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Regulation of Cytokine Secretion in Human CD127$^+$ LTi-like Innate Lymphoid Cells by Toll-like Receptor 2

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SUMMARY

Lymphoid tissue inducer cells are members of an emerging family of innate lymphoid cells (ILC). Although these cells were originally reported to produce cytokines such as interleukin-17 (IL-17) and IL-22, we demonstrate here that human CD127$^+$RORC$^+$ and CD56$^+$CD127$^+$ LTi-like ILC also express IL-2, IL-5, and IL-13 after activation with physiologic stimuli such as common γ-chain cytokines, Toll-like receptor (TLR) 2 ligands, or IL-23. Whereas TLR2 signaling induced IL-5, IL-13, and IL-22 expression in a nuclear factor κB (NF-κB)-dependent manner, IL-23 costimulation induced only IL-22 production. CD127$^+$ LTi-like ILC displayed clonal heterogeneity for IL-13 and IL-5 production, suggesting in vivo polarization. Finally, we identified a role for autocrine IL-2 signaling in mediating the effects of TLR2 stimulation on CD56$^+$CD127$^+$ and CD127$^+$ LTi-like ILC. These results indicate that human LTi-like ILC can directly sense bacterial components and unravel a previously unrecognized functional heterogeneity among this important population of innate lymphoid cells.

INTRODUCTION

Lymphoid tissue inducer cells (LTi) are required for embryonic development of secondary lymphoid tissue in the mouse (Mebius, 2003). Recently, we identified human LTi cells in both fetal and postnatal tissue. These cells express CD127 (IL-7 receptor α) and the transcription factor RORC, as RORγ is called in humans, and produce IL-17 and IL-22 (Cupedo et al., 2009). In this same study we also identified a related cell type that shares characteristics with both LTi and NK cells, in that they express NK cell-associated markers such as CD56 and NKp44, as well as RORC and CD127. Cella et al. (2009) independently identified CD56$^+$ NKp44$^+$RORC$^+$ cells in human tonsils, intestinal lamina propria, and Peyer's patches. These cells produced IL-22 and have been named NK-22 cells. More recent analysis revealed that the CD56$^+$CD127$^+$RORC$^+$ cells we described are identical to NK-22 cells and are developmentally related to LTi-like cells (Crellin et al., 2010). Concurrent to these observations, a population of RORC$^+$ and NKp46$^+$ cells was detected in the human colon (Luci et al., 2009). An analogous population of noncytotoxic IL-22-producing cells expressing both RORγt and NKp46 was also present in mouse gut lamina propria, isolated lymphoid follicles, and Peyer's patches (Luci et al., 2009; Sanos et al., 2009; Satoh-Takayama et al., 2008). These cells mediate an innate response against enteric bacteria such as *Citrobacter rodentium*, which is dependent on IL-22 (Cella et al., 2009; Satoh-Takayama et al., 2008; Zheng et al., 2008). Interestingly, it was recently demonstrated that a population of innate lymphoid cells with LTi-like characteristics mediate pathology in IL-23-driven colitis models in RAG-deficient mice, suggesting that these cells may play an important role in certain inflammatory diseases (Buonocore et al., 2010).

It has now been established, in human and mouse, that the non-T, non-B CD127$^+$RORC$^+$ populations that include the LTi cells, as well as the NK receptor$^+$ LTi-like cells, are distinct from conventional NK cells (Crellin et al., 2010; Satoh-Takayama et al., 2010). However, given the similarities among LTi cells, NK receptor$^+$RORC$^+$CD127$^+$ cells and cNK cells, as well as their developmental dependency on the transcriptional repressor Id2, these cells may all originate from a common precursor and may be considered members of a family of cells that we call here innate lymphoid cells (ILC). Innate cells that may belong to the same ILC family were found in lymphoid clusters interspersed in adipose tissue, and were, like LTi cells and cNK, dependent on the Id2 and the gamma common receptor (Moro et al., 2010). The phenotype of these "natural helper" cells is similar to that of LTi cells, but they develop independently of RORγt. Natural helper cells produce the Th2 cytokines IL-4, IL-5, and IL-13, but not IL-17 or IL-22 (Moro et al., 2010). Another group described a very similar cell type, present in the spleen and lymph nodes of mice, that responded to the Th2-inducing cytokines IL-25 and IL-33, and were named nuocytes (Neill et al., 2010).

The developmental requirements for LTi cells have been explored at the genetic level (Boos et al., 2007; Eberl et al.,

2004; Yokota et al., 1999), but the physiological signals required for LTi cell activation have not been fully characterized. IL-7 can activate LTi cells, leading to an increased number of LTi cells and the development of ectopic lymph nodes and cecal patches in IL-7 transgenic mice (Meier et al., 2007), but it is unclear whether IL-7 acts alone or in concert with other factors. IL-15 activates human NK-22 cells from the tonsil (Cella et al., 2009), but it has yet to be determined what contribution IL-15 and also IL-2 may make to LTi cell activation. LTi and LTi-like ILC may also be activated by IL-23, which has been reported to induce IL-22 production by human NK-22 cells (Cella et al., 2009), and mouse LTi cells strongly express IL-23R and produce IL-17 in response to stimulation (Takatori et al., 2009).

$CD127^+$ LTi-like ILC may also be influenced by the presence of bacteria and bacterial products. The development of $NKp46^+Ror\gamma t^+$ cells in the gut is modified by the presence of commensal bacteria (Sanos et al., 2009; Satoh-Takayama et al., 2008). LTi-induced development of isolated lymphoid follicles in the gut also required colonization with commensal bacteria (Tsuji et al., 2008). Further, mouse LTi cells produced IL-17 and IL-22 in response to in vivo challenge with the yeast cell wall component zymosan (Takatori et al., 2009) or stimulation with TLR5 ligand flagellin (Van Maele et al., 2010). However, Takatori et al. (2009) did not address the issue of whether LTi cell activation was direct or was mediated via antigen presenting cells (APCs), and Van Maele et al. (2010) found that dendritic cells were required. Moreover, Cella et al. (2009) have suggested that human NK-22 cells cannot respond directly to TLR stimulation.

Here we have examined the cytokine production profile of human LTi cells and the physiological signals that promote secretion of cytokines by these cells. We show that human LTi and $CD56^+$ LTi-like cells produce large amounts of IL-2, as well as the Th2 cytokines IL-5 and IL-13. Given that LTi cells and NK $receptor^+RORC^+CD127^+$ ILC are important players in the mucosal immune system, we have addressed the question of whether human $CD127^+$ LTi-like ILC cells are capable of directly sensing and responding to Toll-like receptor (TLR) ligands. We show here that TLR2 is capable of inducing not only IL-22 but also IL-13 and IL-5 secretion from human LTi-like ILC.

RESULTS

$CD56^+CD127^+$ and $CD127^+$ LTi-like Cells Produce IL-13, IL-5, and IL-2

We, and others, have recently observed that human LTi and $CD56^+$ LTi-like cells produce IL-22 (Cella et al., 2009; Cupedo et al., 2009; Hughes et al., 2009) as well as IL-17 and tumor necrosis factor (TNF). Here we expanded our characterization of the cytokines produced by human LTi-like cells, giving particular attention to those cytokines that might play a role in mucosal immunity and homeostasis. We isolated CD117 $(cKit)^+CD56^+CD127^+$ cells, $CD117^+CD127^+$ LTi-like cells, and $CD127^-CD56^+$ cNK cells from lineage (CD3, TCR$\alpha\beta$, TCR$\gamma\delta$, CD19, CD14, and CD34)-negative tonsil cells (Figure S1A available online) The purified LTi-like cells from human tonsil expressed RORC transcripts as previously described (Cupedo et al., 2009), and flow cytometry analysis showed that they also expressed RORC protein. The histogram shows one peak that is shifted compared to the isotype control, suggesting that all cells are RORC positive, but we cannot exclude that there may be cells within the population that do not express RORC protein (Figure 1A).

Although LTi-like cells expressed CD117, they were negative for CD34, indicating that they are distinct from hematopoietic precursor cells. After stimulation with PMA plus ionomycin, LTi-like cells produced substantial amounts of IL-13 (Figure 1B) and high amounts of IL-2 (Figure 1C), which are comparable to IL-2 amounts secreted by naive T cells (data not shown). IL-4, IL-10, and IL-12 were not produced (data not shown). To make sure that IL-2, IL-13, and IL-22 are coproduced by the same cells, we analyzed the production of these cytokines by intracytoplasmic staining of in vitro-expanded LTi-like cells, whose cytokine profile is similar to the fresh cells, and observed coproduction of IL-22 with either IL-13 or IL-2 by $CD127^+$ LTi-like cells, whereas cNK did not produce these cytokines (Figure 1D and data not shown).

IL-13 is often associated with IL-5 expression, and recent studies identified non-T non-B cells innate lymphoid cells that produce high amounts of IL-13 and IL-5 (Moro et al., 2010; Neill et al., 2010; Saenz et al., 2010). Although IL-5 was not detectable in the supernatants of freshly isolated LTi-like cells stimulated with PMA plus ionomycin ex vivo (data not shown), IL-5 was expressed by expanded $CD127^+$ LTi cell lines and clones (Figures 1D and 1E). These clones are representative of $CD127^+$ LTi-like cells, as indicated by the fact that they expressed RORC transcripts, were able to produce IL-22, and showed LTi-like activity in vitro (Crellin et al., 2010).

Recently we reported that there is a clonal heterogeneity in LTi-like cells because we found clones that produced IL-17 but not IL-22, clones that produced both cytokines, as well as clones that produced only IL-22 (Crellin et al., 2010). We also observed clonal heterogeneity with respect to production of IL-2, IL-5, and IL-13 as compared to IL-22. Although the majority of clones produced high IL-22 and low IL-5, some clones (~10%–15%) produced low IL-22 and IL-17, but high IL-13, IL-5, and IL-2 (Figure 1F and data not shown). Further analysis did not reveal a correlation between IL-13 and IL-22 production; however, there was a correlation between IL-13 and IL-2 production ($r^2 = 0.6$) (Figure S1B). These $IL\text{-}22^{lo}IL\text{-}13^{hi}IL\text{-}5^{hi}$ clones expressed similar amounts of RORC protein to $IL\text{-}22^{hi}$ clones (Figure S1C). In addition, these clones expressed GATA3, a transcription factor associated with a Type 2 cytokine response in T cells; however, the amount of expression of GATA3 was not different from $IL\text{-}22^{hi}$ clones (Figure S1D). The clones that produced IL-5 and IL-13 did not produce detectable amounts of IL-4.

Taken together, our results indicate that $lin^-CD117^+CD127^+$ ILC produce not only IL-22 but also the Th2 cytokines IL-5 and IL-13. The isolation of cloned lines of $CD127^+$ ILC that produce IL-13 but not IL-22, and clones that express IL-22 but not IL-13, might suggest that $CD127^+$ ILC can undergo polarization.

Stimulation with IL-2 and IL-15 Induces Cytokine Production by LTi and $CD56^+$LTi-like Cells

To determine which physiological stimuli might stimulate LTi-like cells, we tested the effects of IL-2, IL-7, and IL-15 on

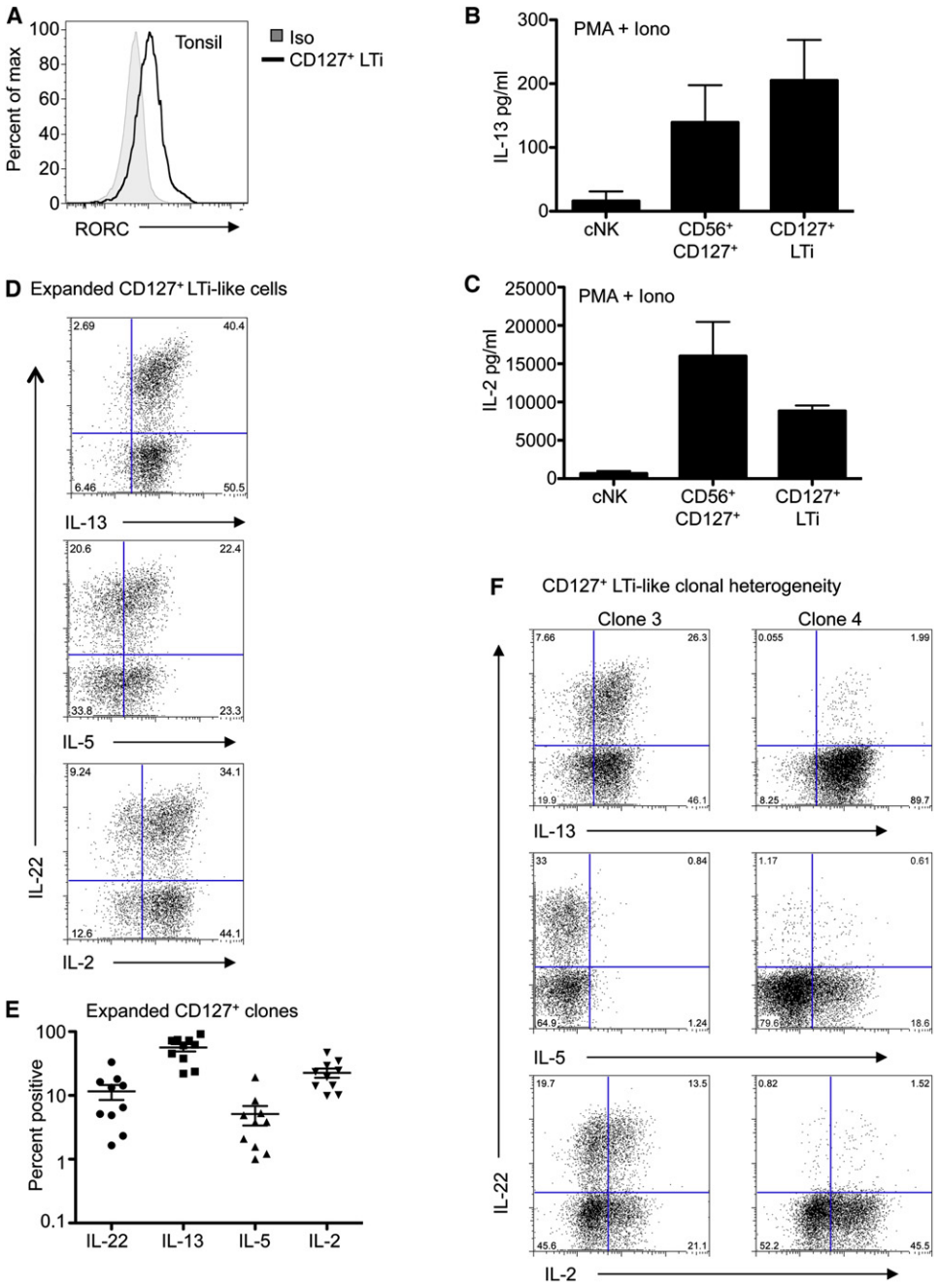

Figure 1. CD56$^+$CD127$^+$ and CD127$^+$ LTi-like Cells Produce IL-13, IL-5, and IL-2

(A) RORC protein expression in CD127$^+$ LTi cells from tonsil, as determined by flow cytometry.

(B and C) Flow cytometry sorted cells were stimulated ex vivo with PMA and ionomycin for 24 hr, and IL-13 (B) and IL-2 (C) secretion was determined by Luminex.

(D) CD127$^+$ LTi-like cells were expanded in vitro, and intracellular cytokine staining was performed after stimulation with PMA plus ionomycin for 6 hr, in the presence of brefeldin A.

(E and F) Single cell clones of CD117$^+$CD127$^+$ LTi-like cells were generated, and intracellular cytokine staining was performed as in (D).

Data shown are as follows. (A and D) A single donor representative of at least three donors. (B) and (C) represent the mean and SEM of at least three donors. (E) Each dot represents a separate clone expanded from a single donor, with mean and SEM indicated by horizontal lines. (F) Two representative clones are shown from one donor. See Figure S1 for additional data.

CD56$^+$CD127$^+$ and CD127$^+$ LTi-like cells because the receptors of these three cytokines are expressed on both populations. We observed that both CD56$^+$CD127$^+$ and CD127$^+$ LTi-like cells proliferated in response to all three cytokines, although the response to IL-2 and IL-15 was more robust than that to IL-7 (Figure 2A). As previously reported for NK-22 cells (Cella et al., 2009), the CD56$^+$CD127$^+$ cells and the CD56$^-$CD127$^+$ produced IL-22 ex vivo after stimulation with IL-15. CD56$^-$CD127$^+$ and

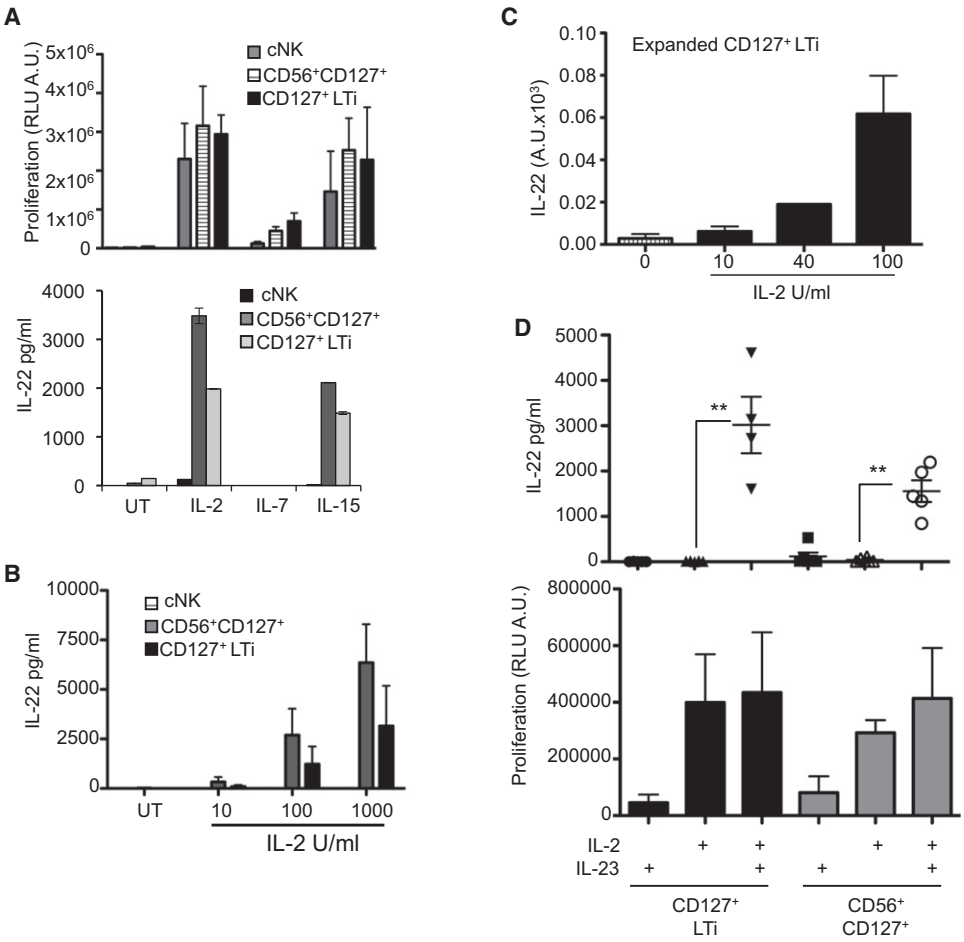

Figure 2. Stimulation with IL-2, IL-15, and IL-23 Induces Cytokine Production by LTi and CD56⁺ LTi-like Cells

(A) Proliferation and cytokine secretion by flow cytometry sorted tonsil cells, either unstimulated or stimulated with IL-2 (1000 U/ml), IL-7 (10 ng/ml), or IL-15 (10 ng/ml) for 6 days.

(B) IL-22 secretion by sorted cells as in (A) stimulated with 10, 100, or 1000 U/ml of IL-2.

(C) IL-22 mRNA expression in cultured CD127⁺ LTi cells stimulated for 18 hr with the indicated doses of IL-2. IL-22 mRNA was analyzed by quantitative RT-PCR. Samples were normalized via 18S expression and expressed as arbitrary units.

(D) IL-22 secretion and proliferation by flow cytometry sorted cells stimulated with IL-2 (10 U/ml), IL-23 (50 ng/ml), or IL-2+IL-23 for 6 days.

The mean and SEM of at least three donors is shown for (A)–(D). See Figure S2 for additional data.

CD56⁺CD127⁺ cells also produced IL-22 after stimulation with IL-2 in a dose-dependent manner (Figures 2B and 2C), whereas IL-7 induced only low or undetectable amounts of IL-22. CD56⁺CD127⁻ cNK cells responded to IL-2 and IL-15 by producing IFN-γ but not IL-22, as expected, but showed no response to IL-7 (Figure 2A). Overall, these data suggest that stimulation with γc cytokines alone is sufficient to induce the activation of LTi-like cells, resulting in proliferation and the production of IL-22.

Human CD127⁺ LTi-like Cells Require Costimulation to Respond to IL-23

It has been reported that NKp44⁺ CD56⁺RORC⁺ cells from the tonsil directly respond to IL-23 (Cella et al., 2009). We observed, however, that IL-23 stimulated IL-22 production of CD56⁺CD127⁺ and CD127⁺ LTi-like cells only in the presence of another stimulus. Coculture with IL-23 and low concentrations of IL-2 (10 U/ml), which by itself did not induce production of cytokines (Figure 2B), induced high amounts of IL-22 secretion from CD127⁺ LTi-like cells, and to a lesser extent from CD56⁺CD127⁺ cells (Figure 2D), but not from cNK cells. IL-23 alone did not provide a proliferative signal to either CD127⁺ LTi-like cells or CD56⁺CD127⁺ cells, and the addition of IL-23 to IL-2 did not induce a greater degree of proliferation as compared to IL-2 alone (Figure 2D). The IL-22 produced by IL-2- and IL-23-activated LTi cells has biological activity because we observed IL-10 production by the epithelial cell line Colo-205 cells after incubation with supernatants from stimulated CD56⁺CD127⁺ or CD127⁺ LTi-like cells. IL-10 production correlated with the amount of IL-22 in the supernatant (Figure S2). Our findings that IL-22 produced by LTi cells induces IL-10 production by Colo-205 cells are in line with findings of others (Cella et al., 2009; Nagalakshmi et al., 2004). Together our data indicate that in our system, CD56⁺CD127⁺ and

$CD127^+$ LTi-like cells require costimulation in order to respond to IL-23.

Human $CD56^+CD127^+$ and $CD127^+$ LTi-like Cells Express TLRs

Cella et al. (2009) reported that bacterial products induce IL-23 production in myeloid cells, which in turn stimulates IL-22 production by enriched $NKp44^+CD56^+RORC^+$ cells. Here, we considered the hypothesis that TLR agonists may also directly act on $RORC^+NKp46^+$ cells. By using quantitative RT-PCR, we observed broad expression of many TLR transcripts, including TLR1, 2, 5, 6, 7, and 9 on ex vivo $CD56^+CD127^+$ and $CD127^+$ LTi-like cells and cNK cells. (Figure 3 and Figure S3A). TLR3 and TLR4 were not expressed at substantial amounts on these cell types. $CD56^+CD127^+$ and $CD127^+$ LTi-like cells expressed TLR2, and the degree of expression was highest on $CD56^+CD127^+$ cells, although still lower than on $CD14^+$ monocytes (Figure 3A). The coreceptors for TLR2, TLR1, or TLR6 were also expressed at substantial amounts, raising the possibility that TLR2 is functional on $CD56^+CD127^+$ and $CD127^+$ LTi-like cells. The TLR2 signal was not due to contamination because TLR2 was also detected on cloned lines of $CD127^+$ LTi-like cells, and indeed correlated with RORC expression (Figure 3B). Additionally, freshly isolated $CD127^+$ LTi-like cells were positive for TLR2 protein as determined by flow cytometry, as were expanded $CD127^+$ LTi-like cell lines and clones (Figure 3C). These data indicate that human LTi-like ILC express a broad range of TLRs including TLR1, 2, 5, 6, 7, and 9, which suggests that these cells can directly respond to microorganisms.

Human LTi-like Cells Produce IL-22 and Proliferate in Response to TLR2 Costimulation

Initial experiments conducted with TLR ligands alone did not reveal any effect on $CD56^+CD127^+$ or $CD127^+$ LTi-like cell proliferation or cytokine secretion (data not shown), in agreement with what was previously reported (Cella et al., 2009). However, in nonmyeloid cells such as T cells and cNK cells, TLR signals are often costimulatory, in that they require additional stimulation through either TCR or cytokine receptors (Crellin et al., 2005; Girart et al., 2007; Komai-Koma et al., 2004; Liu et al., 2006; Sawaki et al., 2007; Sutmuller et al., 2006). Therefore, we cocultured $CD56^+CD127^+$ cells, $CD56^-CD127^+$ cells, and cNK cells ex vivo with the TLR1 and 2 ligand Pam3Cys (Pam3) in the presence or absence of a low dose of IL-2 (10 U/ml) or IL-15 (1 ng/ml) and observed that Pam3 induced IL-22 production from $CD127^+$ LTi-like cells, and to a greater extent $CD56^+CD127^+$ cells, in the presence of IL-2 (Figure 4A) or IL-15 (Figures S4A and S4B). A similar effect was observed with the TLR2 and 6 agonist Pam2Cys in the presence of IL-2 (data not shown), whereas TLR5, 7, or 9 agonists had no effect on IL-22 production (Figure S4C and data not shown). cNK cells did not produce IL-22 after costimulation with Pam3Cys and IL-2 (Figure 4A). Neither $CD56^+CD127^+$ nor $CD127^+$ LTi-like cells produced IFN-γ in response to this stimulus, nor was production of IL-17 or TNF increased (data not shown). Costimulation with low concentrations of IL-2 and Pam3 resulted in a ~2- to 3-fold increase in cell numbers for both $CD56^-CD127^+$ cells and $CD56^+CD127^+$ cells, as compared to IL-2 alone (Figure 4B), whereas Pam3 alone did not induce proliferation. Although the ex vivo culture was relatively lengthy (6 days), we have previously observed that the phenotype of LTi-like cells is very stable in culture (Crellin et al., 2010), and we have never observed differentiation of LTi cells in vitro within this period of time. Taken together, these data suggest that in the appropriate cytokine environment, $CD127^+$ LTi cells can increase in numbers and secrete cytokines in response to bacterial products. We confirmed that expanded $CD127^+$ LTi-like cells produced IL-22 protein, by intracellular staining performed after an overnight stimulation with IL-2 plus Pam3 (Figure 4C). Additionally, stimulation of expanded $CD127^+$ LTi-like cells overnight with IL-2 plus Pam3 increased IL-22 message (Figure 4D) and secreted IL-22 (Figure S4D) above that observed with IL-2 alone. The observation that Pam3 plus IL-2 induces IL-22 production from in vitro expanded $CD56^+CD127^+$ and $CD127^+$ LTi-like cells, which were rigorously assessed for lack of myeloid cells, excludes the possibility that an undetectable contamination with APCs, or other cytokine-secreting cell types, was contributing to the TLR2 response observed with freshly isolated cells. Further, the overnight stimulation resulting in intracellular cytokine production and increased IL-22 message confirms that the stimulation in cytokine production observed with ex vivo cells is not a result of the increased proliferation also observed in these cultures.

Neither IL-23 nor Pam3 could stimulate tonsillar LTi-like cells alone; however, when used in combination, a small increase in IL-22 production from $CD127^+$ LTi-like cells was observed (Figure 4E). In contrast, IL-23 and Pam3 strongly increased IL-22 production by $CD56^+CD127^+$ cells of some but not of all donors (Figure 4E). The reasons for this large donor-to-donor variability are unclear but may be attributed to the various states of inflammation of the tonsils used to isolate the $CD56^+CD127^+$ cells.

Although human LTi-like cells express TLR2 and respond to TLR2 activation, mouse LTi and $NKp46^+Ror\gamma t^+$ cells lack expression of TLR2 transcripts and protein (Figures S5A and S5B), and purified mouse LTi and intestinal $NKp46^+$ cells do not respond to TLR2 stimulation (Figure S5C and data not shown). However, treatment of total splenocytes (containing APCs) from *Rag2*$^{-/-}$ mice with IL-2 plus Pam3 did induce an increase in IL-22 production from LTi cells, most probably in an indirect manner (Figure S5D). Further, no alteration in numbers of intestinal $NKp46^+Ror\gamma t^+$ cells was observed in *Tlr2*$^{-/-}$ mice (data not shown).

Taken together, these data indicate that TLR agonists, in particular TLR2 agonists, directly act on human LTi-like ILC, thus regulating cytokine production by these cells in conjunction with IL-2 and/or IL-23.

IL-13 and IL-5 Are Induced by TLR2 Costimulation with IL-2, but Not with IL-23

Having established that a strong nonphysiological activation was able to induce IL-13 production from $CD127^+$ LTi-like cells, we explored physiological signals serving as triggers for IL-13 and IL-5 production. Stimulation of ex vivo (data not shown) and expanded $CD127^+$ cells with IL-2 induced IL-13 message (Figure 5A), similar to what we observed with IL-22 (Figure 2C). We also examined IL-13 expression in ex vivo $CD56^+CD127^+$, $CD127^+$ LTi-like cells, and cNK cells, costimulated with Pam3. Substantial amounts of IL-13 were produced in response to

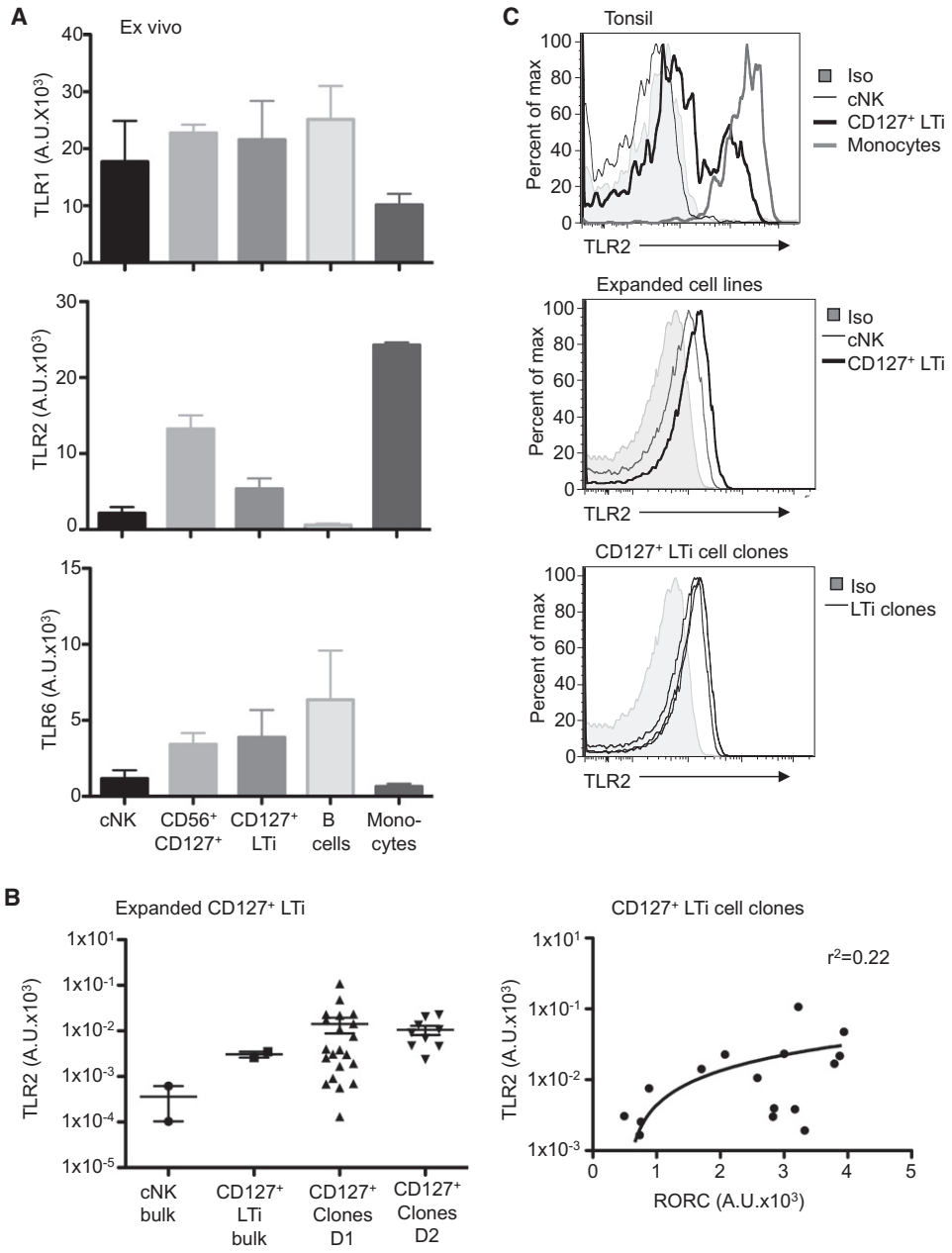

Figure 3. Human CD56⁺CD127⁺ and CD127⁺ LTi-like Cells Express TLRs

(A) The indicated populations were sorted with flow cytometry, and quantitative RT-PCR for TLR1, 2, and 6 mRNAs was performed. Samples were normalized with GAPDH expression levels and expressed as arbitrary units.

(B) Single cell clones of CD127⁺ LTi-like cells were expanded ex vivo, and quantitative RT-PCR for TLR2 and RORC mRNAs was performed. Samples were normalized via 18S expression and expressed as arbitrary units. Left panel of (B) shows TLR2 mRNA levels in individual CD127⁺ LTi-like cell clones from two independent donors, compared to the levels in bulk LTi-like or cNK cultures, while in the right panel the relationship between TLR2 and RORC mRNA levels in individual LTi-like cell clones from a single donor is shown.

(C) TLR2 protein expression on human tonsil cells, expanded CD127⁺ LTi cell lines, and clones, as determined by flow cytometry.

The mean and SEM of three donors is shown in (A), each dot represents a single clone in (B), with mean and SEM indicated by horizontal lines, and one donor representative of at least three is shown in (C). See Figure S3 for additional data.

stimulation with IL-2 plus Pam3 (Figure 5B) or IL-15 plus Pam3 (data not shown). Although ionomycin and PMA did not induce IL-5 by freshly isolated CD127⁺ cells, stimulation with IL-2 plus Pam3 did induce IL-5 (Figure 5B), indicating that ex vivo CD127⁺ LTi-like cells have the capacity to produce IL-5 under the appropriate stimulation.

Strikingly, IL-23 and IL-2 costimulation did not induce IL-13 or IL-5 production from either CD56⁺CD127⁺ or CD127⁺ LTi-like

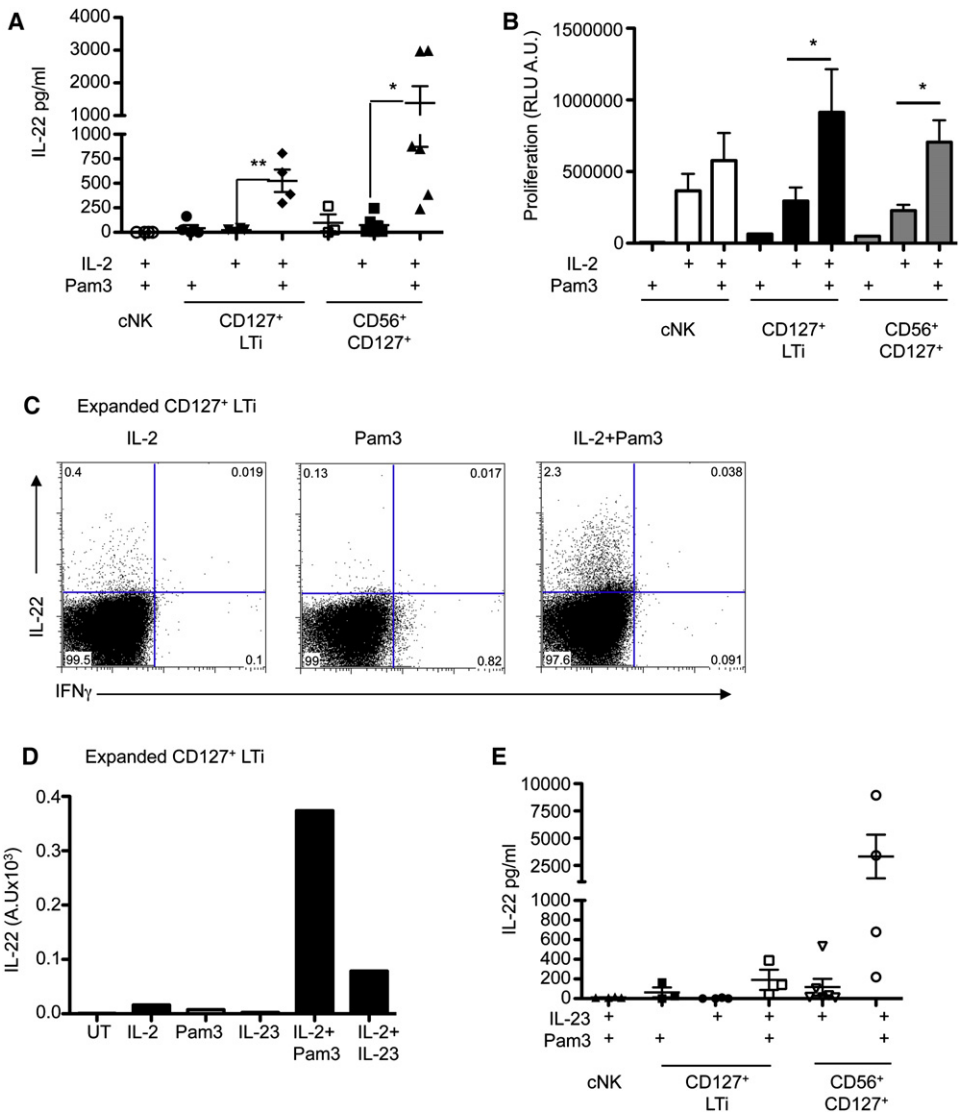

Figure 4. Human LTi-like Cells Produce IL-22 and Proliferate in Response to TLR2 Costimulation

(A) Cytokine secretion of flow cytometry sorted cells stimulated with IL-2 (10 U/ml), TLR2 agonist Pam3Cys (1 μg/ml), or IL-2+Pam3, for 6 days, as determined by ELISA.

(B) Proliferation of cells stimulated in (A).

(C) CD127$^+$ LTi-like cells were expanded in vitro and then stimulated for 24 hr as indicated, in the presence of Brefeldin A for the final 6 hr. Intracellular cytokine production was analyzed by flow cytometry.

(D) CD127$^+$ LTi-like cells were expanded in vitro and then stimulated for 18 hr as indicated, prior to qRT-PCR analysis of IL-22 mRNA levels.

(E) Cytokine secretion of flow cytometry sorted cells stimulated with IL-23 (50 ng/ml), Pam3, or IL-23 + Pam3 for 6 days, as determined by ELISA.

For (A) and (E) each dot represents a single donor, with the mean and SEM indicated by horizontal lines, while the mean and SEM of three donors is shown in (B). (C) and (D) represent a single donor of at least three. See Figures S4 and S5 for additional data.

cells (Figure 5B), even though IL-23 plus IL-2 induced substantial amounts of IL-22. Similarly, coculture with IL-23 plus Pam3 did not induce IL-13 or IL-5 production (Figure 5B). Expanded CD127$^+$ LTi-like cells stimulated with Pam3 and IL-2 increased IL-13 message above that induced by IL-2 alone (Figure 5C), in agreement with cytokine production of the freshly isolated cells. Additionally, overnight stimulation with Pam3 plus IL-2 induced IL-13 and IL-22 production in expanded CD127$^+$ LTi-like cells (Figure 5D). IL-23, however, did not upregulate nor inhibit IL-13 message (Figure 5C). CD127$^+$ LTi cell clones also displayed increased IL-13 production after stimulation with IL-2 plus Pam3, although expression varied between clones (Figure S6). Taken together our data indicate that the composition of the cytokine milieu determines IL-5 and IL-13 production by CD56$^+$CD127$^+$ and CD127$^+$ LTi-like cells.

NF-κB Activation Contributes to TLR2-Costimulated IL-13 and IL-22 Production

As demonstrated above, IL-2 with IL-23 stimulation induced IL-22 but was unable to induce IL-13 production (Figure 5).

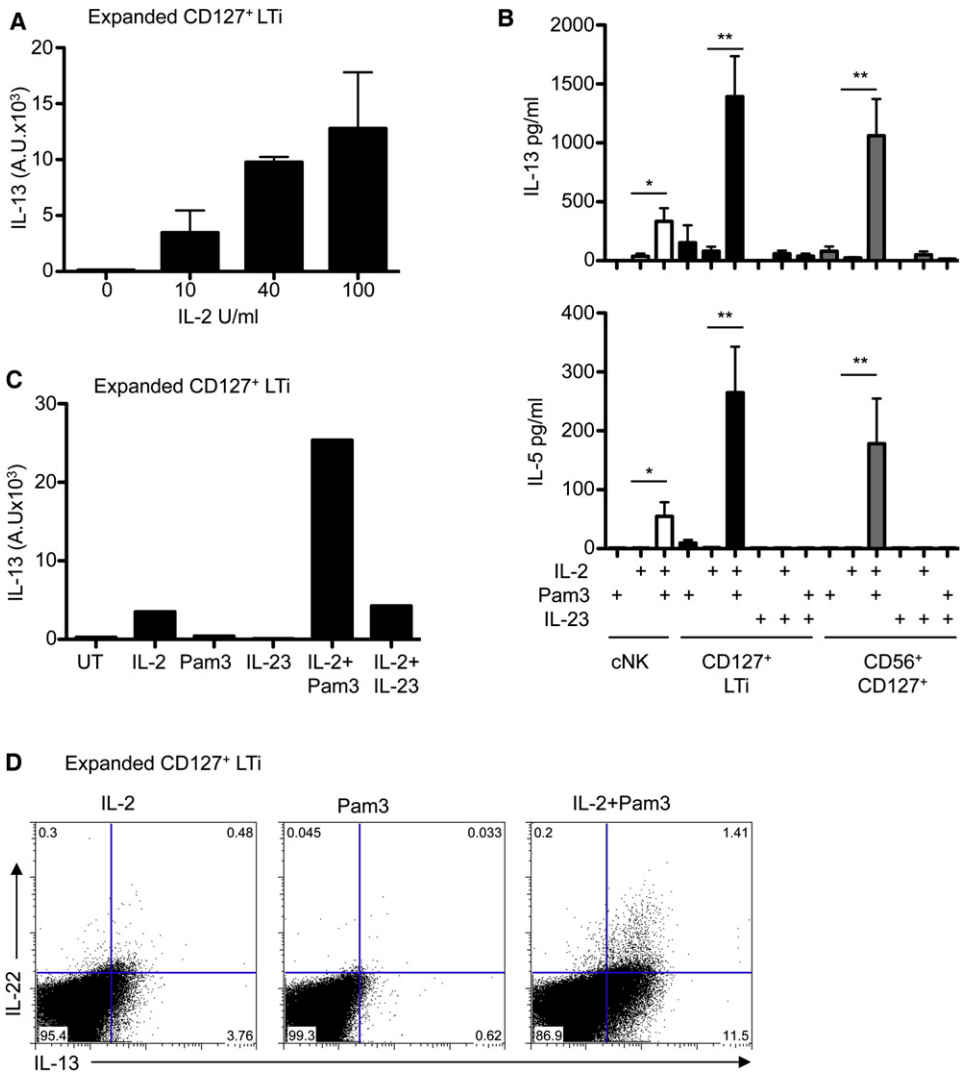

Figure 5. IL-13 and IL-5 Are Induced by TLR2 Costimulation with IL-2, but Not with IL-23

(A) CD127$^+$ LTi-like cells were expanded in vitro and stimulated for 18 hr with IL-2 as indicated. IL-13 mRNA levels were analyzed by quantitative RT-PCR.

(B) Cytokine secretion by flow cytometry sorted cNK, CD127$^+$CD56$^+$, and CD127$^+$ LTi-like cells, stimulated with IL-2 (10 U/ml), Pam3Cys (1 μg/ml), and IL-23 (50 ng/ml), for 6 days, as determined by ELISA and Luminex.

(C) CD127$^+$ LTi-like cells were expanded in vitro, then stimulated for 18 hr as indicated, and IL-13 mRNA levels were analyzed by quantitative RT-PCR.

(D) CD127$^+$ LTi-like cells were expanded in vitro and were stimulated for 24 hr as indicated, in the presence of Brefeldin A for the final 6 hr. Intracellular cytokine production of IL-22 and IL-13 was analyzed by flow cytometry.

For (A) and (B), the mean and SEM of at least three donors are shown. (C) and (D) represent a single donor of at least three. See Figure S6 for additional data.

In contrast, stimulation with TLR2 ligand Pam3Cys, in combination with either IL-2 or IL-15, induced IL-22 and IL-13, as well as IL-5, suggesting that the signaling requirements for production of these cytokines are different. Because TLR ligation is a well-known activator of NF-κB (Kaisho and Akira, 2006), we expected that inhibition of NF-κB would compromise costimulation with Pam3. Indeed, NF-κB inhibition with Bay 11-7082 blocked Pam3 plus IL-2-induced IL-22 and IL-13 mRNA in a dose-dependent manner (Figure 6A). In contrast, inhibiting NF-κB had only a modest, possibly nonspecific, effect on IL-2 plus IL-23-induced IL-22 production (Figure 6B). A pan-Jak inhibitor blocked all responses to IL-2 plus Pam3 or IL-2 plus IL-23 costimulation (Figures 6A and 6B), whereas inhibition of ERK signaling by a MEK inhibitor had no effect (data not shown). These data indicate that TLR2 responsiveness is NF-κB dependent and requires a Jak-dependent costimulatory signal, such as that provided by gamma common cytokines.

Autocrine IL-2 Contributes to TLR2-Induced IL-22 Production

As described above, we observed substantial amounts of IL-2 production from CD56$^+$CD127$^+$ and CD127$^+$ LTi-like cells stimulated with PMA plus ionomycin. Additionally, IL-2 mRNA transcripts were present in ex vivo CD56$^+$CD127$^+$ and CD127$^+$ LTi-like ILC and were ~10-fold higher than cNK, suggesting in situ IL-2 production (data not shown). Given that CD127$^+$

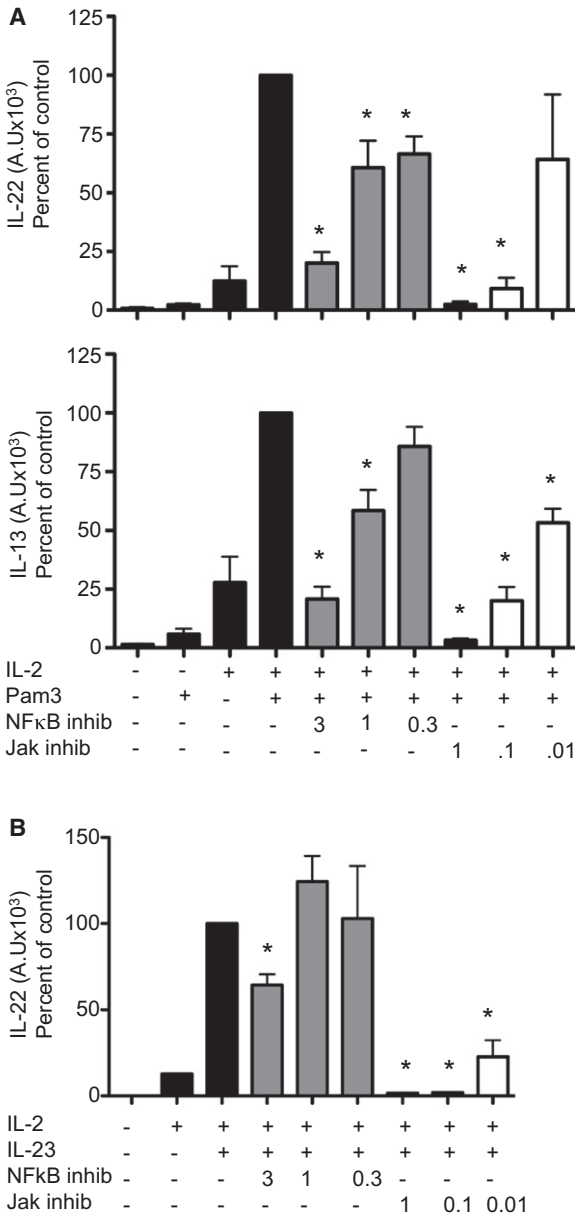

Figure 6. NF-κB Activation Contributes to TLR2 Costimulated IL-13 and IL-22 Production

$CD127^+$ LTi-like cells were expanded in vitro and stimulated with IL-2 (10 ng/ml), Pam3Cys (1 μg/ml), or IL-23 in the presence or absence of inhibitors to NF-κB or JAK kinase, as indicated, for 18 hr. Inhibitor concentrations are indicated in μM. IL-22 or IL-13 mRNA levels were analyzed by quantitative RT-PCR and normalized to 18S. For (A) the level of IL-22 or IL-13 mRNA produced by the cells stimulated with IL-2+Pam3 was set at 100% as reference, while for (B) the IL-2+IL-23 stimulation served as the control. Vehicle was included in control conditions lacking inhibitors. Mean and SEM of at least three donors are shown.

LTi-like cells responded to low amounts of IL-2 costimulation, we examined whether autocrine IL-2 is involved in the response to TLR2 costimulation. We observed a 2-fold increase in IL-2 mRNA expression in ex vivo or expanded $CD56^+CD127^+$ and $CD127^+$ LTi-like cells when stimulated with IL-15 and Pam3, as compared to IL-15 alone (Figure 7A). However, only low amounts of IL-2 protein were detected under these conditions. Nonetheless, these low amounts of IL-2 were biologically active, as indicated by the fact that the addition of a neutralizing IL-2 antibody to $CD56^+CD127^+$ or $CD127^+$ LTi-like cells stimulated with IL-15 and Pam3 reduced IL-22 secretion by ~65%, whereas isotype controls had no effect (Figure 7B). The increased proliferation induced by TLR2 costimulation was reduced by ~25% by the addition of an IL-2 blocking antibody (Figure 7C).

The large discrepancy of the amounts of IL-2 produced by LTi cells after activation with PMA plus ionomycin and IL-15 plus Pam3 may be caused by autocrine consumption of IL-2 under these latter conditions. Indeed, we have observed that ex vivo $CD56^+CD127^+$ and $CD127^+$ LTi-like cells express IL-2Rα (CD25) (Crellin et al., 2010). In light of the autocrine IL-2 production, we questioned whether CD25 expression is influenced by TLR2 stimulation. After culture with either low amounts of IL-2 plus Pam3 (Figure 7D) or IL-15 plus Pam3 (data not shown), we observed that CD25 expression was increased on $CD56^+C127^+$ and $CD127^+$ LTi-like cells, as compared to IL-2 alone. Expression of other activation markers, such as CD69, was not altered, suggesting a specific effect on CD25 expression. Interestingly, the upregulation of CD56 on $CD127^+$ LTi-like cells normally observed after IL-2 stimulation was greatly decreased in the presence of TLR2 costimulation (Figure S7). CD56 expression on $CD56^+CD127^+$ cells was unaffected, indicating that the effect of TLR2 was restricted to the maturation phenotype of $CD127^+$ LTi-like cells. Expression of NKp46 was unchanged, suggesting a specific effect on CD56 expression (data not shown).

Taken together, these data show that IL-2 produced by LTi-like ILC upon activation can amplify an ongoing signal induced by TLR2 signaling in an autocrine manner.

DISCUSSION

LTi and NK receptor-positive LTi-like innate lymphoid cells (ILC) are emerging as new players in the innate immune response (Vivier et al., 2009; Spits and Di Santo, 2010). Both in humans and mouse these ILC have been shown to produce IL-17 (Cupedo et al., 2009; Takatori et al., 2009; Van Maele et al., 2010) and IL-22 (Cella et al., 2009; Cupedo et al., 2009; Hughes et al., 2009; Luci et al., 2009; Sanos et al., 2009; Satoh-Takayama et al., 2008). Given the importance of $CD127^+RORC^+$ ILC in the innate immune response (Cella et al., 2009; Satoh-Takayama et al., 2008; Zenewicz et al., 2008), it is important to determine the cytokine production profile of these cells. We found that in addition to LTα and β, TNF, IL-17, and IL-22, $CD127^+RORC^+$ LTi-like ILC also secrete IL-2, IL-5, and IL-13. Importantly, the production of cytokines that act on cells of the adaptive immune system, such as IL-2, may suggest that $CD127^+RORC^+$ cells are involved in regulating the adaptive immune system as well.

The identification of a non-T cell-derived source of IL-2, IL-13, and IL-22 is of significance, particularly in the context of the gut. T regulatory cells are abundant in the gut and require IL-2 for optimal expansion, and IL-2 drives the development of "induced" T regulatory cells (Curotto de Lafaille and Lafaille, 2009; Josefowicz and Rudensky, 2009). Because autocrine

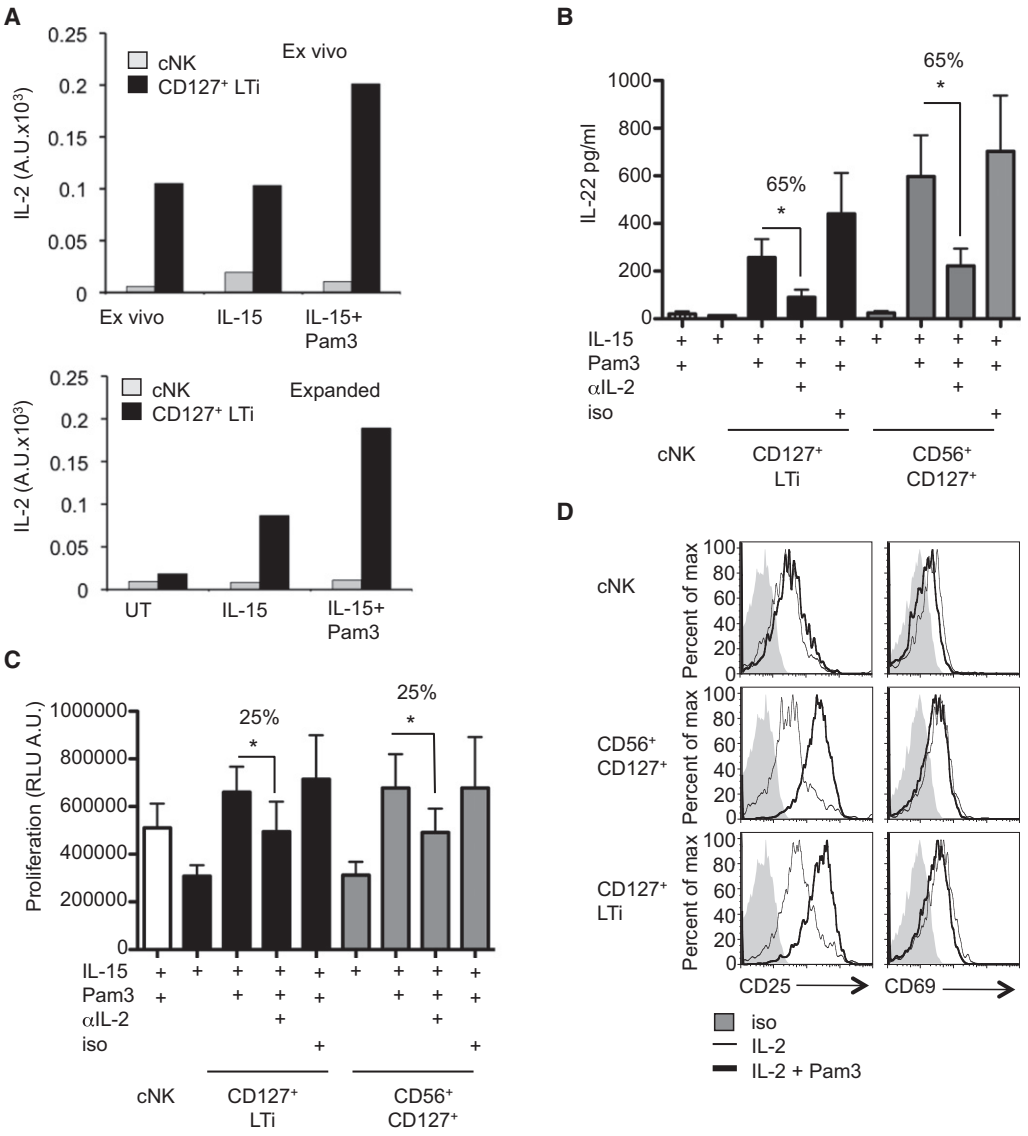

Figure 7. Autocrine IL-2 Contributes to TLR2-Induced IL-22 Production

(A) Ex vivo (top) or in vitro expanded (bottom) cNK and CD127$^+$ LTi-like cells were stimulated with IL-15 (1 ng/ml) or IL-15+Pam3 (1 μg/ml) overnight, and quantitative RT-PCR for IL-2 mRNA was performed. Samples were normalized with 18S expression and expressed as arbitrary units.

(B) Cytokine secretion by freshly isolated sorted cells stimulated with IL-15 (1 ng/ml) + Pam3 (1 μg/ml) in the presence or absence of a neutralizing IL-2 antibody (10 μg/ml) for 6 days, as determined by ELISA.

(C) Proliferation of cells stimulated as in (B).

(D) Expression of CD25 and CD69 on flow cytometry sorted cells after 6 day stimulation with IL-2 (10 U/ml) + Pam3 (1 μg/ml).

A single experiment representative of at least two donors is shown in (A) and (D), while the mean and SEM of three donors is shown in (B) and (C). See Figure S7 for additional data.

IL-2 production contributes to the cytokine response of CD127$^+$RORC$^+$ cells induced by TLR2 costimulation, deficiencies in IL-2 produced by LTi and CD56$^+$ LTi-like cells may result in pathology in the gut. IL-22 and IL-13 are required for normal wound healing in the mouse gut (Pickert et al., 2009; Seno et al., 2009), thus further supporting the idea that LTi-like ILC cells are involved in regulation of the integrity of the epithelial cell layer in the mouse gut. This may also be the case in human gut, because IL-22 has been shown to promote the migration and in vitro "wound healing" of human gut-derived epithelial cell lines (Brand et al., 2006). By using a punch biopsy model, Seno et al. (2009) demonstrated that IL-13 and IL-4 were upregulated in the wound bed and that blocking IL-4 and IL-13 delayed wound healing. Whereas IL-4 and IL-13 may be produced by Th2 cells present in the wound bed, an involvement of IL-13-producing ILC cannot be excluded.

CD127$^+$ innate cell types that are dedicated to the production of the Th2 cytokines IL-4, -5, and -13 have been recently discovered in the mouse. These cells, which were called natural helper lymphocytes in one study (Moro et al., 2010) and nuocytes in

another study (Neill et al., 2010), are RORγt negative (Moro et al., 2010), may be related to RORγt$^+$CD127$^+$ LTi cells because both LTi-like ILC and natural helper lymphocytes express CD127 and cKit, and depend on expression of Id2 (Moro et al., 2010). Our clonal analysis suggests the existence of subpopulations of human LTi-like ILC that may have different functions. In addition to IL-17- or IL-22-specific LTi clones (Crellin et al., 2010), we identified clones that produce IL-5 and IL-13 but no IL-22 or IL-17. These IL-5- and IL-13-producing CD127$^+$ cells may not represent the human equivalents of natural helper lymphocytes or nuocytes, because in contrast to the mouse cells (Moro et al., 2010; Neill et al., 2010), human CD127$^+$ cells express RORC transcripts and protein and fail to produce IL-4. However, we cannot formally exclude that the IL-13$^+$IL-5$^+$IL-22$^-$ clones were derived from cells that were initially RORC^{neg} and that the culture conditions used to expand these cells induced RORC. The relative amounts of IL-5 and IL-13 produced by RORC$^+$CD127$^+$ LTi-like ILC as compared to human nuocytes and natural lymphocytes is as yet unclear and should await further identification and characterization of the human equivalents of these cells. The existence in humans of CD127$^+$RORC$^+$ cells with different cytokine production profiles suggests that one mechanism regulating ILC cytokine production is the polarization into different subpopulations, much as is the case with T cells.

Given that CD127$^+$RORC$^+$ cells are most probably functioning at mucosal interfaces, the possible role of TLRs in regulating cytokine production in these cells seems logical. Indeed, CD56$^+$CD127$^+$ and CD127$^+$ LTi-like ILC express a variety of TLRs including TLR1, 2, and 6; however, only TLR2 agonists, in the presence of cytokines like IL-2, IL-15, and IL-23, consistently increased cytokine production by CD127$^+$RORC$^+$ cells. Costimulation of CD127$^+$RORC$^+$ cells with cytokines and Pam3 was blocked both by NF-κB and Jak inhibitors, confirming that these pathways collaborate inducing cytokine production by these cells. The fact that TLR2 acts as a costimulus, rather than as a trigger on its own, as is the case when TLR agonists stimulate myeloid cells, is not without precedent. TLR2 promotes expansion of Treg cells in conjunction with TCR stimulation (Komai-Koma et al., 2004; Liu et al., 2006; Sutmuller et al., 2006), and TLR2 stimulation enables CD8$^+$ T cells to respond to a suboptimal TCR signal, effectively lowering the threshold for activation (Mercier et al., 2009). Interestingly, TLR2 induced different responses depending on the cytokine milieu. For instance, TLR2 engagement induced only IL-5 and IL-13 in the presence of exogenous IL-2 or IL-15, but not of IL-23. Because IL-2 stimulates Jak3, and IL-23 stimulates Tyk2, it appears that IL-5 and IL-13 can be induced by costimulation of the NF-κB pathway with Jak3, but not by NF-κB and Tyk2 activation. In contrast, IL-22 can be induced by either combination of signaling pathways.

It has been reported that polymorphisms of TLR2 are linked with the disease phenotype in IBD, suggesting that inappropriate signaling through this receptor, perhaps affecting LTi or LTi-like cells, may contribute to the pathology (Henckaerts et al., 2007; Pierik et al., 2006). Increased numbers of lin$^-$CD117$^+$CD127$^+$ cells, which are most probably identical to the CD127$^+$RORC$^+$ cells described here, are present in the gut of patients with Crohn's disease (Chinen et al., 2007). It is possible that CD127$^+$RORC$^+$ cells play a role in the disease process, which would be consistent with the documented involvement of mouse LTi-like ILC in bacteria-driven induced innate colitis in RAG-deficient animals (Buonocore et al., 2010). It will be of interest to investigate the cytokine production profile of LTi cells in inflamed tissues in Crohn's disease and the effects of TLR2 agonists on the activities of these cells.

Overall, it appears that LTi-like ILC produce a broad range of cytokines and that environmental cues may skew their activation and differentiation. This has implications both for our understanding of normal mucosal homeostasis and for directing future investigations into disease pathology.

EXPERIMENTAL PROCEDURES

Cell Purification and Sorting

Human tonsils were digested for 30 min in 0.5 mg/ml collagenase IV (Invitrogen) at 37°C and processed to form a single cell suspension. CD3$^+$ and CD19$^+$ cells were then depleted via StemCell Technologies, Inc. (Vancouver, BC) kits. For flow cytometry sorting, cells were labeled with PE-conjugated anti-CD127 (BD Bioscience, San Jose, CA), PECy7-conjugated anti-CD56 (BD Bioscience), APC-conjugated anti-CD117 (eBioscience, San Diego, CA), Pacific blue-conjugated anti-CD19 (eBioscience), and FITC-conjugated anti-CD3, TCR-αβ, TCR-γδ, and CD14 (eBioscience, San Diego, CA) antibodies. Cells were sorted on a FACSAria (Becton Dickinson) to more than 95% purity. Cells were sorted as lineage$^-$ (CD3$^-$CD19$^-$TCR-αβ$^-$TCR-γδ$^-$CD14$^-$CD34$^-$) CD117$^+$CD127$^+$CD56$^+$ or lineage$^-$ CD117$^+$CD127$^+$CD56$^-$. Tonsils were obtained from Bio-options (Fullerton, CA). Human blood use was approved by the Western Institutional Review Board.

Animal Experiments

Rorc$^{GFP/+}$ BAC transgenic mice (12 weeks old) were used for lamina propria lymphocyte isolation as described (Satoh-Takayama et al., 2008). Small intestine was isolated and Peyer's patchs removed. Epithelial cells were eliminated by incubation with 0.5 mM EDTA. Lamina propria lymphocytes (LPLs) were isolated with 1 mg/ml collagenase (Sigma Aldrich). LPLs were further purified with discontinuous Percoll gradients (40% and 75%). For cell sorting, LPLs were stained with CD3 and NKp46 antibodies. CD3$^-$ subsets differing in NKp46 and RORγt expression were sorted with a FACSAria (Becton Dickinson).

To isolate splenic LTi cells, splenocytes from *Rag2*$^{-/-}$ mice (Taconic) were harvested, and red blood cells were lysed. The splenocytes were then stained with anti-CD11c, B220, CD3, CD8a (all FITC), CD4-APC or A700, and CD127-PE (all antibodies from eBioscience). Lin$^-$(CD11c, B220)CD4$^+$CD127$^+$ LTi cells were then sorted by flow cytometry to more than 95% purity. Splenic LTi cells were cultured in RPMI plus 10% FCS. All animal care and experiments were performed according to protocols approved by the Institutional Use and Care of Animals Committee.

Expansion of LTi Cell Lines and Clones

Flow cytometry-sorted cells were stimulated with irradiated 10^6/ml allogeneic PBMCs (5000 rad) and 1.10^5/ml irradiated JY EBV-transformed B cells (7500 rad), in the presence of 1 μg/mL PHA (Roche) and IL-2 (100 U/ml) (Chiron, Emeryville, CA). IL-2 was replenished every 3 days. Clones were plated at 1 cell/well via limiting dilution and expanded with the same protocol.

Cell Culture Assays

Cells were cultured in Yssel's medium (Gemini Bio-Products, Sacramento, CA, or in-house prepared) plus 1% human AB serum (Gemini Bio-Products). Freshly isolated cells were plated at 1×10^4 cells/ml, and expanded CD127$^+$ cells were plated at 5×10^4 cells/ml, in 96-well round bottom plates. Where indicated, cells were stimulated with TLR ligands such as Pam3CYSK4, all from Invivogen (San Diego, CA). Recombinant human IL-15 and IL-23 used for stimulation were purchased from R&D systems (Minneapolis, MN). Proliferation was determined with CellTiterGlo assay (Promega, Madison, WI). Neutralizing anti-human IL-2 antibody, or isotype control, were purchased

from BD Biosciences and used at 10 μg/ml. The following inhibitors were used as indicated in Figure 6: NF-κB inhibitor Bay 11-7082, and JAK inhibitor 1, both from EMD chemicals/Calbiochem (Gibbstown, NJ, USA).

Quantitative Real-Time PCR

Total RNA was extracted by using either the RNeasy mini or micro kit (QIAGEN, Valencia, CA), according to the manufacturer's instructions. cDNA was reverse transcribed with the high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA). Primers and probes were all predesigned Taqman Gene Expression Assays from Applied Biosystems. Real-time PCR was performed on the 7500 Real Time System (Applied Biosystems) machine. All samples were normalized with either 18S or GAPDH (Figure 2A only) expression level and expressed in arbitrary units.

For mouse cells, mRNA was extracted from isolated cell subsets with the RNeasy micro kit (QIAGEN). cDNA was generated with Superscript III (Invitorogen), and quantitative PCR was performed with the RT^2 Profiler PCR Array System with SYBER Green dye.

Flow Cytometry and Intracellular Cytokine Staining

Cell-surface TLR2 expression was determined with anti-TLR2-PE-Cy7 (eBioscience). Intracellular RORC was detected with anti-RORγt-PE (eBioscience), according to manufacturers' instructions. Intracellular cytokine staining was performed on ex vivo expanded cell lines stimulated for 6 hr with PMA (10 ng/mL) and ionomycin (500 nM) (Calbiochem, Gibbstown, NJ), or overnight with IL-2 (40 U/ml) and Pam3Cys (1 μg/ml), in the presence of 5 mg/ml BFA (BD Bioscience) for the final 4–6 hr of culture. Cells were stained with LIVE/DEAD (violet) viability assay (Invitrogen, Carlsbad, CA), and then fixed and permeabilized according to manufacturer's protocol (eBioscience). The following Abs from BD Biosciences were used: PE-conjugated anti-IL-13 and anti-IL-5 and FITC-conjugated anti-IL-2. AlexaFluor647-conjugated anti-IL-22 (prepared in-house) was also used. Monoclonal mouse anti-human IL-22 antibodies were generated in-house and conjugated with AlexaFluor 647, as previously described (Trifari et al., 2009). Data were acquired on a LSRII machine (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR). During analysis, dead cells were eliminated with the live/dead violet dye and doublets were eliminated based on light scattering parameters.

Luminex and ELISA

Supernatants were collected, and IL-2, IL-5, IL-13, IL-17A, IFN-γ, and TNF-α were measured by Bioplex bead-based assays (Bio-Rad, Hercules, CA) and read by Luminex System (Luminex). IL-22 (R&D Systems, Minneapolis, MN) was measured by ELISA, according to manufacturer's protocol.

Statistics

Statistical significance was determined by the paired Student's t test (difference between two groups or conditions), with 95% confidence intervals. For analysis of correlation, a linear regression analysis was performed. *$p < 0.05$, **$p < 0.01$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at doi:10.1016/j.immuni.2010.10.012.

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