The stage-specific effects of IL-1β on human natural killer cell development.

Dissertation

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Abstract

Our laboratory previously demonstrated that natural killer (NK) cell developmental intermediates can be found in secondary lymphoid tissue (SLT) (Freud, Becknell et al. 2005). Our subsequent study detailed the surface phenotype of SLT NK progenitor and precursor populations, provided a paradigm for NK ontogeny, and demonstrated that NK development progresses in a unidirectional manner through four discrete developmental stages (Freud, Yokohama et al. 2006). Stage 3 cells, which are exclusively committed to the NK lineage, were characterized as a population of immature NK (iNK) cells because they lack certain characteristics of mature NK cells, including some NK cell surface receptors, cytolytic activity, and the capacity for interferon-gamma (IFN-γ) production. It has already been documented that interleukin (IL)-15 is crucial for mature NK cell function (Carson, Giri et al. 1994), survival (Cooper, Bush et al. 2002), and for normal NK cell development (Yu, Fehniger et al. 1998); however, only a small fraction [~2-20%] of stage 3 iNK cells become stage 4 mature NK cells in response to IL-15 in vitro (Freud, Yokohama et al. 2006). Thus, the identity of the immunomodulatory factors regulating stage 3 iNK cell expansion and differentiation may not yet be fully elucidated.

For the past four years, this topic has been the focus of my research.

Through the course of many studies, we (myself along with my collaborators) have discovered that the IL-1 receptor (IL-1R1) is selectively expressed by a subpopulation of
stage 3 iNK cells. We also discovered that the vast majority of stage 3 iNK cells in SLT constitutively and selectively express aryl hydrocarbon receptor (AHR) and IL-22, a T\textsubscript{H}17 cytokine which may have an important role in maintaining mucosal immunity. IL-1R1, AHR, and IL-22 expression were absent during earlier and later stages of NK development, and expression of AHR and IL-22 were largely restricted to the IL-1R1\textsuperscript{hi} subpopulation of stage 3 iNK cells.

These IL-1R1\textsuperscript{hi} stage 3 iNK cells localized proximal to IL-1β-producing conventional dendritic cells (cDC) within SLT, proliferated in direct response to cDC-derived IL-15 and IL-1β, and required continuous exposure to IL-1β to retain AHR and IL-22 expression. In the absence of IL-1β, a substantially greater fraction of IL-1R1\textsuperscript{hi} stage 3 iNK cells differentiated to stage 4 mature NK cells, and acquired the ability to kill and secrete IFN-γ. Thus, cDC-derived IL-1β preserves and expands IL-1R1\textsuperscript{hi}IL-22\textsuperscript{+}AHR\textsuperscript{+} stage 3 iNK cells, potentially influencing human mucosal innate immunity during infection.

These data support a role for IL-1β as an immunomodulatory factor capable of regulating the development of human NK cells in vivo, and also suggest that these “immature” NK cells may have a unique functional role within SLT. Herein, I present these findings and provide an extended discussion relating our new data to current concepts in the field of NK cell developmental biology.

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Dedication

This document is dedicated to Allan Yates.
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I thank my parents and brother for believing in me, and for their unconditional love and support.

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Finally, I wish to acknowledge the generous support of my education through the multiple funding organizations that provide for the Caligiuri Laboratory.
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Chapter 1: Background

1.1 Natural killer cells: innate immune effectors.

Natural killer (NK) cells are CD14−CD3−CD19− large granular lymphocytes that make up about 10% of the peripheral blood (PB) lymphocyte pool and play a critical role in innate immunity (Colucci, Caligiuri et al. 2003). NK cells not only protect the host against invasion by viral pathogens, but also survey for and destroy malignantly transformed tumor cells with their unique ability to directly lyse target cells without prior antigen sensitization (Kiessling, Klein et al. 1975; Brooks, Flannery et al. 1981). Subsequent studies have revealed multiple mechanisms of NK cell cytotoxicity, including the release of cytolytic perforin/granzyme granules and the direct induction of apoptosis in target cells (Liu, Perussia et al. 1986; Atkinson, Gerrard et al. 1990).

In addition, mature NK cells have been shown to elaborate a variety of immunomodulatory cytokines and chemokines, such as interferon-gamma (IFN-γ) (Payne, Linde et al. 1985), which orchestrates the local innate immune response (Cooper, Fehniger et al. 2001; Fehniger, Cooper et al. 2003). This serves to recruit other effectors of innate immunity, as well as members of the adaptive immune system, to the site of infection. Furthermore, data point to a role for NK cells in directing antigen-specific T cell responses in secondary lymphoid tissue (SLT) through the release of IFN-γ, a T\textsubscript{H}1 polarizing cytokine (Maggi, Parronchi et al. 1992; Morandi, Bougras et al. 2006).
Mature NK cells are heterogeneous in nature and accordingly, two subsets have been characterized in the PB. Each of these subsets possess distinct effector functions and can be distinguished by the density at which the surface marker CD56 is expressed. In particular, the CD56\textsuperscript{bright} subset of mature NK cells proliferate and secrete large quantities of IFN-\(\gamma\) in response to monokine co-stimulation, while the CD56\textsuperscript{dim} population of mature NK cells is comprised of cells with high expression of Fc\(\gamma\)RIII (CD16), killer immunoglobulin-like receptors (KIR), and an abundance of cytolytic granules, supporting the role of the CD56\textsuperscript{dim} population of NK cells as cytotoxic effectors (Cooper, Fehniger et al. 2001; Cooper, Fehniger et al. 2001). There is now some evidence to suggest that these two NK populations are developmentally related and, more specifically, that CD56\textsuperscript{bright} NK cells may give rise to CD56\textsuperscript{dim} NK cells (Yu, Mao et al.; Freud and Caligiuri 2006; Annunziato, Cosmi et al. 2007; Chan, Hong et al. 2007).

1.2 Natural killer cell development: general concepts.

While it has been established for some time that T and B cell developmental intermediates reside in the thymus and bone marrow (BM), respectively, a publication from our laboratory was the first to show that human NK cells (Fehniger, Cooper et al. 2003; Ferlazzo, Thomas et al. 2004) and human NK developmental intermediates (Freud, Becknell et al. 2005; Freud, Yokohama et al. 2006), can be found within secondary lymphoid tissue (SLT). Whereas \(\geq 90\%\) of PB NK cells are CD56\textsuperscript{dim}, the majority of NK cells in human SLT – including lymph nodes (LN) and tonsils – are CD56\textsuperscript{bright}, and can
be found within the lamina propria and parafollicular T cell region (Fehniger, Cooper et al. 2003; Ferlazzo, Thomas et al. 2004). Likewise, we discovered that a population of CD56$^{bright}$ NK cells can be reproducibly generated in vitro when a population of CD34$^{dim}$CD45RA$^+$Integrin $\alpha$4$\beta$7$^{bright}$ lymphocytes – which is also selectively enriched in the parafollicular T cell region of SLT – are cultured with interleukin (IL)-15 (Freud, Becknell et al. 2005).

Our discovery of the selective enrichment of both CD34$^{dim}$CD45RA$^+$ NK cell precursors (Freud, Becknell et al. 2005) and CD56$^{bright}$ NK cells (Fehniger, Cooper et al. 2003) within the parafollicular T-cell rich regions of human LN and tonsils suggested that SLT may serve as a site for complete NK cell differentiation, and that a series of developmental intermediates may be present within these organs. Indeed, based on patterns of CD34, CD117, and CD94 expression on the surface of CD3$^-$ lymphocytes, we discovered four discrete stages of NK cell development within human SLT (Freud, Yokohama et al. 2006). These populations can be minimally defined as: CD34$^+$CD117$^-$CD94$^-$ stage 1 pro-NK cells, CD34$^+$CD117$^+$CD94$^-$ stage 2 pre-NK cells, CD34$^-$CD117$^+$CD94$^-$ stage 3 immature NK (iNK) cells, and CD34$^-$CD117$^+$/CD94$^+$ stage 4 mature NK cells.

Our conclusion that these four populations represent sequential stages in NK cell development is supported by the continual and gradual transition between each subset’s immunophenotype; that is, CD34 expression is gradually decreased as CD117 expression
appears, and CD117 expression gradually decreases as cells begin to acquire CD94. Similarly, CD56 expression gradually increases, as it is not seen on stage 1 pro-NK cells, but is highly expressed on stage 4 mature NK cells, which are uniformly CD56^{bright}. Moreover, the expression patterns of CD34 and CD94 in SLT are completely mutually exclusive, suggesting that a developing NK precursor must progress through a CD34^{-}CD94^{+} stage; which we identified as CD117^{+} stage 3 iNK cells (Freud, Yokohama et al. 2006).

In addition to immunophenotyping, our lab characterized these four populations based on lineage commitment, response to cytokines, and NK cell effector function. Whereas CD34^{+}CD117^{-} stage 1 pro-NK cells and CD34^{+}CD117^{+} stage 2 pre-NK cells retain multipotency with T and DC cell potential, CD34^{+}CD117^{+}CD94^{-} stage 3 iNK cells and CD34^{+}CD117^{+/-}CD94^{+} stage 4 mature NK cells are committed exclusively to the NK lineage. Like stage 4 mature NK cells, stage 3 iNK cells are dependent on IL-15 for survival, but stage 3 is classified as “immature” because this population lacks some properties acquired by more differentiated CD94^{+}CD56^{bright} stage 4 mature NK cells, including expression of certain NK cell surface receptors, the capacity for IFN-γ production, and cytolytic activity (Freud, Yokohama et al. 2006; Cupedo, Crellin et al. 2009; Hughes, Becknell et al. 2009). In addition, a fifth and seemingly final stage of NK cell development has recently been described in SLT, characterized by acquisition of CD16 and KIR, with a surface antigen repertoire highly similar to that of PB CD56^{dim} NK cells (Freud and Caligiuri 2006; Annunziato, Cosmi et al. 2007).
1.3 The role of cytokines in regulating natural killer cell development.

The response of NK cells to the cytokines and other signals from their microenvironment appears to be influenced by a combination of multiple factors acting in concert, including the specific combination and concentration of cytokines, the presence of appropriate receptors on the NK cell itself, and cell-to-cell contact with other immune and stromal cells in the vicinity. Each of these parameters contributes to an overall context in which an NK cell “sees” certain stimuli in the microenvironment, which influences how the NK cell will respond.

Although the complete list of immunomodulatory factors influencing NK development remains unknown, it is currently believed that NK cell survival and development requires signaling through the common \( \gamma_c \) chain of the IL-2 receptor (IL-1R\( \gamma \)) (Cao, Shores et al. 1995; Vosshenrich, Ranson et al. 2005), which is triggered by exposure to a family of cytokines including IL-2, IL-7, IL-15, and IL-21 (reviewed in (Kovanen and Leonard 2004)). Situations have been reported in which IL-2 or IL-7 can substitute for IL-15 (Freud and Caligiuri 2006; Satoh-Takayama, Vosshenrich et al. 2008; Cupedo, Crellin et al. 2009), however IL-15 is thought to play the most critical role during NK cell development (Williams, Klem et al. 1998). This has been most compellingly demonstrated through the use of mice lacking expression of either IL-15 (Lodolce, Boone et al. 1998) or the \( \beta \) chain of the IL-2 receptor (IL-2R\( \beta \) or CD122) (Reya, Yang-Snyder et
al. 1996), which is also a essential component of the IL-15 receptor (Giri, Ahdieh et al. 1994).

Accordingly, culture experiments have confirmed that there is likely a role for IL-15 in driving human NK development. In particular, we found that, in the presence of IL-15 in vitro, each of the four populations of SLT NK developmental intermediates underwent a unidirectional progression from stage 1 to 2 to 3 to 4, and acquired a CD56bright surface phenotype (Freud, Yokohama et al. 2006). While IL-15 on its own was sufficient to maintain survival of cells from stages 2-4, nearly all stage 1 pro-NK cells died in the absence of additional cytokines ((Flt 3 ligand (FL), IL-3, IL-7) (Freud, Yokohama et al. 2006), and CD122 mRNA was not detectable in stage 1 pro-NK cells, but expression gradually increased during stages 2-4 (Freud, Yokohama et al. 2006), suggesting that IL-15-responsiveness may be acquired during stage 2 of NK development. Although IL-15 on its own is indeed sufficient to support survival of human stage 3 iNK cells, which represent the most abundant population of SLT NK developmental intermediates, it should be noted that only ~2-20% of stage 3 iNK cells differentiate into IFN-γ-producing CD94+CD56bright stage 4 mature NK cells in the presence of IL-15 in vitro (Freud, Yokohama et al. 2006; Hughes, Becknell et al. 2010). Hence, IL-15 on its own may be inadequate to drive optimal developmental progression from stage 3 to stage 4 in vitro.

Together, these findings suggest that, even among IL-15-responsive populations of NK developmental intermediates, factors other than IL-15 are likely involved in NK
differentiation, thus the conditions required for optimal differentiation of stage 4 mature NK cells *in vitro* remain under investigation.

1.4 Summary.

NK cells comprise a unique, diverse set of lymphocytes with emerging roles in the innate and adaptive immune response (Sun and Lanier 2009). Within 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, Becknell et al. 2005; Freud, Yokohama et al. 2006). Our laboratory initially identified four stages of human NK developmental intermediates in SLT, and characterized each of these populations in terms of lineage commitment, response to cytokines, and NK cell effector function (Freud, Yokohama et al. 2006). It has been long appreciated that cytokines regulate hematopoiesis, and it is likely that IL-15 plays an important role in the development and function of human NK cells. However, a variety of immunomodulatory factors are present within the SLT microenvironment, and how exposure to these factors may influence developing NK cells remains unclear.

Chapters 2 and 3 will focus primarily on investigating the influence of cytokines on homeostasis and differentiation of SLT NK developmental intermediates. We believe that data from these studies will advance current understanding of innate mucosal immunity for the purpose of host defense, and may further implicate SLT as a principal
site for human NK cell development *in vivo*. The data presented in Chapter 2 suggests a potentially important functional role for stage 3 iNK cells, which may serve to promote innate mucosal immunity. The study presented in Chapter 3 allows us to more fully understand the role of certain immunomodulatory factors present in the SLT microenvironment in driving normal human NK cell development. Identifying the mechanisms regulating human NK cell homeostasis and differentiation may lend insight into the first line of defense against cancer and infection, and will likely prove important in developing clinical interventions, such as oral cancer vaccines and novel treatments for autoimmune diseases, including Crohn’s disease and psoriasis.
Chapter 2: Expression of IL-22 by Human Stage 3 Immature Natural Killer Cells

2.1 Introduction.

It was previously established that NK cells share a common progenitor with T cells (Freud, Yokohama et al. 2006), and emerging evidence suggests that the T_{H}17 subset of effector T cells may have a great deal in common with some NK populations. In particular, T_{H}17 cells were shown to develop exclusively from a population of naïve CD4^{+} T cells expressing the NK lineage-associated marker, CD161 (Cosmi, De Palma et al. 2008). CD161 is an NK lineage-associated marker which is uniformly expressed on the surface of SLT stage 3 iNK cells and stage 4 mature NK cells (Freud, Yokohama et al. 2006), PB NK cells, and on subsets of T cells; while absent from B cells, monocytes, and granulocytes (Lanier, Chang et al. 1994).

The T_{H}17 subset of CD4^{+} T cells has often been implicated in autoimmunity (Bettelli, Oukka et al. 2007). T_{H}17 cells differ from T_{H}1 and T_{H}2 populations in that T_{H}17 cells are characterized by expression of cytokines including IL-17 and IL-22 (Harrington, Hatton et al. 2005; Liang, Tan et al. 2006), as well as transcription factors including RAR-related orphan receptor C (RORC) and aryl hydrocarbon receptor (AHR) (Ivanov, McKenzie et al. 2006; Veldhoen, Hirota et al. 2008). While RORC is currently considered an orphan receptor because it has no known ligands, AHR has been reported to bind halogenated aromatic hydrocarbons (Ohtake, Takeyama et al. 2003). Currently described AHR
agonist and antagonist ligands include both naturally occurring substances [derivatives of tryptophan, such as indigo and indirubin, tetapyroles such as bilirubin (Adachi, Mori et al. 2001), the arachidonic acid metabolites lipoxin A4 and prostaglandin G (Seidel, Winters et al. 2001), modified low-density lipoproteins (McMillan and Bradfield 2007), as well as several dietary carotinoids (Denison, Pandini et al. 2002)] and environmental pollutants [(2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), polychlorinated dibenzodioxins, dibenzofurans and biphenyls, and polycyclic aromatic hydrocarbons (3-methylcholanthrene, benzo(a)pyrene, benzantracenes, and benzoflavones) (Poland, Glover et al. 1976)].

AHR is required by T H17 cells for IL-22 production (Veldhoen, Hirota et al. 2008), and is expressed by T cells during development of the T H17 lineage (Quintana, Basso et al. 2008), and at least some of AHR’s functions have been reported to be ligand binding-specific. For example, addition of AHR agonists to culture medium was shown to enhance T H17 differentiation and IL-22 production (Veldhoen, Hirota et al. 2009); therefore, we hypothesize that, by promoting transcription of stage 3-associated genes (such as IL-22), AHR agonists may also maintain stage 3 iNK cell homeostasis.

IL-22 is a member of the IL-10 family of cytokines (Dumoutier, Lejeune et al. 2001), which binds to and signals via a heterodimeric receptor consisting of IL-10Rβ (also involved in IL-10 signaling) and a unique IL-22R1 subunit (Kotenko, Izotova et al. 2001). Interestingly, although IL-22 is produced by immune cells, to date, expression of
IL-22R1 (thus IL-22-responsiveness), has been reported only in cells of non-hematopoietic origin, including endothelial and epithelial cells (Wolk, Kunz et al. 2004). For this reason, it has been suggested that IL-22 mediates cross-talk between the immune system and tissues (Wolk, Kunz et al. 2004; Wolk and Sabat 2006).

Produced in response to microbial pathogens, IL-22 supports mucosal immunity by promoting the integrity of the epithelial cell barrier and the secretion of antimicrobial peptides (Wolk, Kunz et al. 2004; Zheng, Valdez et al. 2008). The findings presented in this Chapter, which relate to our co-discovery of IL-22 expression in an abundant population of resting stage 3 iNK cells from human tonsil (which is an example of SLT and also mucosa-associated lymphoid tissue (MALT)), suggest this population of iNK cells may have an important role in mucosal immunity (Cella, Fuchs et al. 2009; Hughes, Becknell et al. 2009).

2.2 T_{H}17-associated gene transcripts in NK developmental intermediates.

We quantified transcripts for several T_{H}17-associated cytokines, including \textit{IL-17A} and \textit{IL-17F}, \textit{IL-22}, and \textit{IL-26} in resting stage 1-4 human NK developmental intermediates. Due to scarcity of cells from stages 1 and 2, each of the four populations was FACS purified from SLT, then pooled from 6-7 donors to achieve sufficient quantities of mRNA. \textit{IL-17} mRNA was not detected in any of the four stages (\textit{not shown}). Among SLT NK developmental intermediates, \textit{IL-22} mRNA was only detected during stage 3, where it was present at levels which were at least 92-fold greater than the trace quantities found in
stages 1, 2, and 4 (Figure 1A and (Hughes, Becknell et al. 2009)). Since expression levels for each of these genes were similar in stages 1, 2, and 4, and scarcity of stage 1 and 2 cells, which necessitated the pooling of sorted cells from multiple donors, subsequent analyses were performed using only stages 3 and 4, for comparisons within individual donors.

Compared to stage 4 mature NK cells isolated from the same donor, IL-22 mRNA expression was at least $138 \pm 1.6$-fold higher in stage 3 iNK cells ($P \leq 0.0002; n = 7$) (Figure 1B and (Hughes, Becknell et al. 2009)). We also measured IL-17A, IL-17F and IL-26 mRNA in stage 3 and stage 4 cells isolated from SLT of individual donors. Again, no IL-17A or IL-17F was detected in either stage from $> 10$ donors tested (not shown). IL-26 mRNA was expressed in resting stage 3 iNK cells from 5 of 7 donors, while absent or barely detectable in stage 4 mature NK cells from all 7 donors. When present, IL-26 transcript was at least $34.8 \pm 1.6$-fold higher in stage 3 iNK cells when compared to stage 4 mature NK cells ($P \leq 0.002, n = 5$; Figure 1C) (Hughes, Becknell et al. 2009). Finally, in quantitative data not shown, we ascertained that RORC, a gene required for lymph node formation and Th17 differentiation (Sun, Unutmaz et al. 2000; Ivanov, McKenzie et al. 2006), is transcribed during stages 2 and stage 3, while undetectable during stages 1 and 4 (data not shown). RORC mRNA expression was highest during stage 3, at which point RORC transcript could be detected at levels which were at least $4.12 \pm 1.29$-fold higher than that measured in stage 4 ($P = 0.04, n = 3$; Figure 1D). Although RORC is associated with driving Th17 development and cytokine production, a recent study
suggests that $T_H17$ cell expression of AHR, rather than RORC, controls production of IL-22 (Veldhoen, Hirota et al. 2008). In agreement with this study, we observed that, like IL-22, $AHR$ mRNA was also expressed in a stage 3-restricted manner (Figure 1E).

2.3 Production of $T_H17$-associated cytokines by NK populations.

We detected IL-22 protein in resting stage 3 iNK cells using intracellular flow cytometric staining with an anti-IL-22 antibody. The majority of CD117$^+$CD94$^-$ stage 3 iNK cells, a population previously described in our lab as NKp46$^+$2B4$^+$ (Freud, Yokohama et al. 2006), co-express intracellular IL-22, whereas staining is very low in the CD117$^+$/CD94$^+$ stage 4 mature NK population (Figure 2A) (Hughes, Becknell et al. 2009). Indeed, 88.0 ± 5.3% of stage 3 iNK cells were IL-22$^+$ compared to 6.7 ± 3.5% of stage 4 mature NK cells (Figure 2B; $P < 0.00002, n = 6$) (Hughes, Becknell et al. 2009). We utilized an ELISA to confirm that resting and IL-15 stimulated stage 3 iNK cells, but not stage 4 mature NK cells, secrete IL-22 protein (not shown). Figure 3 documents immunohistochemical staining used to identify IL-22$^+$ stage 3 iNK cells in situ within human tonsil. Co-labeling experiments indicated that CD117$^+$IL-22$^+$ iNK cells reside in the epithelia and lamina propria as well as the parafollicular T cell-rich regions of SLT.

Finally, we found that the majority of the Lin$^-$ tonsillar mononuclear cells constitutively producing IL-22 co-expressed CD117 and CD161 (Figure 4A,B), but did not express BDCA-2 (Figure 4C), thus ruling out the inclusion of NK dendritic cells (Dzionek, Sohma et al. 2001). In agreement with a previous report from our group in which stage 3
iNK cells were first characterized (Freud, Yokohama et al. 2006), the majority of IL-22\(^+\) stage 3 iNK cells do not express CD56 or NKp44 (Figure 4B). Thus, in contrast to the NK-22 population, which is >85% IL-22\(^-\) when resting, and by definition, must co-express CD56 and NKp44 (Cella, Fuchs et al. 2009); stage 3 iNK cells are >80% IL-22\(^+\) when resting, and largely CD56\(^-\) and NKp44\(^-\). Together, our data may indicate that the IL-22-producing cells within stage 3 iNK cells and NK-22 cells represent populations which are largely distinct, and stage 3 iNK cells may represent a major population of constitutively IL-22\(^+\) innate immune cells within human SLT.

2.4 Discussion.

In summary, this study showed that the vast majority of resting stage 3 iNK cells in SLT display robust constitutive IL-22 expression \textit{ex vivo}. These findings are distinct from the earlier identification of NKp44\(^+\)CD56\(^+\) cells in SLT that express little or no IL-22 at baseline, yet <10% of these cells were induced to express IL-22, IL-26, and leukemia inhibitory factor (LIF) upon \textit{in vitro} culture with various cytokines or activated monocytes (Cella, Fuchs et al. 2009). The stage 3 iNK population in our study includes a small percentage of cells with the CD56\(^-\)NKp44\(^+\) surface phenotype described to characterize NK-22 cells (Cella, Fuchs et al. 2009), but these markers are neither necessary nor sufficient to unambiguously identify resting IL-22\(^+\) cells within human SLT. Rather, the surface phenotype of stage 3 iNK cells, CD34\(^-\)CD117\(^-\)CD161\(^+\)CD94\(^-\), specifically identifies a larger population of constitutively IL-22\(^+\) NK cells in SLT.
The finding that *IL-22* transcript is absent from stages 1, 2 and 4 during NK development raises two possibilities: (1) *IL-22* expression must be lost prior to differentiation to stage 4; or (2) the subset of *IL-22*⁺ stage 3 iNK cells represent a separate, terminally differentiated population. In support of the latter, < 20% of stage 3 iNK cells differentiate to the IFN-γ-producing stage 4 mature NK cells in the presence of IL-15 *in vitro* (Freud, Yokohama et al. 2006), a finding which may reflect heterogeneity within the population of stage 3 iNK cells.

The abundant intracellular *IL-22* staining in stage 3 iNK cells when freshly isolated from human tonsil is consistent with *in vivo* exposure to a cellular milieu within SLT that is permissive for *IL-22* production. Factors previously implicated in driving T₇ development and *IL-22* synthesis by T cells include RORC, AHR, and inflammatory cytokines such as IL-6, IL-23, and IL-1β, as well as Toll-like receptor (TLR) activation of dendritic cells (DC) and monocyte/macrophages. Accordingly, the work presented in Chapter 3 was undertaken to determine whether T₇-polarizing cytokines, specifically IL-1β, might similarly affect this *IL-22*-producing population of stage 3 iNK cells.

2.5 Experimental procedures.

**Isolation of human NK precursors from SLT.** All procedures were approved by The Ohio State University (OSU) Institutional Review Board. NK developmental intermediates were isolated from fresh normal human tonsil and analyzed as described by Freud et al. (Freud, Yokohama et al. 2006). All populations were isolated using
fluorescence activated cell sorting (FACS) using a FACSVantage (BD Biosciences) to purity >99%.

**cDNA preparation.** RNA was purified from < 5 x 10^5 cells using the Absolutely RNA Nanoprep Kit (Stratagene), or using the RNeasy (Qiagen) when there were > 5x10^5 cells. cDNA was synthesized using MMLV reverse transcriptase kit (Invitrogen) and random hexamers.

**Real-Time PCR.** Taqman primer/probe sets for *IL-22, IL-26, AHR, IL-17A,* and *IL-17F* were purchased from ABI (Applied Biosystems). *RORC* primer and probe sequences were as previously described (Yang, Fan et al. 2006). Real-Time PCR was performed on an ABI Prism 7900HT (Applied Biosystems), analyzed by the ΔΔCt method, and normalized to an *18S* internal control as previously described (Fehniger, Carson et al. 1999).

**Flow cytometric analyses.** Total CD3^-CD19^-CD34^- tonsillar mononuclear cells were isolated as previously described (Freud, Yokohama et al. 2006). All mAbs were purchased from BD Biosciences, except: NKp44 APC, BDCA-2 APC, CD161 APC (Miltenyi Biotec), CD94 FITC (clone 131412, R&D Systems), and CD56 APC (Beckman Coulter). Intracellular staining was performed immediately after CD34 depletion, and without the addition of a protein transport inhibitor, using the Cytofix/Cytoperm Plus Fixation/Permeabilization Kit (BD Biosciences) and anti-IL-22-PE mAb (clone 142928;
R&D Systems). Non-specific staining was detected through the use of an appropriately labeled isotype control antibody in all analyses. Cytometric and data analyses were performed using a FACSCalibur (BD Biosciences) and analyzed as previously described (Freud, Becknell et al. 2005) using CellQuest (BD Biosciences) or WinMDI software (J. Trotter, Scripts Institute).

Immunohistochemistry. Immunohistochemistry was performed as previously described (Fehniger, Cooper et al. 2003). Briefly, the Ultraview Universal (Ventana Medical) system was employed. Serial sections of paraffin-embedded human tonsillar tissue were digested in Protease 1 for 4 minutes, and then incubated with the anti-IL-22 mAb (1:100, R&D systems). Slides were photographed at room temperature, then coverslips were removed, and slides were tested with the anti-human CD117 mAb (DakoCytomation, 1:1000) after antigen retrieval for 30 minutes in CC1. Images were digitally acquired using the following equipment from Olympus (Olympus Imaging America Inc.): DP 12 camera, BX50 microscope, and UPLANF1 objectives at 400x magnification power. Images were analyzed using Adobe Photoshop CS3 software (Adobe Systems, Inc.).

Statistical analysis. Data were analyzed using a Student 2-tailed t-test. $P < 0.05$ was considered significant.
Figure 1: TH17-associated gene expression during NK development. (A-E)
Quantitative (Q) RT-PCR analysis was performed using the following FACS-sorted populations isolated via FACS from human tonsil: (A,E) stage 1-4 NK developmental intermediates (measured in mRNA pooled from multiple donors to achieve sufficient quantities of cDNA from stages 1 and 2; (n = 7)), or (B-D) stage 3 and 4 NK developmental intermediates (mRNA from each donor was measured individually). Relative quantification was performed using the ΔΔCt method, and gene expression levels were normalized to 18S mRNA. Y axis indicates fold increase over level of gene of interest quantified in (A,E) stage 1 pro-NK cells or (B-D) stage 4 mature NK cells, arbitrarily normalized to 1. The average fold change in IL-22, IL-26, and RORC mRNA present in stage 3 iNK cells compared to stage 4 mature NK cells was ~138, ~35, and ~4, respectively. Error bars represent standard error of the mean (SEM) (for B, n = 7, P ≤ 0.0002; for C, n = 5, P ≤ 0.002; for D, n = 3, P = 0.04).
Figure 1: TH17-associated gene expression during NK development.
Figure 2: IL-22 protein expression during NK development. (A,B) Total resting CD3⁻CD19⁻CD34⁻ tonsillar mononuclear cells were isolated (as previously described (Freud, Yokohama et al. 2006)) from human tonsil, then stained for surface expression of lineage markers (“Lin” = CD3, CD19, CD14, CD16), along with CD117 and CD94, followed by intracellular staining for IL-22 protein expression. (A) Intracellular staining with IL-22 mAb (filled) or isotype control antibody (empty) shown in a representative donor (n = 6) after gating on Lin⁻CD117⁺CD94⁻ stage 3 iNK cells or Lin⁻CD117⁻CD94⁺ stage 4 mature NK cells. (B) The mean proportion of IL-22⁺ cells among stage 3 iNK cells or stage 4 mature NK cells was calculated after gating as indicated in A. Error bars represent mean ± SEM (n = 6, P ≤ 0.00002).
Figure 2: IL-22 protein expression during NK development.
Figure 3: IL-22 and CD117 in human tonsil. Immunohistochemical staining depicted in serial sections of human tonsil from a representative donor (n = 3). Magnification, 400x. The different colored arrows indicate the exact same cell after staining for IL-22 (left) and after co-labeling with and CD117 (right). Cells with red staining are IL-22+.

Cells with brown staining are CD117+. CD117+IL-22+ stage 3 iNK cells were typically found within the epithelia and lamina propria, as well as the parafollicular T cell-rich regions, of human tonsil.
Figure 3: IL-22 and CD117 in human tonsil.
Figure 4. Surface phenotype of IL-22+ stage 3 iNK cells. (A-C) Total CD3−CD19−CD34− resting human tonsillar mononuclear cells were stained for surface expression of lineage markers (CD3, CD19, CD14, CD16), in addition to CD117 and CD94, followed by intracellular staining with IL-22 mAb or isotype control. Analyses were performed after gating on Lin− lymphocytes which were also: (A) IL-22+, (B) CD161+ (representing some stage 2 cells, in addition to stages 3-5) or CD94−CD117+ (representing stage 3 iNK cells), or (C) CD94−CD117−IL-22+ (representing IL-22+ stage 3 iNK cells). (A) Graphical summary of the mean proportion of Lin−IL-22+ resting tonsillar mononuclear cells co-expressing the indicated surface marker. Data in A presented as mean ± SEM (n = 4). (B,C) Expression of each indicated surface marker is shown in a representative donor (n = 4). (C) Histograms depict staining with specific mAb (filled), compared to isotype control (empty).
Figure 4: Surface phenotype of IL-22+ stage 3 iNK cells.
Chapter 3: The Effects of IL-1β on IL-1R1hi Stage 3 iNK cells.

3.1 Introduction.

As we showed in Chapter 2, resting stage 3 iNK cells found in human tonsil, constitutively express interleukin-22 (IL-22) (Cupedo, Crellin et al. 2009; Hughes, Becknell et al. 2009), a cytokine which has been implicated in mucosal immunity (Wolk, Kunz et al. 2004). This data suggest that stage 3 iNK cells within SLT may have an important role in mucosal immunity. Conceivably, microbial invasion of MALT requires an expansion of IL-22+ stage 3 iNK cells for effective early mucosal defense (Cupedo, Crellin et al. 2009; Hughes, Becknell et al. 2009). Under such circumstances, expansion of the IL-22+ stage 3 iNK cell 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, lymphoid tissue inducer (LTi) cells must proliferate and upregulate their expression of molecules such as lymphotoxin (LT) α1β2 in order to initiate tissue reorganization (Junt, Scandella et al. 2008). Recently, it was demonstrated that IL-22+ stage 3 iNK cells from human fetal lymph node may be LTi-like, and possess a phenotype identical to that of LTi cells (Cupedo, Crellin et al. 2009).
As we also demonstrated in Chapter 2, among human NK developmental intermediates in SLT, CD34^+CD117^+CD161^+CD94^- stage 3 iNK cells uniquely express AHR. TH17 cells, which also express IL-22 and AHR (Liang, Tan et al. 2006; Veldhoen, Hirota et al. 2008), require exposure to cytokines including IL-1β during development (Reynolds, Pappu et al.). Like IL-22, IL-1β is released within MALT in response to microbial invasion (Ikejima, Dinarello et al. 1984; Zheng, Valdez et al. 2008). Stage 3 iNK cells represent the most abundant NK cell developmental intermediate in human SLT (Figure 5); however, the signal(s) which regulate expansion and differentiation of the stage 3 iNK cell population were unknown, and were investigated in our most recent report (Hughes, Becknell et al. 2010).

In this Chapter, we will present related findings, demonstrating that the IL-1 receptor IL-1R1 was selectively expressed by a subpopulation of stage 3 iNK cells. We found that, in vitro, IL-1β was required to retain AHR and IL-22 expression in IL-1R1^hi iNK cells. In the absence of IL-1β, a substantially greater fraction of IL-1R1^hi iNK cells differentiated to stage 4 mature NK cells capable of cytotoxicity and IFN-γ secretion. These stage 3 iNK cells localized proximal to IL-1β-producing cDC 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-22^+ stage 3 iNK cells, and suggest a potential physiological role for CD11c^hiIL-1β^+ cDC in regulating expansion of the IL-1R1^hi subpopulation of stage 3 iNK cells in SLT.
3.2 Robust expression of IL-R1 protein is restricted to stage 3 iNK cells in SLT.

IL-1β is released by monocytes, macrophages, and DC in direct response to microbial invasion (Ikejima, Dinarello et al. 1984), and promotes T helper 17 (T_{H17}) cell homeostasis (Chung, Chang et al. 2009). We postulated that IL-1β might serve a similar role in stage 3 iNK cell homeostasis within SLT. We therefore assayed FACS purified stage 1-4 NK developmental intermediates from human tonsil for expression mRNA expression of IL-1β’s cognate receptor, IL-1R1 (Dower, Kronheim et al. 1985), using real-time RT-PCR. As shown in Figure 6A, 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 demonstrated IL-1R1 surface protein, ≥ 70% of stage 3 iNK cells expressed IL-1R1 (Figure 6B,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 6B,C).
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.

3.3 IL-22 and AHR are restricted to the IL-1R1\(^{hi}\) subset 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, \(RORC\), and \(AHR\) (Cella, Fuchs et al. 2009; Cupedo, Crellin et al. 2009; Hughes, Becknell 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 7A-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 7D). In contrast, expression of the LTi-associated transcription factor \(RORC\), which is restricted to the stage 3 iNK cells in SLT (Cupedo, Crellin 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, \textit{E4BP4} and \textit{Ets-1} (\textbf{Figure 8A-C}). Flow cytometry revealed that expression of CD127, CD56, CD161, NKp44, CCR7, and LT-\(\alpha\) – markers which are each associated with the LTi-like phenotype (Cupedo, Crellin et al. 2009) – are also relatively restricted to the IL-1R\(^{hi}\) subpopulation of stage 3 iNK cells (\textbf{Figure 7E}). Together, our data suggest that IL-22 and \textit{AHR} are specific to the IL-1R\(^{hi}\) subpopulation of resting human stage 3 iNK cells from SLT, and that these cells also share the phenotype of LTi-like cells. Thus, among stage 3 iNK cells, high density surface expression of IL-1R1 protein coincides with expression of \textit{AHR} and the ability to produce IL-22. Among SLT NK developmental intermediates, all three of these properties are highly specific for stage 3 iNK cells.

\textbf{3.4 Human IL-1R1\(^{hi}\) stage 3 iNK cells display a functional response to IL-1\(\beta\).}

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-1\(\beta\), 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, Legrand et al. 2009), cells treated with IL-1\(\beta\) alone were uniformly dead after 48 hours, 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-1\(\beta\) led to an expansion in the number of viable stage 3 iNK cells when examined after 2, 7, or 14 days. Notably, IL-1\(\beta\) expanded stage 3 iNK cells to 4.7 \pm 0.7-fold relative to the quantity of cells generated after 14 days with IL-15 alone (\textbf{Figure 9A}).
The expansion in the presence of IL-15 and IL-1β was diminished after withdrawal of IL-1β starting at day 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 1991), completely abrogated the IL-1β-mediated expansion observed earlier (Figure 9B).

We determined this IL-1β-induced expansion of stage 3 iNK cells resulted from enhanced proliferation rather than survival (Figure 9C). Importantly, despite the fact that by day 14, nearly all progeny generated from cultures of stage 1 pro-NK cells and stage 2 pre-NK cells had largely adopted a CD34⁺CD117⁺ stage 3-like phenotype – and the fact that a portion of stage 2 pre-NK cells express modest yet measurable amounts of IL-1R1 surface expression ex vivo – the addition of IL-1β had no measurable effect on expansion of stage 1 pro-NK cells or stage 2 pre-NK cells (Figure 9A). 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 9D).

3.5 IL-1β sustains 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 10A,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 10C,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 11A-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-17IL-22^+ human T cells (Trifari, Kaplan et al. 2009).

Although culture of IL-1R1^hi stage 3 iNK cells for 14 days 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 10E,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 9D), but rather resulted from an IL-15-mediated decrease in IL-1R1 on the surface of IL-1R1^hi stage 3 iNK cells (Figure 10G).

3.6 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, Yokohama et al. 2006; Huntington, Legrand et al. 2009; Sanos, Bui 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, sorted 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 12A; 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 12B). These effects, which could be observed within 2 days 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 12C-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 12F) and density of CD56 surface expression (Figure 12G). Figure 12H shows additional maturation markers assessed in IL-1R1 hi stage 3 iNK cells cultured for 14 days in IL-15 or IL-15 and IL-1β.

3.7 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, Yokohama et al. 2006).

Indeed, stage 3 iNK cells incubated in vitro with IL-15 and IL-1β for 14 days 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 days produced substantial IFN-γ, averaging 3,167 ± 734 pg/ml, following co-activation with IL-12, IL-15, and IL-18 (Figure 13A). As shown in Figure 13B, 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 14A), surface expression of the degranulation marker CD107a, which can be used as a functional marker to identify cytotoxic NK cells (Alter, Malenfant et al. 2004), was observed in both CD94+ and CD94− cells generated from culture of stage 3 iNK cells in vitro (Figure 14B). 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 days did not significantly influence CD107a surface expression (Figure 13C; 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 13D; P = 0.01).
3.8 Stage 3 iNK cells proliferate in direct response to 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 15A), IL-1β expression occurred in a large portion of tonsillar CD11c^{hi}CD123^{lo} cDC (Figure 15B and C, top row). In contrast, CD11c^{lo}CD123^{hi} plasmacytoid DC (pDC) and the population of CD11c^{lo}CD123^{lo} cells did not contain intracellular IL-1β (Figure 15B and not depicted, respectively).

Next, we performed immunohistochemical staining to determine whether SLT stage 3 iNK cells are located near IL-1β-producing cells in vivo. These CD117+ stage 3 iNK cells, which are primarily CD161+IL-1R1^{hi}IL-22^{+} (Figure 7; Figure 16), were found to be located in close proximity to IL-1β-producing CD11c^{hi} cDC in the parafollicular T-cell rich region of human tonsil (Figure 15C, bottom row). These CD117+ cells were found in situ in close proximity (within 50-150 μM) to IL-1β-producing CD11c^{hi} cDC in the parafollicular T-cell rich region of human tonsil (Figure 15C, bottom row). Thus, tonsillar cDC 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 cDC, short term coculture of IL-1R1^{hi} stage 3 iNK cells with autologous BDCA-1+CD11c^{hi}CD123^{lo} cDC
enhanced proliferation (measured via 5-ethyl-2’-deoxyuridine (EdU) incorporation assay) of IL-1R1\textsuperscript{hi} stage 3 iNK cells, but not of IL-1R1\textsuperscript{lo} stage 3 iNK cells (\textbf{Figure 15D}). Although neither α-IL-15 nor α-IL-1\textbeta{} antibody altered EdU incorporation as a single blocking reagent (not depicted), addition of α-IL-15 and α-IL-1\textbeta{} antibodies together reduced EdU incorporation to amounts seen in the absence of cDC, implicating specific roles for cDC-derived IL-15 and IL-1\textbeta{} in the proliferative response observed in IL-1R1\textsuperscript{hi} stage 3 iNK cells (\textbf{Figure 15D}). Thus, cDC-derived IL-15 and IL-1\textbeta{} are capable of directly stimulating proliferation of IL-1R1\textsuperscript{hi} stage 3 iNK cells. Similar co-stimulatory effects may also be possible between IL-1R1\textsuperscript{hi} stage 3 iNK cells and IL-1\textbeta{}-producing CD14\textsuperscript{+} monocytes or macrophages, which also produce IL-15.

3.9 Discussion.

The data presented in this Chapter identifies a role for IL-1R1 and its cognate ligand, IL-1\textbeta{}, 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-1\textbeta{} to IL-1R1 expanded IL-1R1\textsuperscript{hi} stage 3 iNK cells and preserved expression of IL-22 and \textit{AHR}. In contrast, culture of IL-1R1\textsuperscript{hi} stage 3 iNK cells in IL-15 alone resulted in a loss of IL-22 and \textit{AHR} expression and enhanced differentiation toward IFN-\gamma{} producing stage 4 mature NK cells. Furthermore, CD117\textsuperscript{+} cells, which co-express CD161 and IL-22, and thus correspond to the IL-1R1\textsuperscript{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-1\textbeta{}, found in...
CD11c^hi cDC, attesting to the physiologic relevance of the IL-1β-IL-1R1 interaction in vivo.

**RORC** is a hallmark of mature T\(_{H}17\) cells (Ivanov, McKenzie et al. 2006), but has also been detected in murine FOXP3^+ regulatory T (Treg) cells (Satoh-Takayama, Vosshenrich et al. 2008) and human FOXP3^+ Treg cells (Ayyoub, Deknuydt et al. 2009). Indeed, unstimulated human stage 3 iNK cells in SLT express **RORC** and **AHR** mRNA (Cupedo, Crellin et al. 2009), IL-22 protein (Hughes, Becknell 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 (**Figure 11C**); 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 (**Figure 8A**), as well as the **RORC**-independent development of MALT reported to occur in murine tear duct (Nagatake, Fukuyama et al. 2009).

Both human T\(_{H}17\) cells and stage 3 iNK cells produce IL-22 (Cella, Fuchs et al. 2009; Hughes, Becknell et al. 2009) and express the transcription factors **RORC** and **AHR** (Cupedo, Crellin et al. 2009; Hughes, Becknell et al. 2009). Murine T\(_{H}17\) cells have
higher expression of *IL-1R1* mRNA than in seen in T_{H1} or T_{H2} cells, and exposure to IL-1β promotes T_{H17} cell proliferation, differentiation, and expression of IL-17 and *RORC* (Chung, Chang et al. 2009). Like T_{H17} cells (Rao, Tracey et al. 2007; Veldhoen, Hirota et al. 2008; Chung, Chang et al. 2009), 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. Importantly, continuous exposure to IL-1β was necessary to sustain *in vitro* expression of *IL-22* and *AHR* mRNA, as well as IL-1R1 and IL-22 protein, each remained restricted to the IL-1R1^{hi} subpopulation of stage 3 iNK cells following culture. This observation lends further support to our notion that *in vivo*, constitutive expression of IL-22 in stage 3 iNK cells likely requires an adequate level of IL-1R1 surface expression, and proximity to cells expressing its cognate ligand, IL-1β.

We previously reported limited differentiation of stage 3 iNK cells to CD94^{+} stage 4 mature NK cells with IL-15 *in vitro* [5-20% (Freud, Yokohama et al. 2006)], but the developmental relationship between stage 3 iNK cells and CD94^{+} stage 4 mature NK cells remains unclear, and was pursued in this study. We have described two subpopulations of stage 3 iNK cells, which can be distinguished by surface expression of functional IL-1R1 (Hughes, Becknell et al. 2010). 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 mature 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 mature 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-1{\beta}. The regulation of the IL-1R1 by IL-15, and the regulation of exposure to its ligand, IL-1{\beta}, 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 populations in SLT.

Together, our data identifies the IL-1R1_{hi} subpopulation of stage 3 iNK cells residing in human tonsil as an innate immune target of IL-1{\beta}, and provides a mechanistic connection between IL-1{\beta} and IL-22 production. In combination with IL-15 – which has a known role in NK cell development – IL-1{\beta} may influence host defense via regulating the relative abundance, as well as the differentiation status, of the IL-1R1_{hi} subpopulation of human stage 3 iNK cells in SLT. These findings have potential implications for mucosal
innate immunity, since IL-22, as well as IL-1β, has been shown to induce mucosal epithelium to secrete antimicrobial peptides, likely acting to promote innate mucosal immunity (Wolk, Kunz et al. 2004). One could imagine a scenario whereby IL-1β is released during microbial invasion of SLT and, in concert with IL-15, expands the stage 3 iNK population while sustaining its anti-microbial properties (via IL-22 secretion).

3.10 Experimental procedures.

**Isolation of human NK precursors from SLT.** All protocols were approved by the OSU Institutional Review Board. Normal human tonsils were obtained within 24 hours 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, Yokohama et al. 2006). Briefly, mononuclear fractions were depleted of CD3⁺ and CD19⁺ cells via magnetic negative selection (Miltenyi Biotec). 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 Biotec) for FACS sorting of stage 1 and 2 cells, while total stage 3 iNK cells and stage 4 mature NK cells were FACS sorted from the CD34⁻ fraction as previously described (Freud, Yokohama et al. 2006). IL-1R1hi and IL-1R1lo 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.
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 Biotec), 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 with an appropriately labeled isotype antibody. IL-1R1 staining was performed on ice for 15 minutes with α-IL-1R1 or goat IgG (1 μg/10⁵ cells) in parallel with any additional directly conjugated antibodies indicated. Samples were washed, and then incubated on ice for 30 minutes with α-goat IgG APC (0.25 μg/10⁵ cells). Intracellular staining was performed after surface staining using the 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 hours incubation with 2 μM GolgiPlug (BD Biosciences). Intracellular staining for perforin, IFN-γ, and IL-1β were performed according to manufacturer’s instructions, IL-22 staining was performed as described for Chapter 2 and previously (Hughes, Becknell et al. 2009). Cells were analyzed immediately as previously described (Freud, Becknell et al. 2005) using a FACS Calibur or LSR II cytometer (BD Biosciences) and FlowJo software (Tree Star, Inc.).
**Real-Time PCR.** mRNA was obtained by lysing a portion of each FACS sorted population in approximately equal quantity (≥ 1x10⁴ cells). For some experiments, mRNA was also obtained after 14 days of *in vitro* culture. Samples from ≤ 5x10⁴ cells were processed using Absolutely RNA Nanoprep Kit (Stratagene); samples with ≥ 5x10⁴ 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, IL-1R1, E4BP4*, and *ETS1* purchased from Applied Biosystems. *RORC* primer and probe sequences were as previously described (Yang, Fan et al. 2006). Gene expression levels were normalized to an *18S* internal control, then analyzed by the ΔΔCt method (Fehniger, Carson et al. 1999).

**Cell culture.** FACS 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 pro-NK 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 hours, half the medium was removed every 2-3 days and replaced with media containing 2x cytokines.
Proliferation assays. Click-iT EdU kit (Invitrogen) was used according to manufacturer’s instructions to assess proliferation of stage 3 iNK cells purified via FACS, 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 cDC were enriched via magnetic selection using BDCA-1-biotin antibody and α-biotin microbeads (Miltenyi Biotec). cDC 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 hours 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 cDC 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 days 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, Malenfant et
al. 2004), was assayed via flow cytometry as previously described (Betts, Brenchley et al. 2003) in response to 8 hour incubation with K562 target cells (4:1 E/T ratio). IFN-γ secretion, in response to 12 hour 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, Fehniger et al. 2001) or ELISA (detection limit 10-30 pg/ml) using commercial antibody pairs (Endogen) (Trotta, Parihar 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, Cooper 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), α-CD11c (Abcam, 1:100), α-CD161 (1:333; Santa Cruz), and α-IL-22 (1:100; R&D Systems). 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<sup>hi</sup> and CD11c<sup>lo</sup> cells. Subsequently, the threshold of signal strength, corresponding specifically to the CD11c chromagen, was increased to display only the CD11c<sup>hi</sup> 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.
Figure 5: Relative abundance of SLT NK developmental intermediates. Immediately after FACS sorting from human tonsil, viable stage 1-4 NK developmental intermediates recovered from each donor were enumerated by Trypan blue exclusion. “Relative population size” was calculated within each donor as the ratio of viable cells isolated from each stage relative to the quantity in stage 2. Stage 2 pre-NK cells were typically outnumbered by stages 1 (2.23 ± 0.73-fold), 3 (25.18 ± 1.74-fold) and 4 (17.35 ± 1.71-fold) (*, P < 0.05; **, P ≤ 0.005; ***, P ≤ 0.0005). Depicted as mean ± SEM (n = 4).
Figure 5: Relative abundance of SLT NK developmental intermediates.
Figure 6: IL-1R1 expression during NK cell development. (A) *IL-1R1* mRNA expression *ex vivo* in FACS purified human stage 1-4 SLT NK developmental intermediates assessed via Real-Time PCR. Fold difference in *IL-1R1* mRNA is relative to that detected in stage 1 pro-NK cells, which was 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 stages 1-4 as described (Freud, Yokohama et al. 2006). (B) Histograms show IL-1R1 (filled) and isotype (empty) surface staining in stage 2 pre-NK cells (top) or stage 3 iNK cells (bottom) from a representative donor (n = 4). (C,D) Shown within each gated SLT NK developmental intermediate populations are average: (C) % of cells positive for IL-1R1 surface expression, 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 ≤ 0.05; **, *P ≤ 0.005; ***, *P ≤ 0.0005).
Figure 6: IL-1R1 expression during NK cell development.
Figure 7: IL-1R1$^{\text{hi}}$ and IL-1R1$^{\text{lo}}$ subpopulations of stage 3 iNK cells ex vivo.  (A-C) Human tonsillar mononuclear cells were stained ex vivo for surface expression of Lineage markers (CD3, CD14, CD16, CD19, CD34, CD94), along with CD117, IL-1R1, and intracellular IL-22.  (A) Gated on Lin$^{-}$ (CD3$^{-}$CD14$^{-}$CD16$^{-}$CD19$^{-}$CD34$^{-}$CD94$^{-}$) lymphocytes, dot plot indicates gates for IL-1R1$^{\text{hi}}$ (upper) or IL-1R1$^{\text{lo}}$ (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 FACS sorted IL-1R1$^{\text{hi}}$ and IL-1R1$^{\text{lo}}$ subpopulations of stage 3 iNK cells. “Fold difference” was quantified relative to expression in the IL-1R1$^{\text{lo}}$ subpopulation, which was 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$^{\text{hi}}$ and IL-1R1$^{\text{lo}}$ 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 7: IL-1R1\textsuperscript{hi} and IL-1R1\textsuperscript{lo} subpopulations of stage 3 iNK cells \textit{ex vivo}.
Figure 8: Transcription factors in IL-1R1\textsuperscript{hi} and IL-1R1\textsuperscript{lo} stage 3 iNK cells \textit{ex vivo}.

IL-1R1\textsuperscript{hi} and IL-1R1\textsuperscript{lo} subpopulations of stage 3 iNK cells were sorted via FACS for quantitative assessment of (A) \textit{RORC} (primer and probe sequences as previously described by (Yang, Fan et al. 2006)), as well as (B) \textit{E4BP4} and (C) \textit{ETS1} (Taqman primer/probe sets purchased from ABI) mRNA expression \textit{ex vivo} via Real-Time PCR. Gene expression levels were normalized to \textit{18S} mRNA, and relative quantification was performed using the \(\Delta\Delta C_t\) method. “Fold difference” is calculated relative to level of expression detected in the IL-1R1\textsuperscript{lo} subpopulation, which was arbitrarily normalized to 1. Results depicted as mean ± SEM (\(n \geq 4\)). \(P > 0.05\) for A-C.
Figure 8: Transcription factors in IL-1R1\textsuperscript{hi} and IL-1R1\textsuperscript{lo} stage 3 iNK cells \textit{ex vivo}. 
**Figure 9: IL-15 and IL-1β promote 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 days of culture in IL-15 and IL-1β divided by the absolute number of cells enumerated after 14 days 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, 55,000, 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 days in the presence of IL-15 or IL-15 and IL-1β. Data in A-D depicted as mean ± SEM.
Figure 9: IL-15 and IL-1β promote expansion of stage 3 iNK cells.
**Figure 10: IL-1β sustains IL-22 and AHR in IL-1R1 hi stage 3 iNK cells.** (A-D) IL-22 and AHR transcript expression following 14 days 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 (\( n \geq 4 \)). (E) Dot plots depict surface IL-1R1 and intracellular staining with IL-22 or isotype control antibody in IL-1R1 hi stage 3 iNK cells from a representative donor cultured for 14 days 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 day 0, and within IL-1R1+ cells remaining after culture for 14 days with IL-15 and IL-1β. (G) IL-1R1 MFI assessed via flow cytometry after FACS sorted IL-1R1 hi or IL-1R1 lo stage 3 iNK cells were cultured for 6 hours in the presence of IL-15 or media alone. Results depicted as the mean ± SEM (\( n = 3 \)).
Figure 10: IL-1β sustains IL-22 and AHR in IL-1R1<sup>hi</sup> stage 3 iNK cells.
Figure 11: *RORC* expression following culture in IL-15 or IL-15 and IL-1β. FACS sorted populations of (A) total stage 3 iNK cells, (B) IL-1R1hi stage 3 iNK cells, or (C) stage 4 mature NK cells were each cultured for 14 days in the presence of IL-15 or IL-15 and IL-1β. Real-Time PCR was then used for quantitative assessment of *RORC* mRNA. Gene expression levels were normalized to 18S mRNA, and relative quantification was performed using the ΔΔCt method. “Fold change” was calculated relative to that in the culture condition resulting in lower level of expression, which was arbitrarily normalized to 1. Results depicted as mean ± SEM (n ≥ 4). *P > 0.05* for A-C.
Figure 11: *RORC* expression following culture in IL-15 or IL-15 and IL-1β.
Figure 12: 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 days of *in vitro* culture of FACS 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 days 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 days 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 10F). Data in A, B, E, F, and G presented as the mean ± SEM (*, P ≤ 0.05; n ≥ 4). Histograms in C, D, and H depict staining in a representative donor (n ≥ 4).
Figure 12: IL-1β inhibits differentiation of IL-1R1^{hi} stage 3 iNK cells.
Figure 13: IL-1β impedes functional maturation of IL-1R1\textsuperscript{hi} stage 3 iNK cells. (A,C) Total or (B,D) IL-1R1\textsuperscript{hi} and IL-1R1\textsuperscript{lo} subpopulations of stage 3 iNK cells were FACS sorted from SLT, and cultured for 14 days in vitro with IL-15 or IL-15 and IL-1β. Cells were replated in equal numbers and either: (A,B) stimulated for 12 hours 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 13: IL-1β impedes functional maturation of IL-1R1\textsuperscript{hi} stage 3 iNK cells.
Figure 14: Effector function of CD94\(^+\) and CD94\(^-\) populations generated \textit{in vitro}.

Total stage 3 iNK cells were isolated from SLT via FACS and cultured for 14 days with IL-15 or IL-15 and IL-1\(\beta\). \textbf{(A,B)} Cells were then stimulated as described for Figure 13 to assess acquisition of effector function. Staining was performed to measure CD94 surface expression (via flow cytometry) in combination with either \textbf{(A)} intracellular IFN-\(\gamma\) or \textbf{(B)} surface expression of the degranulation marker CD107a. \textbf{(A)} While intensity of IFN-\(\gamma\) staining within the CD94\(^-\) population was comparable to isotype control, IFN-\(\gamma\) MFI exceeded isotype within the population which had become CD94\(^+\). When these cells were generated in the presence of IL-1\(\beta\), IFN-\(\gamma\) MFI was reduced (*, \(P < 0.05\)). Results in \textbf{A} and \textbf{B} depicted as mean ± SEM (\(n \geq 3\)). \textbf{(C)} Alternatively, cultured cells were stained for expression of intracellular perforin, or surface expression of TNF-related apoptosis inducing ligand (TRAIL) or Fas ligand (FasL), which were assessed via flow cytometry. Histograms depict staining in a representative donor (\(n \geq 4\)) following culture with IL-15 (\textbf{empty}) or IL-15 and IL-1\(\beta\) (\textbf{shaded}) compared to staining with isotype control (\textbf{dashed}). Staining for perforin and FasL were similar regardless of culture condition, while culture in the presence of IL-1\(\beta\) consistently inhibited IL-15-induced TRAIL surface expression in a minor subset of stage 3 iNK cells.
Figure 14: Effector function of CD94+ and CD94− populations generated in vitro.
Figure 15: Stage 3 iNK cells reside near IL-1β⁺ CD11c⁺ cDC in 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⁺CD11c⁺ pDC and CD123⁺CD11c⁺ cDC 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⁺ and IL-1β (top). CD117⁺ cells did not co-express CD11c, but were located in proximity of CD11c⁺ cDC (bottom). Magnification, 400x. Bar, 30 μM. (D) EdU incorporation in FACS sorted IL-1R1⁺ and IL-1R1⁻ subpopulations of stage 3 iNK cells after 6 hours in α-MEM medium alone, or in the presence of autologous BDCA-1⁺ cDC (4:1 iNK:cDC ratio) with either isotype (IgG) 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.
Figure 15: Stage 3 iNK cells reside near IL-1β+ CD11c^hi cDC in SLT.
**Figure 16: Immunohistochemical analysis of CD117\(^+\) cells within SLT.** Shown in representative donor (n = 4), immunohistochemical staining was performed in serial sections of human tonsil as described for Figure 15 using the ultraView Universal system (Ventana Medical). Antigen retrieval was optimized with the Ventana cell conditioning 1 solution (Ventana Medical) for 30 minutes. The following antibodies were used in combinations indicated: \(\alpha\)-CD117 (1:500; DakoCytomation), \(\alpha\)-IL-22 (1:100; R&D Systems), and \(\alpha\)-CD161 (1:333; Santa Cruz). Magnification power used for images in A and C was 400x. Higher magnifications of the insets in A and C are shown in B and D. Arrows indicate examples of co-labeled cells, which appear yellow. The CD117\(^+\) cells found in SLT in the proximity of CD11c\(^{hi}\)IL-1\(\beta\)^+ cDC (shown in Figure 15C) indeed possessed a phenotype consistent with that of stage 3 iNK cells. In particular, (A,B) the majority of CD117\(^+\) cells co-expressed CD161, and (C,D) IL-22 co-labeled the vast majority of CD161\(^+\) cells. Bars, 30 \(\mu\)M.
Figure 16: Immunohistochemical analysis of CD117$^+$ cells within SLT.
Chapter 4: Unpublished Data and Extended Discussion

4.1 Introduction.

Populations of IL-22-producing NK-like cells have been recently been characterized by several groups. These populations (and sometimes, only subsets thereof) been variably described as: having LTi-like properties and producing IL-17 (Cupedo, Crellin et al. 2009), producing IL-22 in a constitutive (Hughes, Becknell et al. 2009) versus an inducible manner (Cella, Fuchs et al. 2009), and depending on commensal microflora (Sanos, Bui et al. 2009), IL-7 (Cella, Otero et al.), and/or IL-15 (Satoh-Takayama, Lesjean-Pottier et al. 2010) for development/survival. Adding further confusion to this issue is the fact that many of these studies were performed in murine model systems, where several genes which characterize human NK cells (CD56) and the IL-22-producing subset of stage 3 iNK cells (IL-26, NKp44) have no known homologs [phenotypes of human and murine IL-22-producing NK populations are compared and discussed in a recent review by (Vivier, Spits et al. 2009)].

These studies have raised several questions pertaining to the identity of the “true” precursor population which is committed to becoming a stage 4 mature NK cell and whether this population exists, to the characteristics which should be used to identify stage 3 iNK cells, NK-22 cells, and the LTi-like population, as well as to the nature of the developmental relationship between the aforementioned populations (if any).
answers to these questions will undoubtedly have implications in deciphering the
differentiation potential and function of stage 3 iNK cells, and why this population is so
abundant in the SLT (as shown in Figure 5). These issues will be discussed here.

4.2 Heterogeneity within IL-22-producing NK-like populations.
Several recent studies have identified distinct subpopulations of IL-22-producing “LTi-
like” NK precursors, which the authors describe as either terminally differentiated in an
immature state, or containing “true NK progenitors” (Crellin, Trifari et al. 2010; Satoh-
Takayama, Lesjean-Pottier et al. 2010). However, it is important to note that certain
aspects of these studies make them incompatible with our four stage model of NK
development. For example, Crellin et al. recently reported that, when compared to the
CD127− subset, the CD127+ subset of Lin−CD117+ cells is reduced in its propensity to
give rise to “conventional NK cells” in response to IL-15 in vitro (Crellin, Trifari et al.
2010). Importantly, the authors describe “conventional NK cells” as having a
CD56+CD127−CD117− surface phenotype, and never assess surface expression of CD94.

CD94 is expressed by the majority of PB NK cells, and is a requisite feature on the
surface of all stage 4 mature NK cells (Freud, Yokohama et al. 2006). Because the
authors do not demonstrate acquisition of CD94 surface expression, it is difficult to
determine whether the population identified by Crellin et al. contains “true NK
progenitors.” Although our four stage model for NK development has not gained
universal acceptance, in support of our model (and, more specifically, the importance of
CD94 surface expression as criteria for defining functional, mature NK cells), publications from several groups have independently reported that CD94 surface expression is correlated with the capacity to produce IFN-γ (Yu, Mao et al.; Voss, Daley et al. 1998).

Although the omission of CD94 makes many of the findings in the recent report by Crellin et al. difficult to fully reconcile with our own, CD127 is found on the majority of stage 3 iNK cells – and on the majority of IL-1R1 hi stage 3 iNK cells (Hughes, Becknell et al. 2010) – ex vivo (Freud, Yokohama et al. 2006). Thus, the Lin−CD117+CD127+ population Crellin et al. described is likely comprised largely of IL-1R1+ stage 3 iNK cells. As we observed with 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 (Crellin, Trifari et al. 2010). They reported that, compared to the CD127− subpopulation, the CD127+ subpopulation is reduced in its propensity to generate mature NK cells in IL-15.

Likewise, our data indicate that IL-1R1 hi stage 3 iNK cells have a reduced propensity to generate stage 4 mature NK cells (Figure 12D,E). These findings 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 should be explored in future studies, and also emphasizes the heterogeneity within the CD117+CD161+IL-1R1 hi stage 3 iNK cell population. Thus, there appears to be a need for caution in assigning definitive functions and developmental potential.
As we showed in Figure 7E, there is a great deal of heterogeneity within the stage 3 iNK population, particularly with respect to expression of several surface markers, including (but not limited to) CD56, CD127, IL-1R1, and NKp44 (Figure 7E, and (Freud, Yokohama et al. 2006; Hughes, Becknell et al. 2010)). Because the majority of stage 3 iNK cells do not differentiate to become stage 4 mature NK cells in the presence of IL-15 in vitro (Chapter 3 and (Freud, Yokohama et al. 2006; Hughes, Becknell et al. 2010)), this heterogeneity may indicate room for revision to the criteria currently used to define human stage 3 iNK cells.

4.3 “Terminal differentiation” within stage 3 iNK cells.

The data presented in Chapter 3 (specifically, Figures 9, 12, 13, and 14) led us to the conclusion that IL-1β sustains and expands stage 3 iNK cells, while inhibiting the differentiation of CD56^{bright} stage 4 mature NK cells. However, it is unclear how heterogeneous the “true” stage 3 iNK population is, and the identical phenotype described for both stage 3 iNK cells and the LTi-like population suggests that these cells may represent a single population. Alternatively, stage 3 iNK cells may appear to be heterogeneous due to being comprised of several discrete subsets, some of which are capable of differentiating to become mature NK cells, while others may instead be characterized by LTi-like properties and/or maintain a greater propensity to remain immature.
In support of the latter hypothesis, we found that < 20% of stage 3 iNK cells differentiate into stage 4 mature NK cells in the presence of IL-15 in vitro (Hughes, Becknell et al. 2010). Following this line of thought, the ~80% of stage 3 iNK cells which are IL-1R1<sup>hi</sup> and IL-22<sup>+</sup> <i>ex vivo</i> might represent those stage 3 iNK cells which have a reduced propensity to differentiate to stage 4 mature NK cells and/or may have LTi-like activity, while the remaining 20% of stage 3 iNK cells, which is IL-1R1<sup>lo</sup> and IL-22<sup>-</sup> <i>ex vivo</i> might be enhanced in their capacity to generate mature NK cells. As a consequence, culture of total stage 3 iNK cells in the presence of IL-15 would expand the IL-1R1<sup>lo</sup> subset which can give rise to mature NK cells, while adding IL-1β would expand the IL-1R1<sup>hi</sup> subset, which has a greater propensity to remain immature, and may possess LTi-like properties. This alternative model could also explain the data in Figure 12C,D.

Clonal assays were carried out to begin answering some of these questions regarding differentiation potential and heterogeneity within stage 3 iNK cells. To this end, we sorted single cells from the IL-1R1<sup>hi</sup> and IL-1R1<sup>lo</sup> stage 3 iNK subpopulations for parallel cultures in the presence of recombinant human IL-15 or IL-15 and IL-1β (Hughes, Becknell et al. 2010). It should be noted that, in these clonal assays, stage 3 iNK cells required the additional support of the OP9 murine stromal cell line. After 14 days <i>in vitro</i>, we assessed the clonal outgrowth of single IL-1R1<sup>hi</sup> and IL-1R1<sup>lo</sup> stage 3 iNK cells for surface expression of CD94 (which indicates differentiation of stage 4 mature NK cells, and correlates with the ability to express IFN-γ) and intracellular expression of IL-
22 protein (which identifies the IL-1R1\textsuperscript{hi} subpopulation of stage 3 iNK cells). Results of these clonal analyses are depicted in Figure 17.

When cultured in either condition, IL-1R1\textsuperscript{hi} and IL-1R1\textsuperscript{lo} subpopulations of stage 3 iNK cells both gave rise to cells in every well which simultaneously lacked expression of CD94 and IL-22 (Figure 17E, lower left quadrant of each dot plot). As might be expected, the addition of IL-1\(\beta\) had no influence on the differentiation of IL-1R1\textsuperscript{lo} clones. Accordingly, we noted that IL-1R1\textsuperscript{lo} clones in 100% of wells from either culture condition: 1) contained some cells which were CD94\(^+\), and 2) lacked IL-22\(^+\) cells completely. Thus, IL-22\(^+\) cells derived \textit{in vitro} appeared to have been generated exclusively from the IL-1R1\textsuperscript{hi} subpopulation of stage 3 iNK cells, and IL-1\(\beta\) significantly increased the number of wells containing IL-22\(^+\) cells, while decreasing the number of wells containing CD94\(^+\) cells.

Three observations were made when assessing clones derived from single IL-1R1\textsuperscript{hi} stage 3 iNK cells: 1) > 50\% of these wells contained \textit{only} CD94\(^-\) cells (Figure 17C). Although this effect seemed to be enhanced among wells cultured in the presence of IL-1\(\beta\), many wells containing only CD94\(^-\) cells were also generated from parallel cultures with IL-15 alone; 2) regardless of whether culture was performed in the presence of IL-15 or IL-15 and IL-1\(\beta\), a number of the clones derived from single IL-1R1\textsuperscript{hi} stage 3 iNK cells gave rise to both CD94\(^+\) and CD94\(^-\) populations within the same well (Figure 17E); 3) ~50\% of wells cultured in the presence of IL-15, and ~80\% of wells cultured in IL-15 and IL-1\(\beta\)
were scored as positive for IL-22 expression (Figure 17B). Importantly, although CD94+ cells and IL-22+ cells were sometimes generated in the same well, these antigens were expressed in a manner which remained mutually exclusive, and occurred on separate cells (Figure 17G).

Although in some wells, the progeny of IL-1R1 hi stage 3 iNK cells which had been generated in the presence of IL-15 alone included cells which appeared to be IL-22+, it should be noted that the IL-22 staining tended to be barely above background (compared to isotype control staining), both in terms of intensity of IL-22 staining, and in terms of the percentage of cells within each well staining positively for IL-22. In contrast, IL-22+ cells from wells containing IL-15 and IL-1β tended to be clearly above background compared to isotype staining, both in terms of IL-22 MFI, and the percentage of cells within each well which appeared to be IL-22+. This finding is in contrast to results from previous experiments that were performed in the absence of OP9 stroma, which indicated that IL-22 mRNA and protein are no longer detectable following culture of IL-1R1 hi stage 3 iNK cells in the absence of exogenous IL-1β (Figure 10A,C,E).

Importantly, the OP9 stromal cell line, which is required in all wells to provide additional support during this assay, has been previously reported to support hematopoiesis and secrete growth factors (Kodama, Nose et al. 1994; Ueno, Sakita-Ishikawa et al. 2003). Hence it is possible that the IL-22+ cells generated from IL-1R1 hi
stage 3 iNK clones cultured in the absence of exogenous IL-1β might be attributed to the presence of factors secreted by the OP9 cell line or cell-cell contact.

In a further attempt to identify a precursor population with terminal differentiation of CD94 IL-22+ stage 3 iNK cells, we performed additional clonal assays with subpopulations of IL-1R1hi stage 3 iNK cells, sorted based on heterogeneous expression of CD11b, CD56, and NKp44. We found the same three observations noted above for clones derived from single IL-1R1hi stage 3 iNK cells applied equally to the outgrowth of clones from these IL-1R1hi stage 3 iNK subpopulation (not depicted). Together, these clonal assays confirm our interpretation of the bulk cultures, and suggest that the presence of IL-1β does limit the degree of IL-1R1hi stage 3 iNK cell differentiation to stage 4 mature NK cells. Further, while the IL-1R1hi stage 3 iNK cells have an increased propensity to remain as IL-22+ stage 3 iNK cells in the presence of IL-1β, single cells were capable of giving rise to both IL-22+ and CD94+ progeny within the same well, thus it appears that IL-1R1hi stage 3 iNK cells are not necessarily terminally differentiated.

4.4 Stage 3 iNK cells and LTi-like cells.

Excessive inflammation can disrupt SLT architecture, necessitating local tissue regeneration for restoration of the SLT microenvironment (Pemrick, Abarzua et al. 1998). LTi cells have a proposed role in re-establishing the integrity of lymphoid tissues following chronic infections (Scandella, Bolinger et al. 2008). An elegant study by Cupedo et al., which was the first to identify an LTi-like population in humans,
demonstrated that human LTi-like cells reside in the fetal LN and postnatal tonsil (Cupedo, Crellin et al. 2009). Importantly, this work revealed that several of the unique attributes of stage 3 iNK cells were shared by the LTi-like population, which expresses RORC, produces IL-22, and possesses a [Lin\(^-\)CD4\(^-\)CD45\(^{int}\)CD34\(^-\)CD127\(^+\)CD117\(^+\)CD94\(^-\)CD161\(^+\)] surface phenotype indistinguishable from that described for the stage 3 iNK population (Cupedo, Crellin et al. 2009). In a subsequent study, expression of CD127 was used by Crellin et al. to identify a subset of Lin\(^-\)CD34\(^-\)CD117\(^+\)CD161\(^+\) lymphocytes in human tonsil which was enriched for cells possessing LTi-like activity (Crellin, Trifari et al. 2010). As shown in Figure 7E, we found that, among total Lin\(^-\)CD34\(^-\)CD117\(^+\)CD161\(^+\) stage 3 iNK cells, CD127\(^+\) cells were enriched in, and relatively confined to, the IL-1R1\(^{hi}\) subset of stage 3 iNK cells. Indeed, we found very strong congruency between the surface markers on the IL-1R1\(^{hi}\) subpopulation of CD117\(^+\)CD94\(^-\) stage 3 iNK cells (Figure 7E (i.e., CD127, CD161, CCR7, IL-22, and LT-\(\alpha\))) and those associated with human LTi-like cells as identified previously by Cupedo et al. (Cupedo, Crellin et al. 2009).

Because LTi-like activity has been associated with features of the IL-1R1\(^{hi}\) subpopulation of stage 3 iNK cells – specifically, the ability to produce IL-22, and expression of CD127 – it is possible that the IL-1R1\(^{hi}\)IL-22\(^+\) subset of stage 3 iNK cells are LTi-like, and that LTi-like activity is greater than, or restricted to, the IL-1R1\(^{hi}\) subpopulation of stage 3 iNK cells. Although it has not yet been proven whether LTi-like activity is necessarily restricted to the IL-1R1\(^{hi}\)IL-22\(^+\) subset of stage 3 iNK cells, which is enriched for
CD127+ cells, we hypothesized that LTi-like activity might be higher within the IL-1R1hi subset of stage 3 iNK cells.

If stage 3 iNK cells (or the IL-1R1hi subpopulation of stage 3 iNK cells) possess LTi-like activity, the proliferative expansion this population undergoes upon exposure to IL-1β (Figure 9D and (Hughes, Becknell et al. 2010)) may represent a mechanism which serves to regulate the size of this potential reservoir of adult LTi cells. Because modulation of SLT formation and the induction of tertiary lymphoid tissue (in the form of ectopic LNs) has been demonstrated in a variety of autoimmune pathologies and chronic inflammatory conditions (Drayton, Liao et al. 2006; Junt, Scandella et al. 2008), a functional assessment of LTi-like activity among the heterogeneous subpopulations of stage 3 iNK cells is critical to our understanding of human lymph node associated pathologies, as well as human immune reconstitution. Accordingly, we set out to experimentally investigate this matter to the best of our ability.

One of the earliest cellular interactions during lymph node development is that between LTi cells and LTβR+ mesenchymal cells, which results in the induction of expression of VCAM1 (Yoshida, Honda et al. 1999) and ICAM1 (Matsumoto, Iwamasa et al. 1999). Therefore, the studies by Cupedo et al. and Crellin et al. assessed for LTi-like activity by measuring induction of VCAM1 and ICAM1 surface expression on fetal mesenchymal stem cells following co-culture in the presence of fetal LN-derived Lin’CD45intCD127hi NK precursors (Cupedo, Crellin et al. 2009; Crellin, Trifari et al. 2010). It is important to
note that, although that the methods used for the aforementioned studies involve the use of fetal tissue, it is currently illegal to utilize fetal tissue for the purposes of experimental research in the state of Ohio. Because we noted that ectopic LNs can form at variable sites of chronic vascular inflammation [reviewed by Carragher et al. (Carragher, Rangel-Moreno et al. 2008)], we developed an in vitro assay to measure the LTi-like behavior, as well as the potential of fully differentiated endothelial cells to serve as sites of ectopic lymphoid tissue formation, by assessing VCAM1 and ICAM1 induction on fully differentiated primary human umbilical vein endothelial cells (HUVECs) as a possible readout for LTi-like activity. While the assay we used is distinct from that reported earlier, our findings may still be in agreement with those reported by Crellin et al. (Crellin, Trifari et al. 2010).

In the study by Crellin et al., LTi-like activity was restricted to the CD127\(^+\) subpopulation of Lin\(^-\)CD34\(^-\)CD117\(^+\)CD161\(^+\) cells, while the CD127\(^-\) subpopulation possessed greater potential for differentiation to conventional NK cells, which are capable of cytotoxic activity and IFN-\(\gamma\) production (Crellin, Trifari et al. 2010). Similarly, we found that, among total stage 3 iNK cells, the IL-1R1\(^{hi}\) subpopulation is enriched for cells co-expressing CD127 (Figure 7E), and compared to untreated HUVECs or HUVECs co-cultured with CD94\(^+\)CD56\(^{bright}\) stage 4 mature NK cells from the same donor, IL-1R1\(^{hi}\) stage 3 iNK cells induced a higher level of VCAM1 on the surface of HUVECs (Figure 18A). Thus, the CD127\(^+\) subpopulation of Lin\(^-\)CD34\(^-\)CD117\(^+\)CD161\(^+\) cells – which we demonstrated is enriched within the IL-1R1\(^{hi}\) subpopulation of Lin\(^-\)CD34\(^-\)CD117\(^+\)CD161\(^+\)
stage 3 iNK cells (Figure 7E) – may have greater LTi-like activity than CD56\textsuperscript{bright} stage 4 mature NK cells (which also have some co-expression of CD127).

Although some of our findings might support Crellin’s hypothesis that the CD127\textsuperscript{+} subset is enriched for cells with LTi-like activity, both studies also contain some evidence to suggest that the ability to induce VCAM1 and ICAM1 expression on the surface of endothelial cells may not be an accurate means to measure “LTi-like activity” or identify LTi-like populations \textit{in vitro}. For example, in agreement with the study by Crellin et al. (Crellin, Trifari et al. 2010), we found that IL-1R1\textsuperscript{hi} stage 3 iNK cells (which should be LTi-like) and stage 4 CD56\textsuperscript{bright} mature NK cells (which should not be LTi-like) were both able to induce HUVEC expression of ICAM1 (Figure 18B). We also observed that ICAM1 induction was highest on HUVECs co-cultured with stage 4 CD56\textsuperscript{bright} mature NK cells, and ICAM1 induction was nearly equivalent whether HUVECs were co-cultured in the presence of IL-1R1\textsuperscript{hi} or IL-1R1\textsuperscript{lo} stage 3 iNK cells (data not shown), thus the ability to induce endothelial cell expression of ICAM1 \textit{in vitro} does not appear to be restricted to the population reported to have “LTi-activity.” Furthermore, although in positive controls – in which HUVECs were cultured in the presence of recombinant human TNF-α (Matsumoto, Iwamasa et al. 1999; Cinamon, Shinder et al. 2001) – resulted in increased intensity of VCAM1 and ICAM1 surface expression (3-fold or 8-fold, respectively), induction of VCAM1 and ICAM1 surface expression in response to co-culture with CD94\textsuperscript{+}CD56\textsuperscript{bright} stage 4 mature NK cells or IL-1R1\textsuperscript{hi} stage 3 iNK cells was comparatively modest (< 2-fold and < 4-fold, respectively) (Figure 18). This
discrepancy may be accounted for by the responsive difference of HUVECs compared to mesenchymal stem cells. Previous, studies have shown that IFN-γ, which is highly secreted by stage 4 CD56<sup>bright</sup> mature NK cells can induce ICAM-1 expression (Pober, Gimbrone et al. 1986).

4.5 IL-7.

Recent studies in mice have identified an LTi-like population of CD94<sup>−</sup>NKp46<sup>+</sup>NK1.1<sup>int</sup> cells in the gut which mirrors the human stage 3 iNK cell in its expression of IL-22 (Cella, Fuchs et al. 2009), the IL-7 receptor α subunit (IL-7Rα, or CD127), and the transcription factor RORC (Luci, Reynders et al. 2009). Murine studies indicate that RORC<sup>+</sup>CD127<sup>−</sup>NKp46<sup>−</sup>NK1.1<sup>+</sup> 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, Vosshenrich et al. 2008), which is required for IL-2 and IL-15 signaling (Giri, Ahdieh et al. 1994). However, this population is completely absent in IL-2Rγ-deficient animals, which cannot respond to any of the so-called IL-2Rγ common chain (γ<sub>c</sub>) cytokines (Vosshenrich, Ranson et al. 2005). Situations have been reported in which IL-2 or IL-7 may be substituted in place of IL-15 (Freud, Yokohama et al. 2006; Satoh-Takayama, Vosshenrich et al. 2008; Cupedo, Crellin et al. 2009). Given that nearly all human stage 3 iNK cells – as well as the RORC<sup>−</sup>NKp46<sup>−</sup>NK1.1<sup>int</sup> murine population – express CD127 (Luci, Reynders et al. 2009), the role of IL-7 deserves particular attention.
Produced by intestinal goblet epithelial cells (Watanabe, Ueno et al. 1995), IL-7 is essential for maintaining IL-17 production by murine CD4+ T cells (Sawa, Arima et al. 2009). Surface expression of the receptor for IL-7 (CD127) is relatively specific for stage 3 iNK cells among human SLT NK developmental intermediates, and the majority of stage 3 iNK cells are CD127+ (Freud, Yokohama et al. 2006). With this in mind, we assessed the capacity of IL-7, whether in addition to or instead of IL-2 or IL-15, to support survival and differentiation of stage 3 iNK cells in vitro. On its own, IL-7 was much less potent than IL-2 or IL-15 at maintaining stage 3 iNK cell survival in vitro (Figure 19A). Furthermore, we found that stage 3 iNK cells cultured with IL-2 or IL-15 alone did not significantly differ qualitatively compared to parallel cultures supplemented with IL-7 (not depicted). Our findings are in agreement with murine studies, indicating that IL-7 and IL-7Rα deficient mice do not exhibit significant NK developmental defects (Maki, Sunaga et al. 1996; Vosshenrich, Ranson et al. 2005). However, it remains possible that IL-7 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, Crellin et al. 2009). Future studies are needed to fully elucidate the impact of IL-7 on stage 3 iNK cells, as it remains possible that this cytokine plays a role in regulating other properties of this population, such as the LTi-like behavior that has been reported in human stage 3 iNK population (Cupedo, Crellin et al. 2009).
4.6 IL-15.

Beginning at stage 2, human NK developmental intermediates become IL-15-responsive, and depend on IL-15 for survival and differentiation (Freud, Yokohama et al. 2006). The presence of IL-15 is sufficient on its own to stimulate rapid \textit{in vitro} differentiation of stage 2 pre-NK cells, generating a population which is 100\% CD34\(^{+}\) by day 14, with \(~75\%\) of cells already acquiring a CD34\(^{+}\)CD117\(^{+}\)CD94\(^{-}\) stage 3-like phenotype after just 4 days of culture (Freud, Yokohama et al. 2006). Despite the fact that stage 1 pro-NK and stage 2 pre-NK cells efficiently differentiate \textit{in vitro} to an otherwise stage 3-like phenotype (Freud, Yokohama et al. 2006), the progeny of these cells do not expand in response to IL-1\(\beta\) (\textbf{Figure 9A}). Thus, it appears that these \textit{in vitro}-derived stage 3 iNK cells lack IL-1R1, indicating that IL-15 may be insufficient stimulus to induce IL-1R1 expression.

The range of outcomes we observed following culture of NK developmental intermediates, especially IL-1R1\(^{hi}\) and IL-1R1\(^{lo}\) subpopulations of stage 3 iNK cells, might indicate a disparity in the ability of certain populations to respond to IL-15. Since surface expression of IL-15R is not detectable via flow cytometry on SLT NK developmental intermediates until stage 4, to determine whether IL-15R is uniformly expressed on the surface of IL-1R1\(^{hi}\) and IL-1R1\(^{lo}\) subpopulations of stage 3 iNK cells, we made use of biotin-labeled recombinant human IL-15, and flow cytometrically assessed IL-15 binding to IL-15R on the cell surface via secondary fluorescent labeling with streptavidin-APC-Cy7. IL-15 binding within IL-1R1\(^{hi}\) and IL-1R1\(^{lo}\) subpopulations
of stage 3 iNK cells was compared to that in stage 4 mature NK cells (which express IL-15R at levels detectable via flow cytometric staining (Freud and Caligiuri 2006)). As shown in Figure 19B, we found that IL-1R1^hi and IL-1R1^lo subpopulations of stage 3 iNK cells were similar in their capacity to bind IL-15, thus the divergent properties of these subsets are not attributable to differences in capacity to bind/respond to IL-15.

We have previously observed diminishing IL-1R1 expressed on the surface of stage 3 iNK cells, which could be at least partially attributed to IL-15 (Figure 10G and (Hughes, Becknell et al. 2010)). We believe this reduction in IL-1R1 expression levels – thus IL-1-responsiveness – may contribute to the loss of IL-22 and AHR expression we observed following culture of IL-1R1^hi stage 3 iNK cells in the presence of IL-15 (Hughes, Becknell et al. 2010). Accordingly, another group recently reported that culture of human CD3^CD117^CD56^− cells (including stage 3 iNK and LTi-like populations) in the presence of IL-15 down regulates expression of IL-22 and AHR mRNA, while favoring the generation of IFN-γ-expressing CD56^+ cells (Kim, Han et al.).

Although our observations indicate that IL-7 on its own supports survival of stage 3 iNK cells poorly (Figure 19A), Cella et al. reported that culture of NK-22 cells in the presence of IL-7 and IL-1β triggered a transition (from inducible) to constitutive IL-22 production, generated progeny which were almost entirely CD94^−, incapable of IFN-γ production, and remarkably similar to NK-22 cells ex vivo (Cella, Otero et al.). Another recent publication has demonstrated that IL-7 may act in concert with IL-15 to up
regulate IL-1R1 expression on human naïve CD4$^+$ T cells which, like stage 3 iNK cells, can be divided into two distinct subpopulations based on IL-1R1 surface expression (Lee, Kang et al.). Therefore, it remains possible that IL-7 may promote homeostasis of IL-1R1$^{hi}$IL-22$^+$ stage 3 iNK cells, and substituting IL-7 for IL-15 during culture in the presence of IL-1β may support stage 3 iNK cell survival, while potentially ameliorating the IL-15-mediated down regulation of IL-1R1.

4.7 Commensal microflora.

Germ-free mice exhibit severe immune defects, as evidenced by hypoplastic Peyer’s Patches, disorganized B and T cell compartments of the lamina propria, poorly developed B and T cell zones in the spleen and LNs, and cytokine production that is skewed towards a T$_{H2}$-type response (reviewed by (Macpherson and Harris 2004)). Murine studies have demonstrated that the LTi-like population of CD94$^-$NKp46$^+$NK1.1$^{int}$ cells in the gut relies on microflora for its development and homeostasis, as this population was >90% decreased in mice raised in a germ-free facility (Sanos, Bui et al. 2009).

Colonization with segmented filamentous commensal bacteria, which induces differentiation of T$_{H17}$ cells in the lamina propria (Ivanov, Atarashi et al. 2009), is essential for maintenance of an IL-1R1$^+$ population of T$_{H17}$-like γ/δ T cells (Duan, Chung et al.). Similarly, NK-22 cell quantity and IL-22 production are both diminished in germ-free mice, and it has been suggested that commensal microflora may “instruct” the differentiation of IL-22-producing NK cells (Satoh-Takayama, Vosshenrich et al.)
Since IL-1β is released by monocyte/macrophages and cDC in direct response to microbial invasion (Ikejima, Dinarello et al. 1984; Acosta-Rodriguez, Napolitani et al. 2007), future studies should establish whether the development and proliferation these microbes promote occurs in an IL-1β-dependent manner.

Under normal circumstances, mucosal surfaces of the body are continually exposed to bacteria. To maintain commensal microflora while eliminating invading pathogens, mucosal cells respond to microbiota via pathogen recognition molecules, such as toll-like receptors (TLRs) (reviewed in (Lavelle, Murphy et al.)). In particular, TLR2 – whose agonists include bacterial lipoproteins (Aliprantis, Yang et al. 1999), lipoteichoic acid (Schwandner, Dziarski et al. 1999), and yeast zymosan (Ozinsky, Underhill et al. 2000), as well as endogenous ligands including heat shock proteins (Vabulas, Ahmad-Nejad et al. 2001) – is structurally similar to IL-1R1 (Chaudhary, Ferguson et al. 1998).

Like IL-22 (Wolk, Kunz et al. 2004; Zheng, Valdez et al. 2008), TLR2 activation has been shown to promote epithelial barrier integrity (Rakoff-Nahoum, Paglino et al. 2004) and induces antimicrobial peptide expression (Lai, Cogen et al.). TLR2 signaling has also been reported to act on Th17 cells to promote population expansion and IL-22 production (Reynolds, Pappu et al.). Likewise, Crellin et al. demonstrated that TLR2 ligand is capable of stimulating human CD127+ LTi-like cells to produce cytokines including IL-2, IL-5, IL-13, and IL-22 (Crellin, Trifari et al.).
4.8 IL-23 and AHR ligands.

In addition to IL-1β, IL-23 has been reported to act on human T_{H}17 cells to promote T_{H}17 differentiation, and expression of T_{H}17 cell-associated genes, such as IL-22 (Zheng, Valdez et al. 2008), RORC, and IL-26 (Manel, Unutmaz et al. 2008). Although IL-23 represents one of the factors shown to induce IL-22 expression in NK-22 cells (Cella, Fuchs et al. 2009), we found that the presence of IL-23 did not appreciably alter the quantity of cells generated in vitro from stage 3 iNK cells, and did not markedly affect IL-22, IL-26, or RORC mRNA expression, regardless of the presence of IL-15 or IL-15 and IL-1β (Figure 20). Our data suggests that distinct repertoires of cytokines may regulate development and cytokine production in the NK-22 population and stage 3 iNK cells.

Though IL-23 is reported to favor T_{H}17 differentiation and promote expression of IL-22 in T_{H}17 cells, IL-23 neutralization did not affect AHR agonist-mediated IL-22 production (Ramirez, Brembilla et al.). Previous studies have suggested that, though expressed by the T_{H}17 lineage, RORC may not regulate IL-22 production (Veldhoen, Hirota et al. 2009). AHR, which is also expressed by T cells during development of the T_{H}17 lineage (Quintana, Basso et al. 2008), was shown to be required for IL-22 expression (Veldhoen, Hirota et al. 2008). Similarly, AHR is expressed by subsets of NK cells which are IL-22^{+} (Cella, Fuchs et al. 2009), and this gene is found specifically within the IL-22-expressing IL-1R1^{hi} subpopulation of SLT stage 3 iNK cells (Figure 7D). We previously observed
that the presence of IL-1β is required to maintain AHR expression \textit{in vitro} (Figure 10B,D and (Hughes, Becknell et al. 2010)).

The presence of AHR agonists in culture medium has been shown to enhance T_{h}17 differentiation, as well as IL-22 production, in T cells (Veldhoen, Hirota et al. 2009); therefore, we hypothesized that, by promoting transcription of stage 3-associated genes (such as IL-22), AHR agonists may maintain stage 3 iNK cell homeostasis. In the past, we routinely cultured NK developmental intermediates in RPMI in initial studies (Freud, Yokohama et al. 2006). However, we noted that, when stage 3 iNK cells were cultured instead using α-MEM medium (which required for cultures including OP9 stroma), a greater proportion of stage 3 iNK cells differentiated to become CD94⁺ stage 4 mature NK cells \textit{in vitro} (data not shown).

Importantly, compared to RPMI, α-MEM medium may contain a greater concentration of aromatic amino acids, which can be metabolized \textit{in vitro} to produce AHR ligands (Veldhoen, Hirota et al. 2009). For example, the amino acid Tryptophan – which, upon exposure to light, can be metabolized \textit{in vitro} to products including the AHR agonist, formylindolo[3,2-b]carbazole (FICZ) (Rannug, Rannug et al. 1995) – is present at concentrations which are ~2-5-fold higher in α-MEM medium compared to RPMI medium (Veldhoen, Hirota et al. 2009). Together, this suggests that the presence of AHR ligands in culture medium may affect \textit{in vitro} differentiation of stage 4 mature NK cells.
Related experiments to further investigate the potential influence of AHR agonist and antagonist ligands on IL-1R1 hi stage 3 iNK cells are currently underway.

4.9 Stage 3 iNK cells and “natural helper” cells.

The data we have presented in Chapters 2 and 3 indicates that, in the presence of IL-15 in vitro, the vast majority of stage 3 iNK cells do not maintain the stage 3 iNK cell phenotype observed ex vivo. That is to say, though no longer expressing IL-1R1, IL-22, and AHR, the majority of these cells also do not differentiate to become CD94+ stage 4 mature NK cells. For this reason, we wondered whether there might be an additional fate for this population. Crellin et al. showed that stimulation of human RORC+CD127+ LTi-like innate lymphoid cells (ILCs) and CD56+CD127+ NK cells with IL-2 and TLR2 agonist resulted in IL-13, IL-5, and/or IL-22 production, as well as autocrine IL-2 production (Crellin, Trifari et al.). Because, in our lab, we have previously observed that stage 3 iNK cells cultured in the presence of IL-2 for 14 days in vitro are capable of producing GM-CSF, IL-13, and TNF-α (A.G.F., unpublished data), we reasoned that these cells might instead resemble the recently described population of Th2 cytokine-producing “natural helper” cells.

Th2 cytokines are important in the immune response against allergens and helminth invasion of cutaneous or mucosal sites (reviewed by (Paul and Zhu)). Moro et al. first described the “natural helper” population in a murine model as having a Lin- CD45+CD117+CD25+CD127+RORC IL-22- surface phenotype, and resembling the LTi-
like population while lacking LTi-like activity. This population is capable of producing cytokines including GM-CSF, IL-2, IL-4, IL-5, IL-6 and IL-13 in response to stimuli including IL-33 (Moro, Yamada et al.). Relatively little is known about the actions of the most recently characterized member of the IL-1 superfamily, IL-33, but the major source of this cytokine is mucosal epithelial cells, giving it relevance to MALT (Cayrol and Girard 2009).

4.10 The role of IL-22 and IL-1β in autoimmunity and cancer.
Defective NK cell function has a role in a variety of autoimmune conditions (reviewed by (Shi and Zhou)), and 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, Deibel et al. 1990), as well as expression of the IL-1R2 decoy receptor (Colotta, Re et al. 1993). We found that, in vitro, IL-1RA is capable of fully neutralizing the impact of IL-1β on IL-1R1hi stage 3 iNK cell proliferation and differentiation toward CD94+ stage 4 mature 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, Lejeune et al. 2001). These regulatory pathways serve to quickly and precisely adjust biological responses to IL-1β and IL-22.
Indeed, IL-1β has been implicated in the pathogenesis of several inflammatory and autoimmune diseases. In particular, psoriasis (Boniface, Guignouard et al. 2007) and Crohn’s disease (Kleinschek, Boniface et al. 2009) reportedly have excessive production of IL-22 as well as IL-1β. Ablation of IL-1R1 signaling should enhance NK cell differentiation while diminishing IL-22 production from the IL-1R1hi stage 3 iNK cell population. Anti-IL1-therapy, in the form of the drug Kineret® (anakinra, recombinant IL-1RA), is used routinely used in the clinic to treat patients rheumatoid arthritis (Cohen, Hurd et al. 2002). Cancer therapies aimed at modulating function of IL-1 are already being tested in the clinic. For instance, because elevated levels of IL-22 and IL-1RA can be found in the bone marrow of multiple myeloma patients (Cao, Luetkens et al.), Anakinra was used in a recent Phase II clinical trial for treatment of multiple myeloma (Lust, Lacy et al. 2009).

In addition to their roles in autoimmune disease, there is evidence to suggest that IL-1β and IL-22 may also have the capacity to promote tumorogenesis. For example, IL-1-producing tumors have been described, and IL-1β has been shown to promote metastasis of human breast, colon, lung, head and neck cancers, and melanomas (reviewed by (Lewis, Varghese et al. 2006)). Epidemiological studies have reported a strong association with the presence of IL-1β in chronic infection Helicobacter pylori and gastric carcinoma, and polymorphisms in the genes for IL-1β and IL-1RA are both associated with an increased risk for developing gastric cancer (El-Omar, Carrington et al. 2001); likewise, IL-22 polymorphisms have been associated with certain types of
intestinal cancer (Thompson, Plummer et al.). AHR also has reported involvement in carcinogenesis, metabolically activating environmental pollutants create carcinogens, such as benzo[a]pyrene (Shimizu, Nakatsuru et al. 2000).

Because at least some of AHR’s functions have been reported to be ligand binding-specific (Veldhoen, Hirota et al. 2009), some have proposed the use of AHR as a unique “druggable” target for therapeutic immunomodulation (Murray, Morales et al.). In vitro culture with various AHR antagonists (including StemRegenin1, CH-223191, and napthoflavone) all lead to a dose-dependent quantitative expansion of cord blood (CB) CD34⁺ multilineage progenitor cells (Boitano, Wang et al.). Furthermore, Boitano et al. reported that in vitro expansion with StemRegenin1 maintained CB precursors at a less differentiated state, and these expanded cells retained multilineage potential and contributed to both early and sustained engraftment. These findings might be applied to HSC transplant situations to improve transplant outcome by enhancing expansion of HSCs.

In addition to developing new AHR-targeted therapies, there have also been studies identifying AHR binding activity in previously developed drugs that are already being used in the clinic. For example, the anti-inflammatory drug Leflunomide, which is used in the clinic for treatment of rheumatoid arthritis, was recently found to be an AHR agonist (O'Donnell, Saili et al.). Likewise, DuSell et al. found that certain drugs used in the treatment of estrogen receptor (ER) positive breast cancers, including tamoxifen, also
act as AHR agonists (DuSell, Nelson et al.). In this study, the authors reported that certain selective ER modulators and antiestrogens can also act as AHR agonists to induce their own metabolism and excretion (via drug-metabolizing enzymes induced following AHR activation), thereby reducing their own efficacy. This suggests that AHR activation may be antiestrogenic, and could thus have antitumorogenic activity in the breast, indicating that modulation of AHR activation may prove especially important in developing effective breast cancer treatments. This is not surprising, since the majority of breast cancers are ER positive (Kok and Linn), and it is known that AHR and ER signaling pathways engage in cross talk via direct interaction, and also by competing for intracellular signaling mediators (Matthews and Gustafsson 2006). Thus the ability of each therapeutic to induce AHR, in turn up-regulating metabolic enzymes, could contribute to its efficacy in vivo.

4.11 Role of stage 3 iNK cells in MALT.
A previous study from our lab (Freud, Yokohama et al. 2006) indicated that stage 1-4 NK developmental intermediates from human LN and tonsil were phenotypically identical; however, some of the properties of stage 3 iNK cells which we have reported in our more recent studies (especially the ability to produce IL-22) suggest this population (or certain subsets thereof) may have some important role in, or be restricted to, SLT of the mucosa. Because the overwhelming majority of the experiments presented in the previous Chapters were performed using stage 3 iNK cells isolated from human tonsil, we performed some additional immunohistochemical staining of human axillary LN to
determine whether stage 3 iNK cells were present and producing IL-22 outside of MALT. As depicted in Figure 21, we were able to locate cells co-expressing CD117 and IL-22 within the parafollicular T cell region of axillary LN, suggesting that IL-22-producing stage 3 iNK cells may not be confined to the mucosa. Similarly, IL-22-producing stage 3 iNK cells were also recently described within human uterine tissue (Male, Hughes et al.).

Although these findings do not support site-specific role of these IL-22-producing cells in the mucosa, this finding may simply indicate that these cells are trafficking through non-mucosa associated lymphoid tissue, since we have also detected IL-22+ stage 3 iNK cells in circulating blood (data not shown and (Freud and Caligiuri 2006)). It is also possible IL-22+ stage 3 iNK cells may develop and/or reside in non-mucosal LN, producing IL-22 for a purpose other than the support of mucosal immunity.

Indeed, IL-22 is one of the key mediators in psoriasis, where it’s increased in lesional skin (Wolk, Kunz et al. 2004) and serum samples (Lo, Torii et al.). IL-22 is also increased in inflamed tissue from patients with rheumatoid arthritis (Ikeuchi, Kuroiwa et al. 2005), in addition to those with inflammatory bowel disease, ulcerative colitis, and Crohn’s disease (Andoh, Zhang et al. 2005). Although IL-22 has a role in autoimmunity, it is also thought to play a protective role in host defense, promoting epithelial barrier integrity and protecting against extracellular pathogens (Satoh-Takayama, Vosshenrich et al. 2008; Cella, Fuchs et al. 2009).
The role of IL-22 in inflammation/host defense is complex and incompletely understood, but current evidence suggests a dual nature for this cytokine, which can have both pro-inflammatory and anti-inflammatory capacities. The actions of IL-22 appear to depend on an “inflammatory context”, including factors such as the duration of IL-22 expression, the amount of IL-22 present, the overall cytokine milieu, and the tissues involved (Sonnenberg, Nair et al.). It has been hypothesized that during “steady state” conditions, IL-22 has a protective role, and maintains tissue homeostasis. In contrast, during chronic inflammation, IL-22 may take on a more pathological, pro-inflammatory role. Clearly, we are only beginning to understand the functions of IL-22, which appear to be complex, and dependent on a given tissue microenvironment and disease context.

As we continue to develop strategies to immunotherapeutically target human NK developmental intermediates, it will be important to understand the role of cytokines and other immunomodulatory factors in regulating homeostasis and activation of these cells. In the past, IL-2 has been used clinically to expand NK cells in the setting of malignancies (Caligiuri, Murray et al. 1993; Meropol, Porter et al. 1996). We now know that IL-15, not IL-2, is the critical factor for NK cell survival in vivo (Cooper, Bush et al. 2002). Additional investigation of the factors involved in regulating the homeostasis, differentiation, and function of human SLT NK developmental intermediates, such as the studies described in Chapters 2 and 3, may enable us to design therapeutic strategies that preferentially activate the most appropriate population of NK developmental intermediates for treatment of a particular disease state.
One clinically relevant example of this might be the use of recombinant human IL-15 and IL-1β to drive expansion of the IL-22-producing subpopulation of IL-1RIhi stage 3 iNK cells to promote mucosal immunity, which may act to restore integrity of the mucosal barrier. In other settings, this IL-22-producing population might be over-represented, leading to pathology. For example, in the setting of rheumatoid arthritis, it might prove beneficial in some patients to use a combination of IL-15 and IL-1RA drive differentiation of stage 4 mature NK cells. As we continue to understand more about the biology of NK developmental intermediates, the immunomodulatory factors driving their differentiation and activation, and the functions of IL-22, we will be able to design more appropriately targeted strategies for the treatment of human disease.

4.12 Concluding remarks.

The studies presented here were carried out with the intention of elucidating new roles for cytokines in regulating NK cell development by asking basic and important questions regarding the homeostasis, differentiation, and functions of human SLT NK developmental intermediates. The results of these investigations not only contribute to our overall understanding of the biology and development of human NK cells, but also raise many more questions than they answer, as is the case with most good experiments. The underlying goal of this research, as mentioned in the Introduction, was to provide rationale for the treatment of human disease through a better understanding of innate immunity. Thus, it is hoped that the findings we have reported in these studies can be used as a basis for further investigations that may ultimately allow us to manipulate of the
differentiation state of certain NK developmental intermediates for the treatment of
disease, especially Crohn’s disease and psoriasis, both of which have reported
involvement of IL-22 and IL-1β (Wilson, Boniface et al. 2007; Cosmi, De Palma et al.

4.13 Experimental procedures.

**Isolation of human NK precursors from SLT.** All protocols were approved by the
OSU Institutional Review Board. Normal human tonsils were obtained within 24 hours
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, Yokohama et al. 2006). Briefly,
mononuclear fractions were depleted of CD3⁺ and CD19⁺ cells via magnetic negative
selection (Miltenyi Biotec). 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 Biotec)
for FACS sorting of stage 1 and 2 cells, while total stage 3 iNK cells and stage 4 mature
NK cells were sorted from the CD34⁻ fraction as previously described (Freud, Yokohama
et al. 2006). IL-1R1hi and IL-1R1lo 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 iNK cell surface antigens (Freud,
Yokohama 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 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 with an appropriately labeled isotype antibody. Staining was performed as described in Chapter 3. Cells were analyzed immediately as previously described (Freud, Becknell et al. 2005) using a FACS Calibur or LSR II cytometer (BD Biosciences) and FlowJo software (Tree Star, Inc.).

**Cell culture.** FACS 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), IL-2 (1 nM; Hoffman LaRoche), IL-7 (10 ng/ml), and IL-23 (10 ng/ml; R&D Systems). Medium for stage 1 pro-NK 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 hours, half the medium was removed every 2-3 days and replaced with media containing 2x cytokines.
**Real-Time PCR.** mRNA was obtained after FACS sorted stage 3 iNK cells were cultured for 14 days *in vitro* in the presence of IL-15 alone, IL-15 plus IL-23, IL-15 plus IL-1β, or IL-15 plus IL-23 and IL-1β. Cells were lysed, then processed using an RNeasy kit (Qiagen). cDNA was synthesized according to manufacturer’s instructions using an 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* and *IL-26*, which were purchased from Applied Biosystems. *RORC* primer and probe sequences were as previously described (Yang, Fan et al. 2006). Gene expression levels were normalized to an 18S internal control, then analyzed by the ΔΔCt method (Fehniger, Carson et al. 1999).

**Clonal assays.** A FACS Aria II (BD Biosciences) 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 (Costar) containing a layer of GFP^+ murine OP9 stroma in α-MEM medium supplemented with recombinant human IL-15 or IL-15 plus IL-1β. Half the medium was removed every 2-3 days and replaced with fresh media containing 2x cytokines. After 14 days, each of 60 replicate wells was individually stained and 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.

**Immunohistochemical staining of human axillary lymph node.** All protocols were approved by the OSU Institutional Review Board. Normal, non-cancerous, human
axillary (non-mucosal) LN were retrieved fresh from surgically discarded tissue from non-cancer patients by the OSU Tissue Procurement Resource and from the National Disease Research Interchange. CD117 and IL-22 immunohistochemical staining was performed on serial tissue sections from paraffin-embedded axillary LN as indicated for tonsillar tissue in Chapter 3.

**LTi Assay.** Human umbilical vascular endothelial cells (HUVECs) were isolated as previously described (Baudin, Bruneel et al. 2007), and tested for purity via immunoperoxidase staining of acetone-fixed slide cultures for von Willebrand factor. HUVECs were then seeded in a fibronectin-coated 96-well flat bottom tissue culture plate, and cultured to confluence in Medium 199 (M199; Invitrogen). IL-1R1\(^{hi}\) stage 3 iNK cells or CD56\(^{bright}\) stage 4 mature NK cells were isolated via FACS as previously indicated (in Chapter 3 and (Hughes, Becknell et al. 2010)), and added to confluent HUVECs for co-culture at an NK:HUVEC ratio of 10:1 (~100,000 NK cells:10,000 HUVECs). Medium in wells containing stage 3 or stage 4 cells were supplemented with 1 nM recombinant human IL-15 (Amgen). After 5 hours of co-culture, wells were washed twice with sterile PBS (to remove non-adherent lymphocyte populations). HUVECs were then de-adhered from the well (using 0.1% Trypsin (Invitrogen)), and HUVEC induction of VCAM1 and ICAM1 surface expression was assessed via flow cytometric staining. Staining was performed according to manufacturer’s instructions using antibodies from BD Biosciences, and any remaining NK cells were excluded from analysis by gating on CD45\(^{-}\) events. Wells in which IL-1R1\(^{hi}\) stage 3 iNK cells or
CD56^{bright} stage 4 mature NK cells were co-cultured with HUVECs were compared to control wells containing HUVECs cultured in medium alone or medium supplemented with recombinant human TNF-α (300 U/ml; Endogen) (Rice and Bevilacqua 1989).

**IL-15 binding assay.** Since IL-15R is not detectable on the surface of stage 3 iNK cells via flow cytometry (Freud and Caligiuri 2006), IL-15R expression was measured by a cell’s ability to bind IL-15R’s specific ligand, IL-15. To accomplish this, we made use of the Human IL-15 Biotinylated Fluorokine Kit (R&D Systems), which was used according to manufacturer’s instructions. IL-1R1^{hi} and IL-1R1^{lo} subpopulations of stage 3 iNK cells, as well as stage 4 mature NK cells, were FACS sorted from human tonsil as described in Chapter 3. After 6 hours of culture in the presence of biotin-labeled IL-15, flow cytometry was used to assess IL-15 bound to the cell surface via secondary labeling with streptavidin-APC-Cy7 (BD Biosciences). IL-15 binding within each of the stage 3 iNK cell subpopulations (IL-1R1^{hi} and IL-1R1^{lo}) was compared to stage 4 mature NK cells, which served as a positive control, due to expression of IL-15R at levels which are detectable via flow cytometric staining with an anti-IL-15R mAb (Freud and Caligiuri 2006). As a negative control, staining was compared to that in parallel cultures incubated with an irrelevant biotin-labeled protein (“control;” soybean trypsin inhibitor).
Figure 17: Clonal analysis of IL-1R1^{hi} and IL-1R1^{lo} stage 3 iNK cells. (A-G) A 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 or IL-15 and IL-1β. Half the medium was removed every 2-3 days and replaced with fresh media containing 2x cytokines. After 14 days, 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. (A-D) Percentages were calculated relative to the total number of (A,B) wells that grew (a well “grew” when we detected ≥ 10 GFP^{+}CD45^{+} events), and (C,D) quantity of surviving GFP^{+}CD45^{+} lymphocytes detected within each well. Wells were scored as positive for CD94 and/or IL-22 when the proportion of GFP^{+}CD45^{+} lymphocytes which were CD94^{+} or IL-22^{+} exceeded 2% (corresponding to level of background staining detected with isotype control antibodies). Percentages of wells expressing CD94 and/or IL-22 were calculated relative to number of wells that grew. Percentages of cells in each well expressing CD94 or IL-22 represent the proportion of cells which were CD94^{+} or IL-22^{+} among total live cells (GFP^{+}CD45^{+}) detected in that well. (A) Notably, some amount of CD94^{+} cells were generated in 100% of the wells containing outgrowth from IL-1R1^{lo} clones. In contrast, when examining outgrowth from the IL-1R1^{hi} stage 3 iNK subpopulation, CD94^{+} cells were detected in significantly fewer wells, whether cultured in the presence of IL-15, or IL-15 and IL-1β.
(E-G) As shown in a representative donor, although CD94$^+$ cells and IL-22$^+$ cells were sometimes generated in the same well, these antigens were expressed in a manner which remained mutually exclusive, and occurred on separate cells. (E) IL-1R1$^{lo}$ (left) or IL-1R1$^{hi}$ (right) stage 3 iNK cells were cultured as clones on the OP9 cell line with IL-15 in the absence (top) or presence (bottom) of IL-1β. Dot plots depict staining of clonal outgrowth from a representative donor stained for expression of (E,G) CD94 with IL-22 or (F) isotype control. IL-22 protein expression is preferentially maintained by IL-1R1$^{hi}$ stage 3 iNK cells cultured in the presence of IL-1β. (G) Although CD94$^+$ cells and IL-22$^+$ cells were sometimes generated in the same well, these antigens were expressed in a manner which remained mutually exclusive, and occurred on separate cells. Experiments summarized in A-G involved the assessment of each population in over 60 individual wells from each donor (n = 3). Data shown in A-D depict mean ± SEM, and data in E-G shown in a representative donor, from 2 independent experiments with similar results (*, $P \leq 0.05$; **, $P \leq 0.002$; ***, $P \leq 0.0001$; n ≥ 3 donors).
Figure 17: Clonal analysis of IL-1R1<sup>hi</sup> and IL-1R1<sup>lo</sup> stage 3 iNK cells.
**Figure 18: LTI Assay.** (A,B) Induction of (A) VCAM1 and (B) ICAM1 expression on the surface of HUVECs depicted in a representative experiment (n = 3). Mean fluorescence intensity (MFI) of VCAM1 and ICAM1 surface expression was assessed via flow cytometry in adherent HUVECs after attempting to wash away lymphocytes with PBS, then gating out any remaining CD45$^+$ lymphocytes. Following co-culture, FACS sorted IL-1R1$^{hi}$ stage 3 iNK cells were compared to CD56$^{bright}$ stage 4 mature NK cells from the same donor were compared in their ability to induce HUVEC expression of VCAM1 and ICAM1. Also shown for comparison are wells in which VCAM1 and ICAM1 expression were assessed after HUVECs were cultured in medium alone or medium containing recombinant human TNF-α (30 U/ml), included as a negative control and a positive control, respectively.
Figure 18: LTi Assay.
**Figure 19: γc cytokine responsiveness of stage 3 iNK cells.** (A) Total stage 3 iNK cells were FACS purified and cultured for 7 days with IL-15, IL-2, IL-7, or IL-15 plus IL-7. Quantity of viable cells generated *in vitro* (measured via Trypan blue assay) is depicted as “fold change in cell number”, calculated as the ratio of viable cells generated from culture in each experimental condition relative to the number of cells generated from control culture with IL-15 alone. In contrast to IL-15 or IL-2, IL-7 was insufficient to maintain stage 3 iNK cell survival (**, \( P \leq 0.005 \)). (B) Binding of biotin-labeled IL-15 to the cell surface was used to measure IL-15R surface expression. Depicted is the average % of cells FACS sorted from human tonsil as IL-1R1\(^{hi}\) stage 3 iNK cells, IL-1R1\(^{lo}\) stage 3 iNK cells, or stage 4 mature NK cells, which bound to biotinylated IL-15 (“IL-15”) or an irrelevant biotin-labeled protein (“control;” soybean trypsin inhibitor) after 6 hours *in vitro*. Flow cytometry was used to assess for binding of biotin-labeled protein on the cell surface via secondary labeling with fluorescently conjugated streptavidin. Data in A and B presented as mean ± SEM (n = 3 for each).
Figure 19: γc cytokine responsiveness of stage 3 iNK cells.
Figure 20: Stage 3 iNK cells following culture with IL-23. (A-D) Total stage 3 iNK cells were FACS purified and cultured for 14 days with IL-15 alone, IL-15 plus IL-1β, IL-15 plus IL-23, or IL-15 plus IL-1β with IL-23. (A) Quantity of viable cells generated in vitro (measured via Trypan blue assay) is depicted as “fold change in cell number,” calculated as the ratio of viable cells generated from culture in each experimental condition relative to the number of cells generated from control culture with IL-15 alone. In the presence of IL-15 or IL-15 plus IL-1β, the addition of IL-23 had no significant effect ($P \geq 0.05$) on cell quantity. (B-D) Following culture, Real-Time PCR was used for quantitative assessment of: (B) RORC, (C) IL-22, and (D) IL-26 mRNA expression. Gene expression levels were normalized to 18S mRNA, and relative quantification was performed using the $\Delta \Delta Ct$ method. “Fold difference” was calculated relative to the culture condition in which the lowest level of gene expression was detected, which was arbitrarily normalized to 1. Results for A-D depicted as mean ± SEM (n ≥ 4 for each).
Figure 20: Stage 3 iNK cells following culture with IL-23.
Figure 21: IL-22 and CD117 co-labeling in axillary LN. Immunohistochemical staining depicted in serial sections of non-cancerous human axillary (non-mucosal) LN from a representative donor (n = 3). We were able to locate cells positive for CD117 (red) and IL-22 (green) co-expression (yellow) within the lamina propria and parafollicular T cell rich region of human axillary LN, suggesting that human stage 3 iNK cell production of IL-22 may not be confined to the mucosa. Magnification for images in the top row was 400x. Shown in the bottom row are higher magnifications of the insets from the top row.
Figure 21: IL-22 and CD117 co-labeling in axillary LN.
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