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Interleukin-1β selectively expands and sustains interleukin-22^{+} immature human natural killer cells in secondary lymphoid tissue

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SUMMARY

Among human natural killer (NK) cell intermediates in secondary lymphoid tissue (SLT), stage 3 CD34^{-} CD117^{+}CD161^{+}CD94^{-} immature NK (iNK) cells uniquely express aryl hydrocarbon receptor (*AHR*) and interleukin-22 (IL-22), supporting a role in mucosal immunity. The mechanisms controlling proliferation and differentiation of these cells are unknown. Here we demonstrate that the IL-1 receptor IL-1R1 was selectively expressed by a subpopulation of iNK cells that localized proximal to IL-1β-producing conventional dendritic cells (cDCs) within SLT. IL-1R1^{hi} iNK cells required continuous exposure to IL-1β to retain *AHR* and IL-22 expression, and proliferate in direct response to cDC-derived IL-15 and IL-1β. In the absence of IL-1β, a substantially greater fraction of IL-1R1^{hi} iNK cells differentiated to stage 4 NK cells and acquired the ability to kill and secrete IFN-γ. Thus, cDC-derived IL-1β preserves and expands IL-1R1^{hi}IL-22^{+}*AHR*$^{+}$ iNK cells, potentially influencing human mucosal innate immunity during infection.

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INTRODUCTION

Natural killer (NK) cells comprise a unique, diverse set of lymphocytes with emerging roles in the innate and adaptive immune response (Sun and Lanier, 2009). Within secondary lymphoid tissue (SLT), human NK cells appear to proceed through five discrete states of developmental maturity, starting as multipotent $CD34^+$ pro-NK cells, and eventually progressing to functionally competent mature NK cells (Freud et al., 2005; Freud et al., 2006). Our laboratory initially characterized four stages of NK cell development in SLT in terms of lineage commitment, response to cytokines, and NK cell effector function (Freud et al., 2006). $CD34^+CD117^-$ stage 1 pro-NK cells and $CD34^+CD117^+$ stage 2 pre-NK cells retain potential for differentiation into T cells and dendritic cells (DCs), whereas $CD34^-CD117^+CD94^-$ stage 3 immature NK (iNK) cells and $CD34^-CD117^{+/-}CD94^+$ stage 4 mature NK cells appear exclusively committed to the NK cell lineage. In addition, a fifth and seemingly final stage of NK cell development has recently been described in SLT, characterized by acquisition of CD16 and killer cell immunoglobulin (Ig)-like receptors, with a surface antigen repertoire highly similar to that of peripheral blood $CD56^{dim}$ NK cells (Freud and Caligiuri, 2006; Romagnani et al., 2007). Stage 3 cells have been classified as immature because they lack certain NK cell surface receptors, the capacity for IFN-γ production, and the cytolytic activity that are characteristic of mature NK cells including the more differentiated stage 4 NK cells (Freud et al., 2006).

Resting stage 3 iNK cells found in human tonsil constitutively express interleukin-22 (IL-22) (Cupedo et al., 2009; Hughes et al., 2009), a cytokine implicated in mucosal immunity (Wolk et al., 2004). “NK-22” cells found in mucosa-associated lymphoid tissue (MALT) express the aryl hydrocarbon receptor (*AHR*) (Cella et al., 2009). These data suggest that stage 3 iNK cells within SLT may have an important role in mucosal immunity. $IL\text{-}22^+$ iNK cells from human fetal lymph node possess lymphoid tissue inducer (LTi) activity and a phenotype identical to that of LTi cells (Cupedo et al., 2009). More recently, Crellin et al demonstrated LTi-like function within the $CD127^+$ subset of this $Lin^-CD117^+CD161^+$ population (Crellin et al.).

Indeed, stage 3 iNK cells represent the most abundant NK cell developmental intermediate in human SLT (Figure S1). Conceivably, microbial invasion of MALT requires an expansion of the $IL\text{-}22^+$ stage 3 iNK cell for effective early mucosal defense (Cupedo et al., 2009; Hughes et al., 2009). Under such circumstances, expansion of the $IL\text{-}22^+$ stage 3 population would need to occur with minimal differentiation to IFN-γ-producing stage 4 mature NK cells. Likewise, the architecture of SLT can be drastically altered by excessive inflammation caused by acute microbial infections, and needs to be regenerated in order to restore the SLT microenvironment. During this process, LTi cells must proliferate and upregulate their expression of molecules such as lymphotoxin (LT) $\alpha_1\beta_2$ in order to initiate tissue reorganization (Junt et al., 2008). The signal(s) which regulate expansion and differentiation of the stage 3 iNK cell population are unknown and were investigated in this report. Here we show that the IL-1 receptor IL-1R1 was selectively expressed by a subpopulation of stage 3 iNK cells. *In vitro*, IL-1β was required to retain *AHR* and IL-22 expression in $IL\text{-}1R1^{hi}$ iNK cells. In the absence of IL-1β, a substantially greater fraction of $IL\text{-}1R1^{hi}$ iNK cells differentiated to stage 4 NK cells capable of cytotoxicity and IFN-γ secretion. These stage 3 iNK cells localized proximal to IL-1β-producing conventional dendritic cells (cDCs) within SLT, and proliferated in direct response to cDC-derived IL-15 and IL-1β. Together, our findings demonstrate a role for IL-1β as a factor which influences the homeostasis of human $IL\text{-}22^+$ stage 3 iNK cells, and suggest a potential physiological role for $CD11c^{hi}IL\text{-}1\beta^+$ cDCs in regulating expansion of this $IL\text{-}1R1^{hi}$ subpopulation of stage 3 iNK cells in SLT.

RESULTS

Robust expression of IL-R1 surface protein is restricted to stage 3 iNK cells in SLT

IL-1β is released by monocytes, macrophages, and DCs in direct response to microbial invasion (Ikejima et al., 1984), and promotes T helper 17 (Th17) cell homeostasis (Chung et al., 2009). We postulated that IL-1β may serve a similar role in stage 3 iNK cell homeostasis within SLT. We therefore assayed flow cytometry purified stage 1-4 NK developmental intermediates from human tonsil for expression of *IL-1R1* mRNA using real-time RT-PCR. As shown in Figure 1A, significant *IL-1R1* mRNA expression was confined to stage 2 pre-NK cells and stage 3 iNK cells. In particular, *IL-1R1* mRNA increased at least 38 ± 2.6-fold between stages 1 and 2, and increased 49.2 ± 2.46-fold in stage 3 iNK cells over that seen in stages 1 or 4, where expression was negligible. Thus, *IL-1R1* mRNA is selectively and abundantly expressed within stages 2 and 3 of human NK cell development.

Using flow cytometry, we next determined whether stage 2 pre-NK cells and stage 3 iNK cells from SLT display IL-1R1 cell surface protein. Whereas ≤ 20% of stage 2 pre-NK cells IL-1R1 surface protein, ≥ 70% of stage 3 iNK cells expressed IL-1R1 (Figure 1B,C). Notably, < 10% of stage 4 mature NK cells expressed IL-1R1, data consistent with the restricted manner in which *IL-1R1* mRNA was expressed. The ratio of IL-1R1 mean fluorescence intensity (MFI) to isotype control MFI, which was used to gauge the relative density of IL-1R1 expression on IL-1R1^{hi} cells, was more than 3.01 ± 0.31-fold higher on stage 3 iNK cells when compared to stage 2 pre-NK cells (Figure 1D). Collectively, these findings indicate that among human SLT NK developmental intermediates, high density surface expression of IL-1R1 protein is only found on a majority of stage 3 iNK cells.

IL-22 and *AHR* are restricted to the IL-1R1^{hi} subpopulation of stage 3 iNK cells in SLT

It has been shown previously that freshly isolated, unstimulated stage 3 iNK cells may have LTi-like properties, and constitutively express IL-22, RAR-related orphan receptor C (*RORC*), and *AHR* (Cella et al., 2009; Cupedo et al., 2009; Hughes et al., 2009). Flow cytometry performed on unstimulated SLT mononuclear cells indicated that IL-1R1 surface expression identifies two distinct subpopulations of stage 3 iNK cells: an IL-1R1^{hi} subpopulation and an IL-1R1^{lo} subpopulation. We discovered that expression of IL-22 protein was restricted to IL-1R1^{hi} stage 3 iNK cells, which were uniformly IL-22^{+}. In contrast, the minor population of IL-1R1^{lo} stage 3 iNK cells was IL-22^{-}. A donor representative of 5 such analyses is shown in Figure 2A-C. With this in mind, we measured *IL-22*, *AHR*, and *RORC* mRNA expression within sorted resting IL-1R1^{hi} and IL-1R1^{lo} subpopulations of stage 3 iNK cells. Expression of *IL-22* and *AHR* transcripts were restricted to the IL-1R1^{hi} subpopulation of stage 3 iNK cells, where they were present at amounts at least 22 ± 5.75-fold and 3439 ± 860-fold higher, respectively, than those seen in the IL-1R1^{lo} subpopulation of stage 3 iNK cells (Figure 2D). In contrast, expression of the LTi-associated transcription factor *RORC*, which is restricted to the stage 3 iNK cells in SLT (Cupedo et al., 2009), could be detected in both the IL-1R1^{hi} and IL-1R1^{lo} subpopulations of stage 3 iNK cells, as were the NK lineage-associated transcription factors, *E4BP4* and *Ets-1* (Figure S2A-C). Flow cytometry revealed that expression of CD127, CD56, CD161, NKp44, CCR7, and LT-α – associated with the LTi-like phenotype (Cupedo et al., 2009) – are also relatively restricted to the IL-1R1^{hi} subpopulation of stage 3 iNK cells (Figure 2E). Together, our data suggest that IL-22 and *AHR* are specific to the IL-1R1^{hi} subpopulation of resting human stage 3 iNK cells from SLT, and that these cells also share the phenotype of LTi-like cells.

Human IL-1R1^{hi} stage 3 iNK cells selectively display a functional response to IL-1β

To explore the functional relevance of differential IL-1R1 expression in human NK cell development, we isolated stage 1-4 NK developmental intermediates from SLT and treated each with exogenous IL-1β, a physiologic ligand for this receptor. Not surprisingly, given that IL-15 has been demonstrated to serve as a survival factor during NK cell development (Huntington et al., 2008), cells treated with IL-1β alone were uniformly dead after 48 h, regardless of stage. However, when compared to cells from the same stage and donor cultured with IL-15 alone, the combination of IL-15 and IL-1β led to an expansion in the number of viable stage 3 iNK cells when examined after 2, 7, or 14 d. Notably, IL-1β expanded stage 3 iNK cells to 4.7 ± 0.7-fold relative to the quantity of cells generated after 14 d with IL-15 alone (Figure 3A). The expansion in the presence of IL-15 and IL-1β was diminished after withdrawal of IL-1β starting at d 7 of culture (not depicted). Likewise, culture of stage 3 iNK cells in the continuous presence of a 100-fold molar excess of IL-1 receptor antagonist (IL-1RA), a naturally occurring specific competitive inhibitor of IL-1β binding to IL-1R1 (Arend et al., 1991), completely abrogated the IL-1β-mediated expansion observed earlier (Figure 3B).

We determined this IL-1β-induced expansion of stage 3 iNK cells resulted from enhanced proliferation rather than survival (Figure 3C). Notably, despite modest yet measurable amounts of IL-1R1 surface expression, the addition of IL-1β did not substantially effect proliferation of stages 1, 2 or 4. Likewise, parallel cultures comparing IL-1R1^{hi} and IL-1R1^{lo} subpopulations of stage 3 iNK cells revealed that, whereas IL-15 alone maintained survival of both IL-1R1^{hi} and IL-1R1^{lo} subpopulations of stage 3 iNK cells *in vitro*, the addition of IL-1β enhanced proliferation of the IL-1R1^{hi}, but not the IL-1R1^{lo}, subpopulation of stage 3 iNK cells (Figure 3D).

IL-1β sustains expression of IL-22 and *AHR* in IL-1R1^{hi} stage 3 iNK cells

To explore the potential role of IL-1β in regulating expression of IL-22 and *AHR*, which are restricted to IL-1R1^{hi} stage 3 iNK cells *ex vivo*, we measured expression of each transcript after *in vitro* exposure to IL-15 or IL-15 and IL-1β. Whereas *IL-22* and *AHR* mRNA all but disappeared in the presence of IL-15 alone, exposure to IL-1β preserved *IL-22* and *AHR* expression in total stage 3 iNK cells (Figure 4A,B). Furthermore, *IL-22* and *AHR* remained restricted to the IL-1R1^{hi} stage 3 iNK subpopulation, which also required exposure to IL-1β for continued expression *in vitro* (Figure 4C,D). In contrast, culture in the presence or absence of IL-1β resulted in similar expression of *RORC* mRNA, which was sustained in IL-1R1^{lo} and IL-1R1^{hi} subpopulations of stage 3 iNK cells, and induced in stage 4 mature NK cells (Figure S3A-C). These correlative data suggest that human stage 3 iNK cell production of IL-22 may be more dependent on the expression of *AHR* than *RORC*, as has recently reported for IL-17^{-}IL-22^{+} human T cells (Trifari et al., 2009).

Although culture of IL-1R1^{hi} stage 3 iNK cells for 14 d in IL-15 and IL-1β resulted in a decrease in the overall number of cells expressing IL-1R1 and overall expression of IL-22, IL-22 remained restricted to progeny which were also IL-1R1^{+} (Figure 4E,F). Declining numbers of IL-1R1^{+} progeny during culture in IL-15 and IL-1β was not due to overgrowth of the minor IL-1R1^{lo} subpopulation of stage 3 iNK cells (Figure 3D), but rather resulted from an IL-15-mediated decrease in IL-1R1 on the surface of IL-1R1^{hi} stage 3 iNK cells (Figure 4G). Furthermore, we assessed the clonal outgrowth from single IL-1R1^{hi} and IL-1R1^{lo} stage 3 iNK cells and found that the capacity for sustained expression of IL-22 remained restricted to the progeny of IL-1R1^{hi} stage 3 iNK cells and exclusive of CD94 expression (Figure 4H; Table S1). Thus, IL-22^{+} cells derived *in vitro* appear to have been generated exclusively from the IL-1R1^{hi} subpopulation. Together, our data suggest that continued expression of IL-22 by stage 3 iNK cells *in vitro* is dependent on exposure to

IL-1β, and that IL-22 expression remains restricted to the IL-1-responsive subpopulation of IL-1R1^{hi} stage 3 iNK cells.

IL-1β inhibits differentiation of human IL-1R1^{hi} stage 3 iNK cells

IL-15 promotes the differentiation of stage 3 iNK cells to stage 4 mature NK cells, which are characterized by co-expression of CD94 and high density CD56, as well as the potential for IFN-γ production and cytolytic activity (Freud et al., 2006; Huntington et al., 2008; Sanos et al., 2009). If IL-1β expands stage 3 iNK cells for the purposes of sustaining IL-22 expression and mucosal immunity, it should also inhibit differentiation to stage 4 mature NK cells. With this in mind, we assessed expression of CD94 and CD56 via flow cytometry after culture of stage 3 iNK cells, either in bulk, or as IL-1R1^{hi} and IL-1R1^{lo} subpopulations, with IL-15 alone or IL-15 and IL-1β. The presence of IL-1β conferred a 50% decrease in the proportion of stage 3 iNK cells that progressed to stage 4 mature NK cells as measured by CD94 acquisition (Figure 5A; 11.3 ± 2.7% with IL-1β vs. 21.7 ± 2.1% with IL-15 alone), and a concomitant 3.02 ± 0.4-fold decrease in MFI of CD56 surface expression (Figure 5B). These effects, which could be observed within 2 d of *in vitro* culture, were restricted to IL-1R1^{hi} stage 3 iNK cells and were not seen in cultures of the IL-1R1^{lo} subset (Figure 5C-E) or cells from stages 2 or 4 (not depicted). Inclusion of a molar excess of IL-1RA in cultures containing IL-15 and IL-1β fully restored acquisition of CD94 (Figure 5F) and density of CD56 surface expression (Figure 5G). Figure 5H shows additional maturation markers assessed in IL-1R1^{hi} stage 3 iNK cells cultured for 14 d in IL-15 or IL-15 and IL-1β.

IL-1β inhibits the functional maturation of human IL-1R1^{hi} stage 3 iNK cells

We next assessed whether treatment of human stage 3 iNK cells with IL-15 and IL-1β also inhibited the acquisition IFN-γ production and cytotoxic activity seen in stage 4 mature NK cells. Freshly isolated human stage 3 iNK cells do not produce IFN-γ or display natural cytotoxicity against K562 target cells (Freud et al., 2006). Indeed, stage 3 iNK cells incubated *in vitro* with IL-15 and IL-1β for 14 d produced negligible amounts of IFN-γ (~100 pg/ml) after co-activation with IL-12, IL-15, and IL-18. In contrast, stage 3 iNK cells incubated *in vitro* with IL-15 alone for 14 d produced substantial IFN-γ, averaging 3,167 ± 734 pg/ml, following co-activation with IL-12, IL-15, and IL-18 (Figure 6A). As shown in Figure 6B, this IL-1β-mediated reduction in IFN-γ production was restricted to the IL-1R1^{hi} subpopulation of stage 3 iNK cells. As judged by effector cytokine secretion, exposure to IL-1β inhibited the functional maturation of stage 3 iNK cells.

Although the capacity to secrete IFN-γ was restricted to the population which had acquired surface expression of CD94 *in vitro* (Figure S4A), surface expression of the degranulation marker CD107a, which can be used as a functional marker to identify cytotoxic NK cells (Alter et al., 2004), was observed in both CD94^{+} and CD94^{-} cells generated from culture of stage 3 iNK cells *in vitro* (Figure S4B). Surface expression of the degranulation marker CD107a indicated that, compared to bulk stage 3 iNK cells cultured with IL-15 alone, bulk culture of stage 3 iNK cells in the presence of IL-15 and IL-1β for 14 d did not significantly influence CD107a surface expression (Figure 6C; $P = 0.14$), however, exposure to IL-15 and IL-1β significantly reduced degranulation within the IL-1R1^{hi} subpopulation of stage 3 iNK cells (Figure 6D; $P = 0.01$).

Human stage 3 iNK cells proliferate in direct response to proximate cDC-derived IL-15 and IL-1β

To identify potential physiological sources of IL-1β which may interact with stage 3 iNK cells, we used flow cytometry to analyze fresh SLT mononuclear cells for intracellular IL-1β. Although we detected low intracellular IL-1β in a modest fraction of CD3^{+} T cells

and CD14$^+$ monocytes and macrophages (Figure 7A), IL-1β expression occurred in a large portion of tonsillar CD11c^{hi}CD123^{lo} cDCs (Figure 7B and C, **top row**). In contrast, CD11c^{lo}CD123^{hi} plasmacytoid DCs (pDCs) and the population of CD11c^{lo}CD123^{lo} cells did not contain intracellular IL-1β (Figure 7B and not depicted, respectively).

Stage 3 iNK cells were previously identified *in situ* as CD117$^+$ cells with lymphoid morphology, and localized to the lamina propria or the parafollicular T-cell-rich region of the tonsil (Hughes et al., 2009). These CD117$^+$ stage 3 iNK cells, which are primarily CD161$^+$IL-1R1^{hi}IL-22$^+$ (Figure 2; Figure S5), were found to be located in close proximity to IL-1β-producing CD11c^{hi} cDCs in the parafollicular T-cell rich region of human tonsil (Figure 7C, **bottom row**). Thus, tonsillar cDCs appear to be a plausible cellular source of IL-1β for human stage 3 iNK cells *in vivo*.

Indeed, compared to parallel cultures performed in the absence of cDCs, short term co-culture of IL-1R1^{hi} stage 3 iNK cells with autologous BDCA-1$^+$CD11c^{hi}CD123^{lo} cDCs enhanced proliferation (measured via 5-ethynyl-2′-deoxyuridine (EdU) incorporation assay) of IL-1R1^{hi} stage 3 iNK cells, but not of IL-1R1^{lo} stage 3 iNK cells (Figure 7D). Although neither α-IL-15 nor α-IL-1β antibody altered EdU incorporation as a single blocking reagent (not depicted), addition of α-IL-15 and α-IL-1β antibodies together reduced EdU incorporation to amounts seen in the absence of cDCs, implicating specific roles for cDC-derived IL-15 and IL-1β in the proliferative response observed in IL-1R1^{hi} stage 3 iNK cells (Figure 7D). Thus, cDC-derived IL-15 and IL-1β are capable of directly stimulating proliferation of IL-1R1^{hi} stage 3 iNK cells. Similar co-stimulatory effects may also be possible between IL-1R1^{hi} stage 3 iNK cells and IL-1β-producing CD14$^+$ monocytes or macrophages which also produce IL-15.

DISCUSSION

Here we identify a role for IL-1R1 and its cognate ligand, IL-1β, in the homeostasis of stage 3 iNK cells, which selectively expressed IL-1R1 compared to other NK developmental intermediates residing in human tonsil. In the presence of IL-15, binding of IL-1β to IL-1R1 expanded IL-1R1^{hi} stage 3 iNK cells and preserved expression of IL-22 and *AHR*. In contrast, culture of IL-1R1^{hi} stage 3 iNK cells in IL-15 alone resulted in a loss of IL-22 and *AHR* expression and enhanced differentiation toward IFN-γ producing stage 4 mature NK cells. Furthermore, CD117$^+$ cells, which co-express CD161 and IL-22, and thus correspond to the IL-1R1^{hi} subpopulation of stage 3 iNK cells, reside in the lamina propria and parafollicular T cell-rich area of human tonsil, and co-localized with a cellular source of IL-1β found in CD11c^{hi} cDCs, attesting to the physiologic relevance of the IL-1β-IL-1R1 interaction *in vivo*.

RORC is a hallmark of mature Th17 cells (Ivanov et al., 2006), but has also been detected in murine *FOXP3*$^+$ regulatory T (Treg) cells (Lochner et al., 2008) and human *FOXP3*$^+$ Treg cells (Ayyoub et al., 2009). Indeed, unstimulated human stage 3 iNK cells in SLT express *RORC* and *AHR* mRNA (Cupedo et al., 2009), IL-22 protein (Hughes et al., 2009), and as we establish here, IL-1R1. Although IL-1β appears to be required by human stage 3 iNK cells for continued expression IL-22 and *AHR in vitro*, other LTi-associated genes – *IL-26* and *RORC* – were maintained in stage 3 iNK cells by IL-15 alone. Furthermore, culture of stage 4 mature NK cells in the presence of IL-15 induced expression of *RORC in vitro*, suggesting that *RORC* may not be among the subset of LTi-associated genes which are dependent upon IL-1β for continued expression, especially within human stage 3 iNK cells. This is consistent with our observation that unlike IL-22 and *AHR*, constitutive expression of *RORC* is not restricted to the IL-1R1^{hi} subpopulation of stage 3 iNK cells, as well as

RORC-independent development of MALT reported in murine tear duct (Nagatake et al., 2009).

Murine studies indicate that RORC^{+}CD127^{+}NKp46^{+}NK1.1^{+} cells are decreased in the setting of IL-15 gene deficiency as well as in animals lacking the IL-2 receptor beta chain (IL-2Rβ, CD122) (Satoh-Takayama et al., 2008), which is required for IL-2 and IL-15 signaling. However, this population is absent in IL-2Rγ–deficient animals that cannot respond to any of the so-called γ_c cytokines (Vosshenrich et al., 2005). Situations have been reported in which IL-2 or IL-7 may be substituted in place of IL-15 (Cupedo et al., 2009; Freud et al., 2006; Satoh-Takayama et al., ; Satoh-Takayama et al., 2008). Given that nearly all human stage 3 iNK cells – as well as the equivalent RORC^{+}NKp46^{+}NK1.1^{int} murine population – express CD127 (Luci et al., 2009), the role of IL-7 deserves particular attention. Compared to IL-2 or IL-15, IL-7 alone was much less potent at maintaining stage 3 iNK cell survival *in vitro*. Furthermore, stage 3 iNK cells cultured with IL-2 or IL-15 did not significantly differ qualitatively or quantitatively compared to parallel cultures supplemented with IL-7. This is in agreement with murine studies indicating that IL-7 and IL-7Rα deficient mice do not exhibit NK developmental defects (Maki et al., 1996; Vosshenrich et al., 2005). It remains possible that this cytokine plays a role in regulating additional functions of this population in SLT, such as the LTi-like behavior that has been reported in human stage 3 iNK population (Cupedo et al., 2009).

Both human Th17 cells and stage 3 iNK cells produce IL-22 (Cella et al., 2009) and express the transcription factors *RORC* and *AHR* (Cupedo et al., 2009). Murine Th17 cells have higher expression of *IL-1R1* mRNA than in seen in Th1 or Th2 cells, and exposure to IL-1β promotes Th17 cell proliferation, differentiation, and expression of IL-17 and *RORC* (Chung et al., 2009). Like Th17 cells (Chung et al., 2009; Rao et al., 2007; Veldhoen et al., 2008), the IL-1R1^{hi} subpopulation of stage 3 iNK cells selectively expresses *AHR*, expands upon *in vitro* treatment with IL-1β, and depends on IL-1β for expression of *AHR* and IL-22. We also noted that expression of IL-1R1 on stage 3 iNK cells diminishes over time during *in vitro* culture with a proportional decrease in IL-22 expression, and we show that this down regulation of IL-1R1 is mediated by IL-15. This observation lends further support to our notion that *in vivo*, constitutive expression of IL-22 in stage 3 iNK cells likely requires co-expression of IL-1R1 and proximity to cells expressing its cognate ligand, IL-1β.

In addition to IL-1β, we investigated the role of IL-23, which did not markedly alter the quantity of cells generated *in vitro* from stage 3 iNK cells, whether cultured in the presence of IL-15 or IL-15 and IL-1β. IL-23 acts on human Th17 cells to promote expression of Th17 cell-associated genes, such as IL-22 (Zheng et al., 2007), *RORC* and *IL-26* (Manel et al., 2008). We did not observe any marked effect on *IL-22*, *IL-26*, or *RORC* expression when stage 3 iNK cells were cultured with IL-23, regardless of the presence of IL-15 or IL-15 and IL-1β. Together, these findings suggest that distinct repertoires of cytokines regulate the development of human Th17 cells and stage 3 iNK cells found in SLT.

Unabated expression of pro-inflammatory cytokines can lead to chronic inflammatory states, autoimmune disease, and cancer. IL-1β production is subject to intense regulation at the level of its synthesis and excretion. Its interaction with IL-1R1 is further regulated by competitive inhibition by IL-1RA (Carter et al., 1990), as well as expression of the IL-1R2 decoy receptor (Colotta et al., 1993). We found that IL-1RA is capable of fully neutralizing the impact of IL-1β on IL-1R1^{hi} stage 3 iNK cell proliferation and on *in vitro* differentiation toward CD94^{+} stage 4 NK cells. Similarly, IL-22 signaling triggers expression of a naturally occurring antagonist, IL-22 binding protein (IL-22BP), which binds IL-22 to prevent signaling through IL-22R (Dumoutier et al., 2001). These regulatory pathways serve to quickly and precisely adjust biological responses to IL-1β and IL-22. Indeed, the assessment

of anti-IL-1β therapy might be considered in illnesses such as Crohn's disease and psoriasis where excessive IL-22 production is felt to be pathological (Kleinschek et al., 2009; Wilson et al., 2007). Ablation of IL-1R1 signaling should enhance NK cell differentiation and diminish IL-22 production from the IL-1R1^{hi} stage 3 iNK cell population.

Excessive inflammation can disrupt SLT architecture, necessitating local tissue regeneration for restoration of the SLT microenvironment (Kratz et al., 1996). LTi cells have a proposed role in re-establishing the integrity of lymphoid tissues following chronic infections (Scandella et al., 2008). Cupedo et al first identified *RORC*$^{+}$ LTi-like cells in human fetal lymph node as committed iNK cells expressing *IL-22*, CD117, CD127, *LT-α* and *CCR7* (Cupedo et al., 2009). A study by Crellin et al provides strong evidence that LTi-like activity is most robust within a subset of cells co-expressing CD127 and CD161 (Crellin et al.). We found that the IL-1R1^{hi} subpopulation of iNK cells possesses a strikingly similar phenotype. Among total stage 3 iNK cells, the IL-1R1^{hi} subset is enriched for cells co-expressing CD127, CD161, LT-α, CCR7, and IL-22. As we observed in the IL-1R1^{hi} subpopulation of stage 3 iNK cells, Crellin et al reported that CD127^{+} LTi-like cells varied in expression of CD56 and NKp44, and contained cells capable of giving rise to cytotoxic mature NK cells in the presence of IL-15 *in vitro*. They reported that, compared to the CD127^{-} subpopulation, the CD127^{+} subpopulation is reduced in its propensity to generate mature NK cells in IL-15. Our study shows a reduced propensity to generate mature NK cells when IL-1β was added to culture of IL-1R1^{hi} stage 3 iNK cells in the presence of IL-15. These data suggest that the developmental relationship between the CD127^{+}IL-1R1^{hi}, CD127^{+}IL-1R1^{lo}, CD127^{-}IL-1R1^{hi} and CD127^{-}IL-1R1^{lo} populations will need to be explored in future studies. They also serve to emphasize the heterogeneity within the CD117^{+}CD161^{+}IL-1R1^{hi} stage 3 iNK cell population, and the need for caution in assigning definitive functions and developmental potential.

We previously reported limited differentiation of stage 3 iNK cells to CD94^{+} stage 4 NK cells with IL-15 *in vitro* [5-20% (Freud et al., 2006)], but the developmental relationship between stage 3 iNK cells and CD94^{+} stage 4 NK cells remains unclear, and was pursued in this study. The current report describes two subpopulations of stage 3 iNK cells distinguished by surface expression of functional IL-1R1. Predictably, the presence of IL-1β in culture had no appreciable effect on differentiation of IL-1R1^{lo} stage 3 iNK cells, but during culture of IL-1R1^{hi} stage 3 iNK cells, IL-1β limited maturation to stage 4 NK cells and sustained expression of *AHR* and *IL-22* mRNA, as well as IL-22 protein in the remaining IL-1R1^{hi} stage 3 cells. In the absence of IL-1β, culture of IL-1R1^{hi} stage 3 iNK cells with IL-15 had a significantly greater propensity to mature into CD94^{+}CD56^{bright} stage 4 NK cells.

These findings support the hypothesis that the human stage 3 iNK cell population (CD3^{-}CD34^{-} CD117^{+}CD161^{+}CD94^{-}) consists of a minor IL-1R1^{lo} subset that does not express IL-22, as well as an IL-1R1^{hi} subset which constitutively expresses IL-22, yet can also give rise to CD94^{+} mature NK cells. The CD117^{+}IL-1R1^{lo} stage 3 iNK cell may acquire IL-1R1 and then under some circumstances proceed to a CD94^{+}CD56^{bright} stage 4 mature NK cell, or may bypass the acquisition of IL-1R1. The proportion of IL-1R1^{hi} stage 3 iNK cells which differentiate to CD94^{+}CD56^{bright} stage 4 mature NK cells relative to those that continue to express IL-22 appears dependent on their continued expression of IL-1R1, as well as exposure to IL-1β. The regulation of the IL-1R1 by IL-15, and the regulation of exposure to its ligand IL-1β by cell-cell interactions and microbial invasion, may dictate the relative abundance of the CD117^{+}IL-1R1^{hi} stage 3 iNK and the CD94^{+}CD56^{bright} stage 4 mature NK cell in SLT. Finally, this study identifies a stage 3 iNK subpopulation in human tonsil as an innate immune target of IL-1β, and provides a

mechanistic connection between IL-1β and IL-22 production, with implications for mucosal innate immunity.

EXPERIMENTAL PROCEDURES

Isolation of human NK precursors from SLT

All protocols were approved by The Ohio State University (OSU) Institutional Review Board. Normal human tonsils were obtained within 24 h of elective surgery through the NCI-Sponsored Cooperative Human Tissue Network and the Biopathology Center at Nationwide Children's Hospital. NK cell developmental intermediates were isolated as described (Freud et al., 2006). Briefly, mononuclear fractions were depleted of $CD3^+$ and $CD19^+$ cells via magnetic negative selection (Miltenyi). For certain experiments, T and B cell-depleted preparations were stained immediately for phenotypic analyses. Alternatively, $CD34^+$ cells were enriched from $CD3^-CD19^-$ cells using a CD34 progenitor isolation kit (Miltenyi) for flow cytometric sorting of stage 1 and 2 cells, while total stage 3 iNK cells and stage 4 NK cells were sorted from the $CD34^-$ fraction as previously described (Freud et al., 2006). IL-$1R1^{hi}$ and IL-$1R1^{lo}$ stage 3 iNK subpopulations were isolated from the $CD34^-$ fraction using surface staining with goat α-IL-1R1 and α-goat APC (R&D Systems) in addition to the previously described stage 3 cell surface antigens (Freud et al., 2006). Populations were sorted to purity ≥ 95% with a FACS Aria II cell sorter (BD Biosciences).

Flow cytometry

All antibodies used for flow cytometry were purchased from BD Biosciences, except BDCA-1 (Miltenyi), CD56, CD127, CD161, NKp44, NKp46 (Beckman Coulter), goat IgG (Santa Cruz), CD94 (clone 131412), IL-22 (clone 142928), IL-1R1 (MAB269), and α-goat APC (R&D Systems). Unless otherwise indicated, antibodies were used according to manufacturer's instructions. Non-specific staining was detected through the use of an appropriately labeled isotype antibody. IL-1R1 staining was performed on ice for 15 min with α-IL-1R1 or goat IgG (1 μg/10^5 cells) in parallel with any additional directly conjugated antibodies indicated. Samples were washed, then incubated on ice for 30 min with α-goat IgG APC (0.25 μg/10^5 cells). Intracellular staining was performed after surface staining using the BD Cytofix/Cytoperm Plus Fixation/Permeabilization Kit (BD Biosciences). *Ex vivo* staining was performed without additional stimulation, and in the absence of brefeldin A, immediately following CD3- and CD19-depletion. Intracellular staining after culture was performed following 4 h incubation with 2 μM GolgiPlug (BD Biosciences). Intracellular staining for IL-1β was performed according to manufacturer's instructions, and IL-22 staining was as previously described (Hughes et al., 2009). Cells were analyzed immediately as previously described (Freud et al., 2005) using a FACS Calibur or BD LSR II cytometer (BD Biosciences).

Real-Time PCR

RNA was obtained by lysing a portion of each sorted population in approximately equal quantity (≥ 1×10^4 cells). For some experiments, RNA was also obtained after 14 d *in vitro* culture. Samples from ≤ 5×10^4 cells were processed using Absolutely RNA Nanoprep Kit (Stratagene); samples with ≥ 5×10^4 cells were processed using RNeasy (Qiagen). cDNA was synthesized according to manufacturer's instructions using MMLV reverse transcriptase kit (Invitrogen). Real-Time PCR was performed on an ABI Prism 7900HT (Applied Biosystems) using Taqman primer/probe sets for *IL-22*, *AHR*, and *IL-1R1* purchased from Applied Biosystems. Expression levels were normalized to an *18S* internal control, then analyzed by the ΔΔCt method (Fehniger et al., 1999).

Cell culture

Flow cytometry purified NK developmental intermediates were cultured in a round-bottom 96-well plate (Costar) at a starting density of 25,000 cells/ml in α-MEM medium containing 10% FBS, penicillin G (100 μg/ml), and streptomycin (100 μg/ml) (Invitrogen). Cells from stages 2-4 were cultured in the indicated recombinant human cytokines, including IL-15 (1 nM; Amgen), IL-1β (10 ng/ml; Peprotech), and IL-1RA (100 ng/ml; R&D Systems). Medium for stage 1 cells was also supplemented with IL-7 (10 ng/ml), IL-3 (10 ng/ml; R&D Systems), and FL (100 ng/ml; Peprotech). For cultures lasting more than 24 h, half the medium was removed every 2-3 d and replaced with media containing 2x cytokines.

Clonal assays

FACS Aria II was used to deposit single IL-1R1^{hi} or IL-1R1^{lo} stage 3 iNK cells directly into each well of a 96-well flat bottom plate containing GFP^{+} murine OP9 stroma in α-MEM medium containing IL-15 plus IL-1β. After 14 d, each of 60 replicate wells was individually assessed via flow cytometry for human CD45 and CD94 surface expression in addition to intracellular IL-22. OP9 cells were excluded by gating on GFP^{-} events expressing human CD45.

Proliferation assays

Click-iT EdU kit (Invitrogen) was used according to manufacturer's instructions to assess proliferation of stage 3 iNK cells purified via flow cytometry, either in bulk, or as IL-1R1^{hi} and IL-1R1^{lo} subpopulations, in response to cDC-derived or recombinant IL-15 or IL-15 and IL-1β. After CD3- and CD19-depletion, SLT cDCs were enriched via magnetic selection using BDCA-1-biotin antibody and α-biotin microbeads (Miltenyi). Purity was ≥ 87% via flow cytometric staining. Autologous IL-1R1^{hi} and IL-1R1^{lo} stage 3 iNK subpopulations were sorted from BDCA-1-depleted fractions. Co-cultures were performed at 4:1 iNK:cDC ratio. After 6 h in the presence of 5 mM EdU, the proportion of CD117^{+} cells positive for EdU uptake (corresponding to DNA replication, and S phase of the cell cycle) was assessed via flow cytometry. Controls included cells cultured in the absence of EdU and samples without AlexaFluor 488 azide (used to fluorescently label EdU). Additional controls included for cDCs co-cultures included: iNK cell monocultures performed in media alone or media recombinant IL-15 and IL-1β. For neutralization experiments, iNK/cDC co-cultures were performed in the presence of α-IL-15 (1 μg/ml) and/or α-IL-1β (0.5 μg/ml) blocking antibodies (R&D Systems) and/or an equal concentration of isotype IgG (Santa Cruz).

Effector function assays

After 14 d of culture with IL-15 or IL-15 and IL-1β, cells were counted and replated in equal numbers for each assay. Surface staining for CD107a, a degranulation marker used to identify cytotoxic NK cells (Alter et al., 2004), was assayed via flow cytometry as previously described (Betts et al., 2003) in response to 8 h incubation with K562 target cells (4:1 E/T ratio). IFN-γ secretion, in response to 12 h stimulation with recombinant monokines [IL-15 with IL-12 (10 ng/ml; Genetics Institute) and IL-18 (50 ng/ml; BASF)], was measured as previously described via intracellular flow cytometry (Cooper et al., 2001) or ELISA (detection limit 10-30 pg/ml) using commercial antibody pairs (Endogen) (Trotta et al., 2005). Controls for effector function assays included staining with isotype control antibody, and parallel cultures performed in the absence of K562 or monokines.

Immunohistochemical staining

Immunohistochemistry was performed on human tonsils as previously described (Fehniger et al., 2003). The ultraView Universal system (Ventana Medical) was employed to assess paraffin-embedded tonsillar tissue in serial sections (0.5 μM) stained with the indicated

antibodies, including: α-CD117 (1:500; DakoCytomation), α-IL-1β (1:50), and α-CD11c (Abcam, 1:100). Images were digitally acquired using: DP 12 camera, BX50 microscope, and UPLANF1 objectives (Olympus) and analyzed using Photoshop CS3 software (Adobe). The Nuance FX system (Cambridge Research & Instrumentation) digitally converted staining with DAB or fast red to fluorescent green or red, respectively. Using the Nuance System's multispectral image analysis, we were able to identify both $CD11c^{hi}$ and $CD11c^{lo}$ cells. Subsequently, the threshold of signal strength, corresponding specifically to the CD11c chromagen, was increased to display only the $CD11c^{hi}$ cells within tissue sections.

Statistical analysis

For comparison of two conditions, data were analyzed using paired t-tests. Linear mixed models were used for analysis when more than two treatment conditions were used. All tests were two sided. Holm's method was used to correct for multiple comparisons when needed. $P < 0.05$ was considered significant for single comparisons, and after adjustment for multiple comparisons.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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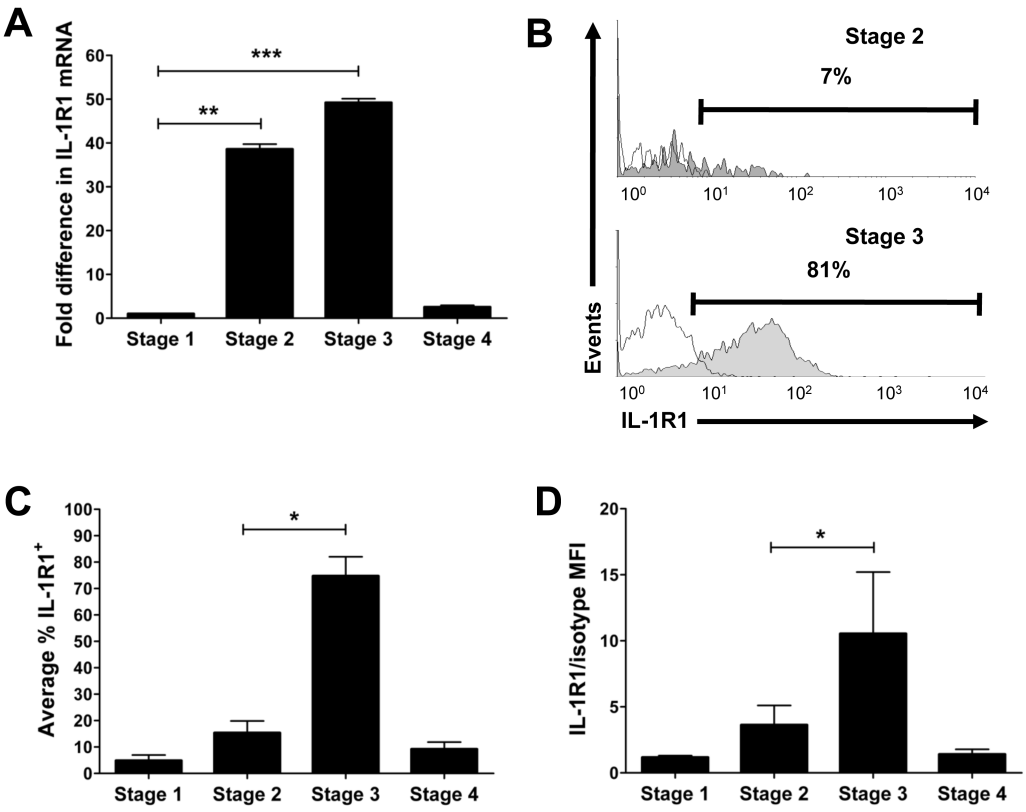

Figure 1. IL-1R1 expression during NK cell development

(A) *IL-1R1* mRNA expression *ex vivo* in flow cytometry purified human stage 1-4 SLT NK developmental intermediates assessed via Real-Time PCR. Fold difference in *IL-1R1* mRNA is relative to that in stage 1, arbitrarily normalized to 1 (n = 4). **(B-D)** Total $CD3^{-}CD19^{-}$ tonsillar mononuclear cells were stained for surface expression of CD34, CD117, CD94, and IL-1R1, and gated for each stage as described (Freud et al., 2006). **(B)** Histograms show IL-1R1 **(filled)** and isotype **(empty)** surface staining in stage 2 **(top)** or stage 3 **(bottom)** cells from a representative donor (n = 4). **(C,D)** Shown within each gated SLT NK developmental intermediate populations are average: **(C)** % of cells with IL-1R1 surface expression (n = 4), and **(D)** density of IL-1R1 surface expression, as represented by the ratio of IL-1R1 mean fluorescence intensity (MFI) to isotype MFI (n = 6). Data in **A**, **C**, **D** presented as mean ± SEM (*, $P \leq 0.05$; **, $P \leq 0.005$; ***, $P \leq 0.0005$).

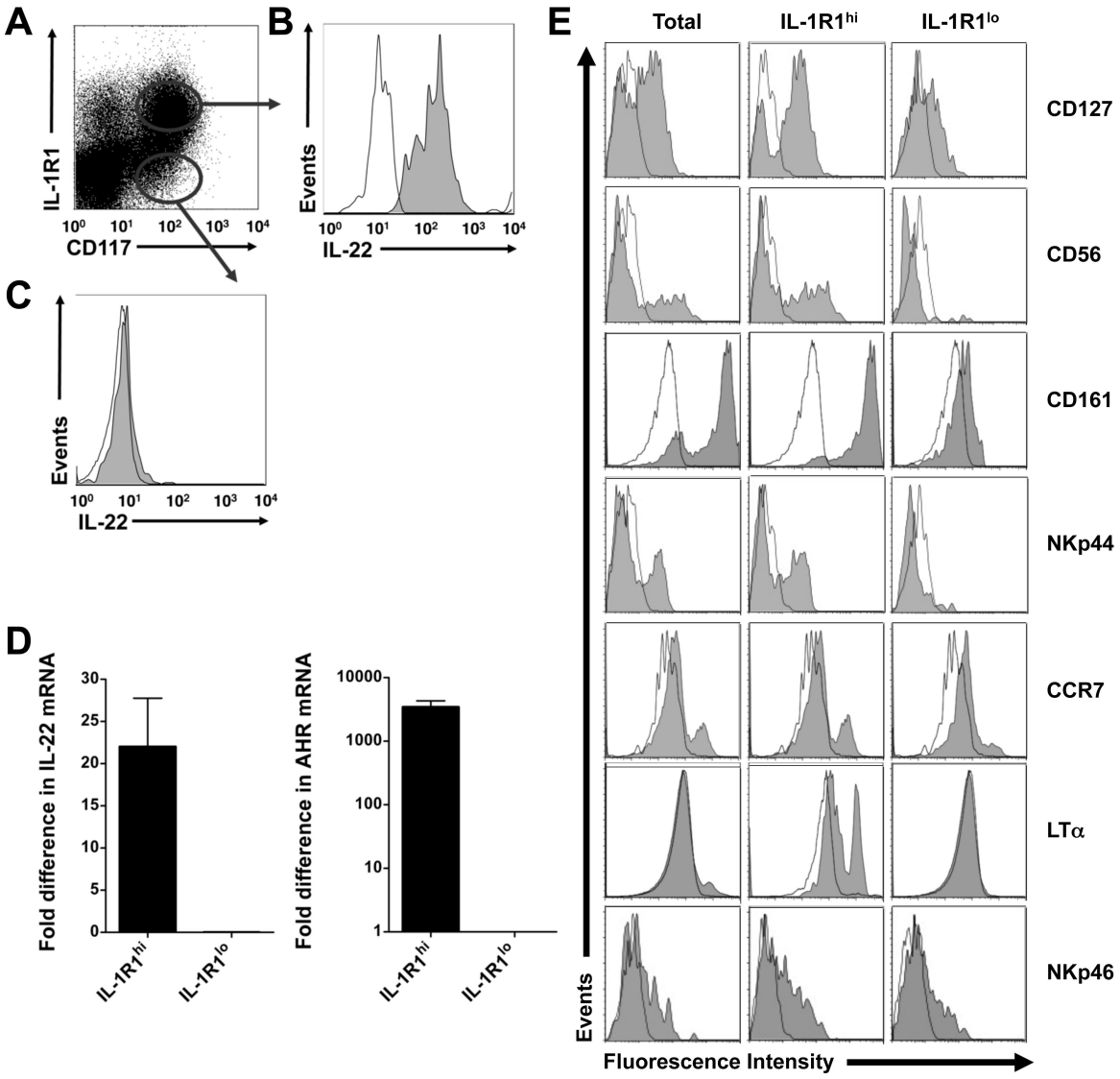

Figure 2. IL-1R1^{hi} and IL-1R1^{lo} subpopulations of stage 3 iNK cells *ex vivo*

(A-C) Tonsillar mononuclear cells were stained *ex vivo* for surface expression of CD117, IL-1R1, and **(B,C)** intracellular IL-22. **(A)** Gated on Lin$^-$ (CD3$^-$CD14$^-$CD16$^-$CD19$^-$CD34$^-$CD94$^-$) lymphocytes, dot plot indicates gates for IL-1R1^{hi} **(upper)** or IL-1R1^{lo} **(lower)** subpopulations of CD117$^+$ stage 3 iNK cells as sorted in a representative donor (n = 18). **(B,C)** Gating as indicated in **(A)**, histograms depict staining with isotype **(empty)** or α-IL-22 **(filled)** antibody in a representative donor (n = 5). **(D)** *IL-22* and *AHR* mRNA measured via Real-Time PCR *ex vivo* in flow cytometry sorted IL-1R1^{hi} and IL-1R1^{lo} subpopulations of stage 3 iNK cells. "Fold difference" was quantified relative to expression in the IL-1R1^{lo} subpopulation, arbitrarily normalized to 1 ($P \leq 0.05$). Data in **D** presented as mean ± SEM (n = 6). **(E)** Using gating as indicated in **(A)**, histograms depict a representative donor stained *ex vivo* with isotype **(empty)** or antibody specific for the indicated antigen **(filled)**, within total stage 3 iNK cells or IL-1R1^{hi} and IL-1R1^{lo} subpopulations of stage 3 iNK cells (n ≥ 4). The IL-1R1$^-$CD117$^-$ and

IL-1R1^{+}CD117^{-} subsets noted in **(A)** were both non-reactive for intracellular IL-22 protein staining.

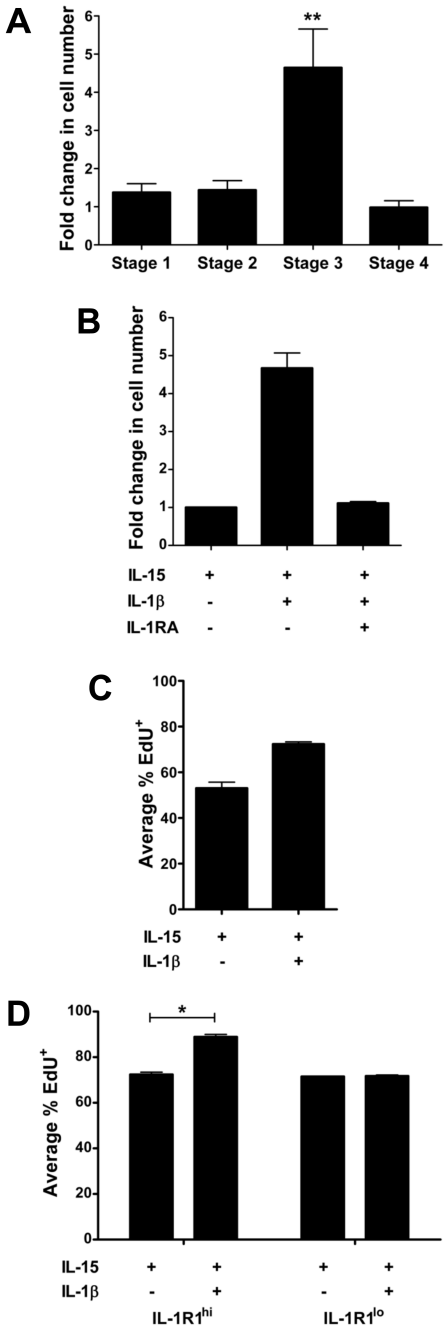

Figure 3. Treatment with IL-15 and IL-1β promotes the selective expansion of stage 3 iNK cells

(A,B) "Fold change" was calculated and averaged from 11 donors as: absolute number of cells enumerated (by Trypan blue exclusion) after 14 d of culture in IL-15 and IL-1β divided by the absolute number of cells enumerated after 14 d of culture in IL-15 alone. For example, starting with 5,000 stage 3 iNK cells/well, four representative donors cultured in IL-15 and IL-1β expanded to 90,000, 285,000, 162,000, and 40,000, while the same four donors' stage 3 iNK cells cultured in IL-15 alone expanded to 20,000, 55,0000, 45,000 and 10,000 cells, respectively. Results revealed a selective growth advantage among stage 3 iNK cells in response to culture with IL-15 and IL-1β (**, $P < 0.005$; n = 11), **(B)** which was

completely abrogated in the presence of IL-1RA ($P < 0.05$; n = 4). **(C,D)** Proliferation assessed via EdU incorporation in **(C)** total stage 3 iNK cells ($P < 0.05$; n = 8) or **(D)** IL-1R1^{hi} and IL-1R1^{lo} subpopulations of stage 3 iNK cells (*, $P = 0.01$; n = 3) after culture for 14 d in the presence of IL-15 or IL-15 and IL-1β. Data in **A**-**D** depicted as mean ± SEM.

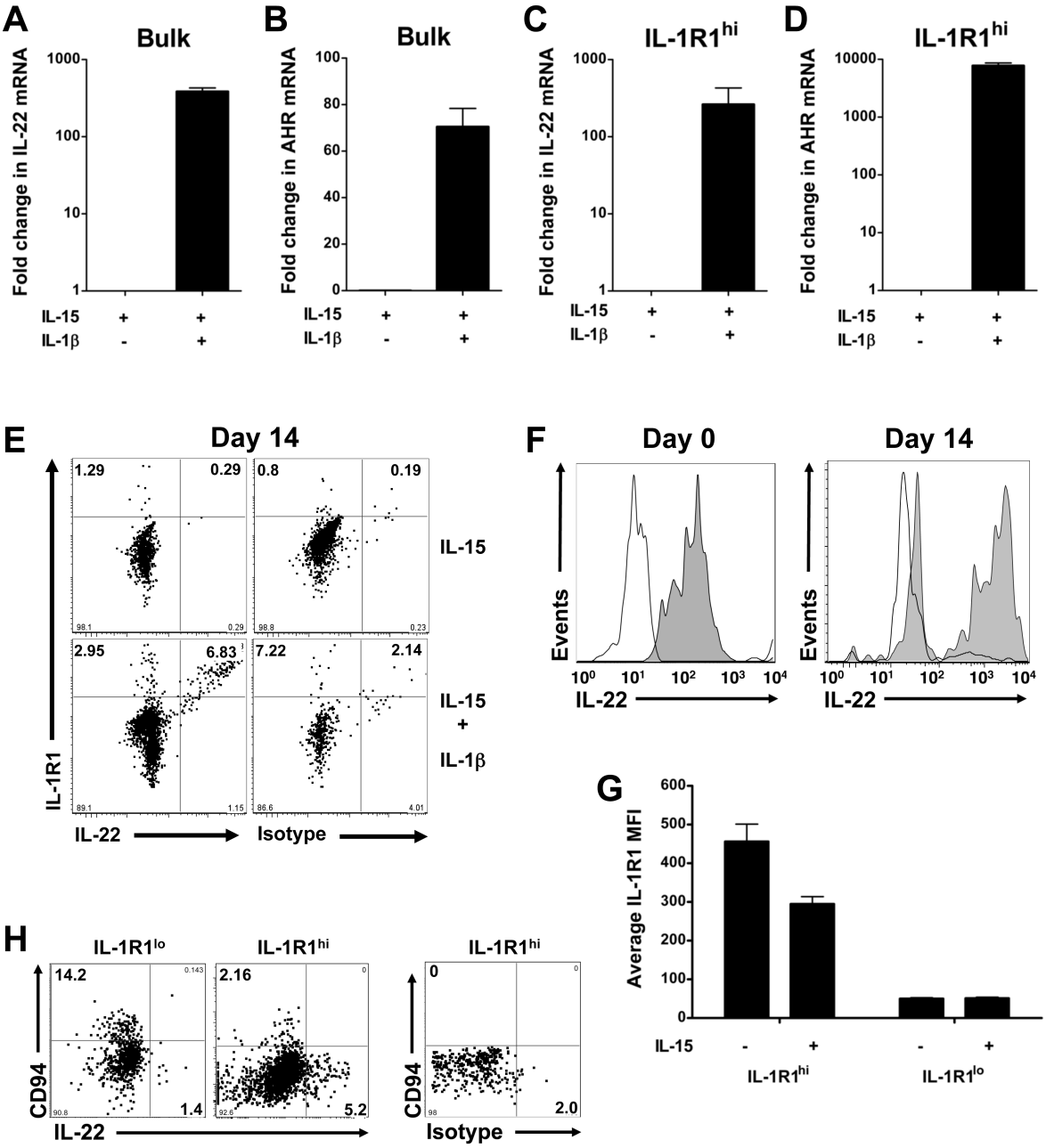

Figure 4. IL-1β sustains IL-22 and *AHR* expression in IL-1R1^{hi} stage 3 iNK cells

(A-D) *IL-22* and *AHR* transcript expression following 14 d in the presence of IL-15 or IL-15 and IL-1β, shown in **(A,B)** total ("bulk") stage 3 iNK cells, or **(C,D)** the IL-1R1^{hi} subpopulation of stage 3 iNK cells. Results depicted as the mean ± SEM, and $P \leq 0.05$ in each (n ≥ 4). **(E)** Dot plots depict surface IL-1R1 and intracellular staining with IL-22 or isotype antibody in IL-1R1^{hi} stage 3 iNK cells from a representative donor cultured for 14 d in the presence of IL-15 or IL-15 and IL-1β (n = 6). All IL-22^{+} cells co-expressed IL-1R1. **(F)** Histograms from a representative donor (n = 4) depict staining with IL-22 **(filled)** or isotype **(empty)** antibody in the IL-1R1^{hi} subpopulation of stage 3 iNK cells d 0, and within

IL-1R1^{+} cells remaining after culture for 14 d with IL-15 and IL-1β **(G)** IL-1R1 MFI assessed via flow cytometry after a 6 h culture in the presence of IL-15 or media alone (n = 3). **(H)** Representative staining for surface CD94 and intracellular IL-22 protein at d 14 in cells cloned from either a single IL-1R1^{lo} stage 3 iNK cell or an IL-1R1^{hi} stage 3 iNK cell. Isotype staining on far right.

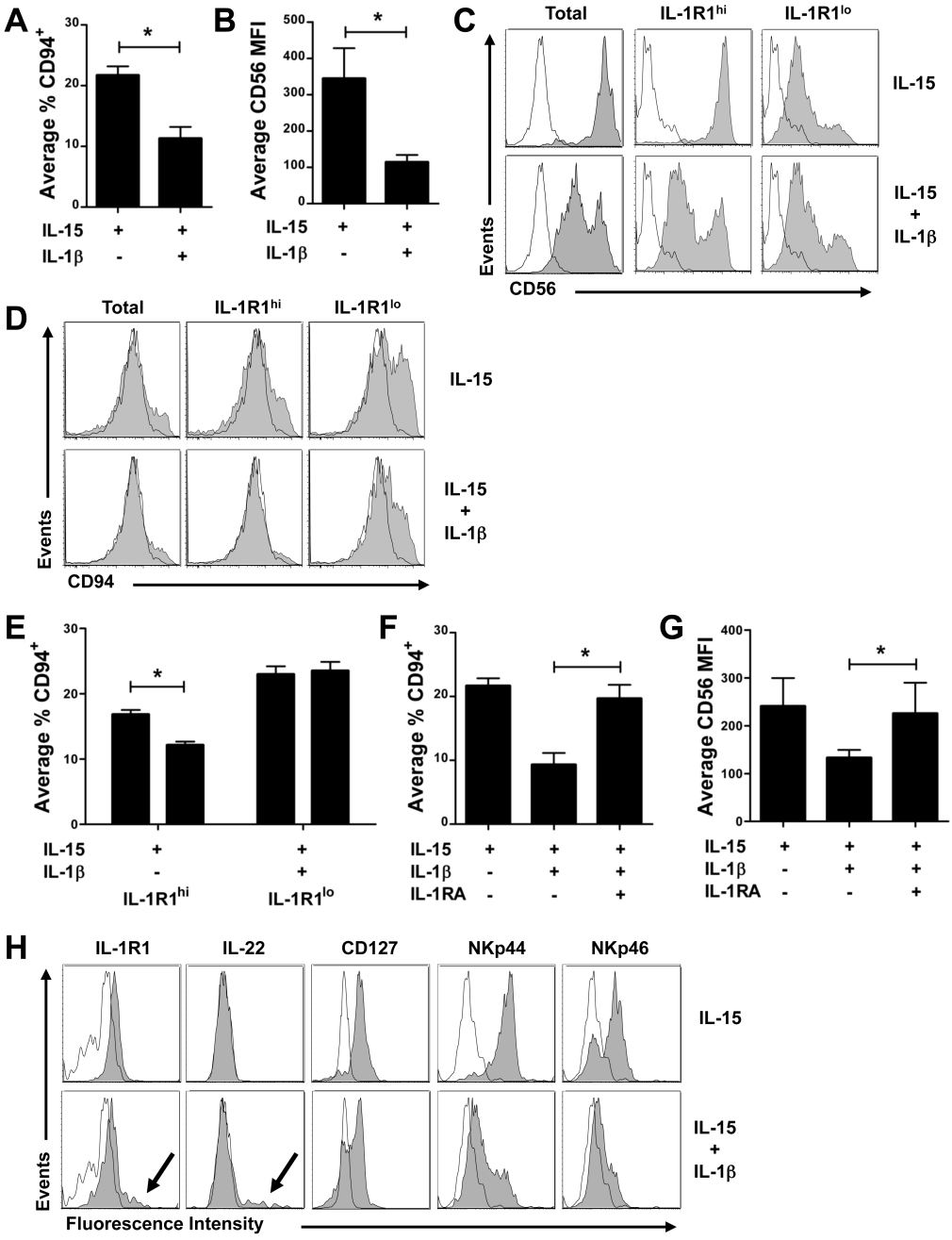

Figure 5. IL-1β inhibits differentiation of IL-1R1^{hi} stage 3 iNK cells

Surface expression of **(A,D,E)** CD94 and **(B,C)** CD56 assessed after 14 d of *in vitro* culture of sorted **(A,B)** total, or **(C,D,E)** IL-1R1^{hi} and IL-1R1^{lo} subpopulations of stage 3 iNK cells with IL-15 or IL-15 and IL-1β. **(F,G)** CD94 and CD56 were also assessed after bulk stage 3 iNK cells were cultured for 14 d in the presence of IL-15, IL-15 and IL-1β, or IL-15 with IL-1β and IL-1RA. **(H)** Histograms depict staining with isotype **(empty)** or antibody specific for the indicated antigen **(filled)** after culture of IL-1R1^{hi} stage 3 iNK cells for 14 d in the presence of IL-15 or IL-15 and IL-1β. Arrows indicate high density expression for IL-1R1 and IL-22 (also see Figure 4F). Data in **A, B, E, F, and G** presented as the mean ±

SEM (*, $P \leq 0.05$; $n \geq 4$). Histograms in **C, D, and H** depict staining in a representative donor ($n \geq 4$).

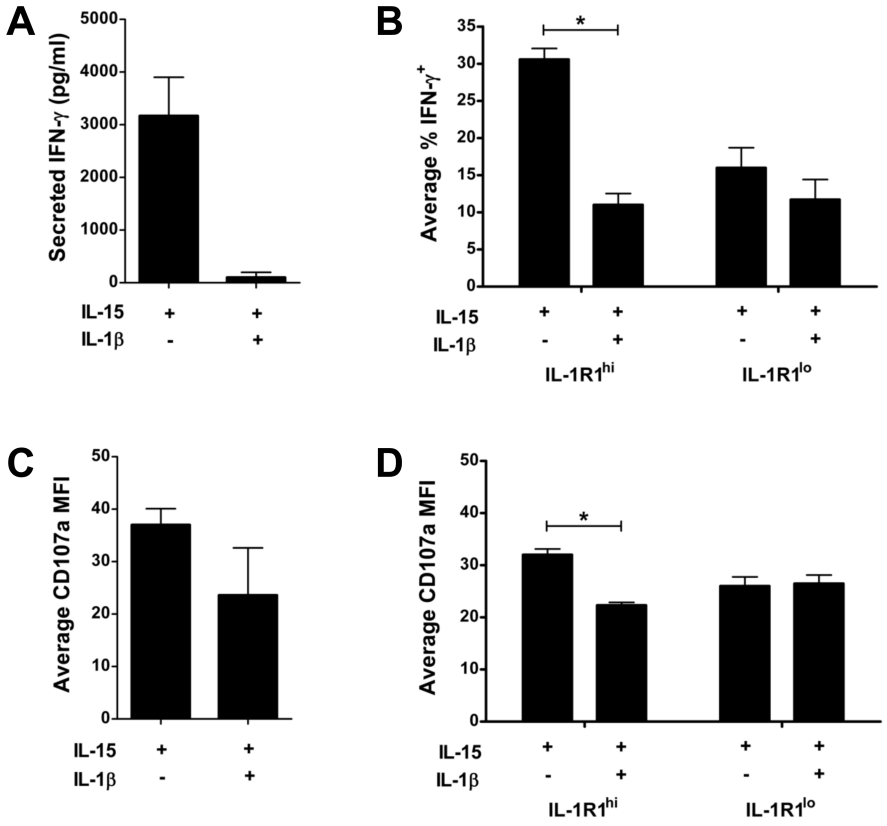

Figure 6. IL-1β impedes the IL-1R1^{hi} subpopulation of stage 3 iNK cells from acquiring IFN-γ production and degranulation

(A,C) Total or **(B,D)** IL-1R1^{hi} and IL-1R1^{lo} subpopulations of stage 3 iNK cells sorted from SLT were cultured for 14 d *in vitro* with IL-15 or IL-15 and IL-1β. Cells were replated in equal numbers and either: **(A,B)** stimulated for 12 h with IL-15 + IL-12 + IL-18 to assess IFN-γ secretion via **(A)** ELISA ($P < 0.005$; n = 7) or **(B)** intracellular flow (n = 8); or **(C,D)** incubated overnight with K562 targets (4:1 E/T ratio) and assessed for surface expression of the degranulation marker CD107a by flow cytometry (n = 3; for **C**, $P = 0.14$). Data in **B** depicts IFN-γ expression after gating on the population which had acquired CD94 surface expression. Data presented as mean ± SEM (*, $P \leq 0.01$).

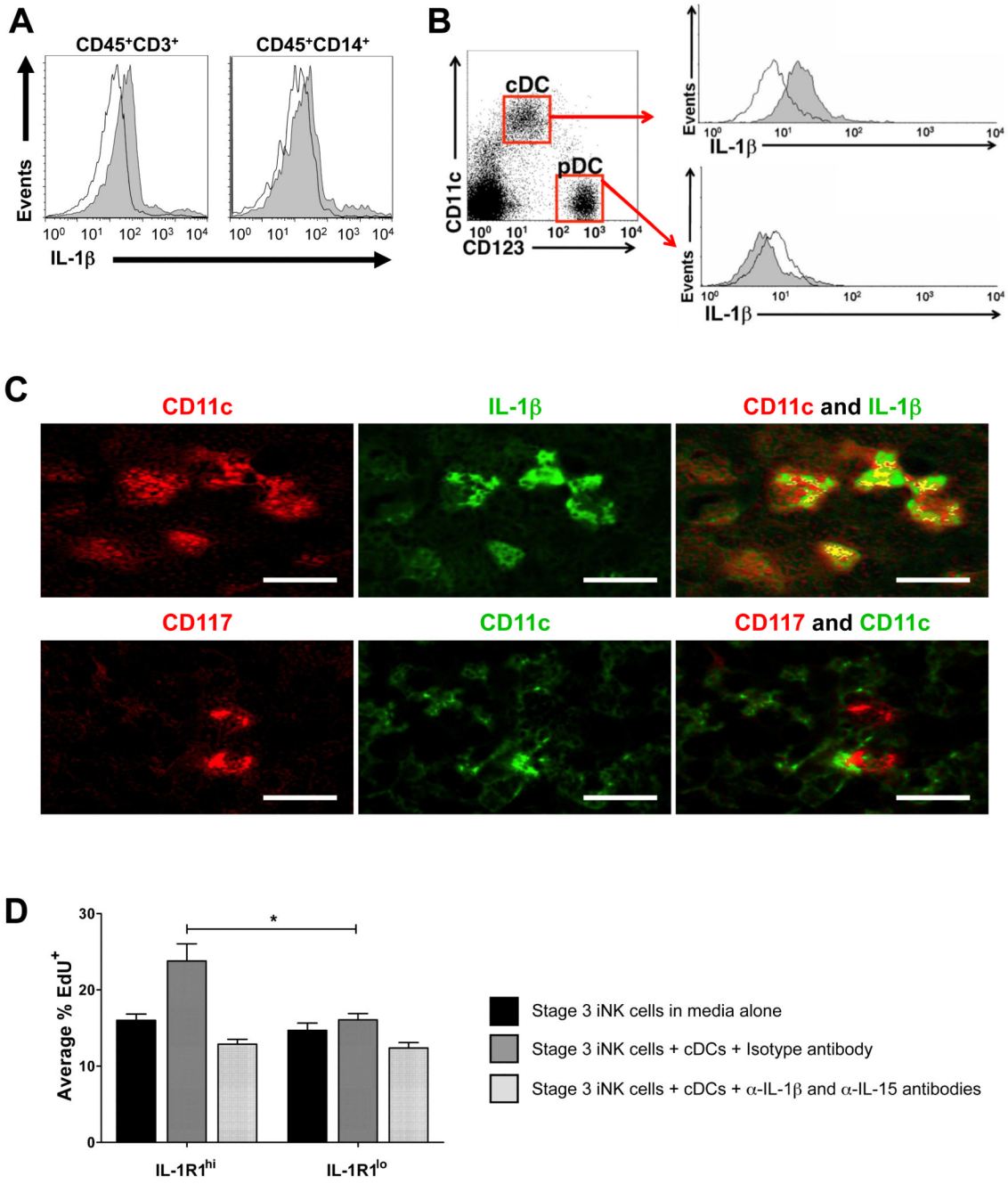

Figure 7. Stage 3 iNK cells reside in the vicinity of IL-1β^{+} CD11c^{hi} cDCs within SLT

(A,B) Intracellular staining of unstimulated SLT mononuclear cells from a representative donor (n = 3) with isotype **(empty)** or α-IL-1β **(filled)** antibody shown after gating on **(A)** CD45^{+}CD3^{+} T cells and CD45^{+}CD14^{+} monocytes and macrophages, or **(B)** CD123^{hi}CD11c^{lo} pDCs and CD123^{lo}CD11c^{hi} cDCs subsets. **(C)** Immunohistochemical staining depicted in serial sections of human tonsil from a representative donor (n = 4). Yellow areas indicate co-expression, as shown for CD11c^{hi} and IL-1β (**top**). CD117^{+} cells did not co-express CD11c, but were located in proximity of CD11c^{hi} cDCs (**bottom**). Magnification, 400x. Bar, 30 μM. **(D)** EdU incorporation in sorted IL-1R1^{hi} and IL-1R1^{lo}

subpopulations of stage 3 iNK cells after 6 h with α-MEM medium alone, or in the presence of autologous BDCA-1$^+$ cDCs (4:1 iNK:cDC ratio) with either isotype or α-IL-15 and α-IL-1β blocking antibodies. Depicted is % of CD117$^+$ lymphocytes which were also EdU$^+$ (*, $P = 0.05$; n = 3). Data in **D** presented as mean ± SEM.