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) $2^{-/-}$ splenocytes but not Rag2$^{-/-}$ common γ chain$^{-/-}$ splenocytes. We found that a major source of IL-17 was CD4^{+}CD3^{-}NK1.1^{-}CD11b^{-}Gr1^{-}CD 11c^{-}B220^{-} 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.

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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-β1 are critical factors that promote Th17 cell differentiation (1, 3). Although IL-23 was originally thought to be important for inducing naive CD4^{+} T cells to become Th17 cells, naive CD4^{+} 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 γt (RORγt), 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 RORγt, 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 γ chain (γc)–dependent non–T, non–B cells

To determine if there are considerable proportions of innate immune cells that produce IL-17, we examined $Rag2^{-/-}$ mice that have few T and B cells. We first assessed whether IL-17 production occurred in $Rag2^{-/-}$ 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 $Rag2^{-/-}$ mice, the effect of IL-23 was reduced in $Rag2^{-/-}$ splenocytes (Fig. 1 A). However, $Rag2^{-/-}$ 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 $Rag2^{-/-}$ splenocytes (Fig. S1 A, available at http://www.jem.org/cgi/content/full/jem.20072713/DC1). In addition to memory $CD4^+$ T cells, iNKT cells are another important source of IL-17 (7), but such cells are lacking in $Rag2^{-/-}$ mice. Accordingly, anti-CD3/28 or α-galactosylceramide (α-GalCer) stimulation induced IL-17A production by WT splenocytes but not $Rag2^{-/-}$ 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 $Rag2^{-/-}$ splenocytes, we examined another genetic model, namely $Rag2^{-/-}\gamma c^{-/-}$ mice. In addition to lacking T and B cells, $Rag2^{-/-}\gamma c^{-/-}$ mice also lack NK cells. We therefore cultured WT, $Rag2^{-/-}$, or $Rag2^{-/-}\gamma c^{-/-}$ 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 $Rag2^{-/-}$ splenocytes (Fig. 1 B). In contrast, IL-17A production by $Rag2^{-/-}\gamma c^{-/-}$ splenocytes was essentially abrogated (Fig. 1 B). These results indicate that γc-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). $Rag2^{-/-}$ 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.

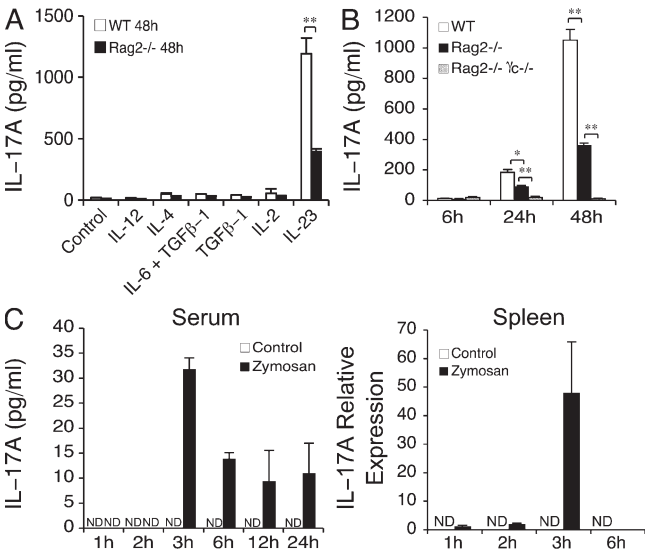

Figure 1. IL-23 and zymosan induce IL-17 production in a population of non–B, non–T cells. (A) WT or $Rag2^{-/-}$ splenocytes were cultured in the presence of the indicated cytokines for 48 h, and IL-17A in culture supernatants was measured by ELISA. The data are means ± SD from duplicate cultures and are representative of four independent experiments ($n = 8$). **, $P < 0.01$. (B) WT, $Rag2^{-/-}$, or $Rag2^{-/-}\gamma c^{-/-}$ splenocytes were cultured in the presence of IL-23 alone, and IL-17A in culture supernatants was measured by ELISA. The data are means ± 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 $Rag2^{-/-}$ mice after injection of 5 mg zymosan or PBS (Control). (left) IL-17A in sera was measured by ELISA. (right) The relative expression levels (/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 ± SD of results from two mice per group per time point and are representative of two independent experiments. ND, not detected.

IL-17 production by $CD4^+CD3^-CD11c^-B220^-$ 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 $Rag2^{-/-}$ 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 γc-dependent lineage that rapidly produces cytokines upon stimulation is the NK cell. Based on the response to IL-23 in $Rag2^{-/-}$ splenocytes and loss in $Rag2^{-/-}\gamma c^{-/-}$ splenocytes, we thought that NK cells could be IL-17 producers. However, contrary to our expectations, the majority of IL-17A–producing $CD3^-$ cells did not express NK1.1 (Fig. 2 A). Thus far, our results have indicated that neither T, NKT, NK cells (all γc dependent) nor γc-independent myeloid cells were a major source of IL-17 production in $Rag2^{-/-}$ spleens. We did observe that in $Rag2^{-/-}$ 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).

$CD4^+CD3^-$ cells in the $Rag2^{-/-}$ spleen comprise three populations, including $CD11C^{low}B220^+$ plasmacytoid DCs, $CD11C^+B220^-$ conventional DCs, and $CD4^+CD3^-CD11c^-B220^-$ cells (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20072713/DC1). As indicated in Fig. S2,

we did not detect IL-23–mediated IL-17A production by CD11C$^+$ DC populations. The latter, CD4$^+$CD3$^-$CD11c$^-$B220$^-$ 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$^+$CD3$^-$CD11c$^-$B220$^-$ 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$^+$ 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.

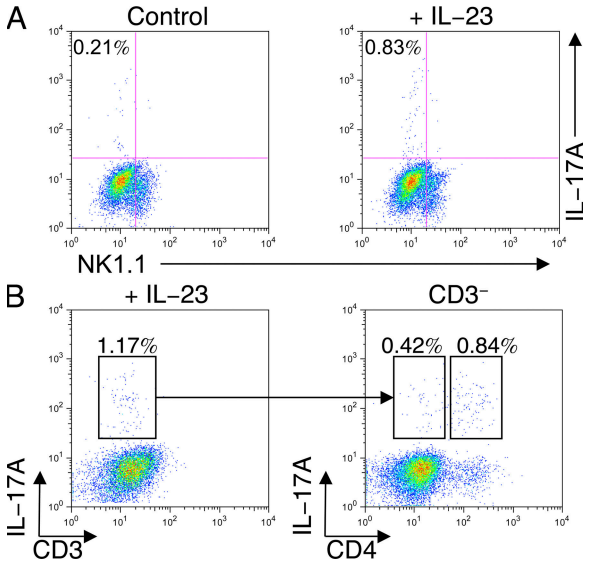

Figure 2. CD4$^+$CD3$^-$lineage$^-$ cells produce IL-17. Rag2$^{-/-}$ splenocytes were cultured in the absence (Control) or presence of IL-23 for 24 h. The proportion of IL-17A–producing CD3$^-$ cells was evaluated by intracellular staining. (A) NK1.1$^+$ versus IL-17A$^+$ cells gated on CD3$^-$ cells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

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$^{fl/fl}$; MMTV-Cre* or *Stat3$^{fl/fl}$; CD4-Cre* splenocytes compared with those of *Stat3$^{fl/fl}$* 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$^+$ 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$^+$ 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$^+$ 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$^+$OX40L$^+$γc$^+$IL-7Rα$^+$Thy1.2^{high} CD44^{high}CD62L^{low} (Fig. S6). LTi-like cells are also known to express the chemokine receptors CXCR5$^+$ and CCR7$^+$ (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

memory CD4+ 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+ LTi-like cells coexpressed 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$^{-/-}$ 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$^{-/-}$ 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 proportion 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$^{-/-}$ 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$^{-/-}$ mice (Fig. 5 A). In vitro stimulation with

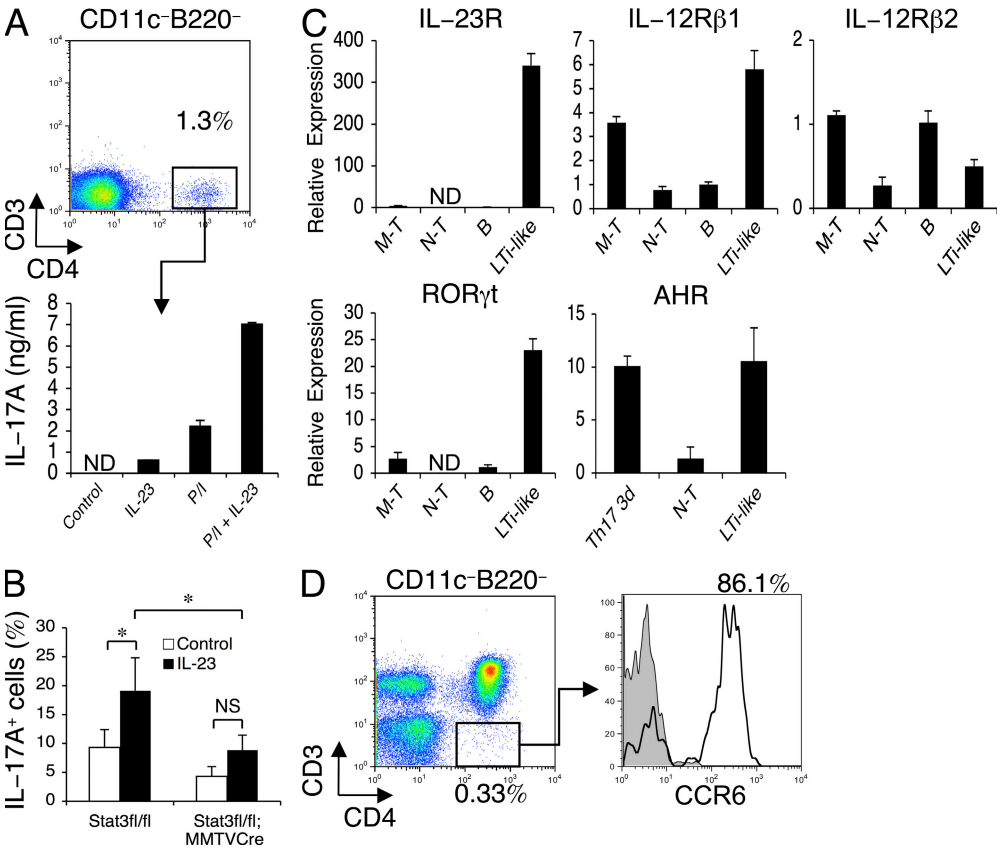

Figure 3. Isolated CD4+CD3−CD11c−B220− LTi-like cells produce IL-17 and constitutively express IL-23R, RORγt, AHR, and CCR6. (A) Isolated CD4+CD3−CD11c−B220− LTi-like subsets from Rag2$^{-/-}$ splenocytes (top) were cultured in the absence (Control) or presence of IL-23 (or with PMA/Iono) for 24 h, and 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*$^{fl/fl}$ or *Stat3*$^{fl/fl}$; *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$^{-/-}$ 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.

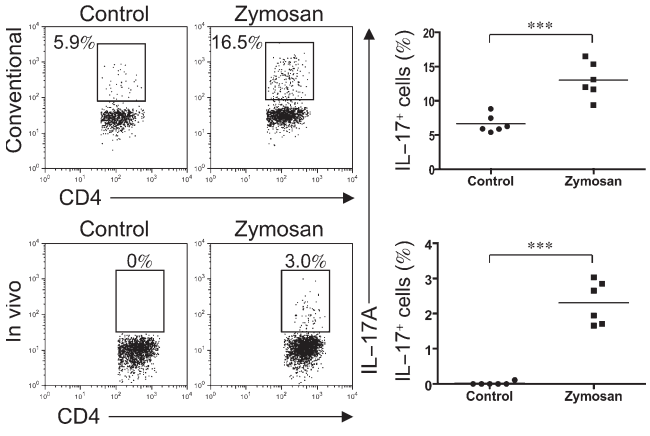

Figure 4. Zymosan induces IL-17 production in splenic LTi-like cells in vivo. (left) Intracellular staining of IL-17A was performed with in vitro conventional staining (top) or in vivo staining (bottom). For conventional staining, Rag2$^{-/-}$ mice were challenged with PBS (Control) or 12.5 mg zymosan 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, γδ 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$^{-/-}$γc$^{-/-}$ 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 γc cytokines for development (17). Before our studies, it was known that these cells expressed RORγt, which was required for their function in LNs (16, 18). RORγt 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 RORγt (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 RORγt, 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 α, lymphotoxin β, and TNF-α (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

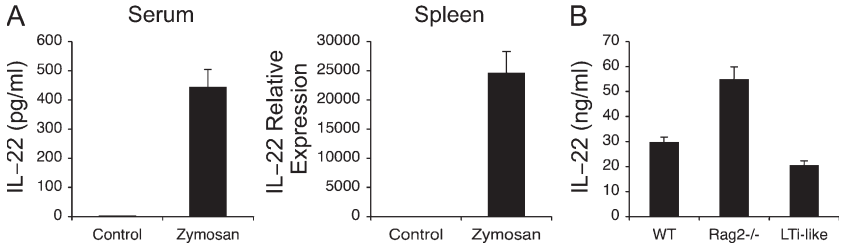

Figure 5. LTi-like cells produce high levels of IL-22. (A) Sera and splenocytes were harvested from Rag2$^{-/-}$ mice 3 h after injection of PBS (Control) or 5 mg zymosan. (left) IL-22 in sera was measured by ELISA. (right) The relative expression levels (/18S) of IL-22 mRNA were determined by q-PCR and are depicted as fold induction relative to control cells. The data are means ± 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 ± 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$^{-/-}$ 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$^{-/-}$ mice, and Rag2$^{-/-}$γc$^{-/-}$ mice (Taconic) were purchased as indicated. *Stat3*$^{fl/fl}$ mice were bred with mice expressing Cre under the control of the MMTV-LTR (*MMTV-Cre*) to produce *Stat3*$^{fl/fl}$*; MMTV-Cre* mice (provided by L. Hennighhausen [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% formyl 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$^{-/-}$ 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$^+$CD3$^+$CD62L$^-$CD44$^+$ memory T cells; CD4$^+$CD3$^+$CD62L^{high}CD44^{low} naive T cells; CD19$^+$ B cells, Th17 cells polarized with IL-6, TGFβ-1, and anti-CD3/-CD28 for 3 d; and CD4$^+$CD3$^-$CD11c$^-$B220$^-$ 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 18SrRNA (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 4×10^6 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$^{-/-}$ 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$^-$ cells do not express myeloid markers. Fig. S3 shows three populations in CD4$^+$CD3$^-$ cells from Rag2$^{-/-}$ 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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