensions from the thymus, spleen, bone marrow, and mesenteric lymph node (MLN) tissues and LP lymphocytes were prepared as previously described (10, 46) and treated with 10 ng/ml PMA (Sigma-Aldrich), 500 ng/ml calcium ionophore (Sigma-Aldrich), and GolgiStop (BD Pharmingen) for 6 h. Fcγ III/II receptors were blocked with 0.5 μg of anti-CD16/CD32 and the cell suspension was incubated with an optimal concentration of mAbs. Intracellular staining was performed using Fix & Perm cell permeabilization reagents (Caltag Laboratories) according to manufacturer's instructions. Flow cytometry analysis was performed on a BD FACSCalibur or LSR II apparatus (BD Biosciences) and data were analyzed using FlowJo software (Tree Star). Gates were set by isotype controls. Alternatively, live thymocytes were stained and sorted for DN1 population using BD FACSVantage SE Turbo Sorter (BD Biosciences) under aseptic conditions. IL-17A protein in the cell culture and thymus supernatants was measured by Quantikine M mouse IL-17A ELISA kit (R&D Systems). Blood counts were taken via tail bleed into EDTA-coated capillary tubes and analyzed by automatic analyzer (Hemavet 850; CDC Technologies) and confirmed by Kimura-stained manual counts using a hemocytometer.

Adoptive transfer experiments

Single-cell suspensions from thymi of *Rag1*$^{-/-}$ or CD45.1^{+} WT mice were created by straining tissues through a 70-μm nylon mesh. CD45.1^{+} or *Rag1*$^{-/-}$ thymocytes were depleted of myeloid cells using CD11b microbeads (Miltenyi Biotec), and 10^7 cells suspended in 500 μl of sterile PBS were injected i.v. into recipient nude mice under sterile conditions. Cell-sorted DN1 thymocytes (1.5×10^5) suspended in 500 μl of sterile PBS were injected into nude mice recipients. Nude mice were given autoclaved water supplemented with antibiotics (5 mM sulfamethoxazole, and 0.86 mM trimethoprim (Sigma-Aldrich). Neutropenic rescue experiments were analyzed 8–9 wk after adoptive transfer. Homing experiments were analyzed at 1 wk after transfer. For *Rag1*$^{-/-}$ homing experiments, the thymi from *Rag1*$^{-/-}$ mice were pooled (6–10 mice) and labeled with 2 μM CFSE (Molecular Probes) as previously described (47) before injection into nude mice. Single cell suspensions of pooled WT thymocytes (3–8 mice) were depleted of CD4^{+} and CD8^{+} cells with anti-CD4- and anti-CD8-conjugated beads (Miltenyi Biotec) according to manufacturer's instructions to enrich for DN cell populations.

Immunohistochemistry

The MLN and spleen were removed from *Rag1*$^{-/-}$ mice. Immunohistochemistry was performed on 5-μm paraffin sections following heat-induced Ag retrieval with unmasking solution (Vector Laboratories). Sections were probed with goat anti-mouse CD3 (M-20); Santa Cruz Biotechnology) and detected using Vectastain Elite kit (Vector Laboratories). Positive cells were identified by staining with diaminobenzidine (DakoCytomation). Counterstaining was performed using Harris hematoxylin (Richard-Allen Scientific).

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

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Table I. *Blood neutrophil counts and serum IL-17A levels for each mouse strain*[a]

Facility	Mice	Blood Neutrophil Counts (K/μl)	IL-17A (pg/ml)
UVA	$Rag1^{-/-}$	1.5 ± 0.1	ND
	WT	1.9 ± 0.2	ND
	Nude	0.4 ± 0.2§	ND
LIAI	$Rag1^{-/-}$	1.7 ± 0.2	Not done
	WT	2.1 ± 0.2	ND
	Nude	2.8 ± 0.2*	ND
	$Il17a^{-/-}$	3.0 ± 0.2*	ND

[a] Mean ± SEM, n = 4–9. ND, Not detectable (<12 pg/ml).

§, $p < 0.01$, significantly different from all other groups at the University of Virginia (UVA) facility.

*, $p < 0.05$, significantly different from $Rag^{-/-}$ at the La Jolla Institute for Allergy and Immunology (LIAI) facility.

Statistical analysis

Data were expressed as mean ± SEM. Statistical significance between groups was set at $p < 0.05$ using a two-tailed Student's t test or a non parametric Mann-Whitney U test.

Results

TCR expression or engagement is not required for the production of IL-17A

The number of blood neutrophils was assessed in WT mice and mice lacking mature T cells. WT and $Rag1^{-/-}$ mice were found to have normal circulating neutrophil numbers, which was confirmed in a second animal facility at the La Jolla Institute of Allergy and Immunology (Table I). BM cellularity was similar in $Rag1^{-/-}$ mice and WT mice (supplemental Fig. 1). C57BL/6$^{nude/nude}$ mice at the University of Virginia showed an 80% reduction in neutrophil counts compared with WT mice and were considered neutropenic (Table I). Serum IL-17A was not detectable in any mice used in the present study (Table I). Nevertheless, IL-17A-producing cells were readily detectable in the spleen, MLN (10), and LP of WT mice and in the MLN of $Rag1^{-/-}$mice following activation with PMA/ionomycin (Fig. 1, *a* and *b*). The IL-17A-producing cells detected in the MLN of $Rag1^{-/-}$ mice were Thy-1$^+$, NK1.1$^-$, and GR-1$^-$ suggesting that they did not originate from myeloid progenitors (Fig. 1*c*). Most IL-17A-producing cells found in the MLN of $Rag1^{-/-}$ mice expressed intracellular CD3 (Fig. 1, *d* and *e*), indicating that the IL-17A-producing cells in $Rag1^{-/-}$ mice are of T cell lineage. IL-17A-producing cells were not detectable in the MLN, spleen, or LP of nude mice housed at University of Virginia (supplemental Fig. 2).

Although nude mice were consistently neutropenic at the University of Virginia, nude mice from the same source (The Jackson Laboratory) showed consistently elevated neutrophil counts at the La Jolla Institute of Allergy and Immunology (Table I). In nude mice, at both the University of Virginia and the La Jolla Institute of Allergy and Immunology IL-17A protein was below the detection limit in plasma (Table I), but plasma IL-17F was detectable (33.9 pg/ml ± 14.5).

To test whether cells lacking a TCR were capable of secreting IL-17A, we stimulated splenocytes from WT, $Rag1^{-/-}$, and nude mice for 3 days in the presence of PMA/ionomycin, with and without IL-23 and plate-bound anti-CD3ε. IL-23 did not induce IL-17A secretion in cell supernatants from nude mice (Fig. 1*f*), but WT and $Rag1^{-/-}$ splenocytes produced IL-17A protein at equal levels in response to PMA/ionomycin and IL-23. As expected, plate-bound anti-CD3ε enhanced IL-17A secretion from WT, but not $Rag1^{-/-}$ splenocytes. These findings show that TCR engagement is not required for the secretion of IL-17A in secondary lymphoid tissues.

IL-17A-producing Rag1$^{-/-}$ *thymocytes can rescue neutropenia*

Mature T cells can regulate granulopoiesis as demonstrated by increased blood neutrophil numbers after engraftment of WT thymi or adoptive transfer of CD4$^+$ T cells into nude mice (33). In these studies it was assumed that functional TCR was required for this function, but this was not formally investigated. To directly test whether thymus-derived cells of $Rag1^{-/-}$ mice could also normalize neutrophil numbers in nude mice, myeloid cell-depleted (>99.9%) $Rag1^{-/-}$ thymocytes or CD45.1$^+$ WT thymocytes (as a positive control) were adoptively transferred into nude mice. At 8 wk, nude mice that received $Rag1^{-/-}$ or WT thymocytes had significantly elevated blood neutrophil counts (Fig. 2*a*). Total bone marrow cellularity remained unaltered in recipient nude mice receiving $Rag1^{-/-}$ thymocytes but was elevated in mice receiving CD45.1$^+$ WT thymocytes (Fig. 2*b*). Nude mice that received $Rag1^{-/-}$ thymocytes had significantly more GR-1$^+$ BM neutrophils and neutrophil precursors compared with the saline control (compare Fig. 2, *c* and *d*). All neutrophils in the nude mice were CD45.2$^+$ recipient-derived (99.8 ± 0.1% CD45.2$^+$), and not from contaminating donor-derived (CD45.1$^+$) myeloid cells (Fig. 2*e*). These results demonstrate that thymocytes lacking a functional TCR can regulate granulopoiesis to the same extent as WT thymocytes.

After the adoptive transfer of thymocytes, IL-17A-producing cells were found in the spleen and LP of nude mice receiving $Rag1^{-/-}$ thymocytes, although a lower proportion was detected than that in nude mice receiving WT thymocytes (Fig. 2, *f* and *g*). IL-17A-producing cells were not found in the periphery of nude mice reconstituted with saline (supplemental Fig. 3). The largest number of IL-17A$^+$ cells was found in the LP of the adoptively transferred nude mice, suggesting that IL-17A-producing cells preferentially home to GALT. IL-17A-producing cells found in nude mice reconstituted with $Rag1^{-/-}$ thymocytes were Thy-1$^-$, CD11b$^-$, CD117$^-$, and NK1.1$^-$ (Fig. 2*h*). The majority of IL-17A-producing cells found in nude mice reconstituted with WT thymocytes also lost Thy-1 expression and were CD25$^-$CD69$^+$CD4$^+$ T cells (Fig. 2*i* and data not shown).

IL-17A-producing thymocytes home to GALT

We reasoned that homing to GALT might be important in correcting neutrophil numbers after adoptive transfer. To compare the homing of WT CD45.1$^+$ (Fig. 3*a*) and $Rag1^{-/-}$ (Fig. 3*b*) thymocytes, we injected CFSE-labeled $Rag1^{-/-}$ thymocytes into nude mice. After 1 wk, the majority of $Rag1^{-/-}$CFSE$^+$ thymocytes were found in the spleen, with a lower number detected in the MLN and LP (Fig. 3*b*). As expected (48), most IL-17A-producing donor-derived T cells from WT mice were found in the LP (Fig. 3*c*). All of the transferred IL-17A-producing CD45.1$^+$ T cells in the MLN were CD44^{high}, and 87% expressed β_7 integrin (Fig. 3*d*).

IL-17A-producing cells are found in the DN1 compartment of WT and Rag1$^{-/-}$ *thymi*

The ability of IL-17A-producing thymocytes from $Rag1^{-/-}$ mice to reconstitute normal granulopoiesis in nude mice suggests that a normal thymic epithelium, but not TCR gene rearrangement, is required for the differentiation of these cells. IL-17A protein was detectable in thymus supernatants from WT (90 ± 16 pg/ml) and $Rag1^{-/-}$ (174 ± 23 pg/ml) mice (Fig. 4*a*). Among the unfractionated thymocytes of WT mice, IL-17A-producing T cells were detectable at a low frequency (data not shown). These IL-17A-producing T cells were found among mature $\gamma\delta^+$, CD4$^+$, and CD8$^+$

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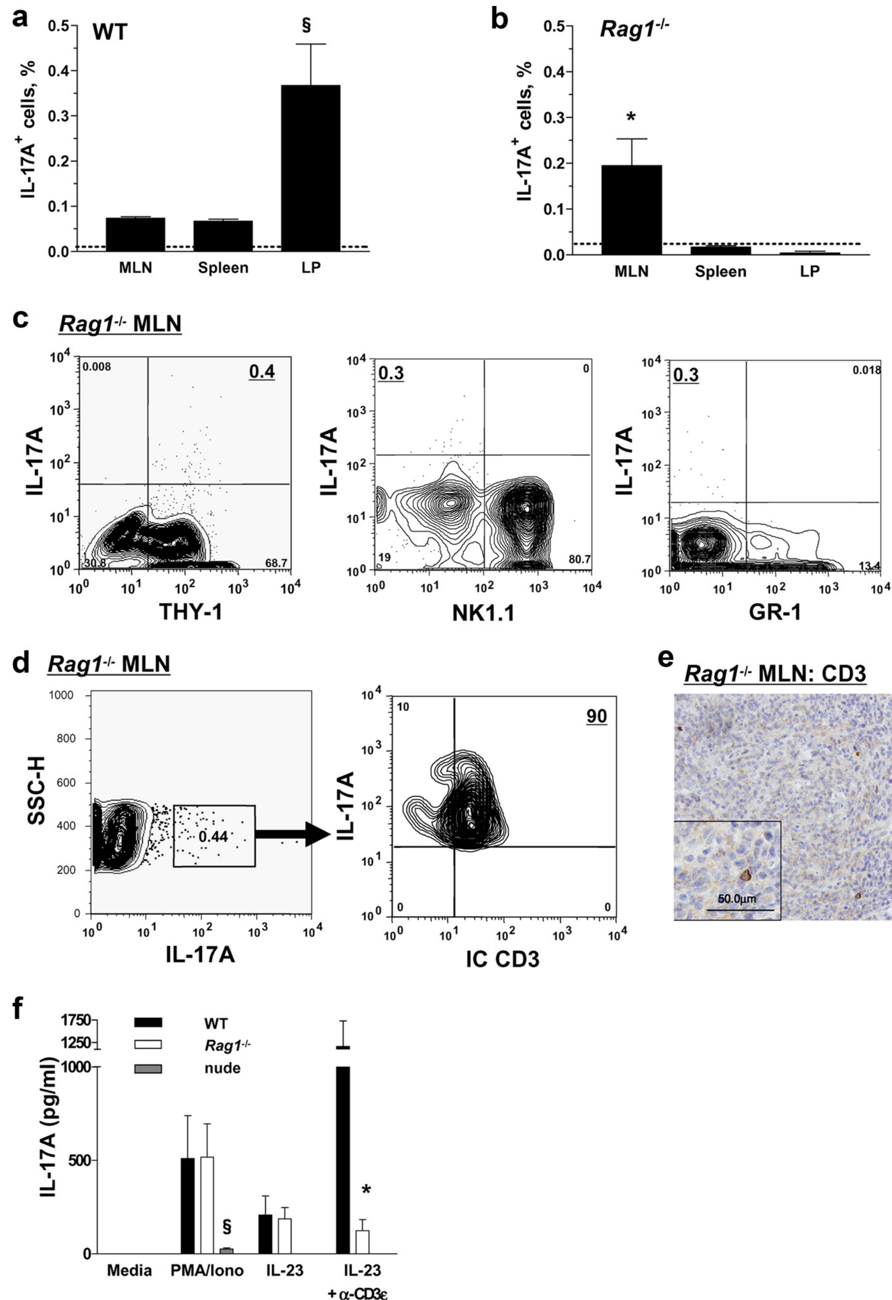

FIGURE 1. Rag1$^{-/-}$ but not nude mice contain IL-17A-secreting cells and have normal blood neutrophil numbers. *a* and *b*, IL-17A-producing cells were found in the MLN, spleen, and LP of WT (n = 3–7) mice (*a*) and in the MLN of *Rag1*$^{-/-}$ (n = 3–7; dashed line, isotype control) mice (*b*). §, $p < 0.05$, significantly different from MLN and spleen; ∗, $p < 0.05$, significantly different from spleen and LP. *c* and *d*, IL-17A-producing cells in the MLN of *Rag1*$^{-/-}$ mice were Thy-1^{+}, NK1.1^{-}, and GR-1^{-} (*c*) and 90% of the IL-17A^{+} population in the *Rag1*$^{-/-}$ MLN expressed intracellular (IC) CD3 (*d*). *e*, CD3^{+} cells in the MLN of *Rag1*$^{-/-}$ mice were identified by immunoperoxidase staining. SSC-H, Side scatter height. *f*, Unfractionated splenocytes from WT, *Rag1*$^{-/-}$, or nude mice (n = 4 each) were stimulated with IL-23 (20 ng/ml), PMA (10 ng/ml) and ionomycin (500 ng/ml) with or without plate-absorbed anti-CD3ε (α-CD3ε; 10 μg/ml) for 3 days. ∗, $p < 0.01$, significantly different from WT mice with same treatment; §, $p < 0.01$, significantly different from all other mice with same treatment.

single-positive and double-positive cells (data not shown) and also in the DN1 compartment of WT mice (Fig. 4, *b* and *c*). Thymocytes were gated on the lineage-negative population and labeled with Abs to CD44 and CD25 (Fig. 4*b*). The DN1 IL-17A-producing cells were lineage^{-}CD25^{-}, CD44^{high}, CD24^{-}, CD117$^{+/-}$, and CD127$^{low/+}$ (Fig. 4, *b* and *c*, and data not shown). The proportion of IL-17A-producing thymocytes was enriched ~5-fold in the *Rag1*$^{-/-}$ thymi compared to WT thymi, possibly due to the decrease in total thymus cellularity seen in these mice. IL-17A-producing thymocytes in *Rag1*$^{-/-}$ mice were also CD25^{-}, CD44^{high}, CD24^{-}, and CD117^{+} (Fig. 4*d*), consistent with the DN1 stage of development.

Rag1$^{-/-}$ mice have a developmental block at the DN3 to DN4 transition but also have a defect in B cell development. To confirm that IL-17A-producing cells indeed arise before this block in a second model, we used *Myb*$^{f/f}$ cwLckCre mice. In these mice, Cre under the *Lck* promoter inactivates both alleles of the floxed c-Myb gene, which leads to a block at the DN3 to DN4 stage of thymocyte development (42). IL-17A-producing thymocytes in the *Myb*$^{f/f}$*c-wLckCre* mice were 5-fold elevated compared with those in the littermate controls (Fig. 4, *e* and *f*) (42). As in *Rag1*$^{-/-}$ mice, blood neutrophil counts were normal in *Myb*$^{f/f}$ *cwLckCre* mice (data not shown).

DN1 thymocytes can correct neutropenia in nude mice

To directly test whether DN1 thymocytes contained IL-17A-producing cells that were able to correct neutropenia, we reconstituted nude mice with flow cytometry-sorted DN1 thymocytes (CD25^{-}CD44^{high}CD24^{-}) from WT CD45.1^{+} mice. These cells normalized blood neutrophil counts (Fig. 5*a*) and were able to home to secondary lymphoid tissue with the largest proportion found in the LP (Fig. 5*b*), where some of these cells expressed IL-17A (Fig. 5*c*). A small population of CD45.1^{+} DN1 cells (1.0% ± 0.3) was found in the BM of the treated

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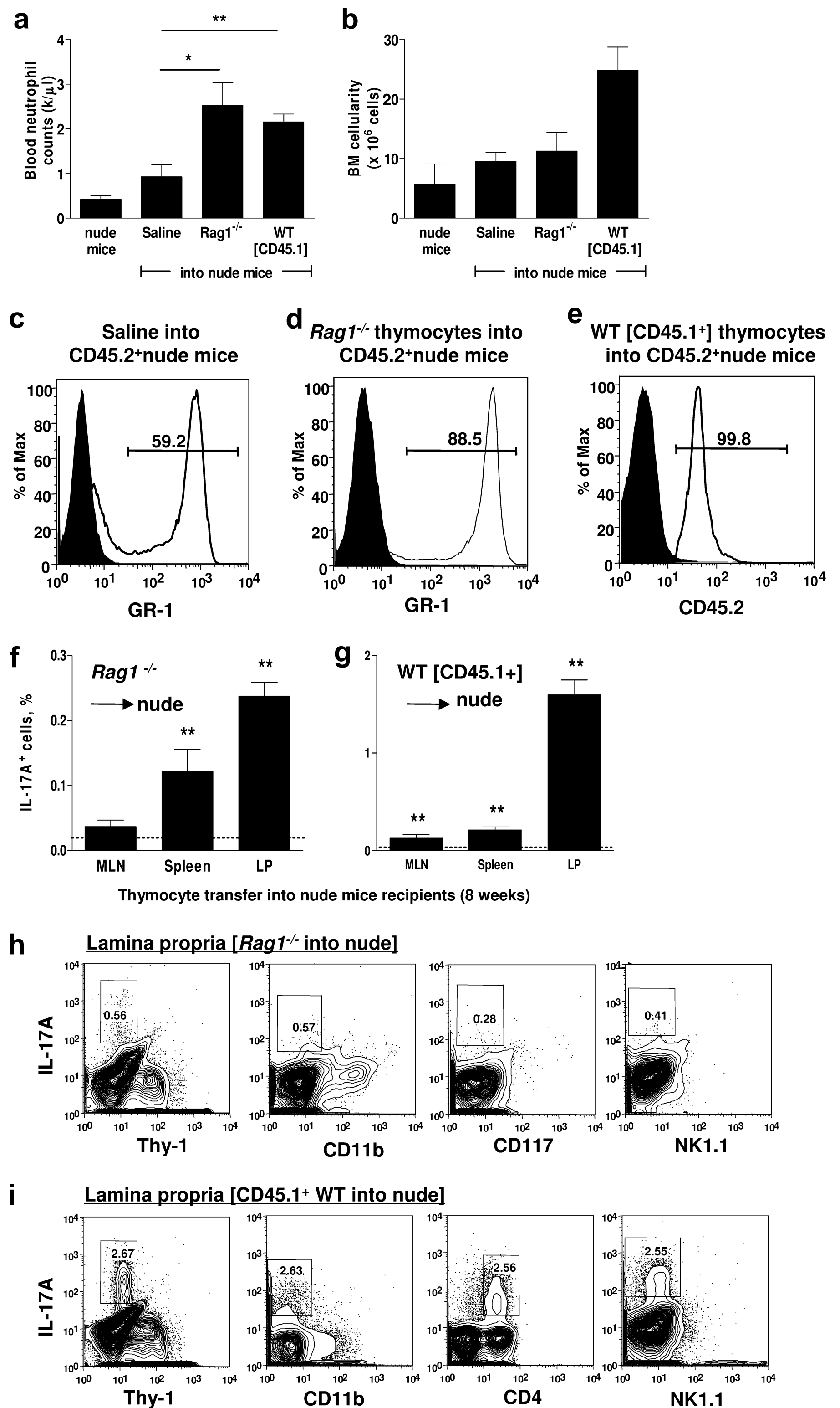

FIGURE 2. IL-17A-producing $Rag1^{-/-}$ thymocytes regulate granulopoiesis. *a* and *b*, Myeloid cell-depleted thymocytes from $Rag1^{-/-}$ mice or CD45.1$^+$ WT mice (as positive controls) were adoptively transferred by i.v. injection into nude mice, and blood neutrophil levels (*a*) and BM cellularity (*b*) were measured after 8 wk (n = 4 each). *c* and *d*, Gr-1$^+$ cells in the BM of nude mice receiving saline (63 ± 6%) (*c*) or $Rag1^{-/-}$ thymocytes (85 ± 2%; $p < 0.05$) (*d*). *e*, GR-1$^+$ neutrophils and precursors in the BM (99.8%) were derived from the recipient (CD45.2$^+$) and not the WT donor (CD45.1$^+$). Filled histogram indicates the isotype control. *f* and *g*, IL-17A-producing cells were analyzed in the MLN, spleen, and LP of nude mice injected with $Rag1^{-/-}$ (*f*) or WT thymocytes (*g*). **, $p < 0.01$, significantly different from isotype control (dashed line). *h* and *i*, Eight weeks after the adoptive transfer of $Rag1^{-/-}$ thymocytes (*h*) or CD45.1$^+$ thymocytes (*i*) into nude mice, IL-17A-producing cells in the LP were analyzed for Thy-1, CD11b, CD117, and NK1.1 expression or CD4 expression. Cells were gated on the lymphocyte population by forward and side scatter.

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nude mice, but they did not secrete detectable IL-17A (Fig. 5). These data suggest that contact with thymic epithelium, but not V(D)J rearrangement, is needed for the development of IL-17A-producing cells and the regulation of granulopoiesis.

Discussion

We demonstrate that IL-17A can be released from thymus-derived cells without TCR gene rearrangement and therefore without TCR engagement. IL-17A-producing T cells were found in the DN1 compartment of WT mice, but also $Rag1^{-/-}$ and $Myb^{f/f}$ cwLckCre mice, which have a complete and partial block at the DN3 to DN4 stages of thymocyte development, respectively (41, 42). IL-17A-producing cells were also detected in the MLN of RAG-1 deficient mice, where they expressed intracellular CD3, but not myeloid markers. This suggests that these cells are of T cell lineage. Unfractionated thymocytes from $Rag1^{-/-}$ mice were able to fully restore normal neutrophil levels in neutropenic nude mice, as were cells isolated from the DN1

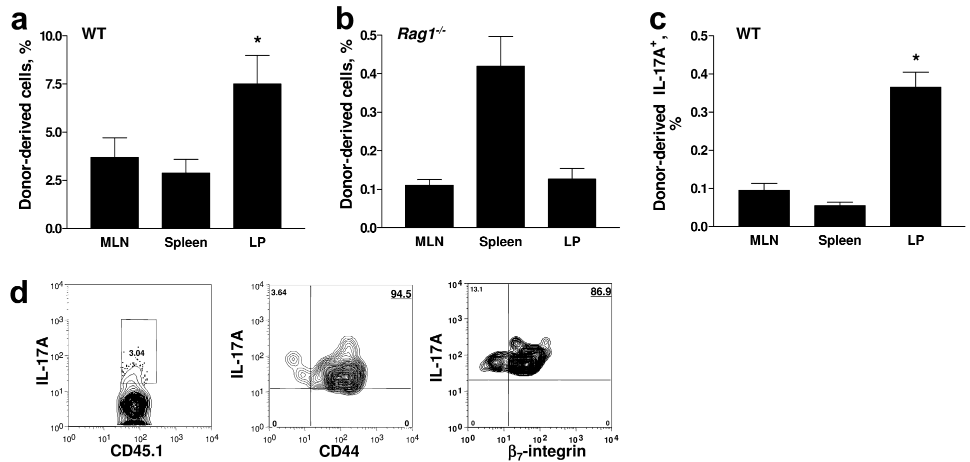

FIGURE 3. Thymocytes home to lymphoid tissues. *a* and *b*, Thymocytes from WT CD45.1$^+$ mice (n = 4) (*a*) or *Rag1*$^{-/-}$ mice (n = 6–10 pooled per experiment) (*b*) were adoptively transferred by i.v. injection into nude mice (n = 2–4) and analyzed 1 wk after injection. *c*, IL-17A-producing neutrophil regulatory T cells were measured in nude mice transferred with WT thymocytes (n = 4). *, $p < 0.05$ from other organs. *d*, CD44 and β_7 integrin expression on IL-17A$^+$ WT thymocytes that had homed to the LP of nude mice.

compartment of WT mice. Taken together, our data suggest that thymus-derived T-lineage cells that lack a TCR can secrete IL-17A and regulate granulocyte homeostasis.

Perhaps the most surprising finding of our study is that nude mice are neutropenic in one specific pathogenic-free facility but not in another. This finding may explain previous reports of neutropenic (33) and neutrophilic nude mice (38, 39). This difference in phenotype is most likely due to different commensal flora (44, 49, 50), although formal experiments to address the role of commensals were not part of this study. IL-17A production in nude mice was undetectable by RT-PCR, flow cytometry, and plasma ELISA at the University of Virginia and the La Jolla Institute of Allergy and Immunology vivaria, but plasma IL-17F was detectable in nude mice at the La Jolla Institute of Allergy and Immunology, the facility where nude mice are not neutropenic. This finding supports the concept that IL-17F can compensate for IL-17A with respect to blood neutrophil numbers (27). The effect of vivarium housing on IL-17A production has been previously reported in WT mice, with mice imported from Taconic Farms showing a 10-fold increase in Th17 cells in the LP compared with WT mice supplied from The Jackson Laboratory (44).

Although CD4$^+$ T cells were the first cells to be identified as active secretors of IL-17A (17) and continue to receive the most attention, the most numerous IL-17A-producing T cells in normal mice are $\gamma\delta^+$ T cells (10). A recent study has shown that naive IL-17A-producing $\gamma\delta^+$ T cells escape the thymus and are polarized to produce IL-17A (51). These CD122$^{\text{low}}$ cells populated the thymus, lymph nodes and spleen. Taken together with a prior study (52), this finding suggests that positive selection is not required for these $\gamma\delta^+$ T cells. IL-17A-producing $\gamma\delta^+$ T cells can be derived from the fetal thymi of normal mice (53). These cells express CD25 but not CD122 and are detected in the thymus before CD25 is expressed. Although these authors did not investigate IL-17A expression in DN1 thymocytes, our data suggest that polarization toward IL-17A expression occurs earlier than previously reported (53). The primitive IL-17A-producing T-lineage cells colonize secondary lymphoid tissues of *Rag1*$^{-/-}$ mice, which lack mature T cells and are fully able to correct neutrophil counts in nude mice. Cells expressing intracellular CD3 have been previously described in lymphoid organs of *Rag1*$^{-/-}$ mice (54), although the function of these cells was not investigated. In the present study, we show that at least some of these cells produce IL-17A and support granulopoiesis in *Rag1*$^{-/-}$ mice.

FoxP3 is the defining transcription factor of regulatory T cells (55). In a recent study of FoxP3 expression in human thymocytes, FoxP3-expressing cells were found among DN1 thymocytes (56). This may reflect another example where T cell lineage commitment precedes TCR engagement. Regulatory and IL-17A-producing T cell lineages are related to each other (20, 57), and both may undergo early lineage commitment before expressing a functional TCR. In the present study, we found that splenocytes from WT and *Rag1*$^{-/-}$ mice were equipotent in their ability to secrete IL-17A in response to IL-23 in the absence of anti-CD3ε/CD28 stimulation. As expected, TCR ligation greatly augmented IL-17A release from WT cells but not *Rag1*$^{-/-}$ cells. This suggests a two-tiered model of IL-17A control: lower levels sufficient to maintain granulopoiesis may be secreted in response to cytokines such as IL-23, whereas large amounts, such as those seen in autoimmune diseases, depend on TCR engagement.

The restoration of normal levels of circulating neutrophils in neutropenic nude mice reconstituted with myeloid-depleted WT or *Rag1*$^{-/-}$ DN1 thymocytes shows that homeostatic levels of IL-17A can be produced without the need for TCR expression or ligation. Elegant studies by Monteiro et al. showed that Ag-activated CD4$^+$ T cells in the BM regulate granulopoiesis, but these studies predated the discovery of Th17 cells (33). *Rag1*$^{-/-}$ mice had normal circulating neutrophil counts (Table I) and no block in myeloid progenitor cell differentiation in the BM (supplemental Fig. 1). Monteiro et al. showed that D011.10 RAG$^{-/-}$ mice (which only contain naive CD4$^+$ T cells) were neutropenic and that neutrophil counts were only restored following treatment with OVA (33). Taken together with our finding of normal neutrophil counts in *Rag1*$^{-/-}$ mice, this suggests that naive T cells may actively suppress granulopoiesis in the D011.10 RAG1$^{-/-}$ mice. We hypothesize that under "normal" conditions Ag-activated, IL-17A-producing CD4$^+$ T cells regulate granulopoiesis in mice. In the absence of mature CD4$^+$ T cells, other cells, possibly at the DN1 stage of development, are able to escape the thymus and regulate granulopoiesis without TCR engagement.

The fact that neutrophil levels in reconstituted nude mice become normal and not exaggerated suggests that IL-17A-producing cells from *Rag1*$^{-/-}$ mice are still subject to regulation similar to that of their WT counterparts. BM cellularity was also not altered between the reconstituted mouse groups. Previous studies have shown that adenovirus overexpression of IL-17A in WT mice also does not affect BM cellularity, although myeloid precursor CFUs (CFU-GM/CFU-GEMM, where GEMM is granulocyte-erythrocyte-megakaryocyte-monocyte) were significantly raised (11). We have previously hypothesized that the regulation of granulopoiesis by the IL-17A-IL-23 cytokine axis is likely to occur in the GALT (10, 18, 58). Following the adoptive transfer of unfractionated thymocytes, IL-17A$^+$ cells from WT and *Rag1*$^{-/-}$ mice were found

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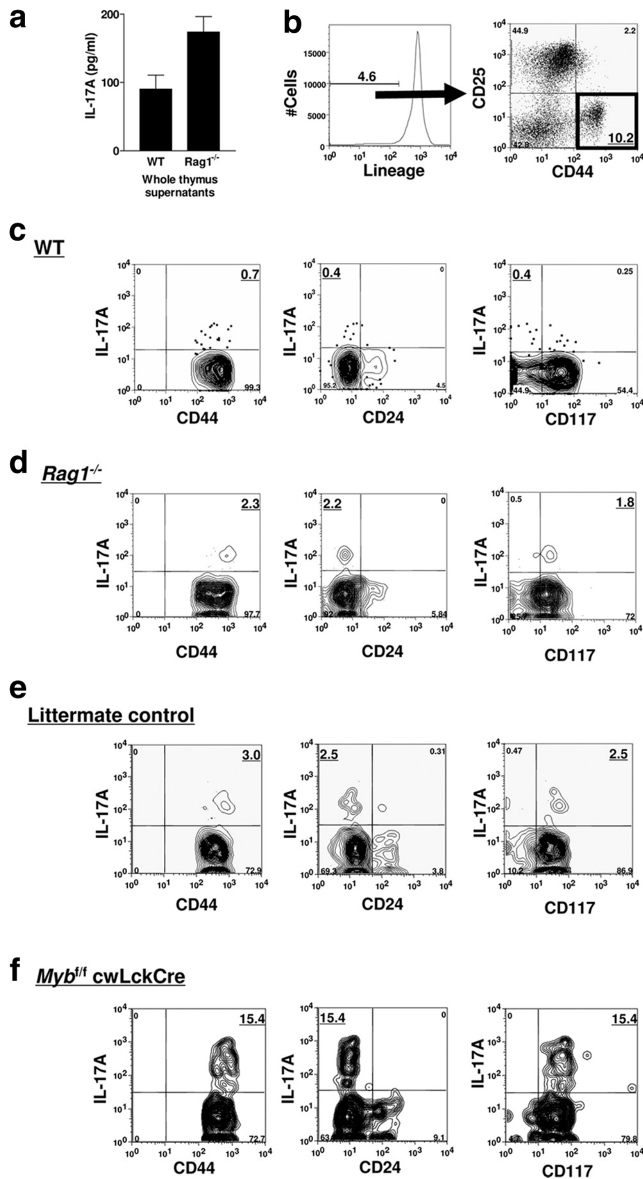

FIGURE 4. IL-17A-producing cells are found in the DN1 compartment of the thymus. *a*, IL-17A protein was detectable in the supernatants of homogenized thymi of WT and $Rag1^{-/-}$ mice. *b*, Unfractionated thymocytes were stimulated with PMA and ionomycin in the presence of GolgiStop for 5 h before staining, gated on the lymphocyte population by forward/side scatter and the DN population by the lineage negative (GR-1^-, $B220^-$, $CD11b^-$, $CD3^-$, $CD4^-$, $CD8^-$, $Ter119^-$) population and then gated for DN ($CD44^+CD25^-$). *c–f*, In WT (*c*) and $Rag1^{-/-}$ (*d*) mice, IL-17A-producing cells were $CD44^{high}$ and $CD24^-$. Most IL-17A-producing cells in $Rag1^{-/-}$ mice were $CD117^+$, whereas these cells in WT mice expressed mixed levels of CD117. IL-17A-producing $CD44^{high}CD24^-CD117^+$ thymocytes were found at higher levels in the DN1 compartment of $Myb^{f/f}$ cwLckCre (*f*) mice than their littermate controls ($Myb^{f/f}$ cwLckCre are on a mixed genetic background) (*e*).

predominantly in the LP after 8 wk. A small number of $CD45.1^+$ cells were found in the BM; however they did not secrete detectable IL-17A. IL-17A regulates G-CSF secretion, most likely from BM stromal cells, and thus controls circulating neutrophil numbers (18). As part of a negative feedback loop, IL-17A inhibits its own production and that of IL-17F, but IL-17F cannot serve this function (26, 27). One way to directly address the role of IL-17A in regulating blood neutrophil numbers would be to adoptively transfer DN1 thymocytes from $Il17a^{-/-}$mice. However, interpretation of any transfer experiments with these mice is hampered by the overexuberant production of IL-17F and the consequently elevated neutrophil numbers in these mice (27), making this approach uninformative.

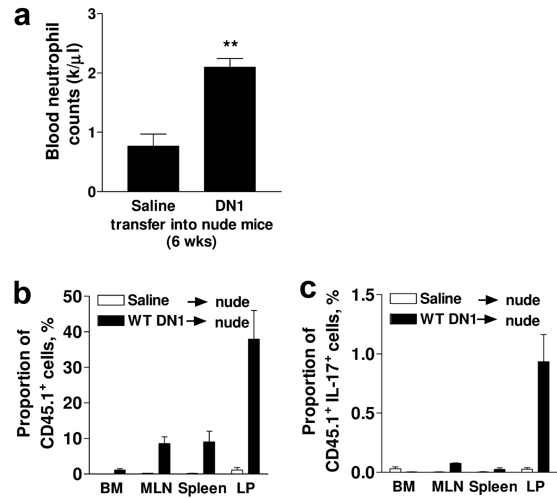

FIGURE 5. DN1 cells can regulate granulopoiesis. *a*, Cell-sorted WT $CD45.1^+CD24^-$ DN1 thymocytes were adoptively transferred into nude mice and neutrophil counts were assessed after 6 wk. *b* and *c*, $CD45.1^+$ cells were found predominantly in the LP (*b*), where some expressed IL-17A as detected by intracellular staining (*c*).

After adoptive transfer, IL-17A-producing $CD45.1^+$ WT T cells lost Thy-1 expression but showed an organ distribution in recipient nude mice that was similar to the distribution of Thy-1^+IL-17A-producing T cells in WT mice. However, whereas $Rag1^{-/-}$ thymocytes also lost Thy-1 expression on their cell surface, the distribution of $Rag1^{-/-}$ IL-17A-producing cells transferred into nude mice followed a different pattern, with the largest population found in the spleen (after 1 wk) and LP (after 8 wk). This may reflect differences in the homing patterns between endogenous $Rag1^{-/-}$ cells able to escape thymic selection as seen in $Rag1^{-/-}$ mice and adoptively transferred bulk $Rag1^{-/-}$ thymocytes. The mechanisms by which TCR^- IL-17A-producing cells escape the thymus remain to be investigated.

The fact that nude mice fail to develop IL-17A-producing cells suggests that the thymic epithelium is required for their development. The thymic epithelium is known to provide essential Notch ligands needed for thymocyte development (59). Based on the present data, we speculate that IL-17A-producing T-lineage cells may have originated in phylogeny before the advent of thymic selection. $Rag1^{-/-}$ mice express no functional TCR and therefore do not show thymic selection, yet these mice have IL-17A-producing cells in the thymus and elsewhere. IL-17 family members are found in all mammals and most vertebrates down to zebrafish (60) and even cartilaginous fishes (61, 62). IL-17R and a form of primordial IL-17 were found up-regulated in skin cells following LPS stimulation of lamprey (*Lethenteron japonicum*), which are jawless vertebrates (63, 64). Interestingly, lampreys do not express *Rag1* or *Rag2* genes and are unable to undergo recombinatorial diversification. This suggests that the IL-17A-IL-17R system predates the advent of thymic selection and TCR expression (65). The precise orthologous relationships between different members of the IL-17 family of cytokines in different species remains to be investigated, so it is unclear whether the ancestral IL-17 may correspond to IL-17A, IL-17F, or even another family member.

To investigate the role of V(D)J recombination, we used $Rag1^{-/-}$ mice for most of our experiments and show that their

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thymi produce copious amounts of IL-17A, their splenocytes respond to IL-23 by secreting IL-17A, and their thymocytes home to the spleen and LP of nude mice, where they secrete sufficient amounts of IL-17A to restore normal blood neutrophil counts. This raises the question of whether such cells exist in immunocompetent mice. We addressed this in two ways. First, DN1 thymocytes from WT mice also restored blood neutrophil counts in nude mice. Second, we found IL-17A-producing $CD4^-CD8^-$ DN T cells in secondary lymphoid organs of immunocompetent mice. Because these cells are extremely rare in normal WT mice (18), we used neutrophilic *Itgb2*$^{-/-}$ mice for these experiments. Two recent reports show that lymphoid LTi-like cells also produce IL-17A without TCR engagement. $CD4^+CD3^-NK1.1^-CD11b^-Gr1^-CD11c^-B220^-$ LTi cells can produce IL-17A in response to zymosan or IL-23 (25), and human fetal LTi cells are IL-17-producing precursors to $RORC^+CD127^+$ NK-like cells (66). The cells described in the present report are not LTi cells because they do not express CD4 but intracellular CD3, suggesting that they are of T cell lineage.

In conclusion, adoptive transfer of thymocytes from WT or *Rag1*$^{-/-}$ mice corrects neutropenia in nude mice. The maintenance of normal neutrophil levels in *Rag1*$^{-/-}$ mice together with normal IL-17A secretion from splenocytes in response to IL-23 indicates that V(D)J recombination is dispensable for the generation of IL-17A-producing cells. IL-17A-producers are found among DN1 thymocytes of normal WT mice and at elevated levels in *Rag1*$^{-/-}$ and *Myb*$^{f/f}$ cwLckCre mice. Although the mechanisms behind the thymic egress of these cells remain unknown, we show that DN1-derived T-lineage cells populate the secondary lymphoid organs of nude mice and produce enough IL-17A to correct neutropenia.

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Disclosures

The authors have no financial conflict of interest.

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