

# Lymphoid tissue inducer–like cells are an innate source of IL-17 and IL-22

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The interleukin (IL) 17 family of cytokines has emerged to be critical for host defense as well as the pathogenesis of autoimmune and autoinflammatory disorders, and serves to link adaptive and innate responses. Recent studies have identified a new subset of T cells that selectively produce IL-17 (Th17 cells; Bettelli, E., T. Korn, and V.K. Kuchroo. 2007. *Curr. Opin. Immunol.* 19:652–657; Kolls, J.K., and A. Linden. 2004. *Immunity.* 21:467–476), but the regulation of IL-17 production by innate immune cells is less well understood. We report that *in vitro* stimulation with IL-23 induced IL-17 production by recombination activating gene (Rag) 2<sup>-/-</sup> splenocytes but not Rag2<sup>-/-</sup> common  $\gamma$  chain<sup>-/-</sup> splenocytes. We found that a major source of IL-17 was CD4<sup>+</sup>CD3<sup>-</sup>NK1.1<sup>-</sup>CD11b<sup>-</sup>Gr1<sup>-</sup>CD11c<sup>-</sup>B220<sup>-</sup> cells, a phenotype that corresponds to lymphoid tissue inducer–like cells (LTi-like cells), which constitutively expressed the IL-23 receptor, aryl hydrocarbon receptor, and CCR6. *In vivo* challenge with the yeast cell wall product zymosan rapidly induced IL-17 production in these cells. Genetic deletion of signal transducer and activator of transcription 3 reduced but did not abrogate IL-17 production in LTi-like cells. Thus, it appears that splenic LTi-like cells are a rapid source of IL-17 and IL-22, which might contribute to dynamic organization of secondary lymphoid organ structure or host defense.

Naive T cells undergo differentiation on signals received from the TCR and cytokine receptors and differentiate to specialized subsets characterized by their production of signature cytokines. Th17 cells produce IL-17 or IL-17A and IL-17F, which are major mediators of inflammation and are critical for host defense against extracellular bacteria and fungi (1, 2). IL-6, IL-21, and TGF- $\beta$ 1 are critical factors that promote Th17 cell differentiation (1, 3). Although IL-23 was originally thought to be important for inducing naive CD4<sup>+</sup> T cells to become Th17 cells, naive CD4<sup>+</sup> T cells do not express IL-23Rs (4). Rather, IL-23 is now thought to affect the expansion, maintenance, and pathogenicity of Th17 cells (5). IL-23 also induces IL-17 production from  $\gamma\delta$  T cells and invariant NKT (iNKT) cells (6, 7). Regardless of exactly how IL-23 works, current

evidence clearly argues that IL-23–mediated IL-17 production is crucial in host defense and in the pathogenesis of autoimmune diseases (8–10).

IL-6, IL-21, and IL-23 share the ability to activate Stat3, which was shown to be critical for Th17 cell differentiation in mouse and man (3, 11, 12). Stat3 directly regulates the *Il17* and *Il21* genes but also regulates IL-23R expression (3, 12). Furthermore, the aforementioned cytokines acting via Stat3 induce the retinoic acid–related orphan receptor  $\gamma$ t (ROR $\gamma$ t), the master regulator of Th17 cell differentiation (13).

In contrast to T cells, much less is known about the ability of innate cell subpopulations to produce IL-17. We report that splenic lymphoid tissue inducer–like cells (LTi-like cells) constitutively express ROR $\gamma$ t, IL-23R, CCR6, and aryl hydrocarbon receptor (AHR) (14), and produce IL-17 and IL-22. Interestingly,

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the yeast wall product zymosan elicited IL-17 production by LTI-like cells in vivo. Whether the production of IL-17 and IL-22 influences the architecture of secondary lymphoid organs (SLOs) and contributes to host defense will be important issues to examine in the future.

**RESULTS AND DISCUSSION**

**IL-23 induces IL-17 production by a population of common  $\gamma$  chain ( $\gamma$ c)-dependent non-T, non-B cells**

To determine if there are considerable proportions of innate immune cells that produce IL-17, we examined Rag2<sup>-/-</sup> mice that have few T and B cells. We first assessed whether IL-17 production occurred in Rag2<sup>-/-</sup> splenocytes after stimulation with various cytokines. As shown in Fig. 1 A, IL-23 alone induced IL-17A production by WT splenocytes, presumably indicative of an effect on memory T cells. Consistent with the idea that memory T cells are lacking in Rag2<sup>-/-</sup> mice, the effect of IL-23 was reduced in Rag2<sup>-/-</sup> splenocytes (Fig. 1 A). However, Rag2<sup>-/-</sup> splenocytes still produced about one third as much IL-17A as WT splenocytes (Fig. 1 A). Other cytokines used separately or in combination

with IL-23 had no effect on IL-17A production; however, IL-4 suppressed IL-23-induced IL-17A production by WT and Rag2<sup>-/-</sup> splenocytes (Fig. S1 A, available at <http://www.jem.org/cgi/content/full/jem.20072713/DC1>). In addition to memory CD4<sup>+</sup> T cells, iNKT cells are another important source of IL-17 (7), but such cells are lacking in Rag2<sup>-/-</sup> mice. Accordingly, anti-CD3/28 or  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer) stimulation induced IL-17A production by WT splenocytes but not Rag2<sup>-/-</sup> splenocytes (Fig. S1, B and C). Collectively, these data suggest that although T and NKT cells are both major producers of IL-17, populations of non-T, non-B cells can also produce IL-17.

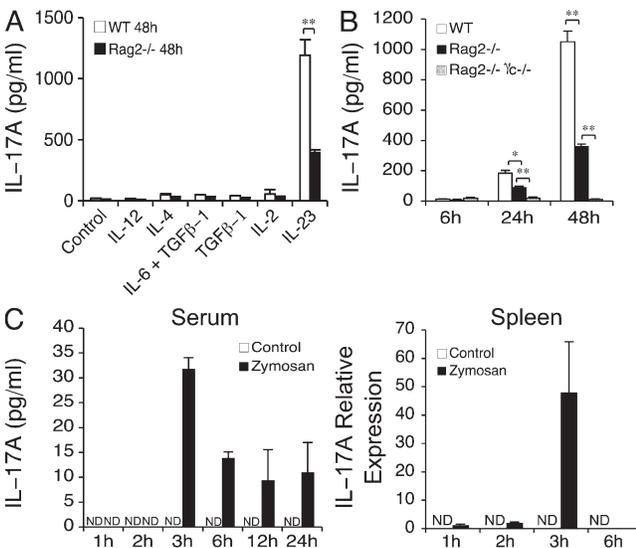
To identify which population of cells might be responsible for IL-23-dependent IL-17 production by Rag2<sup>-/-</sup> splenocytes, we examined another genetic model, namely Rag2<sup>-/-</sup> $\gamma$ c<sup>-/-</sup> mice. In addition to lacking T and B cells, Rag2<sup>-/-</sup> $\gamma$ c<sup>-/-</sup> mice also lack NK cells. We therefore cultured WT, Rag2<sup>-/-</sup>, or Rag2<sup>-/-</sup> $\gamma$ c<sup>-/-</sup> splenocytes in the presence of IL-23. Consistent with Fig. 1 A, IL-17A production was present in WT splenocytes and reduced but not absent in Rag2<sup>-/-</sup> splenocytes (Fig. 1 B). In contrast, IL-17A production by Rag2<sup>-/-</sup> $\gamma$ c<sup>-/-</sup> splenocytes was essentially abrogated (Fig. 1 B). These results indicate that  $\gamma$ c-dependent, non-T, non-B cell populations in the spleen can produce IL-17 in vitro.

IL-23 has been shown to be induced by certain microbial products (15). Rag2<sup>-/-</sup> mice were therefore injected with zymosan to induce IL-23 production by DCs, which resulted in rapid IL-17A appearance in sera and spleens (Fig. 1 C). Thus, these data indicate that innate immune cells can generate IL-17 in response to the fungal product in vivo.

**IL-17 production by CD4<sup>+</sup>CD3<sup>-</sup>CD11c<sup>-</sup>B220<sup>-</sup> LTI-like cells**

To delineate the population of IL-17-producing cells, we next used intracellular cytokine staining. We used markers for a variety of innate immune cells expected to be present in Rag2<sup>-/-</sup> spleens but failed to detect IL-23-mediated IL-17A production in granulocytes, macrophages, or DCs (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20072713/DC1>). A prominent  $\gamma$ c-dependent lineage that rapidly produces cytokines upon stimulation is the NK cell. Based on the response to IL-23 in Rag2<sup>-/-</sup> splenocytes and loss in Rag2<sup>-/-</sup> $\gamma$ c<sup>-/-</sup> splenocytes, we thought that NK cells could be IL-17 producers. However, contrary to our expectations, the majority of IL-17A-producing CD3<sup>-</sup> cells did not express NK1.1 (Fig. 2 A). Thus far, our results have indicated that neither T, NKT, NK cells (all  $\gamma$ c dependent) nor  $\gamma$ c-independent myeloid cells were a major source of IL-17 production in Rag2<sup>-/-</sup> spleens. We did observe that in Rag2<sup>-/-</sup> splenocytes stimulated with IL-23 (Fig. 2 B, left), a considerable proportion of the IL-17A-producing non-T cells expressed CD4 (Fig. 2 B, right).

CD4<sup>+</sup>CD3<sup>-</sup> cells in the Rag2<sup>-/-</sup> spleen comprise three populations, including CD11c<sup>low</sup>B220<sup>+</sup> plasmacytoid DCs, CD11c<sup>+</sup>B220<sup>-</sup> conventional DCs, and CD4<sup>+</sup>CD3<sup>-</sup>CD11c<sup>-</sup>B220<sup>-</sup> cells (Fig. S3, available at <http://www.jem.org/cgi/content/full/jem.20072713/DC1>). As indicated in Fig. S2,



**Figure 1. IL-23 and zymosan induce IL-17 production in a population of non-B, non-T cells.** (A) WT or Rag2<sup>-/-</sup> splenocytes were cultured in the presence of the indicated cytokines for 48 h, and IL-17A in culture supernatants was measured by ELISA. The data are means  $\pm$  SD from duplicate cultures and are representative of four independent experiments ( $n = 8$ ). \*\*,  $P < 0.01$ . (B) WT, Rag2<sup>-/-</sup>, or Rag2<sup>-/-</sup> $\gamma$ c<sup>-/-</sup> splenocytes were cultured in the presence of IL-23 alone, and IL-17A in culture supernatants was measured by ELISA. The data are means  $\pm$  SD from duplicate cultures and are representative of three independent experiments ( $n = 6$ ). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . (C) Sera and splenocytes were harvested from Rag2<sup>-/-</sup> mice after injection of 5 mg zymosan or PBS (Control). (left) IL-17A in sera was measured by ELISA. (right) The relative expression levels ( $I/18S$ ) of IL-17A mRNA were analyzed by quantitative PCR (q-PCR) and are depicted as fold induction relative to cells 1 h after treatment with zymosan. The data are means  $\pm$  SD of results from two mice per group per time point and are representative of two independent experiments. ND, not detected.

we did not detect IL-23-mediated IL-17A production by CD11c<sup>+</sup> DC populations. The latter, CD4<sup>+</sup>CD3<sup>-</sup>CD11c<sup>-</sup>B220<sup>-</sup> cells, have been shown to be present in SLOs of fetal and adult mice and are termed LTi's and LTi-like cells, respectively (16). Importantly, LTi-like cells are greatly reduced in  $\gamma c^{-/-}$  mice (17).

We next determined whether isolated CD4<sup>+</sup>CD3<sup>-</sup>CD11c<sup>-</sup>B220<sup>-</sup> LTi-like subsets produced IL-17 (Fig. 3 A, top). In fact, as shown in Fig. 3 A (bottom), we observed IL-17A production by isolated LTi-like cells in response to IL-23. Moreover, directly isolated LTi-like cells produced more IL-17A in the presence of PMA/ionomycin (Iono), which was dramatically enhanced by IL-23 (Fig. 3 A, bottom). In contrast, LTi-negative subsets produced minimal IL-17A (Fig. S4, available at <http://www.jem.org/cgi/content/full/jem.20072713/DC1>). Consistent with Fig. S2, isolated CD11c<sup>+</sup> cells did not produce IL-17A with IL-23 stimulation (unpublished data). Another source of LTi-like cells in an adult mouse is the intestinal lamina propria (16). Interestingly, LTi-like cells in the lamina propria from WT mice produced IL-17A after stimulation with PMA/Iono (Fig. S5). These results suggest that the ability to produce IL-17 is not unique to the splenic population.

One possible explanation for our observations is that IL-23 promoted in vitro survival of LTi-like cells rather than enhancing IL-17 production by itself. LTi-like cells have been shown to express IL-7Rs (Fig. S6, available at <http://www.jem.org/cgi/content/full/jem.20072713/DC1>) (16). Therefore, we modified the cell-culture conditions by adding IL-7

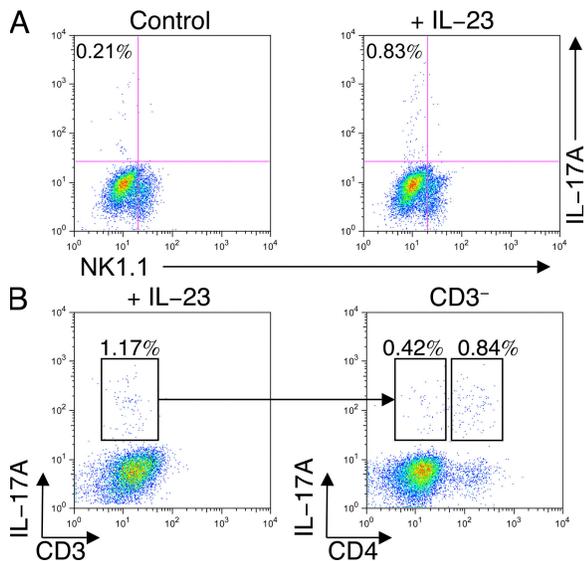
to maintain cells for the initial 48 h, followed by IL-23 for another 6 h. As shown in Fig. S7 A, IL-17A and IL-17F mRNA expression was potently induced by IL-23 in the presence of IL-7. These results suggest that LTi-like cells quickly enhance the production of IL-17 in response to IL-23, and this cytokine does not simply serve to keep these cells alive.

It is well known that Stat3 is activated by IL-23 and other inducers of Th17 cell differentiation, and the absence of Stat3 abrogates IL-17 production by T cells (12). We therefore assessed the proportion of IL-17A-producing LTi-like cells in Stat3-deficient mice. Not surprisingly, we found that IL-23-mediated IL-17A production was decreased in Stat3-deficient LTi-like cells of *Stat3<sup>β/β</sup>*; *MMTV-Cre* or *Stat3<sup>β/β</sup>*; *CD4-Cre* splenocytes compared with those of *Stat3<sup>β/β</sup>* splenocytes, although IL-17A production was not completely abrogated (Fig. 3 B and not depicted). Because Cre-mediated deletion of STAT3 is >90% in both systems (unpublished data), there seems to be both STAT3-dependent and -independent pathways for the production of IL-17 in LTi-like cells. We also observed that IL-23 up-regulated mRNA expression of Stat3 and Stat4 but not GATA3 in LTi-like cells (Fig. S7 B). These results highlight the notion that the IL-17 production by LTi-like cells might be dependent on these factors.

#### LTi-like cells constitutively express IL-23R, AHR, and CCR6

The complete receptor for IL-23 comprises the IL-12Rβ1 associated with the ligand-specific subunit IL-23R, and both subunits are required for the action of this cytokine (4). As memory CD4<sup>+</sup> T cells respond to IL-23 because of constitutive expression of its receptors (4), we evaluated mRNA expression of IL-23R and IL-12Rβ1 in LTi-like cells. Consistent with their rapid responsiveness to IL-23, LTi-like cells constitutively expressed IL-23R mRNA at levels significantly greater than T cells (Fig. 3 C). LTi-like cells also expressed slightly higher levels of IL-12Rβ1 mRNA compared with those of memory CD4<sup>+</sup> T cells (Fig. 3 C). In contrast, expression levels of IL-12Rβ2 mRNA in LTi-like cells were low compared with those in memory CD4<sup>+</sup> T cells (Fig. 3 C). Also consistent with previous studies (16, 18), we noted higher expression levels of RORγt mRNA in LTi-like cells compared with T cells (Fig. 3 C). In contrast, expression of IL-6Rα and IL-17RA mRNA in LTi-like cells was much lower compared with that in T and B cells (unpublished data). Recently, the AHR has been reported to be expressed in Th17 cells (14). Interestingly, LTi-like cells also expressed AHR mRNA at levels equivalent to those in Th17 cells polarized for 72 h (Fig. 3 C). Furthermore, IL-23 up-regulated mRNA expression levels of IL-23R, RORγt, and AHR (Fig. S7 C).

Consistent with previous studies (16, 18), splenic LTi-like cells were found to be CD30L<sup>+</sup>OX40L<sup>+</sup>γc<sup>+</sup>IL-7Rα<sup>+</sup>Thy1.2<sup>high</sup>CD44<sup>high</sup>CD62L<sup>low</sup> (Fig. S6). LTi-like cells are also known to express the chemokine receptors CXCR5<sup>+</sup> and CCR7<sup>+</sup> (16, 18), and we again confirmed expression of these in the splenic LTi-like cells (Fig. S6). Additionally, it has recently been shown that CCR6 identifies a population of human



**Figure 2. CD4<sup>+</sup>CD3<sup>-</sup>lineage<sup>-</sup> cells produce IL-17.** Rag2<sup>-/-</sup> splenocytes were cultured in the absence (Control) or presence of IL-23 for 24 h. The proportion of IL-17A-producing CD3<sup>-</sup> cells was evaluated by intracellular staining. (A) NK1.1<sup>+</sup> versus IL-17A<sup>+</sup> cells gated on CD3<sup>-</sup> cells are shown. (B) IL-17A<sup>+</sup> versus CD3<sup>+</sup> cells (nongated; left) and IL-17A<sup>+</sup> versus CD4<sup>+</sup> cells gated on CD3<sup>-</sup> cells (right) are shown. Data are representative of three independent experiments.

memory CD4<sup>+</sup> T cells that selectively produce IL-17 (19), we found that most of the splenic LTI-like cells express CCR6 (Fig. 3 D). Approximately half of the CCR6<sup>+</sup> LTI-like cells coexpress CXCR5 and CCR7 (unpublished data), suggesting that the expression of those chemokine receptors on LTI-like cells is heterogeneous, as previously reported (20). On the other hand, the expression of CXCR3, CCR5, or CCR4, which are preferentially expressed on Th1 or Th2 cells (19), was low on splenic LTI-like cells (Fig. S6 and not depicted).

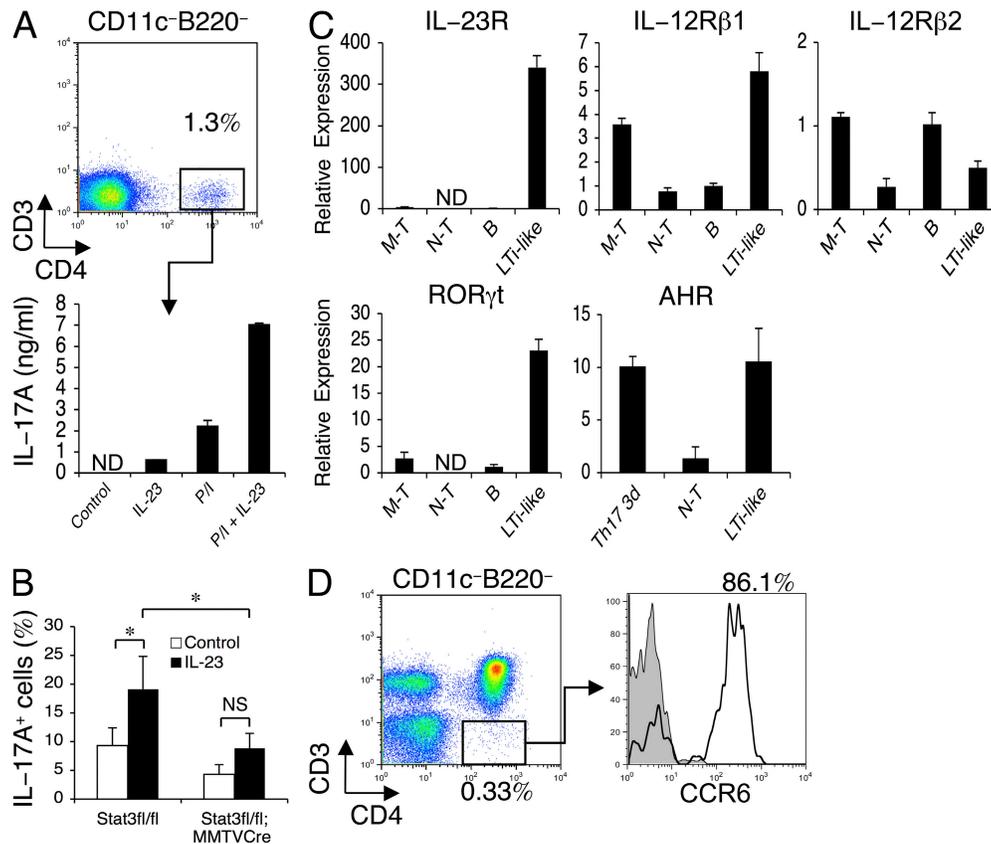
**LTI-like cells produce IL-17 during zymosan-induced inflammation in Rag2<sup>-/-</sup> mice**

We next determined whether LTI-like cells produced IL-17 in vivo in an inflammatory setting. We approached this problem in two ways: first, we injected Rag2<sup>-/-</sup> mice with zymosan i.p. to induce inflammation and harvested splenocytes 2 h later. The cells were then stimulated ex vivo with PMA/Iono and Brefeldin A (BFA) for 2 h, and the propor-

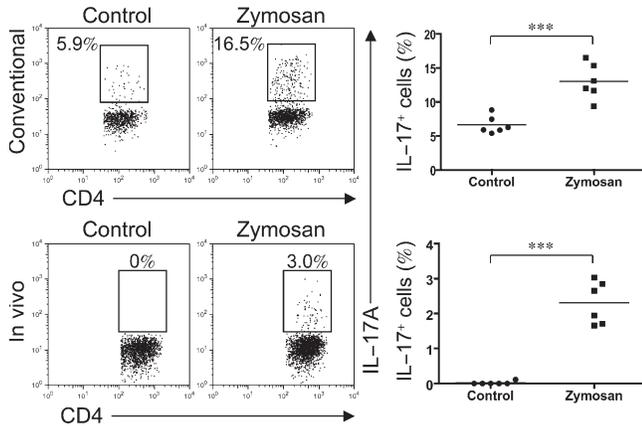
tion of IL-17A-producing LTI-like cells was determined by FACS. As shown in Fig. 4 (top) the proportion of IL-17-producing LTI-like cells was significantly greater in zymosan-treated mice compared with control mice. To further establish the ability of LTI-like cells to produce IL-17 in vivo, we treated Rag2<sup>-/-</sup> mice with BFA i.v. (21) and challenged them with zymosan i.p. for 6 h. We observed few IL-17A-producing LTI-like cells in control mice, whereas zymosan-treated mice had a significant increase in IL-17A-producing cells (Fig. 4, bottom). These results establish that LTI-like cells can rapidly produce IL-17 in vivo when challenged with the product of fungal pathogens.

**LTI-like cells also produce IL-22**

It has been argued that Th17 cells also preferentially produce IL-22 (22). Interestingly, we found that treatment with zymosan induced not only IL-17A but also IL-22 in the sera and spleens of Rag2<sup>-/-</sup> mice (Fig. 5 A). In vitro stimulation with



**Figure 3. Isolated CD4<sup>+</sup>CD3<sup>-</sup>CD11c<sup>-</sup>B220<sup>-</sup> LTI-like cells produce IL-17 and constitutively express IL-23R, RORγt, AHR, and CCR6.** (A) Isolated CD4<sup>+</sup>CD3<sup>-</sup>CD11c<sup>-</sup>B220<sup>-</sup> LTI-like subsets from Rag2<sup>-/-</sup> splenocytes (top) were cultured in the absence (Control) or presence of IL-23 (or with PMA/Iono) for 24 h, and IL-17A in culture supernatants was measured by ELISA (bottom). Data are means ± SD from duplicate cultures and are representative of two independent experiments. (B) Stat3<sup>fl/fl</sup> or Stat3<sup>fl/fl</sup>; MMTV-Cre splenocytes were cultured in the absence (Control) or presence of IL-23 for 24 h. The proportion of IL-17A-producing LTI-like cells was evaluated by intracellular staining. Data are means ± SD from duplicate cultures and are representative of four independent experiments (n = 8). \*, P < 0.05. (C) Total RNA was prepared from LTI-like cells isolated from Rag2<sup>-/-</sup> spleens. The relative expression levels (18S) of the indicated mRNA was analyzed by q-PCR and are depicted as fold induction relative to fresh B cells (or fresh naive T cells for AHR mRNA expression). Data are representative of three independent experiments. M-T, memory T cells; N-T, naive T cells. (D) The expression of CCR6 on LTI-like cells of WT splenocytes was analyzed by FACS. The shaded histogram indicates staining with isotype-matched control antibodies. The continuous line histogram indicates the surface expression level of CCR6 on LTI-like cells. Data are representative of three independent experiments. ND, not detected.



**Figure 4. Zymosan induces IL-17 production in splenic LTI-like cells in vivo.** (left) Intracellular staining of IL-17A was performed with in vitro conventional staining (top) or in vivo staining (bottom). For conventional staining,  $Rag2^{-/-}$  mice were challenged with PBS (Control) or 12.5 mg zymosan i.p. Splenocytes were isolated 2 h later and stimulated with PMA/Iono and BFA for 2 h in vitro. The proportion of IL-17A-producing LTI-like cells was evaluated by intracellular staining. For the in vivo staining,  $Rag2^{-/-}$  mice were injected with PBS (Control) or 12.5 mg zymosan i.p. together with 0.25 mg BFA i.v. (reference 22). The proportion of IL-17A-producing LTI-like cells in spleens was directly evaluated by intracellular staining. (right) The mean values (horizontal bars) for IL-17A-producing LTI-like cells were calculated for in vitro staining (top,  $n = 6$ ) and in vivo staining (bottom,  $n = 6$ ). Data are representative of three (top) or two (bottom) independent experiments. \*\*\*,  $P < 0.001$ .

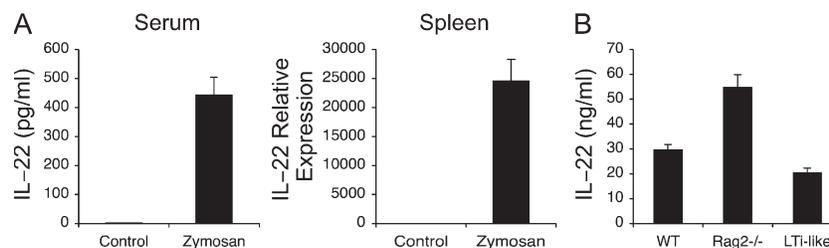
IL-23 induced more IL-22 production by  $Rag2^{-/-}$  splenocytes than WT splenocytes (Fig. 5 B). Furthermore, isolated LTI-like cells also produced IL-22 (Fig. 5 B and Fig. S7 D). Although we observed that  $NK1.1^{+}CD3^{-}$  NK cells failed to produce IL-17A (Fig. 2 A), isolated NK cells produced small amounts of IL-22 in response to IL-23 (Fig. S8, available at <http://www.jem.org/cgi/content/full/jem.20072713/DC1>). These findings illustrate the potentially distinct modes of regulation for IL-17 and IL-22.

The IL-23–IL-17 axis has emerged to be important in host defense and in models of autoimmunity such as experimental autoimmune encephalomyelitis and inflammatory bowel disease (10, 23). Despite the importance of Th17 cells,

it is becoming increasingly clear that they are not the only source of IL-17. Clearly,  $\gamma\delta$  T and iNKT cells produce IL-17 to protect against bacterial infection (6, 7). In addition, a recent study provides evidence that IL-23 can drive IL-17 production by innate immune cells in animal models of inflammatory bowel disease (23).

We were very impressed by the loss of IL-17 production in  $Rag2^{-/-}\gamma c^{-/-}$  mice (Fig. 1 B). We initially suspected that this pointed to production of IL-17 by NK cells but were surprised by the failure to see IL-17 production by  $NK1.1^{+}CD3^{-}$  NK cells (Fig. 2 A). However, LTI-like cells are also dependent on  $\gamma c$  cytokines for development (17). Before our studies, it was known that these cells expressed ROR $\gamma t$ , which was required for their function in LNs (16, 18). ROR $\gamma t$  has several important functions, but it is now clear that one function is the regulation of Th17 cell differentiation (13). We confirmed that splenic LTI-like cells constitutively express ROR $\gamma t$  (Fig. 3 C) and that IL-23 further up-regulated its expression (Fig. S7 C), arguing that this factor is essential in controlling IL-17 and IL-22 production by non-T cells. Interestingly, fresh LTI-like cells also constitutively expressed more AHR than polarized Th17 cells (Fig. 3 C). LTI-like cells also constitutively expressed IL-23R and CCR6 (Fig. 3, C and D), similar to  $CCR6^{+}IL-23R^{+}$  human memory  $CD4^{+}$  T cells, which are major producers of IL-17 (19, 24). Thus, the expression of ROR $\gamma t$ , AHR, CCR6, and IL-23R seems to be the signature to define IL-17- and IL-22-producing cells in both adaptive and innate immune cells.

LTI-like cells are involved in the proper formation of peripheral LNs, the spleen, and gut-associated lymphoid tissue by highly expressing lymphotoxin  $\alpha$ , lymphotoxin  $\beta$ , and TNF- $\alpha$  (16, 25). Our data indicate that splenic and gut LTI-like cells also produce IL-17 and IL-22 (Figs. 3 A and 5 B; and Fig. S7, A and D) (26). It has been recently shown that blocking IL-17 results in disruption of the germinal center-like formation in the spleen, and restoration of the lymphoid microanatomy is dependent on the proliferative accumulation of LTI-like cells in SLOs during viral infection (27, 28). In addition, the cross talk between bacteria, LTI cells, stromal cells, DCs, and B cells seems to be essential for isolated lymphoid follicle formation in the gut (29). LTI-like cells might



**Figure 5. LTI-like cells produce high levels of IL-22.** (A) Sera and splenocytes were harvested from  $Rag2^{-/-}$  mice 3 h after injection of PBS (Control) or 5 mg zymosan. (left) IL-22 in sera was measured by ELISA. (right) The relative expression levels (/18S) of IL-22 mRNA were determined by q-PCR and are depicted as fold induction relative to control cells. The data are means  $\pm$  SD from two mice and are representative of two independent experiments. (B) WT or  $Rag2^{-/-}$  splenocytes or isolated LTI-like cells were cultured in the presence of IL-23 for 24 h, and IL-22 in culture supernatants was measured by ELISA. The data are means  $\pm$  SD from duplicate cultures and are representative of two independent experiments.

help organize the development of lymphoid tissues through the production of IL-17. In addition, IL-17 and IL-22 act on epidermal cells, producing antimicrobial products that protect the host from extracellular bacteria and fungi (22). Our *in vivo* experiments in which Rag2<sup>-/-</sup> mice challenged with zymosan produced IL-17 (Fig. 4) are consistent with the notion that splenic LTI-like cells might participate in host defense. It is therefore possible that splenic LTI-like cells exist not only for the development of SLOs but also for the rapid response to pathogens that elicit IL-17 and IL-22. Thus, our report brings to light new potential roles of LTI-like cells in host defense in addition to their known roles in regulating lymphoid architecture.

## MATERIALS AND METHODS

**Recombinant cytokines and antibodies.** Mouse IL-6, IL-12, IL-21, IL-23, and TGFβ-1 were purchased from R&D Systems. Mouse IL-2, IL-4, IL-7, and IL-15 were purchased from PeproTech. Antibodies to CD4 (GK1.5), CD3 (145-2C11), CD28 (37.51), CD11c (HL3), B220 (RA3-6B2), γc (4G3), IL-7Rα (SB199), Thy1.2 (30-H12), CD44 (IM7), CD62L (MEL-14), CCR5 (C34-3448), CXCR5 (2G8), and IL-17 (TC11-18H10; for IL-17A) were purchased from BD. Antibodies to CD30L (RM153), OX40L (RM134L), and CCR7 (4B12) were purchased from eBioscience. Anti-CCR6 antibody (clone 140706) was obtained from R&D Systems. Both anti-CXCR3 and -CCR4 antibodies were purchased from Abcam. α-GalCer was obtained from AXXORA, LLC.

**Mice.** C57BL/6J WT mice (The Jackson Laboratory), Rag2<sup>-/-</sup> mice, and Rag2<sup>-/-</sup>γc<sup>-/-</sup> mice (Taconic) were purchased as indicated. Stat3<sup>fl/fl</sup> mice were bred with mice expressing Cre under the control of the MMTV-LTR (*MMTV-Cre*) to produce Stat3<sup>fl/fl</sup>; *MMTV-Cre* mice (provided by L. Hennighausen [National Institute of Diabetes and Digestive and Kidney Diseases] and D. Levy [New York University, New York, NY]) (30). All animal experiments were performed according to the National Institutes of Health (NIH) guidelines for laboratory animals and were approved by the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) Animal Care and Use Committee.

**Isolation of cells and cell culture.** Single-cell suspensions were prepared from spleens of healthy 8–10-wk-old mice. All cell cultures were performed in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 5 mM Hepes, 100 U/ml Pen-Strep, and 2.5 μM 2-ME at 37°C for 4, 24, or 48 h. Cells stained with the appropriate antibodies were isolated by flow cytometric cell sorting using a Mo-Flo cell sorter (Dako). Whole splenocytes or isolated cells were cultured in the presence of 20 ng/ml IL-23, 20 ng/ml IL-4, 10 ng/ml IL-6, 20 ng/ml IL-7, 20 ng/ml IL-15, 20 ng/ml IL-2, 20 ng/ml IL-12, 100 ng/ml IL-21, or 5 ng/ml TGFβ-1.

**Flow cytometric analysis and intracellular cytokine staining.** Cells were stimulated for 2 h with 50 ng/ml PMA and 1 μg/ml Iono, followed by incubation with BFA (GolgiPlug; BD) for an additional 2 h. Cells were fixed in 4% formal saline and permeabilized with 0.1% saponin permeabilization buffer after surface staining. PE-conjugated anti-IL-17 antibody was used to detect intracellular cytokine levels (BD). Stained cells with the appropriate antibodies were analyzed on a flow cytometer (FACSCalibur; BD). Events were collected and analyzed with FlowJo software (Tree Star, Inc.). To evaluate production of IL-17A *in vivo*, Rag2<sup>-/-</sup> mice were injected i.p. with 5 or 12.5 mg zymosan (Sigma-Aldrich). Control animals received PBS. To assess *in vivo* intracellular cytokine levels, 0.25 mg BFA (Sigma-Aldrich) was simultaneously injected i.v., as previously described (21).

**RNA isolation and measurement of cytokines.** Total RNA was isolated using TRIzol reagent (Invitrogen) from freshly isolated

CD4<sup>+</sup>CD3<sup>+</sup>CD62L<sup>-</sup>CD44<sup>+</sup> memory T cells; CD4<sup>+</sup>CD3<sup>+</sup>CD62L<sup>high</sup>CD44<sup>low</sup> naive T cells; CD19<sup>+</sup> B cells, Th17 cells polarized with IL-6, TGFβ-1, and anti-CD3/-CD28 for 3 d; and CD4<sup>+</sup>CD3<sup>-</sup>CD11c<sup>-</sup>B220<sup>+</sup> LTI-like cells. cDNA was synthesized with the TaqMan Reverse Transcription kit (Applied Biosystems). TaqMan primers and probes for mouse IL-17A, IL-17F, IL-22, IL-23R, IL-12Rβ1, IL-12Rβ2, RORc (for RORγt), IL-17RA, IL-6Rα, CCR6, AHR, GATA3, and 18S rRNA (as endogenous control) were purchased from Applied Biosystems. Samples were analyzed by using a sequence detection system (ABI PRISM 7700; Applied Biosystems).

The amounts of cytokines in the culture supernatant were measured using mouse IL-17 Quantikine assay kits (for IL-17A; R&D Systems) and the mouse IL-22 ELISA construction kit (Antigenix America Inc.) according to the manufacturers' instructions. IL-23-mediated cell stimulations were performed at cell concentrations of 4 × 10<sup>6</sup> cells/ml. Samples were measured in duplicate against the standard curve of the assay.

**Data analysis.** Statistical significance was determined by the Student's *t* test. *P* < 0.05 was considered to indicate a significant difference.

**Online supplemental material.** Fig. S1 shows IL-17A production by WT or Rag2<sup>-/-</sup> splenocytes in the presence of IL-23 with γc-dependent cytokines, anti-CD3/-CD28, or α-GalCer. Fig. S2 shows that IL-17A-producing CD3<sup>-</sup> cells do not express myeloid markers. Fig. S3 shows three populations in CD4<sup>+</sup>CD3<sup>-</sup> cells from Rag2<sup>-/-</sup> spleens. Fig. S4 shows that LTI-negative subsets produce minimal IL-17A. Fig. S5 shows IL-17A production by LTI-like cells in the gut. Fig. S6 shows the surface expression of various markers on LTI-like cells. Fig. S7 shows mRNA expression of various factors in LTI-like cells after IL-23 stimulation with IL-7. Fig. S8 shows IL-22 and IL-17A production by isolated NK cells. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20072713/DC1>.

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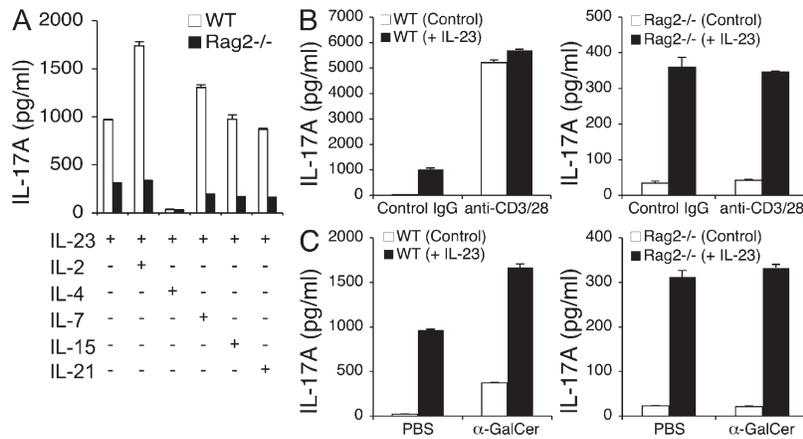
## REFERENCES

- Betelli, E., T. Korn, and V.K. Kuchroo. 2007. Th17: the third member of the effector T cell trilogy. *Curr. Opin. Immunol.* 19:652–657.
- Kolls, J.K., and A. Linden. 2004. Interleukin-17 family members and inflammation. *Immunity.* 21:467–476.
- Wei, L., A. Laurence, K.M. Elias, and J.J. O'Shea. 2007. IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner. *J. Biol. Chem.* 282:34605–34610.
- Parham, C., M. Chirica, J. Timans, E. Vaisberg, M. Travis, J. Cheung, S. Pflanz, R. Zhang, K.P. Singh, F. Vega, et al. 2002. A receptor for the heterodimeric cytokine IL-23 is composed of IL-12Rβ1 and a novel cytokine receptor subunit, IL-23R. *J. Immunol.* 168:5699–5708.
- McGeachy, M.J., K.S. Bak-Jensen, Y. Chen, C.M. Tato, W. Blumenschein, T. McClanahan, and D.J. Cua. 2007. TGF-β and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology. *Nat. Immunol.* 8:1390–1397.
- Shibata, K., H. Yamada, H. Hara, K. Kishihara, and Y. Yoshikai. 2007. Resident Vδ1+ γδ T cells control early infiltration of neutrophils after *Escherichia coli* infection via IL-17 production. *J. Immunol.* 178:4466–4472.
- Michel, M.L., A.C. Keller, C. Paget, M. Fujio, F. Trottein, P.B. Savage, C.H. Wong, E. Schneider, M. Dy, and M.C. Leite-de-Moraes. 2007. Identification of an IL-17-producing NK1.1<sup>int</sup> iNKT cell population involved in airway neutrophilia. *J. Exp. Med.* 204:995–1001.
- Cua, D.J., J. Sherlock, Y. Chen, C.A. Murphy, B. Joyce, B. Seymour, L. Lucian, W. To, S. Kwan, T. Churakova, et al. 2003. Interleukin-23

- rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature*. 421:744–748.
9. Murphy, C.A., C.L. Langrish, Y. Chen, W. Blumenschein, T. McClanahan, R.A. Kastelein, J.D. Sedgwick, and D.J. Cua. 2003. Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *J. Exp. Med.* 198:1951–1957.
  10. Langrish, C.L., Y. Chen, W.M. Blumenschein, J. Mattson, B. Basham, J.D. Sedgwick, T. McClanahan, R.A. Kastelein, and D.J. Cua. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* 201:233–240.
  11. Milner, J.D., J.M. Brenchley, A. Laurence, A.F. Freeman, B.J. Hill, K.M. Elias, Y. Kanno, C. Spalding, H.Z. Elloumi, M.L. Paulson, et al. 2008. Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature*. 452:773–776.
  12. Chen, Z., A. Laurence, Y. Kanno, M. Pacher-Zavisin, B.M. Zhu, C. Tato, A. Yoshimura, L. Hennighausen, and J.J. O’Shea. 2006. Selective regulatory function of Socs3 in the formation of IL-17-secreting T cells. *Proc. Natl. Acad. Sci. USA*. 103:8137–8142.
  13. Ivanov, I.I., B.S. McKenzie, L. Zhou, C.E. Tadokoro, A. Lepelley, J.J. Lafaille, D.J. Cua, and D.R. Littman. 2006. The orphan nuclear receptor ROR $\gamma$  directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell*. 126:1121–1133.
  14. Veldhoen, M., K. Hirota, A.M. Westendorf, J. Buer, L. Dumoutier, J.C. Renauld, and B. Stockinger. 2008. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature*. 453:106–109.
  15. Gerosa, F., B. Baldani-Guerra, L.A. Lyakh, G. Batoni, S. Esin, R.T. Winkler-Pickett, M.R. Consolaro, M. De Marchi, D. Giachino, A. Robbiano, et al. 2008. Differential regulation of interleukin 12 and interleukin 23 production in human dendritic cells. *J. Exp. Med.* 205:1447–1461.
  16. Ivanov, I.I., G.E. Diehl, and D.R. Littman. 2006. Lymphoid tissue inducer cells in intestinal immunity. *Curr. Top. Microbiol. Immunol.* 308:59–82.
  17. Kim, M.Y., G. Anderson, A. White, E. Jenkinson, W. Arlt, I.L. Martensson, L. Erlandsson, and P.J. Lane. 2005. OX40 ligand and CD30 ligand are expressed on adult but not neonatal CD4+CD3<sup>-</sup> inducer cells: evidence that IL-7 signals regulate CD30 ligand but not OX40 ligand expression. *J. Immunol.* 174:6686–6691.
  18. Lane, P.J., F.M. Gaspal, and M.Y. Kim. 2005. Two sides of a cellular coin: CD4+CD3<sup>-</sup> cells regulate memory responses and lymph-node organization. *Nat. Rev. Immunol.* 5:655–660.
  19. Acosta-Rodriguez, E.V., L. Rivino, J. Geginat, D. Jarrossay, M. Gattorno, A. Lanzavecchia, F. Sallusto, and G. Napolitani. 2007. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat. Immunol.* 8:639–646.
  20. Kim, M.Y., S. Rossi, D. Withers, F. McConnell, K.M. Toellner, F. Gaspal, E. Jenkinson, G. Anderson, and P.J. Lane. 2008. Heterogeneity of lymphoid tissue inducer cell populations present in embryonic and adult mouse lymphoid tissues. *Immunology*. 124:166–174.
  21. Liu, F., and J.L. Whitton. 2005. Cutting edge: re-evaluating the in vivo cytokine responses of CD8+ T cells during primary and secondary viral infections. *J. Immunol.* 174:5936–5940.
  22. Liang, S.C., X.Y. Tan, D.P. Luxenberg, R. Karim, K. Dunussi-Joannopoulos, M. Collins, and L.A. Fouser. 2006. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J. Exp. Med.* 203:2271–2279.
  23. Hue, S., P. Ahern, S. Buonocore, M.C. Kullberg, D.J. Cua, B.S. McKenzie, F. Powrie, and K.J. Maloy. 2006. Interleukin-23 drives innate and T cell-mediated intestinal inflammation. *J. Exp. Med.* 203:2473–2483.
  24. Annunziato, F., L. Cosmi, V. Santarlasci, L. Maggi, F. Liotta, B. Mazzinghi, E. Parente, L. Fili, S. Ferri, F. Frosali, et al. 2007. Phenotypic and functional features of human Th17 cells. *J. Exp. Med.* 204:1849–1861.
  25. Kim, M.Y., F.M. McConnell, F.M. Gaspal, A. White, S.H. Glanville, V. Bekiaris, L.S. Walker, J. Caamano, E. Jenkinson, G. Anderson, and P.J. Lane. 2007. Function of CD4+CD3<sup>-</sup> cells in relation to B- and T-zone stroma in spleen. *Blood*. 109:1602–1610.
  26. Ivanov, I.I., L. Frutos Rde, N. Manel, K. Yoshinaga, D.B. Rifkin, R.B. Sartor, B.B. Finlay, and D.R. Littman. 2008. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe*. 4:337–349.
  27. Hsu, H.C., P. Yang, J. Wang, Q. Wu, R. Myers, J. Chen, J. Yi, T. Guentert, A. Tousson, A.L. Stanus, et al. 2008. Interleukin 17-producing T helper cells and interleukin 17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice. *Nat. Immunol.* 9:166–175.
  28. Scandella, E., B. Bolinger, E. Lattmann, S. Miller, S. Favre, D.R. Littman, D. Finke, S.A. Luther, T. Junt, and B. Ludewig. 2008. Restoration of lymphoid organ integrity through the interaction of lymphoid tissue-inducer cells with stroma of the T cell zone. *Nat. Immunol.* 9:667–675.
  29. Tsuji, M., K. Suzuki, H. Kitamura, M. Maruya, K. Kinoshita, I.I. Ivanov, K. Itoh, D.R. Littman, and S. Fagarasan. 2008. Requirement for lymphoid tissue-inducer cells in isolated follicle formation and T cell-independent immunoglobulin a generation in the gut. *Immunity*. 29:261–271.
  30. Wagner, K.U., K. McAllister, T. Ward, B. Davis, R. Wiseman, and L. Hennighausen. 2001. Spatial and temporal expression of the Cre gene under the control of the MMTV-LTR in different lines of transgenic mice. *Transgenic Res.* 10:545–553.

SUPPLEMENTAL MATERIAL

Takatori et al., <http://www.jem.org/cgi/content/full/jem.20072713/DC1>



**Figure S1.**  $\gamma$ c cytokines, anti-CD3/28, and  $\alpha$ -GalCer do not enhance IL-23-mediated IL-17 production by Rag2<sup>-/-</sup> splenocytes. WT or Rag2<sup>-/-</sup> splenocytes were stimulated with (A) various  $\gamma$ c-dependent cytokines, (B) 5  $\mu$ g/ml anti-CD3/CD28, or (C) 100ng/ml  $\alpha$ -GalCer in the absence or presence of IL-23 for 48 h. IL-17A in culture supernatants was measured by ELISA. The data are means  $\pm$  SD.

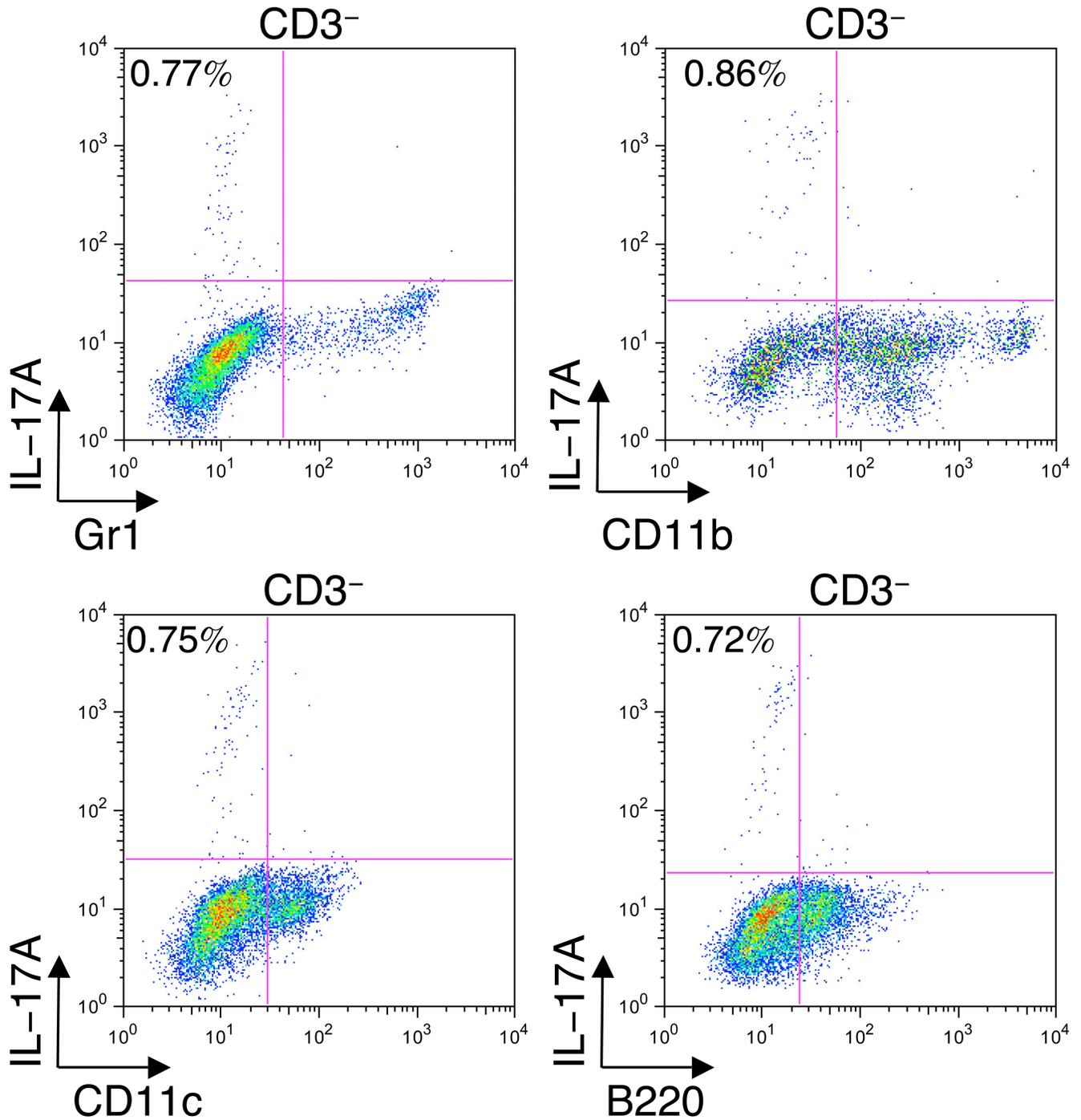


Figure S2. IL-17-producing CD3<sup>-</sup> cells do not express myeloid cell markers. The surface expression of presented markers and the proportion of IL-17A-producing CD3<sup>-</sup> cells were analyzed as described in Fig. 2.

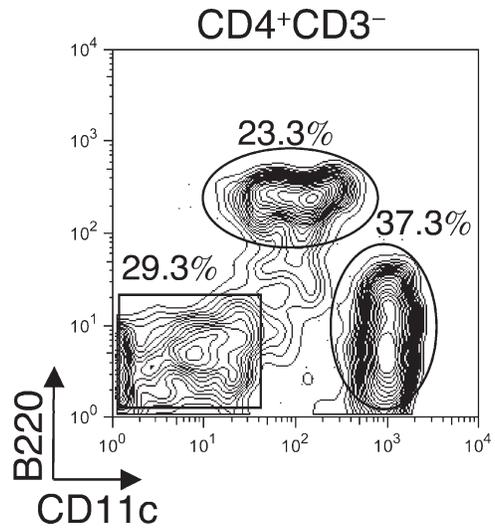


Figure S3. CD4<sup>+</sup>CD3<sup>-</sup> cells comprise LTI-like cells and DCs. Rag2<sup>-/-</sup> splenocytes were stained, and B220<sup>+</sup> versus CD11c<sup>+</sup> cells were gated on CD4<sup>+</sup>CD3<sup>-</sup> cells were analyzed by FACS.

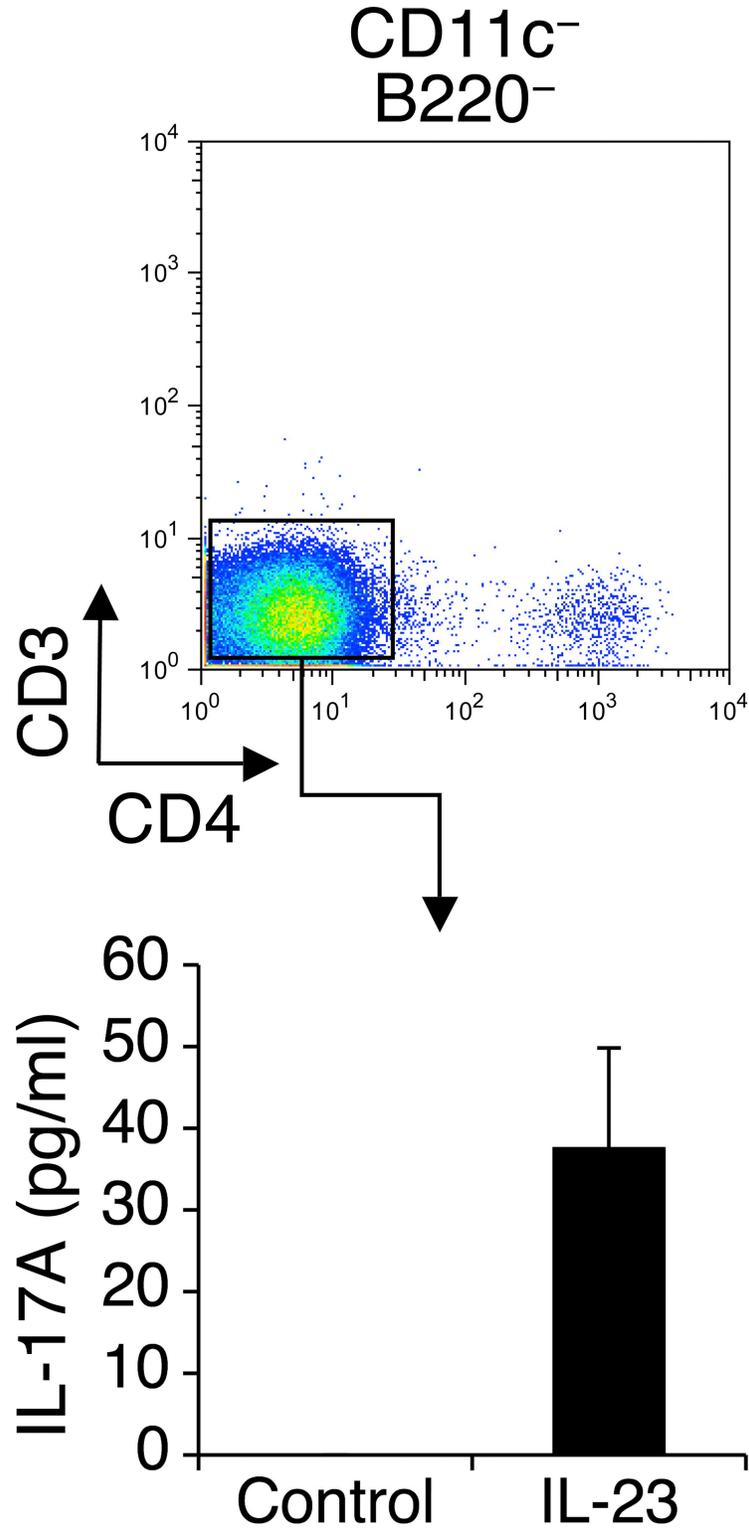
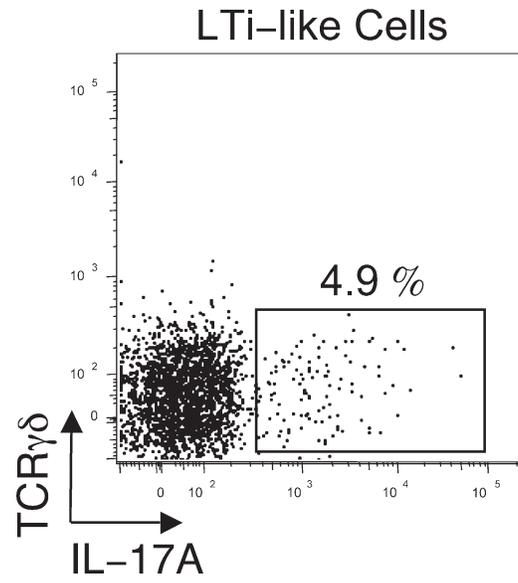


Figure S4. LTI-negative subsets produce minimal IL-17. IL-17A production by isolated CD4<sup>-</sup>CD3<sup>-</sup>CD11c<sup>-</sup>B220<sup>-</sup> cells were analyzed as described in Fig. 3 A. The data are means ± SD.



**Figure S5. Intestinal LTi-like cells also produce IL-17.** Lamina propria cells were isolated from the small intestines of WT mice. After stimulation of PMA/Iono, the proportion of IL-17A-producing LTi-like cells was analyzed by intracellular staining.

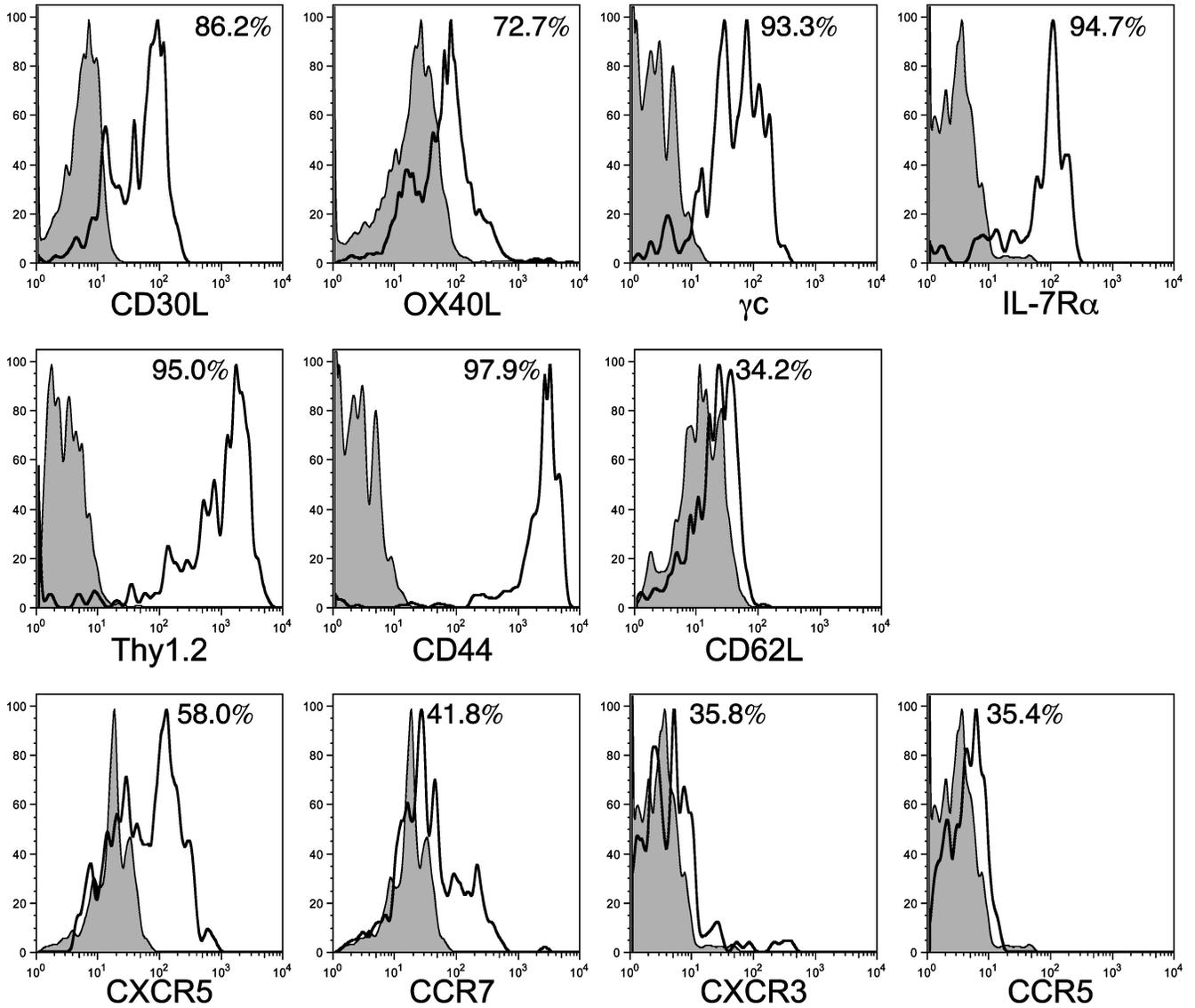
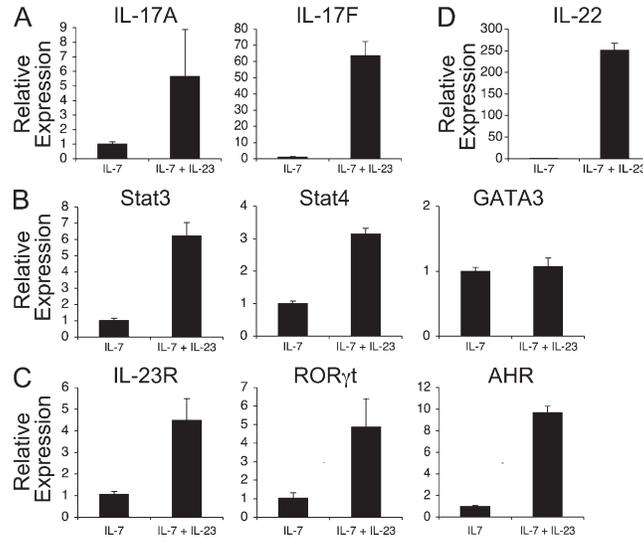
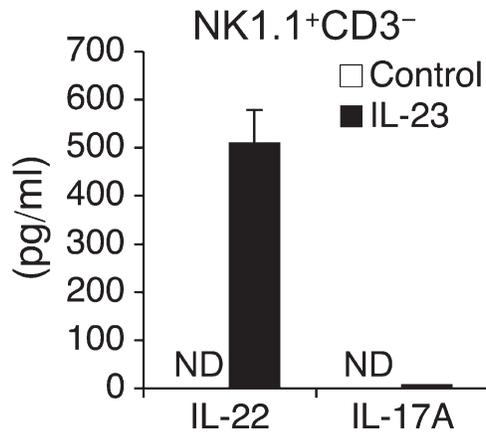


Figure S6. Splenic LTi-like cells are CD30L<sup>+</sup>OX40L<sup>+</sup> $\gamma$ C<sup>+</sup>IL-7R $\alpha$ <sup>+</sup>Thy1.2<sup>high</sup>CD44<sup>high</sup>CD62L<sup>low</sup>. The surface expression of the indicated markers on LTi-like cells was analyzed as described in Fig. 3 E.



**Figure S7.** IL-23 induces expression of Th17-related factors in LTI-like cells. Isolated LTI-like cells were cultured with IL-7 for 48 h, after which IL-23 was added for 6 h. The mRNA expression was measured by q-PCR and is depicted as fold induction relative to cells cultured with IL-7 alone. The data are means  $\pm$  SD.



**Figure S8.** Conventional NK cells produce small amounts of IL-22. Isolated NK1.1<sup>+</sup>CD3<sup>-</sup> cells were cultured in the absence or presence of IL-23, and cytokines in supernatants were measured by ELISA. The data are means  $\pm$  SD.