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INTERLEUKIN-15:
A CRITICAL REGULATOR OF THE DEVELOPMENT
AND FUNCTION OF THE INNATE IMMUNE SYSTEM

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Todd Arthur Fehniger, B.S.

*****

The Ohio State University

2000

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To me, every hour of the light and dark is a miracle,
Every cubic inch of space is a miracle,
Every square yard of the surface of the earth is spread with the same,
Every foot of the interior swarms with the same;
Every spear of grass—the frames, limbs, organs, of men and women, and all that concerns them,
All these to me are unspeakably perfect miracles

Walt Whitman
ABSTRACT

Interleukin (IL)-15 is a pleiotropic cytokine that is important for both innate and adaptive immune cell homeostasis, as well as peripheral immune functions. IL-15 shares signaling receptor (R) components with IL-2 (IL-2/15Rβ, γc), but uses a private IL-15Rα for high affinity binding. IL-15 transcript is widely expressed by multiple cell types including activated antigen presenting cells (e.g. macrophages, dendritic cells), epithelial cells, and is constitutively produced by bone marrow stromal cells. Here, studies are presented that address key questions regarding the biology of IL-15 as a regulator of innate immune development and function.

Early in vitro studies have identified an important role for IL-15 during human NK cell development from stem cells in the bone marrow. However, a complete understanding of how IL-15 and the IL-15R complex fits into the complex framework of NK cell ontogeny remains unknown. Here, studies demonstrate that class III receptor tyrosine kinase ligands, flt3 ligand (FL) and c-kit ligand (KL), potentiate IL-15-induced NK cell differentiation. Moreover, FL and KL act early on NK cell progenitors to induce an IL-2/15Rβ+ NK cell precursor, which is then responsive to IL-15 for differentiation into mature NK cells. These novel findings identify critical interactions between bone marrow stromal cell-derived factors that orchestrate step-wise differentiation from NK progenitors, to NK precursors, and finally mature NK cells. This in vitro culture system
will be a useful model for advancing our understanding of the molecular events governing NK cell development, and identifies factors (FL, IL-15) that are potential therapeutic agents to expand NK cells in patients with cancer or immunodeficiency.

Studies with human NK cells have shown that IL-15 costimulates IFN-γ protein production, however the in vivo significance of these findings were unknown. Therefore, utilizing severe combined immunodeficient mice (SCID) mice, which lack antigen-specific T and B cells but contain NK cells and macrophages, we examined whether IL-15 was an important costimulator of innate immune IFN-γ production in vivo. Pre-treatment with either blocking anti-IL-2/15Rβ or neutralizing anti-IL-15 antibodies significantly reduced serum IFN-γ protein measured 6 hours after LPS administration to SCID mice. In addition, anti-IL-2/15Rβ or anti-IL-15 antibodies reduced the amount of IFN-γ protein produced by NK cells in the spleen following LPS challenge, as measured by intracellular flow cytometry. Finally, pre-treatment with anti-IL-15 antibodies before the IFN-γ-dependent priming phase of the LPS-elicited Shwartzman reaction reduced lethality in this model of cytokine-induced shock. This is the first study to provide evidence that IL-15 costimulates NK cell IFN-γ production in vivo, and indicates that IL-15 may be useful to augment IFN-γ production during infection.

Early in vitro, and more recent in vivo experiments describing the phenotypes of IL-15⁻/⁻ and IL-15Rα⁻/⁻ mice have documented a critical role for IL-15 in the development of NK cells and the expansion and/or survival of CD8⁺ T cells. Interestingly, the expression of IL-15 protein is tightly controlled by multiple posttranscriptional mechanisms, suggesting
that over-expression may somehow be deleterious to the host. We therefore engineered a transgenic mouse to globally over-express IL-15 by eliminating three primary posttranscriptional checkpoints. IL-15 transgenic mice have early (e.g. 6 weeks of age) expansions in NK and memory-phenotype CD8$^+$ T lymphocytes. Later, these mice develop lymphocytic leukemia with massive multi-organ lymphocytic infiltrates and premature death. Thus, this transgenic mouse model provides in vivo data documenting the importance of IL-15 in NK and CD8$^+$ T cell homeostasis. Further, these data provide novel evidence that leukemia, like certain other cancers, can arise as the result of chronic stimulation by a proinflammatory cytokine.

Collectively, these studies provide novel advancements in our understanding of NK cell development, the immunoregulatory role of IL-15 during the innate immune response, and the effects of IL-15 over-expression in mice in vivo. While extending our basic understanding of immune regulation, these studies also provide novel experimental tools, and point toward potential clinical applications of IL-15.
Dedicated to my family and friends

and

In loving memory of Robert and Elaine Mortimer
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CHAPTER 1

INTRODUCTION

1.1 The Innate Immune System and Natural Killer Cells

The immune system, a collection of all cells and molecules responsible for immunity, may be divided into two main branches: the innate (natural) and adaptive (antigen-specific) immune systems. These two branches differ in their cellular constituents, general properties, and major immune functions (Table 1.1). The innate immune system is phylogenetically older, and in mammals has two critical features: 1) to rapidly limit spread of infectious pathogens and 2) prepare the adaptive immune system to effectively clear the pathogens. Thus, through understanding the regulation and development of the innate immune system strategies to boost inadequate responses (e.g. immunodeficiency) or attenuate inappropriate responses (e.g. autoimmunity) may be developed. As our understanding of the immune response grows, a greater appreciation of the importance of innate immunity in shaping the subsequent adaptive response has come to light. One central immunoregulatory player in the innate immune response is the natural killer (NK) cell, and expanding our knowledge of NK cell development...
(Chapters 2 and 4), and effector cytokine production (Chapter 3) are a major themes of this dissertation.

NK cells\textsuperscript{7,8} are large granular lymphocytes (LGL) (Fig. 1.1) that demonstrate cytotoxicity against tumor and virally-infected cells, produce immunoregulatory cytokines and chemokines, and are an important component of the innate immune defense against viruses, fungi, bacteria, and protozoa.\textsuperscript{9-13} Biron et al. first reported a patient that lacked NK cells and suffered from recurrent infections with herpes viruses including varicella and cytomegalovirus, highlighting the importance of this cell type in our defense against viral infections.\textsuperscript{14} NK cells, unlike T and B lymphocytes, do not require the recombinase activating genes (RAGs) for development, and do not express clonally rearranged receptors. NK cells do express a number of cell surface receptors that recognize MHC class I ligands and regulate NK cell activation and lysis of target cells,\textsuperscript{15,16} and are likely to be important for the control of some cancers.\textsuperscript{17} Phenotypically, NK cells are identified in humans as CD56\textsuperscript{+}CD3\textsuperscript{-} lymphocytes and in mice as NK1.1\textsuperscript{+}CD3\textsuperscript{-} or DX5\textsuperscript{+}CD3\textsuperscript{-} lymphocytes.\textsuperscript{7,8} The majority (~90\%) of human NK cells have low density surface expression of CD56 (CD56\textsuperscript{dim}), express high surface density of CD16 (Fc\gammaRIII), and have a low proliferative capacity. CD56\textsuperscript{bright} NK cells represent a minor subset (~10\%) of human NK cells, are low or negative for CD16, and are capable of high proliferation.\textsuperscript{8} The development and function of NK cells is discussed in greater detail below in the context of interleukin (IL)-15 (Section 1.4).
1.2 Cytokines and Cytokine Receptors

Cytokines are small glycoproteins that mediate local intercellular communication between cells of the immune system.\textsuperscript{18,19} They mediate their effects by binding to specific cell surface receptors, that in turn transduce intracellular signals within the target cell. Several major families of cytokines have been described based upon common structural components: type 1 cytokines (including most interleukins), type II cytokines (interferons, IL-10) and pro-inflammatory cytokines (including the TNF family, IL-1 family, and chemokines).\textsuperscript{18,19} Type 1 cytokines are characterized by a four $\alpha$-helical structure, and are further divided into short-chain (IL-2, IL-3, IL-4, IL-5, IL-7, IL-9, IL-13, IL-15, GM-CSF, M-CSF, SCF) and long-chain (IL-6, IL-11, oncostatin M, LIF, CNTF, cardiotropin-1, growth hormone, prolactin, EPO, TPO, leptin, G-CSF) type 1 cytokines. As major mediators of immune cell communication, cytokines typically operate in complex cascades and networks that are not fully understood.\textsuperscript{20} Cytokines exhibit several properties including pleiotropy -- one cytokine mediating multiple biologic functions, and redundancy -- multiple cytokines mediating the same biological function. Initially, cytokines were thought to act on and be produced by only immune cells. It is now appreciated that many non-immune cell types (e.g. endothelial cells, bone marrow stromal cells) produce and respond to cytokines, thereby communicating with immune cells and contributing to host defense and immune cell development. IL-15 is a type 1 cytokine central to innate immune development and function. The molecular and cellular biology of this cytokine is comprehensively reviewed in Section 1.4, while IL-15’s relevance to human disease is discussed in Section 1.5.
Cytokine receptors are type 1 transmembrane proteins that have multiple functional domains that bind extracellular cytokines and then initiate intracellular signaling cascades that typically result in changes in gene expression and cellular function. Similar to cytokines, their receptors may also be sub-divided into multiple families based upon similar structure. Typically, cytokine receptors are multimeric complexes, and one receptor subunit may be shared by multiple cytokines. For example, the common γ chain (γc) is shared by a family of type 1 cytokines, each of which have a private α chain that provides binding specificity (Table 1.2). An in depth discussion of the IL-15 receptor complex and its signaling is provided below (Section 1.4).

1.3 Hematopoiesis

Hematopoiesis is the process whereby self renewing pluripotent stem cells in the bone marrow differentiate into all blood lineages. This process is regulated by numerous soluble and membrane bound factors including cytokines and colony stimulating factors. Lymphopoiesis refers to the development and differentiation of T, B, and NK cells. Remarkable progress over the past quarter century has been made in our understanding of T and B lymphocyte development, which occurs in the thymus and bone marrow, respectively. However, much remains unknown about NK cell development. A summary of our knowledge of NK cell development is provided below (Section 1.4), and the development of the human NK cell lineage is the topic of Chapter 2.
1.4 Molecular and Cellular Biology of IL-15

1.4.1 The Discovery of IL-15 and its Relationship to IL-2

IL-15 was identified by two independent groups based upon its ability to stimulate proliferation of the IL-2-dependent CTLL-2 T cell line in the presence of neutralizing anti-IL-2 antibodies. The activity within cell culture supernatants of the simian kidney epithelial cell line CV-1/EBNA was purified, molecularly cloned, and designated IL-15. The activity identified in supernatants of the human T cell leukemia virus-1 (HTLV-1) cell line, HuT-102, was purified and called IL-T.

Scientists at the Immunex Corporation isolated the 14-15 kD protein responsible for the CTLL proliferation within CV-1/EBNA supernatants utilizing anion-exchange and high pressure liquid chromatography, and sequenced the NH2-terminal residues. Degenerate oligonucleotide primers were generated from this partial protein sequence, and were used to obtain the full-length simian IL-15 cDNA from a CV-1/EBNA cDNA library. Utilizing simian IL-15 cDNA as a probe, the full-length human IL-15 cDNA was cloned from the IMTLH bone marrow stromal cell line. The IL-T protein identified by researchers at the NIH within HuT-102 supernatants was later cloned and shown to be a chimera composed of the HTLV-1 long terminal repeat (LTR) and human IL-15. The HTLV-1 LTR was fused in frame immediately upstream of IL-15 thereby deleting a portion of its 5' untranslated region (UTR).

As IL-15 was initially identified through its ability to mimic IL-2-induced T cell proliferation, the biochemical and functional relationship between these two cytokines was quickly examined. Comparisons of the primary protein and cDNA sequences of...
human or simian IL-15 yielded little primary homology to IL-2, however, computer modeling of IL-15’s secondary structure suggested that IL-15 belonged to the 4 α-helix bundle cytokine family\(^{27}\) including human growth hormone, IL-2, IL-3, IL-6, IL-7, G-CSF and GM-CSF.\(^{31,32}\) Functional studies utilizing antibodies that blocked the various IL-2R subunits determined that IL-15 utilized the IL-2Rβ subunit and the common gamma chain (\(\gamma_c\)) (but not the IL-2Rα). Because signaling via IL-2 and IL-15 appears to occur exclusively via the same \(\beta\gamma\) chains, IL-15 mediates functions similar to IL-2 in vitro.\(^{27,29,33}\) However, in vivo it is the distribution of the distinct IL-15Rα and IL-2Rα chains that direct when and where each ligand will bind and activate via this \(\beta\gamma\) signaling pathway. Through development of mice with targeted disruption of the IL-15\(^{34}\) and IL-15Rα\(^{35}\) genes, it is now apparent that IL-15 and IL-2 mediate very different in vivo functions. The similarities and differences between IL-15 and IL-2 are highlighted where appropriate below.

1.4.2 Structure of the Genomic Locus Encoding IL-15

The IL-15 gene spans \(\geq 34\) kb, mapping to human chromosome 4q31 and the central region of mouse chromosome 8.\(^{36,37}\) The genomic structure of human IL-15 contains 9 exons (7 coding exons), with a similar intron/exon structure and estimated size between the murine and human IL-15 gene.\(^{36,37}\) More recently, an alternative exon has been described in human\(^{38-40}\) and murine\(^{41,42}\) IL-15, consisting of an additional sequence between exons 4 and 5 that encodes an alternative leader peptide. The structure of the human IL-15 genomic locus is diagramed in Fig. 1.2, and includes the recently described alternative exon (4A).\(^{37,38,40}\) The IL-2 gene is located on human chromosome 4q26-28.
and consists of 4 exons and 3 introns. Of note, the overall intron/exon structure of the portion of the IL-15 gene encoding the mature IL-15 protein (4 exons and 3 introns) is similar to that of the IL-2 gene and other four α-helix bundle cytokines. However, consistent with other family members, there is little primary homology between IL-2 and IL-15 at the nucleotide or protein levels (Fig. 1.3). Thus, while it is doubtful that the IL-2 and IL-15 gene have a direct, recent ancestral relationship, the gene structure of all members of the 4 α-helix bundle cytokine family appears similar.

1.4.3 IL-15 mRNA Isoforms, Signal Sequences, and Intracellular Trafficking

The originally identified human IL-15 cDNA contains a 5' UTR of at least 316 base pairs (bp), a 486 bp open reading frame, and a 3' UTR of at least 400 bp, encoding a precursor IL-15 with an unusually long 48 amino acid (AA) leader peptide, and a 114 AA mature protein. Identification of human, simian, and murine IL-15 indicated that IL-15 was conserved between species (97% identity comparing human and simian, 73% identity comparing human with murine). An alternative human IL-15 cDNA was identified containing an additional exon. This variant IL-15 human cDNA contains 119 nucleotides (nt) within intron 4, but lacks exons 1 and 2 of the original cDNA, resulting in a smaller spliced mRNA product (Fig. 1.2). However, this exonic sequence encoded three premature stop codons, and a novel downstream ATG translation start site, resulting ultimately in an IL-15 precursor protein with a 21 AA short signal peptide (SSP), compared to the original 48 AA long signal peptide (LSP). A similar alternative transcript has been described in the mouse. In human and mouse, both IL-15
isoforms encode an identical mature IL-15 protein, containing differences only within the signal sequence. The regulation of the IL-15 mRNA species expressed may occur through alternative splicing and/or an additional uncharacterized IL-15 promoter driving the expression of the SSP-IL-15.

The two IL-15 mRNA isoforms have significant differences in the N-terminal portions of their leader peptides (Fig. 1.2). Leader peptides determine the intracellular localization and potential secretion for the associated mature protein. Both LSP-IL-15 and SSP-IL-15 appear to have low secretion potential compared to well-secreted cytokines such as IL-2, as replacement of either endogenous IL-15 signal peptide by CD33 or IgVκ signal peptides resulted in efficient secretion of bioactive IL-15 protein. Both LSP-IL-15 and SSP-IL-15 isoforms had 2 to 3 log-fold less secretion compared to the IL-2 SP, and analysis with IL-15-green fluorescent protein (GFP) fusion proteins demonstrated that LSP-IL-15 was targeted to the secretory pathway (ER/golgi) while SSP-IL-15 appeared restricted to the cytoplasm and nucleus. This differential trafficking is discussed below in the context of the regulation of IL-15 gene expression. SSP-IL-15 mRNA is expressed in the heart, thymus, appendix and testis, while LSP-IL-15 is in skeletal muscle, placenta, heart, lung, liver, thymus, and kidney, but the biological significance of these observations will require further investigation. Recently Nishimura et al. generated transgenic mice that express both isoforms of IL-15 under the control of an MHC class I promoter, documenting differential in vivo roles for LSP- and SSP-IL-15 (see below).
1.4.4 Structure of the Mature IL-15 Protein

The 14-15 kD, 114 AA mature IL-15 protein is encoded by exons 5-8 of the IL-15 gene (Fig.1.2).\textsuperscript{27,36,37} IL-15 contains two disulfide bonds at positions Cys\textsuperscript{42}-Cys\textsuperscript{88} and Cys\textsuperscript{35}-Cys\textsuperscript{85}, the former being homologous to the Cys-Cys within IL-2. There are two N-linked glycosylation sites at the C-terminus of the IL-15 protein, at asparagines 79 and 112. The mature IL-15 protein has been predicted to have strong helical moments at AA1-15, 18-57, 65-78, and 97-114, supporting a theoretical model of a four α-helix bundle structure (Fig. 1.3).\textsuperscript{27,48}

1.4.5 The IL-15 Receptor Complex and its Relationship to the IL-2 Receptor Complex

Three different IL-2R complexes exist: the isolated IL-2Rα binds IL-2 with low affinity ($K_a \sim 10^8 M^{-1}$) without transducing a signal, the heterodimeric IL-2Rβγ binds IL-2 with intermediate affinity ($K_a \sim 10^9 M^{-1}$) and transduces intracellular signals, while the heterotrimeric IL-2Rαβγ binds IL-2 with high affinity ($K_a \sim 10^{11} M^{-1}$) and also signals.\textsuperscript{44,49,50} The IL-2Rγ, also referred to as the common gamma chain ($\gamma_c$), is shared by receptors for IL-2, IL-4, IL-7, IL-9 and IL-15, and has been implicated in X-linked severe combined immunodeficiency (SCID).\textsuperscript{51,52} Early experiments utilizing specific blocking mAb documented the participation of the IL-2Rβ and $\gamma_c$, but not the IL-2Rα, in IL-15 binding and function, suggesting the presence of an additional subunit for high affinity IL-15 binding to the IL-15R complex.\textsuperscript{29,33,53,54}
The murine and human IL-15Rα subunits were subsequently cloned and characterized, and shown to be highly homologous to their IL-2Rα counterparts. The full length human IL-15Rα is a type I transmembrane protein with a signal peptide of 32 AA, an extracellular domain of 173 AA, a transmembrane domain of 21 AA, a 37 AA cytoplasmic tail, and multiple N- or O-linked glycosylation sites. Comparison of the IL-2Rα and the IL-15Rα revealed the presence of a conserved protein binding motif (sushi domain or GP-1 motif), and similar intron/exon structure, placing IL-2Rα and IL-15Rα as the founding members of a new receptor family. Through transfection experiments it was established that the full length IL-15Rα alone was sufficient for high affinity (K_a ≥ 10^{11} M^{-1}) binding of IL-15, but similar to IL-2Rα played no role in signal transduction. This high affinity of the isolated IL-15Rα for IL-15 is in stark contrast to the IL-2Rα, which has low affinity for IL-2 (K_a ~10^8 M^{-1}) in the absence of the IL-2Rβγ. Thus, the IL-15Rα binds IL-15 with high affinity, but transduces signals only in the presence of the IL-2/15Rβ and γc (Fig. 1.3). IL-15, like IL-2, may also bind and signal through the heterodimeric IL-2/15Rβγc with intermediate affinity (K_a ~10^9 M^{-1}), in the absence of IL-15Rα.

Eight splicing variants of the hIL-15Rα have been identified, including all combinations of exon 2 deletion, exon 3 deletion, and alternative use of exon 7 or 7'. Isoforms that lacked exon 2 (Δ2) were unable to bind IL-15, raising the possibility that Δ2IL-15Rα may associate with IL-2/15Rβγc removing them from participation in high affinity IL-15R complexes. While all isoforms of IL-15Rα were detected within the plasma
membrane, ER, and golgi, only IL-15Rα forms containing exon 2 localized to the nuclear membrane.\textsuperscript{56} The localization of both IL-15Rα and SSP-IL-15 to the nuclear membrane/nucleus calls for additional studies to better characterize its biological significance.

The originally identified full-length IL-15Rα transcript (~1.7 kb) was detected in numerous tissues and cell lines, demonstrating a much wider distribution than the IL-2Rα.\textsuperscript{55} Expression of all eight IL-15Rα isoforms was observed in multiple tissues (e.g., brain, intestine, liver, PBMC) and cell lines, however the relative expression of each isoform varied.\textsuperscript{56} Due to IL-15Rα's high affinity for IL-15, it has been hypothesized that the IL-15Rα may act as a molecular sink for excess IL-15, or possibly associate with other yet to be identified receptor components.\textsuperscript{55,57} A distinct high affinity binding receptor (60-65 kD, IL-15RX) has been identified on mast cells that does not include the identified IL-2/15Rβ, γc, or IL-15Rα, and transduces different intracellular signals than IL-15Rαβγ.\textsuperscript{58} Further studies are required to understand the functional importance of IL-15's binding to IL-15RX in mast cell biology, as no mast cell developmental defects were found in IL-15\textsuperscript{-/-} mice.\textsuperscript{34}

1.4.6 Pathways of IL-15 Signal Transduction

As IL-2 and IL-15 share common signaling components (IL-2/15Rβγc), most evidence to date suggests that the interaction of IL-15 with its receptor complex in various cell types leads to a series of signaling events that are similar, if not identical, to those elicited by IL-2.\textsuperscript{59} These include activation of the Janus kinase (Jak)/signal transducer and activator
of transcription (STAT) pathway. IL-2/15Rβ is associated with Jak1 and the γc is associated with Jak3, resulting in STAT3 and STAT5 phosphorylation, respectively, following ligation with IL-15 (Fig. 1.3). Additional signaling pathways through the IL-2/15R complexes include the src-related tyrosine kinases, induction of Bcl-2, and stimulation of the Ras/Raf/MAPK pathway that ultimately results in fos/jun activation. In neutrophils IL-15 has been shown to activate NF-κB but not AP-1, while IL-15 stimulation of bulk human PBL activated both transcription factors. However, reports of IL-15- but not IL-2-induced signals in an IL-15RxIL-2/15Rβ− colonic epithelial cell line leaves open the possibility of alternative IL-15 signaling mechanisms. The alternative receptor system in mast cells (IL-15RX) has been shown to induce phosphorylation of Jak2 and STAT5.

1.4.7 Physiologic Expression of IL-15

IL-15 mRNA is produced by multiple tissues (placenta, skeletal muscle, kidney, lung, heart, monocyte/macrophages), and numerous cell types through various stimulatory conditions. The first cell type to be implicated as a functionally relevant source of IL-15 in the context of the immune response were members of the monocyte/macrophage lineage. Other antigen presenting cells (APCs), such as blood-derived dendritic cells, have been shown to produce IL-15 mRNA and protein, suggesting a role in the attraction and stimulation of T cells. IL-15 is also produced by bone marrow (BM) stromal cell lines, primary human BM stromal cells, thymic epithelium, and fetal intestinal epithelium consistent with IL-15’s role during hematopoiesis. Epithelial and fibroblast cells from various tissues have been documented to produce IL-15 mRNA and/or protein,
including kidney epithelial cell lines, epidermal skin cells and keratinocytes, fetal skin, retinal pigment epithelium, and intestinal epithelial cells. Other cells producing IL-15 with a less obvious function requiring further investigation include kidney proximal tubule cells, as well as astrocytes and microglia. While not detected initially, T cells have later been shown to express IL-15 mRNA by more sensitive techniques. Considering the extensive posttranscriptional control of IL-15 now evident (see below), it will be important to document IL-15 protein production, either intracellularly, at the cell surface, or secreted extracellularly, to better understand the true role of IL-15 during normal homeostasis and immune defense.

1.4.8 Regulation of IL-15 Gene Expression

IL-15 transcription, translation and secretion have proved to be regulated through multiple, complex mechanisms. This topic has been recently reviewed in depth elsewhere, and only the major points of interest are summarized below. While transcriptional control of IL-15 is important, the principle level of IL-15 regulation appears to be posttranscriptional.

1.4.8.1 Control of transcription

Both human and murine 5' regulatory regions upstream of exon 1 have been cloned, sequenced, and analyzed for consensus transcription factor binding motifs. These studies revealed some common consensus binding sites within the mouse and human promoter regions including α-INF-2, NF-IL-6, γ-IRE, myb, GCF, and NF-κB. The NF-κB site located -75 to -65 relative to the transcription start site of the human IL-15
promoter was shown to be important for HTLV-1 Tax protein-induced IL-15 mRNA upregulation, and for LPS-induced IL-15 gene expression in murine macrophages. The region -201 and -141 in the human IL-15 promoter was reported to contain an unidentified site responsible for negative regulation of IL-15 expression, as 5' deletion of this region resulted in a dramatic increase in IL-15 promoter activity.

An essential interferon regulatory factor (IRF)-E consensus-binding site was identified at -348 to -336 relative to the cap site of the murine IL-15 promoter, following the observation that IRF-1-/- mice simultaneously lack inducible IL-15 expression and natural killer (NK) cells. It remains unclear what upstream signals are responsible for IRF-induced IL-15 expression during the normal physiologic process of NK cell development in the BM (discussed below).

1.4.8.2 Primary regulation of IL-15: translation, intracellular trafficking, and secretion

Three primary checkpoints have been identified that regulate IL-15 mRNA translation into the IL-15 precursor protein: multiple start codons (AUGs) in the 5' UTR, the unusual LSP and SSP, and a negative regulator near the C-terminus of the precursor proteins. The LSP-IL-15 5' UTR is relatively long (greater than 316 nt in humans) and contains multiple (12 in humans) AUGs upstream of the actual translation start site, that have been shown to dramatically reduce translational efficiency. Bamford et al. demonstrated that removal of 8 of 10 upstream AUGs in the 5' UTR of the human LSP-IL-15 by fusion with the HTLV-1 R region resulted in enhanced IL-15 protein production in the HuT-102 cell line (approximately 5 to 10-fold). However, when the upstream AUGs were...
circumvented, IL-15 translation and secretion still appeared lower than other cytokines, such as IL-2.

Tagaya et al. showed that replacement of endogenous IL-15 signal peptides with the human IL-2 signal peptide resulted in dramatically elevated IL-15 levels detectable in supernatants from COS-7 transfectants. LSP-IL-15 was shown to have a lower translational efficiency compared to SSP-IL-15 or IL-2SP-IL-15. Utilizing IL-15-green fluorescent protein (GFP) fusion constructs, it was evident that SSP-IL-15 was restricted to the cytoplasm and nucleus, while LSP-IL-15 was detected within the ER/Golgi. Thus, it appears that SSP-IL-15 is translated efficiently but not secreted, while LSP-IL-15 is translated less efficiently, but traffics through the ER/Golgi pathway and is secreted from the cell at low levels. Similarly, Gaggero et al. showed that LSP-IL-15 and exogenous IL-15 were detectable in CHO cell endosomes, indicating that rapid uptake by IL-15R-bearing cells may have a regulatory effect on the action of IL-15. The CTLL bioactivity of LSP-IL-15-GFP construct was significantly higher compared to the LSP-IL-15 without a 3' tag, suggesting that a signal in the carboxyl portion of the mature IL-15 protein results in inefficient secretion, possibly through a retention signal.

Through the systematic elimination of these three check-points, i.e. removing upstream AUGs, replacing the endogenous human IL-15 leader with that of IL-2, and fusing the C-terminus of the IL-15 mature protein with the FLAG epitope tag, the synthesis of bioactive IL-15 increased 250-fold. Such complex and tight control of the IL-15 gene product is unusual for most cytokines thus far characterized, and may indicate that IL-15, if over-produced, is somehow dangerous to the host. Evidence supporting this hypothesis
is provided in Chapter 4, as transgenic mice that express an IL-15 cDNA lacking posttranscriptional checkpoints develop lymphocytosis, and eventually lymphocytic leukemia.

Recently, Musso et al. detected bioactive IL-15 protein constitutively expressed on the surface of human monocyte/macrophage cell lines and primary human monocytes. Cell surface expression was increased upon stimulation with IFN-$\gamma$, suggesting a mechanism by which IL-15 could exert a biological effect while being undetectable in culture supernatants. It is unclear as to whether this membrane expression of IL-15 depends upon signal peptide expression. Another recent report demonstrated constitutive IL-15 protein expression in human PBMC by western analysis and flow cytometry, which was upregulated by *C. neoformans*, LPS or IFN-$\gamma$. Further study should reveal the exact cell types, tissue distribution, and functional significance of constitutive cell surface expression of IL-15. IL-15's function in promoting the survival of IL-15-responsive cell types, such as NK cells and memory T cells may be one hypothesis explaining the reason for low, constitutive protein expression. Then, in response to infectious insult, cytoplasmic protein may be translocated to the cell surface, to further stimulate IL-15R bearing cells, in combination with additionally induced monokines such as IL-12.

In other physiologic settings such as NK cell development (discussed below), it will be interesting to examine BM stromal cells for constitutive cell-surface expression of IL-15, and test if any signals induce the movement of cytoplasmic IL-15 protein to the cell surface. In both of these settings, it seems likely that activation of monocyte/macrophages (and other cells, such as dendritic cells and epithelium) or
appropriate stimulation of BM stromal cells could result in more efficient IL-15 synthesis and secretion through removal of the multiple posttranscriptional control points.

1.4.9 An Essential Role for IL-15 in NK Cell Development

It has long been appreciated that NK cells require the BM microenvironment for complete maturation, based upon studies examining mice with BM ablation by 89-strontium or β-estradiol. However, the precise factors and events responsible for orchestrating NK cell development within the BM remained elusive for decades. Long term BM cultures that included stromal cells but lacked exogenous cytokines were able to differentiate low numbers of the NK cell lineage. Moreover, through the addition of recombinant (r) IL-2, NK cells were produced in cultures that lacked stroma cells, and when added to cultures containing stroma, rIL-2 produced NK cells with high efficiency. In addition, provision of rIL-2 to mice and humans in vivo results in an expansion of NK cells. However, the physiologic relevance of these IL-2 effects were a mystery during NK cell development, as IL-2 is produced primarily by antigen-activated T cells located in the periphery, and mice deficient in IL-2 contain functional NK cells. In addition, mice and humans that lack the γc subunit of the IL-2R lack NK cells, as do mice that lack the IL-2/IL-15Rβ. Collectively, these data suggested that a factor other than IL-2 was produced in the BM and utilized signaling components of the IL-2R to induce NK cell development. Numerous studies from many laboratories have now convincingly shown that this factor critical for NK cell development is IL-15, acting through the IL-15Rαβγ. Two broad lines of evidence support this assertion: 1) experiments utilizing in vitro models of human and murine NK
cell differentiation and 2) genetically targeted mice with disruption of IL-2, IL-15, IL-2/15 receptors, their signaling components, or transcription factors that regulate gene expression critical to NK cell development (Table 1.3).

1.4.9.1 IL-15 differentiates human NK cells in vitro

First, for IL-15 to be the major physiologic growth factor responsible for NK cell ontogeny, it must be expressed at the anatomical site of NK cell differentiation, i.e., the BM. In support of this, the human IL-15 cDNA was first cloned from the IMTLH BM stromal cell line. Mrozek et al. directly demonstrated that primary human BM stromal cells produced IL-15 at the transcript and protein levels, while lacking any expression of IL-2. Further, a 3-week culture of CD34+ hematopoietic progenitor cells (HPCs) supplemented with rIL-15 induced the differentiation of functional CD56+ NK cells, in the absence of stroma or other cytokines. The CD56+ NK cells generated in these cultures lysed MHC class I tumor targets, produced cytokine and chemokines upon stimulation, and expressed cytoplasmic CD3-ζ chain protein similar to mature peripheral blood NK cells.

An additional BM stromal cell factor, the ligand for the class III receptor tyrosine kinases (RTK) c-kit (c-kit ligand, KL) has been shown to potentiate the expansion of other hematopoietic cell lineages, usually in combination with a lineage-specific growth factor. KL alone induce no NK cell differentiation, but when combined with IL-15 each of these factors potentiated IL-15-induced expansion of NK cells from CD34+ HPCs. Additional human culture systems have also supported a central role for IL-15 in human NK cell development from various starting progenitor populations including
cord blood (CB) CD34⁺,⁹¹²,⁹¹³ adult BM CD34⁺,⁹¹³ fetal liver CD34⁺CD38⁻,⁹¹⁴ and thymocyte T/NK progenitors.¹¹⁵ Collectively, these in vitro human culture systems demonstrate a central role for IL-15 in NK cell development, from various adult and fetal progenitors. However, additional studies were needed to clarify how IL-15 acts in concert with signals from early acting growth factors and stromal cells to facilitate physiologic human NK cell development. The data presented in Chapter 2 identify the mechanism whereby class III RTK ligands combine with IL-15 to temporally orchestrate NK cell development, and provide the first framework of human NK cell developmental intermediates (Fig. 2.7). Such basic understanding of how human NK cells develop can also be directly translated to optimizing the ex vivo¹¹⁶ and in vivo expansion of this lymphocyte subset for therapeutic intervention.

1.4.9.2 IL-15 differentiates murine NK cells

In vitro murine culture systems have also demonstrated a critical role for IL-15 during NK cell ontogeny. Leclercq et al. showed that bipotential T/NK progenitors¹¹⁷ isolated from the thymus selectively differentiate into NK cells in the presence of IL-15. Addition of high concentrations of IL-15 to progenitors in fetal thymic organ cultures (FTOC) blocked TCRαβ T cell development, and shifted differentiation towards the NK cell lineage.⁷² A series of studies by Kumar and colleagues have examined the role of IL-15 during murine NK cell ontogeny from adult BM progenitors.¹¹⁸ First, Puzanov et al. demonstrated that IL-15 corrected the lytic defect of immature NK1.1⁺ cells within mice rendered osteopetrotic by β-estradiol treatment. This suggested that IL-15 may replace the BM microenvironment deficits resulting in such immature NK cells.¹¹⁹
Williams et al. established a murine stroma-free culture system in which an NK progenitor population (c-kit\(^+\)Sca2\(^+\)Lin\(^-\)IL-2/15R\(\beta^-\)) cultured in IL-6/IL-7/KL/FL differentiated into an NK cell precursor population (NK1.1\(^-\)IL-2/15R\(\beta^+\)), which in turn responds to IL-15 for differentiation into mature, lytic NK1.1\(^+\) NK cells. These in vitro murine culture systems confirmed a central role for IL-15 in NK cell development in mice.

1.4.9.3 Mice with targeted genetic alterations demonstrate that IL-15 is a critical NK cell differentiation factor in vivo

As introduced above, the phenotypes of mice with targeted disruption of IL-2, IL-2R\(\alpha\), and \(\gamma_c\) first suggested that cytokines other than IL-2, acting through IL-2R components, may be important for NK cell development. Following the identification and cloning of IL-15, the aforementioned in vitro studies provided the first evidence that IL-15 could indeed serve as the central physiologic NK cell hematopoietic factor. Consequently, additional gene targeting experiments in mice have confirmed this postulate at the level of the whole animal (Table 1.3). Mice with targeted disruption of the IL-2/15R\(\beta\), shared only by IL-2 and IL-15, have a dramatic reduction in peripheral NK1.1\(^+\)CD3\(^-\) cells, and an absence of NK cytotoxicity in vitro. In addition, Fuji et al. recently demonstrated that the IL-2/15R\(\beta\) H-region, that activates STAT5/STAT3, selectively affects the development of NK and \(\gamma\delta\) T cells, through reconstitution of IL-2/15R\(\beta^+\) mice with IL-2/15R\(\beta\) transgenes containing specific mutations in the cytoplasmic domain. Mice that have genetic disruption of the \(\gamma_c\) have multiple lymphoid defects, including a
dramatic decrease in NK cells.\textsuperscript{108,122} Consistent with these models, mice that have disrupted signaling components that operate downstream of IL-15R, such as Jak3\textsuperscript{−/−}, STAT5\textsubscript{a/b}\textsuperscript{−/−}, or STAT5\textsubscript{b}\textsuperscript{−/−} also have NK cell defects.\textsuperscript{123-126}

Mice with disruption of the IRF-1 gene fail to induce IL-15 in the BM, and have an NK cell deficiency.\textsuperscript{82,83} Progenitor cells from IRF-1\textsuperscript{−/−} mice develop into functional NK cells upon culture in IL-15 or transfer into lethally irradiate wild type mice, suggesting that IRF-1 is required for the expression of IL-15 in the BM microenvironment. Indeed, IRF-1 binding sites have been identified in the 5' regulatory region of the IL-15 gene, supporting this hypothesis.\textsuperscript{82} At present it is unclear what provides the endogenous signal to activate IRF-1 within BM stromal cells, that in turn induces the IL-15 expression important for NK cell development.

Mice with targeted disruption of the Ets-1 transcription factor were shown to have a selective deficit in NK cells.\textsuperscript{127} Ets-1\textsuperscript{−/−} mice do not appear to have defects in IL-15R\textalpha\beta\gamma, IL-15, or IL-2 expression, leaving the precise mechanism responsible for this NK deficiency unknown. As Ets-1 is expressed in mature NK cells, it is possible that upstream IL-15-derived signals may induce Ets-1 that may then orchestrate the expression of an NK cell genetic program. Further investigation into the genetic changes at the molecular level during NK cell differentiation will help to clarify the gene programs activated during NK ontogeny, and the transcription factors involved in this process.
Lodolce et al. recently generated mice with targeted disruption of the IL-15Rα, providing direct evidence that the IL-15/IL-15Rαβγ system is critical for murine NK cell development. These mice contain multiple defects in innate immune effectors, including an absence of splenic NK cells and NK cytotoxic activity. Moreover, IL-15−/− mice also lack any phenotypic or functional NK cells in the spleen and liver, a defect that is reversible upon administration of exogenous IL-15 for one week. Exogenous IL-15 treatment of normal mice increases NK cell activity and both the percentage and absolute number of splenic NK cells. Thus, in vivo evidence demonstrates that IL-15 is requisite for murine NK cell development, and exogenous IL-15 supports the differentiation of human NK cells in BM culture systems. In Chapter 4, the phenotype of transgenic mice that over-express murine IL-15 is described, providing novel information about the effects of prolonged IL-15 exposure in vivo. Consistent with the phenotype of IL-15−/− and IL-15Rα−/− mice these mice have early expansion in NK cells. These basic observations provided invaluable insight into the critical, non-redundant role of IL-15 during NK cell development, and suggest potential utility of IL-15 therapy to expand NK cells in patients. Further, by identifying additional stromal cell factors, as described in Chapter 2, combination growth factor therapy may yield more consistent and prolonged NK cell expansions.

1.4.10 IL-15 Activates NK Cell Proliferation, Cytotoxicity, Cytokine Production and Regulates NK Cell/Macrophage Interaction

NK cells constitutively express cytokine receptors and produce abundant cytokines and chemokines during the early, innate immune response to infection. Such NK cell-derived
immunoregulatory factors may be vital in the orchestration of the innate immune response, as well as influence the developing adaptive response.\textsuperscript{4,11,130} NK cells constitutively express IL-2/15R\(\beta\)\(\gamma\), but the lack of abundant IL-2 during the early innate immune response suggests that IL-15 is the more important physiologic ligand for NK cell proliferation, cytotoxicity, and cytokine production in this setting.

\textbf{1.4.10.1 NK cell proliferation and cytotoxicity}

IL-15 induced the proliferation of CD56\textsuperscript{bright} NK cells in a dose-dependent fashion to a similar extent as IL-2, yet required a nanomolar concentration to activate the IL-2/15R\(\beta\) for proliferative activity.\textsuperscript{33} However, picomolar amounts of IL-15 were effective at maintaining NK cell survival in serum free media.\textsuperscript{89} IL-15 was found to activate cytotoxicity and antibody dependent cellular cytotoxicity (ADCC) by sorted CD56\textsuperscript{bright} and CD56\textsuperscript{dim} human NK cell subsets.\textsuperscript{33} Incubation of purified, resting CD56\textsuperscript{dim} NK cells with IL-15 resulted in significant, dose-dependent increases in lymphokine activated killing (LAK) activity against the NK resistant COLO 205 cell line, and ADCC against the P815 murine mastocytoma cell line. IL-15 and IL-2 induce nearly identical levels of cytotoxicity, and both depend upon signals through the IL-2/15R\(\beta\), as enhanced NK activity was abrogated in the presence of an anti-IL-2/15R\(\beta\) mAb.\textsuperscript{33} Infection of human PBMC with herpesviruses resulted in endogenous, IL-15-dependent increases in NK cell cytotoxicity, suggesting that IL-15 participates in normal innate host defense against viral infections.\textsuperscript{131,132}
1.4.10.2 IL-15 costimulates NK cell cytokine and chemokine production and regulates interactions between macrophages and NK cells

IL-15 acts in concert with IL-12 to induce the macrophage-activating factors IFN-γ and TNF-α, while IL-15 alone appears a potent stimulus for GM-CSF production by resting CD56⁺ human NK cells. Interestingly, human CD56bright NK cells stimulated with IL-15 plus IL-12 produce approximately 10-fold greater amounts of IFN-γ protein, compared to an equal number of CD56dim NK cells (ref. 135). Thus, the CD56bright NK cell may have a unique biological role for the production of immunoregulatory cytokines within the innate immune system.

Macrophages activated with lipopolysaccharide (LPS), IFN-γ, mycobacteria, Toxoplasma gondii, C. neoformans and Salmonella have been shown to express IL-15. In human co-cultures of LPS-stimulated macrophages and NK cells, endogenous IL-15 produced by the activated macrophages, working in concert with IL-12, was shown to be critical for optimal IFN-γ production by human NK cells. In mice, both in vitro and in vivo. This novel data is the first to demonstrate a requisite role for IL-15 as a costimulator of proinflammatory cytokine release in vivo. Therefore, in both mice and humans, activated macrophages and NK cells interact through a paracrine feedback loop, with macrophages producing monokines (e.g. IL-15 and IL-12), that bind to surface receptors constitutively present on NK cells, resulting in the production of macrophage activating factors (e.g. IFN-γ). NK cell-derived macrophage activating factors in turn feed back upon the macrophage to further augment their activation.
Thus, macrophage-derived IL-15 contributes with other monokines (especially IL-12) to the pro-inflammatory cascade leading to innate immune IFN-γ production. Additionally, Ross et al. demonstrated that continuous stimulation of CD56+ NK cells with IL-15 + IL-12 in vitro results in NK cell apoptosis mediated through an autocrine TNF-α-dependent mechanism, initiated after 24 hours of monokine stimulation. This may represent one means whereby the innate immune system limits itself after prolonged activation.\textsuperscript{133}

NK cells also produce the C-C chemokines MIP-1α and MIP-1β, following stimulation with IL-15, which is augmented with the addition of IL-12.\textsuperscript{134,138,139} As C-C chemokines also serve as chemoattractants for NK cells,\textsuperscript{140} IL-15 + IL-12-induced MIP-1α and MIP-1β production may be one mechanism to properly traffic additional NK cells to the site of infectious insult. In addition, chemokine production may have implications in the interactions between macrophages and NK cells, as MIP-1α has been shown to potentiate IFN-γ-inducible secretion of inflammatory cytokines by macrophages such as IL-1β.\textsuperscript{141}

Further, NK cells costimulated with IL-15 + IL-12 produce soluble factors (including C-C chemokines) that inhibit HIV-1 replication in vitro.\textsuperscript{139} The cytokine and chemokine profiles produced by human NK cells in response to monokines IL-15 + IL-12 were recently compared to that induced by monokines IL-18 + IL-12.\textsuperscript{135} Alone, IL-18, IL-15 or IL-12 induced little or none of the cytokines examined, with the exception of IL-15-induced GM-CSF, MIP-1α, and MIP-1β. IL-18 + IL-12 induced extremely high amounts of IFN-γ by CD56+ NK cells, with the majority produced by the CD56\textsuperscript{bright} NK cell subset. IL-15 + IL-12 induced moderate amounts of IFN-γ, but was also the optimal
stimulus for IL-10, TNF-α, GM-CSF, MIP-1α and MIP-1β. This suggests that NK cell cytokine production may be governed in part by the monokine milieu (including IL-15) induced during the early pathogen-dependent response to infection, as well as the NK cell subset present at the site of inflammation.  

As described above, there is now in vitro and in vivo evidence for IL-15’s participation in the early innate immune cytokine response, especially those resulting in IFN-γ production by NK cells. Thus, IL-15 acts as a costimulator of IFN-γ production by NK cells and may therefore be important to the control of infections that require IFN-γ for clearance. Additional in vivo studies of IL-15’s role during such infections may yield information to direct therapies aimed at boosting the immune response to infection or decreasing improper immune activation or inflammation. As mice genetically targeted to delete functional IL-15 or IL-15Ra gene products concurrently lack NK cells, it will be difficult to assess the role of IL-15 during the innate immune response in these models. Utilization of experimental systems that neutralize IL-15, such as those in Chapter 3, will be important to complement such IL-15/IL-15R knockout mice.

1.4.11 IL-15 and Uterine NK cells

In mice, the metrial gland is a uterine tissue that develops adjacent to the placenta, and contains granulated metrial gland (GMG) cells that are phenotypically and functionally uterine NK cells. Similar NK-like cells have also been identified in the human uterus, and have the phenotype CD56[^142] CD16[^143,144]. These NK cells are located at the maternal/fetal junction and are thought to play a role in regulating implantation and
trophoblast invasion, but the factors governing their localization and/or differentiation are unclear. Uterine NK cells express IL-15R components, and IL-15 increases expression of NK cytolytic mediators (perforin and granzymes) in pregnant uterine tissue explanted in vitro, as well as in primary mouse uterine NK cells. Moreover, IL-15 (but not IL-2) was detected in uterine macrophages, amnion, chorion, and decidual cells, suggesting a possible source of IL-15 during pregnancy. IL-15 expression was shown to peak in the mid-secretory phase of normal human menstruation, and was upregulated during progesterone-induced decidualization. As IL-15 costimulates blood NK cell cytokine production, IL-15 may also be involved in cytokine production by uterine NK cells, which have been proposed to regulate placental changes during pregnancy. Thus, IL-15 and IL-15R components are expressed in murine and human uterine tissues and may play a role in the differentiation or homeostasis of specialized uterine NK cells. Further studies may illuminate potential roles for abnormal IL-15/IL-15R expression during pathological pregnancies.

1.4.12 IL-15 Regulation of Monocyte/Macrophages and Neutrophils

Human macrophages express high affinity binding sites for IL-15, that are upregulated upon activation with LPS. Monocytes have been shown to express the IL-2/15Rβ, γc, and the IL-15Rα, suggesting the possibility for IL-15 to act in an autocrine fashion. Human monocytes treated with IL-15 (10-1000 ng/ml) produced IL-8 and MCP-1, that was chemotactic for neutrophils and monocytes, respectively. Alleva et al. demonstrated autocrine IL-15 regulation of macrophage proinflammatory cytokine production that was highly dependent upon the concentrations of IL-15 available to the
macrophage. Extremely low (picomolar to attomolar) concentrations of IL-15 suppressed macrophage proinflammatory (TNF-α, IL-1, IL-6) cytokine production, while at high concentrations enhanced production of these mediators.

Human neutrophils express the IL-2Rβ and γc subunits of the IL-2/15R complexes, as well as IL-15Rα. IL-15 activates human neutrophils, with effects including morphological changes, increased phagocytosis, increased de novo RNA and protein synthesis, delayed apoptosis, and stimulation of the growth-inhibitory effects of neutrophils against C. albicans. Overall, these studies further suggest that IL-15 may have a role establishing innate immune responses, and maintaining neutrophil-mediated inflammatory processes. However, defects in these cell types were not reported in IL-15Rα<sup>-/-</sup> or IL-15<sup>-/-</sup> mice, suggesting that IL-15/IL-15R interactions are not critical to their development or expansion.

1.4.13 IL-15 Plays a Role in the Development, Homeostasis, and/or Activation of TCRγδ Dendritic Epidermal T Cell, Intestinal Intraepithelial Lymphocytes, and NK-T Cells

As IL-15 binds to and signals through the shared IL-2/15Rβγc, cell-types that respond to ligation of these receptors with IL-2 were logical potential targets for IL-15. In addition to NK cells, several innate immune T cell populations have been shown to express the IL-2/15Rβ and/or the γc. These include dendritic epidermal TCRγδ T cells (DETC), intestinal intraepithelial lymphocytes (i-IEL), and NK1<sup>+</sup> T cells (NK-T cells).
DETC are a skin-specific member of the TCRγδ T cell population that migrate to the skin during fetal life in mice. The DETC population in skin was absent following treatment with anti-IL-2/15Rβ in utero, yet are present in normal numbers in IL-2−/− mice. Further, Vγ3 DETC are absent in IL-2/15Rβ−/− mice, and γc−/− mice. IL-15 (but not IL-2) expression was detected in fetal skin, during the time frame that DETC traffic to the skin microenvironment. Thy-1+ murine DETC proliferated in response to IL-15, following activation with mitogens that induced IL-15R components. When low concentrations of exogenous IL-15 were added to NK/T progenitors in alymphoid fetal thymic organ cultures (FTOC), the greatest expansion occurred in the Vγ3 DETC compartment. The status of Vγ3 DETC was not reported in IL-15Rα−/− or IL-15−/− mice. Collectively, these studies suggest that IL-15 is critical to DETC growth and survival following activation, and may be important in selective localization of these cells in the skin.

Intestinal intraepithelial lymphocytes (i-IEL) consist of both TCRαβ and TCRγδ T cells located at the basolateral surfaces of intestinal epithelial cells, and are thought to play a key role in mucosal immunity. TCRγδ i-IEL lymphocytes express the CD8αα homodimer, while TCRαβ express CD8αβ, CD8αα, or CD4. Thy-1+CD8αα+ i-IEL are thought to develop extrathymically, express the IL-2/15Rβγc receptor components, yet are able to proliferate in an IL-2-independent fashion. Thy-1+CD8αβ+ or CD4+ i-IEL are thought to depend on the thymus for development.
TCRγδ i-IEL were shown to express IL-15Rα mRNA, proliferate in response to IL-15, and IL-15 protected these cells against growth factor deprivation-induced apoptosis through the upregulation of Bcl2.\textsuperscript{169} Listeria monocytogenes infection of rats resulted in increased IL-15 mRNA within intestinal epithelial cells that correlated with i-IEL accumulation and IFN-γ production.\textsuperscript{170} Further, mice with targeted disruption of the IL-2/15Rβ\textsuperscript{110} or γc\textsuperscript{171} have a dramatic reduction TCRγδ and TCRαβ/CD8αα i-IEL. Mice deficient in IRF-1 (IRF-1\textsuperscript{−/−}) fail to induce IL-15 expression, and exhibit a similar deficit in CD8αα i-IEL.\textsuperscript{83} A definitive demonstration of the requisite IL-15/IL-15R complex participation in the development of CD8αα i-IEL was provided by mice with targeted disruption of the IL-15Rα and IL-15. IL-15Rα\textsuperscript{−/−} mice had 2-fold reduction in total i-IEL, and a 5- to 10-fold reduction in TCRγδ CD8αα i-IEL.\textsuperscript{35} IL-15\textsuperscript{−/−} mice had a 2-fold decrease in total i-IEL numbers, a 2-fold increase in the ratio of TCRαβ:TCRγδ i-IEL, and a dramatic reduction in TCRαβCD8αα i-IEL.\textsuperscript{34} IL-15 also stimulates the proliferation, cytotoxicity, and IFN-γ production by human TCRγδ IEL.\textsuperscript{171} Thus, IL-15 plays a critical role in the expansion and function of i-IEL and DETC cells, thereby contributing to mucosal immune defense. While, IL-15Rα\textsuperscript{−/−} and IL-15\textsuperscript{−/−} mice demonstrate changes in DETC and i-IEL TCRγδ T cells, there are no apparent numerical or phenotypic defect in other TCRγδ compartments.\textsuperscript{34,35} However, these results do not preclude a role for IL-15 in TCRγδ proliferation and cytokine production in response to infectious pathogens.\textsuperscript{136,172-174}

Murine T cells that exhibit a restricted TCR repertoire (invariant Vα14 with Vβ8, 2 or 7) that co-express NK1.1 and TCRαβ have been defined as NK1\textsuperscript{+} T cells (or NK-T
Analogous NK-T cells have also been described in humans. Similar to NK cells, NK-T cells lyse MHC class I and YAC-1 target cells, express NK1.1, NKRPs, and the IL-2/15Rβ, and are readily detectable in athymic mice. Murine NK-T cells proliferate in response to IL-15, and their numbers are severely reduced in IL-2/15Rβ−/−, IRF-1−/−, IL-15Rα−/− and IL-15−/− mice, yet are normal in IL-2−/− mice. The few NK-T cells present in IL-15−/− mice underwent normal thymic selection, suggesting that IL-15 is important for the expansion, survival, or functional maturation of committed NK-T cells. Therefore, NK-T cells depend upon IL-15/IL-15R complex mediated signals for expansion and/or homeostasis, in contrast to NK cells that appear to have an absolute requirement for IL-15/IL-15R for development.

1.4.14 Function of IL-15 on TCRαβ T cells

IL-15 was first identified as a T cell growth factor through its ability to promote the proliferation of CTLL cells and mitogen-stimulated T cells in a fashion similar to exogenous IL-2. Of note, resting T cells do not appear to respond to IL-15, and it is likely that TCR ligation induces the expression of IL-15Rα, conferring responsiveness to IL-15. Such action on T cells suggests that IL-15 expressed by APCs may be important for the early activation of T cells at sites of inflammation, immediately following TCR ligation. Stimulation of TCR-engaged T cells with IL-15 has been shown to induce various activation antigens such as IL-2Rα (CD25), IL-2/15Rβ (CD122), FasL (CD95), CD30, TNFRII, CD40L, CD69 and CD94/NKG2A, while down-regulating the IL-15Rα. The in vitro proliferation of resting T lymphocytes in response to anti-CD3 plus IL-15 was greatly reduced in IL-15Rα−/− lymphocytes, compared to wild type, 31
supporting the idea that IL-15 requires induction of the IL-15Rα to optimally stimulate resting T cells. Further, IL-15Rα^− T cells showed a lower response to CD3 ligation plus IL-2, suggesting that IL-15Rα may be important for the induction of IL-2Rα and hence responsiveness to IL-2. IL-15 also protected ConA-activated human T lymphoblasts from undergoing apoptosis following Fas or CD3 cross-linking, or treatment with dexamethasone in vitro and in vivo.

IL-15 acts as a potent chemoattractant for T cells isolated from human blood. This observation has led to a number of studies examining the role of IL-15 during chronic inflammatory and autoimmune diseases such as rheumatoid arthritis (see below). IL-15Rα^− mice have defects in T cell homing to peripheral lymph nodes, providing additional evidence that IL-15 has a physiologic role in T cell trafficking in vivo. IL-15 may also operate through indirect mechanisms such as chemokine production and activation of human endothelial cell expression of hyaluronan, to regulate T cell trafficking.

Jonuleit et al. demonstrated that unstimulated human DC cultures produced low levels of IL-15 protein that was detectable by ELISA and CTLL bioassay, and stimulated-DC supernatants were shown to be chemoattractant for T cells. The production of IL-15 protein following phagocytosis or CD40 ligation provides an intriguing mechanism for initial attraction and stimulation of T cells in the absence of IL-2, as is likely the case for other APCs, such as macrophages.
IL-15 stimulated the proliferation of human memory (CD45RO⁺) CD4 and CD8, and naïve (CD45RO⁻) CD8⁺ human T cells in vitro, while having no effect on naïve CD4 T lymphocytes, consistent with IL-2/15Rβ expression. Zhang et al. documented selective high IL-2/15Rβ expression on CD44hiCD8⁺ memory T cells in mice, and IL-15 selectively stimulated this cell type both in vitro and in vivo. In Chapter 4, data is provided that documents an increase in memory-phenotype CD8⁺TCRαβ T cells in the peripheral blood and spleen. Further, in mice transgenic for the LSP-IL-15 cDNA under the control of an MHC class I promoter, CD44hiLy-6C⁺CD8⁺ memory-phenotype T cells were increased in lymph nodes. IL-15Rα⁻/⁻ mice have a selective deficit in CD8⁺ T cell numbers in both the thymus and periphery. Specifically, thymic CD8⁺ single positive and peripheral CD8⁺CD44hi and CD44int lymphocytes were reduced. IL-15⁻/⁻ mice have reduced numbers of memory-phenotype CD8⁺ T cells in the spleen and lymph nodes, that was reversible upon provision of exogenous IL-15. Because IL-15⁻/⁻ mice had normal numbers of single positive CD8 thymocytes, IL-15 may not be requisite for the development of CD8⁺ T cells, but critical for their expansion or survival. The subtle differences in the thymic CD8 single positive cells between IL-15Rα and IL-15 knockout mice warrants additional investigation. IL-15⁻/⁻ mice maintained good health when housed under specific pathogen free conditions, however demonstrate a dramatic lethal sensitivity to vaccinia virus infection, compared to control mice. As both NK cells and CD8⁺ T cells are important for protection against vaccinia, failure to mount a protective host response is likely due to the deficiencies in these lymphocyte populations. Examining IL-15⁻/⁻ in various models of infectious disease may provide novel information about the requirement for these cell types in host defense.
1.4.15 Function of IL-15 on Non-Immune Cells

IL-15 expression has been detected in numerous tissues, many not sites of immune responses, indicating the potential for additional non-immune functions.27 Indeed, IL-15 also affects cells outside of the immune system. IL-15 serves as an anabolic agent for skeletal muscle,194 and may support muscle cell differentiation.195 Intestinal epithelial cells signal and proliferate in response to IL-15 in vitro.77 Vascular endothelial cells express IL-15Rαβγ mRNA, respond to IL-15 with intracellular signals, and IL-15 induced angiogenesis in vivo.196 However, IL-15Rα and IL-15γ−/− mice do not appear to have defects in muscle, bone, or vasculature suggesting that IL-15 is not critical to the development or function of these non-immune tissues.34,35 IL-15’s broad, pleiotropic effects on multiple tissues and cell types outside of the immune system are unusual for a cytokine, and further understanding of how IL-15 may affect non-immune cells could provide information relevant to the design of potential therapeutic applications.

1.5 Relevance of IL-15 to Human Disease

1.5.1 Role of IL-15 in Autoimmune and Inflammatory Disease

1.5.1.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic degenerative condition of synovial membranes, which is thought to be mediated in part by aberrant cytokine regulation that ultimately results in abnormally high levels of proinflammatory cytokines, such as TNF-α, within joints. Current hypotheses suggest that abnormal T cell trafficking to joints may be a key early step in this process.197 Based upon work describing IL-15 as a potent T cell
Mclnnes et al. suggested that IL-15 may play a primary role in the development of RA. IL-15 protein was demonstrated in the synovial fluids and synovial membranes of patients with active RA. Synovial fluids were found to promote the chemoattraction and activation of T cells, which was partially abrogated by addition of anti-IL-15 antibodies. In addition, injection of a single dose of IL-15 resulted in a lymphocytic inflammatory infiltrate in vivo.

The presence of multiple chemoattractants in synovial fluid, including IL-15, IL-8, MCP-1, and MIP-1α suggest some redundancy in the factors responsible for T cell extravasation into RA synovial membranes. Subsequent studies also demonstrated that IL-15-activated T cells from RA patients stimulated macrophage cell lines and primary monocyte/macrophages to produce TNF-α in vitro. This effect was cell-contact dependent, and antibodies to CD69, LFA-1, and ICAM-1 inhibited the T cell-induced production of TNF-α by macrophages. An increased expression of IL-15 protein in the synovium of RA, compared to osteoarthritis patients, has also been documented.

In the mouse, administration of soluble IL-15Rα prevents collagen-induced arthritis, suggesting that development of effective IL-15 blocking agents such as soluble receptors or monoclonal antibodies may be useful in the treatment of RA.

1.5.1.2 Sarcoidosis

Sarcoidosis is a chronic granulomatous condition of unclear etiology that progressively affects multiple organs, especially the lungs. Agostini et al. suggested a potential role for IL-15 during the pathogenesis of pulmonary sarcoidosis as alveolar macrophages...
isolated from patients with active sarcoidosis expressed IL-15 mRNA and cytoplasmic or membrane IL-15 protein, while patients with inactive disease or normal donors did not. In addition, CD4+ T cells isolated from bronchoalveolar lavage of active sarcoidosis patients expressed components of the IL-2/15R complexes and proliferated in response to IL-15, suggesting that macrophages may provide a proliferative signal to T cells in the lungs during this disease process.

1.5.1.3 Inflammatory bowel disease

Two major types of inflammatory bowel disease (IBD) commonly occur: ulcerative colitis (UC) and Crohn's disease (CD), and are thought to be inappropriate chronic inflammatory processes. Kirman et al. demonstrated that patients with UC or severe CD had an increased percentage of IL-15 protein expressing PBMC, which was decreased following successful symptomatic treatment. Serum IL-15 was detectable in several UC patients with moderate to severe disease (5/8 cases, range 0-490 pg/ml), but was not detectable in CD patients or normal donors. In vitro, LPS activation of UC or CD patients' PBMC resulted in further increases of intracellular IL-15 protein levels. IL-15 protein was detected from supernatants of rectal mucosal biopsies, and IL-15 mRNA was detected in macrophages and epithelial cells by in situ hybridization, from patients with active CD/UC, but not from normal controls. Further, lamina propria mononuclear cells from IBD patients proliferate in response to rIL-15. Recent studies have confirmed IL-15 protein production by macrophages in the mucosa of IBD patients, and provided evidence for T cell modulation by IL-15 in this setting. These studies
suggests that IL-15 released during chronic bowel inflammation may contribute to the pathogenesis of UC, and possibly CD.

1.5.1.4 Other autoimmune or inflammatory diseases

Increased production of IL-15 during several other chronic inflammatory conditions has also been observed, including hepatitis C-induced liver diseases and multiple sclerosis. Elevated levels of serum IL-15 protein have been measured in Hepatitis C virus-infected patients with chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma, compared to asymptomatic carriers and normal controls. The highest levels of IL-15 were found in hepatocellular carcinoma patients, who had significantly higher IL-15 levels compared to all other groups studied (n=11, mean ± SD: 77.4 ± 78 pg/ml, \( P < 0.05 \)). Treatment with IFN-α resulted in resolution of liver inflammation measured by transaminase levels, concurrent with suppression of serum IL-15 levels.

Multiple sclerosis (MS), an inflammatory disease of the central nervous system, is thought to have an etiology involving autoimmunity and cytokine dysregulation. MS patients were found to have higher numbers of IL-15 mRNA-expressing blood mononuclear cells compared to patients with aseptic meningo-encephalitis \( (P < 0.02) \) or healthy controls \( (P < 0.01) \). Increased numbers of IL-15 mRNA expressing cells were found in the CNS, compared to the blood in MS patients. In addition, patients with chronic-progressive MS had higher IL-15 expression compared to relapsing-remitting MS. Another study confirmed this, and correlated it with the duration and durability of MS. The role of IL-15 in these diseases needs to be further evaluated in appropriate animal models.
1.5.2 IL-15 and Transplant Rejection

The immunologic rejection of allografted solid organs by the recipient is a complicated process that includes acute, subacute, or chronic subtypes, and is a major obstacle in transplant medicine. Cytokines are thought to contribute to allograft rejection by promoting the infiltration and activation of recipient immune cells within the transplanted organ. IL-15 mRNA expression was detected in 45/45 biopsies from transplanted kidneys (IL-2 in only 3/45), and was significantly increased in rejecting, compared to non-rejecting grafts. While IL-15 mRNA expression was detected in the majority of post-transplanted livers, and upregulated compared to non-transplanted liver tissue, no significant correlation was found between IL-15 expression and rejection. The failure of an antibody (BT563) that selectively blocks the high affinity IL-2Rα pathway to prevent acute rejection of heart allografts suggests that other cytokines, such as IL-15, may be responsible for the T cell proliferation during this process.

Further studies demonstrated IL-15 mRNA and protein expression within CD68+ macrophages infiltrating transplanted myocardium, with or without anti-IL-2Rα treatment. While the percentage of IL-15+ cells invading the grafts did not directly correlate with rejection grade, these studies suggest that IL-15 may be involved in T cell activation during heart allograft rejection in the absence of IL-2. Li et al. observed that mice with targeted disruption of IL-2 and IL-4 (IL-2+/IL-4+ double knockout) rejected islet allografts, with robust intragraft expression of both IL-15 and IL-7. In this animal model, blockage of the γc receptor significantly prolonged survival, suggesting that signals mediated by IL-15 or IL-7, in the absence of IL-2 and IL-4, participate in acute
graft rejection. Collectively, these studies demonstrate a potential role for IL-15 as a therapeutic target during the rejection of transplanted solid organs that requires further investigation.

1.5.3 IL-15 and Cancer

1.5.3.1 HTLV-1-mediated adult T cell leukemia

Infection with HTLV-1 can result in adult T cell leukemia (ATL), a malignancy in which the early phases are associated with autocrine production of IL-2 and expression of IL-2R components. However, later phases lack IL-2 production while IL-2R components remain on the surface of these leukemic cells. As described above, IL-15 was co-discovered as the IL-T fusion protein involving the R region of HTLV-1 fused with the 5' UTR of the IL-15 gene, and the ATL cell line Hut-102 produced abundant IL-T/IL-15 protein within cell culture supernatants. Later it was discovered that HTLV-1 Tax protein transactivates IL-15 gene transcription through a NF-κB site, and cell lines infected with HTLV-1 or transfected with HTLV-1 Tax had significantly higher IL-15 mRNA levels. In addition, IL-15 mRNA expression is elevated in ex vivo leukemic cells from ATL patients, compared to normal peripheral blood T cells. Yamada and coworkers demonstrated that ATL cell lines expressed IL-15Rα, bound [125I]IL-15 with high affinity (170 pM), and proliferated for months in response to rhIL-15. However, these cell lines did not produce measurable IL-15 protein in cell culture supernatants, and plasma from ATL patients (n=31) did not contain detectable IL-15 protein with a few exceptions (4.9 pg/ml, 1.7 pg/ml, and 1.7 pg/ml). Thus, the unique property of abundant
IL-15 protein secretion by Hut-102 cells does not appear to be a general phenomenon of all ATL cell lines or primary leukemic cells isolated from ATL patients.

Based upon these studies, the authors suggest a possible role for IL-15 in the pathogenesis or maintenance of ATL, especially during the relatively common infiltration into IL-15-expressing tissues such as the skin, lungs, liver and GI tract. This hypothesis is consistent with findings that in later IL-2-independent stages of ATL, there is constitutive activation of IL-2/15R signals Jak1/Jak3 and STAT3/STAT5. Further study of IL-15 in patients with ATL are warranted and will be required to definitively determine whether IL-15 may participate in the initiation, maintenance, or progression of this disease.

1.5.3.2 Other lymphoid malignancies

Cutaneous T cell lymphoma (CTCL) broadly delineates a group of related lymphoproliferative disorders of the skin, including mycosis fungoides (MF) and the Sezary syndrome (SS). Both IL-2 and IL-7 have been implicated as an important growth factors for CTCL cells, however the etiology of these disorders is actively being investigated. Indeed, treatment of these patients with an IL-2-fusion toxin, thereby targeting IL-2/15Rβ+ cells, appears promising. Dobbeling et al. demonstrated that IL-15 may act as a growth and viability promoting factor for CTCL. IL-15 promoted growth and prolonged survival of the SeAx Sezary cell line as well as malignant T cells isolated from SS patients, and IL-15 mRNA was present within these cells. IL-15 protein was detected by immunohistochemistry in the basal cell layer keratinocytes and in the infiltrating lymphocytes of SS and MF patients’ skin biopsies. IL-15 was also shown
to stimulate AP-1 and JunD DNA binding within CTCL cell lines, and may utilize such pathways to promote CTCL growth. Of note, HTLV-1 infection has also been implicated in such CTCL, consistent with the above involvement of HTLV-1/ATL and IL-15 expression. In agreement with IL-7's ability to promote CTCL growth in vitro, transgenic mice engineered to over-express IL-7 develop cutaneous lymphoproliferation and lymphoma. Intriguingly, mice with a global over-expression of a human IL-2 transgene, via a MHC class I promoter, develop a mild cutaneous lymphocytic infiltration. However, this phenotype is not lethal or transforming. In Chapter 4, transgenic mice that globally over-express IL-15 develop are shown to develop a lymphocytic skin infiltrate with alopecia, and eventually leukemia.

Lymphoproliferative disorder of granular lymphocytes (LDGL) is characterized by an expansion of LGL consisting of either CD3+ (T cell) or CD3- (NK cell) populations. Zambello et al. showed that both CD3+ and CD3- cells isolated from LDGL patients expressed all three of the IL-15 receptor components: IL-2/15Rβ, γc, and IL-15Rα, and proliferated in response to IL-15 in vitro. While no IL-15 protein was detected in the serum or CD14+ cell culture supernatants of these cells, membrane bound IL-15 was demonstrated on the surface of CD3+ and CD3- cells from LDGL patients, but not normal controls, by flow cytometry. This is in contrast to the lack of IL-15 mRNA expression by RT-PCR in sorted LGL from patients, leaving the significance of surface IL-15 detected by flow cytometry unclear. However, the chronic nature of this leukemia and the clear role of IL-15 in the development of CD3+ NK-T and CD3- NK cells would suggest that the possible deregulation of IL-15 expression in these patients should be pursued.
Indeed, in Chapter 4 the phenotype of IL-15 transgenic mice supports a role for IL-15 in the pathogenesis of chronic lymphocytic leukemia, especially LGL leukemia (see below).

IL-15 has also been shown to induce the proliferation of normal B cells, malignant B cells obtained from B cell chronic lymphocytic leukemia (B-CLL) and hairy cell leukemia (HCL), and the M-07e AML cell line. IL-15R components are expressed on multiple myeloma cells, and IL-15 may play a role in the autocrine propagation of this malignancy.

1.5.3.3 Solid tumors

IL-15 is normally expressed at the mRNA level in several organs including skeletal muscle, skin, lung, and kidney. Their malignant counterparts, including osteosarcoma, Ewing's, rhabdomyosarcoma, melanoma, small cell lung cancer, renal cell carcinoma, glioblastoma, neuroblastoma, and mesothelioma, also express the IL-15 transcript. Intracellular protein was observed in melanoma cell lines and renal cancer cells, and low secretion of IL-15 was observed in a subset of osteosarcoma and rhabdomyosarcoma lines (4-8 pg/ml). However, a definitive role for IL-15 in the pathogenesis of solid tumors has not been demonstrated.

1.5.4 IL-15 and Infectious Disease

1.5.4.1 Human immunodeficiency virus (HIV)

IL-15, through its ability to mimic the actions of IL-2, has two opposing effects in the HIV-infected patient: a potential beneficial augmentation of immune function.
and a potential detrimental activation of HIV replication. As both of these effects have been observed in several experimental systems, any proposed use of IL-15 in vivo to boost immunity in this immunocompromised population must include careful analysis of HIV replication. Initial studies by Seder et al. demonstrated that IL-15 enhanced the proliferative response of PBMC from HIV-infected patients when stimulated with polyclonal mitogens, tetanus toxoid, or HIV-specific antigens. Patki et al. showed that while IL-2 enhanced both spontaneous and antigen-induced lymphocyte proliferation in HIV-infected patients, IL-15 enhanced antigen-induced lymphocyte function only with significantly less HIV-1 replication. IL-15, alone and in combination with IL-12, enhanced deficient in vitro NK cell activity in HIV-1 infected patients, and IL-15 + IL-12-stimulated NK cells isolated from HIV-infected patients produced C-C chemokines and inhibited HIV-1 infection in vitro. Thus, a large body of evidence now suggests that IL-15 significantly improves innate and antigen-specific immune cell function in HIV-1-infected patients in vitro, in some cases better than IL-2. While in vitro evidence documents that IL-15 may increase HIV-1 replication in PBMC and cell lines in vitro, it remains unclear whether the benefits of IL-15 may be achieved at concentrations that do not effect viral loads in vivo. Encouragingly, IL-2 has been used successfully in vivo at low doses to boost immune function in HIV-infected patients, without increases in HIV viral load. Thus, IL-15 is poised as a potential candidate for cytokine therapy in HIV-1 patients that may provide the benefits of IL-2, and additional immune modulation because of distinct IL-2/15Raβγ expression patterns.
1.5.4.2 Other viral and bacterial pathogens

IL-15 mRNA levels are elevated in PBMC from patients with another HTLV-1-mediated disease, HTLV-I Myelopathy/Tropical Spastic Paraparesis (HAM/TSP), compared to normal donors. Antibodies against IL-15 or its receptor blocked spontaneous proliferation of HAM/TSP PBMC, and when combined with anti-IL-2 antibodies, completely abrogated proliferation. This suggests that dual autocrine loops, including both IL-15/IL-15R and IL-2/IL-2R, may promote lymphocyte proliferation in this disorder. In vitro infection of human PBMC with human herpes virus (HHV)-6, HHV-7, HSV-1, or EBV resulted in IL-15-dependent increases in NK cell cytotoxicity and IFN-γ production. Such studies suggest that IL-15 may be involved in early, innate immune defense against viral infections in humans.

Serum levels of IL-15 were significantly elevated in patients with bacteremic (mean 49.4, range 12.4-338.8 pg/ml) and non-bacteremic (mean 31.3, range 11.6-2743 pg/ml) *Burkholderia pseudomallei* infection (melioidosis), compared to controls (mean 12.8, range <8.2-122.2 pg/ml), with significantly higher levels in patients with positive blood cultures. Several reports have indicated that exogenous administration of rIL-15 prior to infection, such as *Salmonella*, *P. falciparum*, or *C. neoformans*, improves host defense against, and clearance of the invading organism. Nishimura et al. generated two lines of transgenic mice that express both isoforms of IL-15 under the control of an MHC class I promoter. LSP-IL-15 transgenic mice expressed serum IL-15 protein and had increased memory-phenotype CD8+ T cells in lymph nodes. These mice were resistant to *Salmonella* infection via a mechanism dependent upon CD8+ T cell IFN-γ production. In
contrast, mice transgenic for the SSP-IL-15 expressed intracellular IL-15 protein, had
defective T cell IFN-γ production, and were susceptible to *Salmonella* infection. Thus, it
appears that in vivo, over-expression of the LSP-IL-15 leads to IL-15 secretion, enhanced
CD8+ memory type cell development and enhanced host-defense against *Salmonella*,
while over-expression SSP-IL-15 remains intracellular and limits T cell IFN-γ
responses.47 rIL-15 has been successful as a vaccine adjuvant in animals, through
boosting the production of *Toxoplasma gondii*-specific CD8+ T cells.256 Continued
exploration of additional animal models of infection, as well as delineation of the
mechanism(s) of the action of IL-15 during host defense are needed to fully understand
its role during both innate and specific immune responses.

1.5.5 IL-15 and Toxicity

For IL-15 to be considered for use as an immunomodulator in humans, it must be
determined whether such exogenous provision will result in toxicity for the patient.
While IL-15 administration (3 x 10^5 U/mouse/day) did not result in mortality when
provided alone to mice over the course of at least 10-14 days, direct co-administration of
IL-15 + IL-12 (1 ug/day) to mice results in a lethal cytokine-induced shock cascade,
indistinguishable from the lethal effects of IL-2 + IL-12.257 Depletion of NK cells or
macrophages prior to administration of IL-15 and IL-12 provided protection from
lethality, suggesting that these innate immune effector cells were the primary effectors
during this cytokine-induced shock. Selective elimination of various inflammatory
mediators (TNF-α, IFN-γ, MIP-1α, IL-1, IL-1 converting enzyme, Fas, perforin, iNOS,
or STAT1) utilizing mice with targeted disruptions or neutralizing antibodies revealed
that none of these, even in combinations, were responsible for the lethal shock-like reaction. Therefore it is possible that novel proinflammatory mediators are produced in this cascade. Of note, in this model modest doses of IL-15 only become toxic when combined with IL-12. The generalized Shwartzman reaction is a lethal cytokine-induced shock response elicited by sequential priming and challenge with bacteria or bacterial components (e.g. LPS). IL-12-induced IFN-γ is critical for sensitization of macrophages during LPS priming. After initial IFN-γ-dependent priming, a subsequent i.v. LPS challenge 18-24 h later results in cytokine-induced shock and death, largely due to the release of tumor necrosis factor (TNF)-α and IL-1. In Chapter 3, data is provided that IL-15 is a cofactor in IL-12-induced IFN-γ critical for the Shwartzman reaction.

Several chemotherapy agents (e.g. 5-fluorouracil [5-FU], irinotecan [CT-711]) have documented clinical efficacy against colon cancer, but severe mucosal toxicity remains a significant clinical problem. In rat models of colon carcinoma, exogenous administration of IL-15 protected against diarrhea, stomatitis, weight loss, and death induced by 5-FU and irinotecan, and increased maximal tolerated doses (MTD) and overall CR. In contrast, exogenous IL-2 potentiated toxicities with no beneficial anti-tumor effect when used in combination with either chemotherapy agent. While the clinical benefit observed in these two animal model systems requires additional study for confirmation of the apparent toxicity-limiting activity, these pre-clinical models suggest that IL-15 may be useful as a chemoprotective agent to certain therapies.
1.6 Conclusion

This introductory chapter has attempted to comprehensively review the extensive knowledge base accumulated on IL-15 over the past six years. The work presented in the following chapters focuses on several facets of IL-15 biology, and fills gaps in our knowledge as identified throughout the literature review above. First, Chapter 2 further examines the role of IL-15/IL-15R, in cooperation with other stromal cell factors, in the development of human NK cells from progenitors in the bone marrow. This Chapter extends the initial finding that IL-15 is produced by bone marrow stromal cells and alone can inefficiently differentiate NK cells from CD34+ HPCs. The study asks questions about the mechanism operative when class III RTK ligands (FL, KL) work in concert with IL-15 for the efficient differentiation of human NK cells, and provides a novel model of step-wise NK cell differentiation through NK progenitor and NK precursor developmental intermediates. Armed with such an in vitro model system, and the critical cytokine (IL-15) in the final differentiation step, new approaches to understanding the human NK cell genetic program are open for exploration.

While in vitro studies have shown that IL-15 can costimulate NK cell cytokine production (especially IFN-γ), in vivo evidence for this action was lacking. Chapter 3 utilizes simulated infection of SCID mice to perform such in vivo experiments utilizing antibodies that block IL-15 and the IL-15R. These findings open the doorway to explore the role of IL-15 within models of infection dependent upon IFN-γ for clearance, and point to novel therapeutic uses of IL-15 as a vaccine adjuvant, and potential immunotherapy for intracellular pathogens.
Finally, in an attempt to understand the importance of IL-15's tight posttranscriptional regulation, Chapter 4 describes the phenotype of mice that globally over-express an IL-15 transgene that lacks the three primary posttranscriptional checkpoints. This in vivo transgenic model shows that abundant IL-15 protein results initially in a benign lymphocytosis that consists mainly of NK cells and CD8⁺CD44hi T cells. After months of chronic over-production of IL-15, lymphocytic leukemia evolves from a background of expanded NK and CD8⁺ T cells. This model provides novel information demonstrating that IL-15 expands NK cells and memory-phenotype CD8⁺ T cells in vivo, complementing phenotypes of IL-15⁻/⁻ and IL-15Ra⁻/⁻ mice. Further, these mice provide the first evidence that the normal posttranscriptional regulation of IL-15 is critical for the homeostasis of the lymphocyte compartment.

Collectively, the studies described here provide novel information extending our knowledge of IL-15's role in the development (Chapters 2, 4) and function (Chapters 3, 4) of the innate immune system, especially NK cells. Based upon these findings, exciting new avenues of research are now available to better understand both the molecular and cellular biology of IL-15 and NK cells. Further, these studies in combination with those discussed above (Section 1.5) point toward potential clinical applications for IL-15 in the near future.
Table 1.1 Properties and components of the innate and adaptive immune systems.

<table>
<thead>
<tr>
<th></th>
<th>Innate</th>
<th>Adaptive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Diversity</td>
<td>Limited</td>
<td>High</td>
</tr>
<tr>
<td>Memory?</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Time of action within</td>
<td>Early</td>
<td>Late</td>
</tr>
<tr>
<td>immune response</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Physical Barriers</td>
<td>Skin, mucosal epithelium</td>
<td></td>
</tr>
<tr>
<td>Proteins in Blood</td>
<td>Complement, Defensins</td>
<td>Antibodies</td>
</tr>
<tr>
<td>Cellular Constituents</td>
<td>Natural killer cells</td>
<td>T lymphocytes</td>
</tr>
<tr>
<td></td>
<td>Macrophages</td>
<td>B lymphocytes</td>
</tr>
<tr>
<td></td>
<td>Dendritic cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Neutrophils</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eosinophils</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Basophils</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mast cells</td>
<td></td>
</tr>
</tbody>
</table>
Table 1.2  Cytokines that utilize the common γ chain.

While all five cytokines bind to and utilize the γc, only IL-2 and IL-15 share the IL-2/15Rβ for binding and signaling. A complete discussion of the IL-15 and IL-2 receptors is provided in Section 1.4.5.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Alpha</th>
<th>Beta</th>
<th>Gamma</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>IL-2Rα</td>
<td>IL-2/15Rβ</td>
<td>γc</td>
</tr>
<tr>
<td>IL-4</td>
<td>IL-4Rα</td>
<td></td>
<td>γc</td>
</tr>
<tr>
<td>IL-7</td>
<td>IL-7Rα</td>
<td></td>
<td>γc</td>
</tr>
<tr>
<td>IL-9</td>
<td>IL-9Rα</td>
<td></td>
<td>γc</td>
</tr>
<tr>
<td>IL-15</td>
<td>IL-15Rα</td>
<td>IL-2/15Rβ</td>
<td>γc</td>
</tr>
<tr>
<td>Mutation</td>
<td>Major phenotypes</td>
<td>References</td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>----------------------------------------------------------------------------------</td>
<td>------------</td>
<td></td>
</tr>
<tr>
<td>IL-15&lt;sup&gt;−&lt;/sup&gt;</td>
<td>NK cells absent</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NK-T cell deficiency (thymic and peripheral)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Memory CD8&lt;sup&gt;+&lt;/sup&gt; T cell deficiency</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>i-IEL TCRγδ decreased</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>i-IEL CD8αα decreased</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Decreased weight, cellularity of peripheral LN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRF-1&lt;sup&gt;−&lt;/sup&gt;</td>
<td>NK cells deficient, NK cell activity absent</td>
<td>82,83</td>
<td></td>
</tr>
<tr>
<td>(Lacks inducible IL-15)</td>
<td>NK-T cells deficiency</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>i-IEL CD8αα deficient</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-15Rα&lt;sup&gt;−&lt;/sup&gt;</td>
<td>NK cells absent</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NK-T cell deficiency (30% of wt)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Single Positive CD8&lt;sup&gt;+&lt;/sup&gt; T cell deficiency (thymus)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD8&lt;sup&gt;+&lt;/sup&gt; T cell deficiency (periphery)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Memory CD8&lt;sup&gt;+&lt;/sup&gt; T cell deficiency</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TCRγδ i-IEL decreased 5-10-fold</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2/15Rβ&lt;sup&gt;−&lt;/sup&gt;</td>
<td>NK cells absent</td>
<td>75,110,180</td>
<td></td>
</tr>
<tr>
<td></td>
<td>i-IELs (TCRαβ CD8αα, TCRγδ) reduced</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NK-T cells deficient</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DETCs absent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2/15Rβ&lt;sup&gt;−&lt;/sup&gt;→tgΔHIL-2/15Rβ</td>
<td>NK cells deficient</td>
<td>121</td>
<td></td>
</tr>
<tr>
<td>IL-2/15Rβ&lt;sup&gt;−&lt;/sup&gt;→tgΔAIL-2/15Rβ</td>
<td>i-IEL TCR γδ deficient</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NK cells normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>i-IEL TCR γδ normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Decreased induced NK cytotoxicity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>γc&lt;sup&gt;−&lt;/sup&gt;</td>
<td>NK cells deficient (&gt; 350-fold reduction)</td>
<td>108,122</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T and B cells deficient (10-fold reduction)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jak3&lt;sup&gt;−&lt;/sup&gt;</td>
<td>NK cell deficient</td>
<td>123,124</td>
<td></td>
</tr>
<tr>
<td>(γc signal)</td>
<td>DETC absent</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>i-IEL TCRγδ deficient</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STAT5a/b&lt;sup&gt;−&lt;/sup&gt;</td>
<td>NK cell deficient</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>(γc signal)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STAT5b&lt;sup&gt;−&lt;/sup&gt;</td>
<td>NK cell proliferation defect</td>
<td>126</td>
<td></td>
</tr>
<tr>
<td>(γc signal)</td>
<td>NK cell cytotoxicity defect</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.3** Summary of major phenotypes affecting immune cell compartments exhibited by mice with targeted disruption of IL-15, IL-15R components, IL-15R downstream signals, and transcription factors affecting IL-15 expression

IL, interleukin; R, receptor; tgΔH, transgenic for IL-2/15Rβ with deletion of the cytoplasmic H region; tgΔA, transgenic for IL-2/15Rβ with deletion of the cytoplasmic A region; i-IEL, intestinal intraepithelial lymphocyte; DETC, dendritic epidermal T cell; γc, common gamma chain; IRF, interferon regulatory factor; LN, lymph node.
Fig. 1.1 Photomicrograph of human large granular lymphocytes.

Photomicrograph (100X) of a human peripheral blood smear with Wright/Giemsa staining. Two LGL are located on the left and right side of the photomicrograph. In contrast, a small lymphocyte is located below, and a monocyte above.
Fig. 1.2  Human IL-15 gene, mRNA, and protein structure.

The human IL-15 locus consists of 9 exons and 8 introns and is located on chromosome 4q31.36,37 Two IL-15 mRNA isoforms have been described, the classical LSP and alternative SSP, with both encoding an identical IL-15 mature protein of 114 AA (see text for details). Translational start sites (→) and stop codons (*) are indicated. (in2, intron 2)
Fig. 1.3 Comparison of IL-15 and IL-2 homology at the DNA, primary protein, and tertiary protein levels.

The model of IL-2's tertiary folding structure was generated from known crystal structures from protein data bank data file 3INK. The theoretical model of IL-15's tertiary structure was generated utilizing IL-2's 3D structure as a template and the SwissProt software program, and yielded results similar to those described.
IL-15 is pleiotropic cytokine that operates in NK cell development, lymphocyte homeostasis, and peripheral immune functions.

Multiple cell types elaborate IL-15, including activated (e.g., virus- or bacteria-infected) monocyte/macrophages, dendritic cells, epithelium, as well as constitutive production by BM stromal cells. IL-15 has a critical role in the development of the NK lineage, as well as its survival, expansion, and function. Other innate lymphocytes (NK-T, CD8αα i-IEL, TCRγδ DETC) and memory-phenotype CD8+ T cells depend upon IL-15 for survival and/or expansion. IL-15 also plays multiple roles in peripheral innate and adaptive immune cell function.
CHAPTER 2

HUMAN NATURAL KILLER CELL DEVELOPMENT:
IDENTIFICATION OF EARLY (FLT3 LIGAND) AND LATE (IL-15)
ACTING FACTORS AND NOVEL HUMAN NK CELL
DEVELOPMENTAL INTERMEDIATES

2.1 Introduction

Natural killer (NK) cells are large granular lymphocytes (LGL) that play an important role in the innate or antigen non-specific immune response to infection.\textsuperscript{8,11,13} Although it is known that NK precursor cells reside in the bone marrow (BM), their phenotype(s) and the factors that regulate their differentiation into mature NK cells are incompletely understood.\textsuperscript{267,268} IL-2 has been used extensively to study NK cell development from CD34\textsuperscript{+} hematopoietic progenitor cells (HPCs) in vitro,\textsuperscript{95-97,269} however, IL-2 is produced exclusively by antigen-activated T cells and is not found within the BM stroma.\textsuperscript{44,71}
Further, NK cells develop normally in mice lacking T cells and in mice which bear a disrupted IL-2 gene, yet are absent in mice and humans which lack either the β or γc signaling components of the IL-2 receptor (IL-2R). Collectively, these data suggest that other factors that bind to the IL-2R are critical for NK cell development.

IL-15 is produced by human BM stromal cells, binds to and signals through the IL-2Rβγ and can induce the differentiation of NK cells from CD34+ HPCs in vitro. Mice that cannot express IL-15 in their bone marrow stroma lack NK cells. Another stromal cell factor, c-kit ligand (KL) can significantly enhance the expansion of NK cells from CD34+ HPCs in combination with IL-15, but alone has no effect on NK cell differentiation. However, mice that lack KL have not been reported as having NK cell deficiencies, suggesting that other stromal factors may contribute to the expansion of NK cells. c-Kit, the KL receptor, is a member of the Class III receptor tyrosine kinase (RTK) family that also includes Flt3. Flt3 is expressed almost exclusively on early hematopoietic CD34+ stem cells, while c-kit appears to be expressed on primitive and mature hematopoietic cells as well as blood NK cells. Flt3 ligand (FL) has strong homology with KL and is also produced by stromal cells. Several studies have shown that FL maintains and stimulates the proliferation of primitive murine and human HPCs and synergizes with a number of other growth factors including IL-3, IL-6, IL-7, IL-11, IL-12, and granulocyte (G)-CSF. Interestingly, mice with a genetically disrupted FL gene exhibit a deficiency in early lymphopoiesis, with low to absent mature NK cells, implicating FL as an important factor for murine NK cell development. Recently, Williams et al showed that c-kit+Sca2+IL-2/15Rβ-Lin- murine BM cells
incubated in a combination of IL-6, IL-7, KL, and FL subsequently developed into functional NK1.1⁺ NK cells when cultured in IL-15. In this chapter the role of FL in the regulation of human NK cell development from BM-derived CD34⁺ HPCs in vitro was investigated, and compared the effect of FL to that of KL. A novel population of human CD34⁺ HPCs that express IL-2/15Rβ after culture in FL or KL are identified. This cell type is IL-15 responsive, and thus appears to represent a distinct intermediate in human NK cell development.

2.2 Materials and Methods

Growth factors

Purified recombinant human (rh) FL, rhKL (specific activity >10⁵ U/mg), and rhIL-15 (specific activity 1.49 x 10⁹ U/mg) were kindly provided by Immunex Research and Development Corp, Seattle, WA. rhIL-2 (specific activity of 1.53 x 10⁷ U/mg) was obtained from Hoffmann LaRoche, Nutley, NJ. rhIL-7, rhIL-9, TGF-β1, and TNF-α were purchased from Peprotech Inc., Rocky Hill, NJ. rhIL-12 (specific activity of 4.5 x 10⁶ U/mg) was a gift of Dr. Stanley Wolf (Genetics Institute, Cambridge, MA).

Monoclonal antibodies (mAb)

The following mAb were purchased from Becton Dickinson (San Jose, CA): anti-CD34-PE, anti-CD34-, anti-CD2- (Leu-5b), and anti-CD3- (Leu-4) FITC, non-reactive mouse immunoglobulin (MsIg)-FITC (control-FITC), and MsIg-PE (control-PE). The anti-CD16-FITC mAb was obtained from CalTag Laboratories (San Francisco, CA), and anti-CD56- (NKH-1) PE from Coulter Immunotech (Hialeah, FL). The mouse-anti human
natural killer cell receptor (NKR) mAbs utilized for this study were EB6 (anti-CD158a), GL 183 (anti-CD158b), Zin 276 (anti-p70/CDw159), FSTR 172 (anti-p50.3) and Zin 270 (anti-NKG2A), kindly provided by Drs. Alessandro and Lorenzo Moretta (Università di Genova, Italy).294-296

**Purification of human BM HPCs**

BM was aspirated from the posterior iliac crest of healthy adult donors after obtaining informed consent. BM mononuclear cells (MNC) were separated by density centrifugation over a Ficoll-Hypaque (Sigma, St. Louis, MO) gradient at 400g for 30 minutes. CD34+ cells were enriched from MNC by affinity chromatography using the Ceprate LC device (CellPro, Bothel, WA), following the manufacturer's instructions. The enriched cells were then stained with anti-CD34-PE, (CD34+LIN-) population on a FACStar Plus cell sorter (Becton Dickinson). Cells obtained in this manner were routinely ≥ 98% pure.71

**Long-term suspension culture of CD34+ HPCs**

Sorted CD34+LIN- HPCs were cultured in 96-well microplates at a concentration of 2 x 10^4 in 200 µl of complete RPMI-1640 medium: RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated human AB serum (HAB) (C-six, Diagnostics, Mequon, WI), and antibiotics (Sigma, St. Louis, MO), and the indicated growth factors: rhFL, IL-15, and KL: 100 ng/ml; rhIL-2, IL-7, IL-9: 10 ng/ml; rhIL-12: 10 U/ml; TNF-α, TGF-β1: 20 ng/ml. At day 7, day 14, and every 3 days thereafter, half of the culture medium was replaced with fresh medium containing 10% HAB and the same
concentration of growth factors. After 21 days, the cells were enumerated and analyzed for morphology, cell surface phenotype, cytotoxic activity, and cytokine production. Cell enumeration was performed in triplicate by vital dye exclusion using a hemacytometer. In some experiments, after culture for 21 days, cells were cultured for an additional 14 days in presence or absence of additional cytokines as indicated.

**Immunophenotype analyses**

Cultured cells were harvested, incubated on ice with an excess of non-reactive mouse IgG for 10 minutes, and then stained on ice for 15-30 minutes with various combinations of the following directly conjugated mAb: anti-CD56-PE and FITC-conjugated mAb reactive against CD2, CD3, CD16. For analysis of NKR expression, cultured cells were stained with unconjugated mouse-anti human NKR mAb, washed, stained with a secondary FITC-conjugated goat anti-mouse IgG (Sigma, MO), washed twice, and then stained with directly conjugated anti-CD56-PE. Background fluorescence was determined by analysis of cells identically stained with non-reactive isotype control mAbs. Cells were analyzed using a MNC gate on a FACScan (Becton Dickinson) with the Lysis II software program.

**Cytotoxicity and proliferation assays**

Cytotoxic activity of cultured cells was determined in a standard $^{51}$Cr-release cytotoxicity assay against the NK sensitive K562 cell line as previously described, at an E:T ratio of 8:1. Results of cytotoxicity assays represent the mean ± SEM of triplicate wells. Cell proliferation was measured by $[^{3}H]$-thymidine incorporation during the final 24 hours of
a 96-hour incubation at 37°C. Results of proliferation assays represent the mean ± SEM of triplicate wells, and are expressed as cpm of [3H]-thymidine incorporation.

**Measurement of NK cell cytokine production**

Three-week cultures of CD34+ HPCs in FL plus IL-15, or IL-15 alone were harvested and separated by FACS into CD56+CD3− and CD56−CD3− subsets. Sorted (>98% purity) CD56+CD3− and CD56−CD3− populations were plated at 50,000 cells/well in complete RPMI-1640 medium. After resting overnight in the absence of any cytokines, cells were costimulated with 100 ng/ml rhIL-15 and 10 U/ml of rhIL-12. After a 48-hour incubation, cell-free culture supernatants were collected and frozen at -70°C. Commercial ELISA kits were used for determination of IFN-γ (Endogen, Woburn, MA), TNF-α, TGF-β (Genzyme, Cambridge, MA), and MIP-1α (R & D Systems, Minneapolis, MN) levels, following the manufacturer's instructions. All supernatants were assayed simultaneously.

**Limiting dilution analysis (LDA)**

As shown below, there is a striking positive correlation between the expression of CD56 in these cultured cells and the expression of NKRs, LGL morphology, cytotoxic activity, and the potential for cytokine production, all of which define NK cells. Further, CD56− cells in culture lack these properties. Therefore, CD56 expression was used as an accurate readout for NK cell development in the LDA of NK cell precursor frequency. Human CD34+ HPCs were isolated as described above, plated by limiting dilution and either assayed for NK cell precursor frequency 2 weeks after being plated in 100 ng/ml
IL-15 (day 0 LDA), or were first cultured in 100 ng/ml KL or FL for 21 days, washed, and then cultured for two weeks in IL-15 (day 21 LDA). For the LDA, cells were plated in a limiting dilution fashion from a concentration of 5000 cells/well to 21 cells/well in 96 well plates with 15 replicates per cell concentration. One half of the cell culture medium was removed every 5 days and replaced with fresh IL-15-supplemented medium. Following the 2-week culture in IL-15, the cells from each well were stained with anti-CD56-PE, washed, analyzed for CD56 expression by flow cytometry, and wells were scored positive or negative compared to identical wells stained with the non-reactive PE-conjugated isotype control mAb. BM-derived NK cells have very high (i.e., \( \geq 10^5 \) PE log fluorescence) surface density expression of CD56 (Fig. 2.1A). Thus, CD56\(^+\) wells were clearly and easily demarcated from identical wells stained with isotype control mAb. In some experiments CD34\(^{bright}\)CD122\(^+\) and CD34\(^{dim/neg}\)CD122\(^-\) cells were sorted following CD34\(^+\) HPC culture in FL alone, and plated in an LDA for NK cell progenitor frequencies as described above. The NK cell progenitor frequency was calculated as the reciprocal of the concentration of cells that resulted in 37% negative wells using Poisson statistics and the weighted mean method\(^{297,298}\).

Cell cycle analysis

Cell cycle status was examined by staining cells with either propidium iodide (PI) as described previously\(^{133}\) or Hoechst 33342 as follows. One million viable cells were stained with 2 \( \mu g/ml \) Hoechst 33342 in 1 ml of complete RPMI-1640 medium, and incubated at 37\( ^\circ C \) for 45 minutes. After centrifugation supernatants were decanted and cells were stained with CD56-PE mAb for 15 minutes on ice, and then washed with PBS.
containing 2 µg/ml Hoechst 33342. After being fixed in PBS with 2% formaldehyde and 2 µg/ml Hoechst 33342, cells were simultaneously analyzed for cell cycle status and CD56 expression by flow cytometry.

Analysis of flt3, c-kit and IL-15Rα expression by RT-PCR

Total RNA was prepared using RNAzol in accordance with the method of Chomczynski and Sacchi.299 cDNA synthesis for RT-PCR was carried out at 42°C for 1 hour in a 30 µl reaction mixture containing 2 µg of total RNA, 2 µmol/L of random hexamer primers, 300 U MMLV reverse transcriptase (Gibco), and dNTPs at 0.2 mmol/L each (Gibco), followed by a 5 minute incubation at 95°C, then incubated on ice for 5 minutes. Five µl of the resultant cDNA product was amplified by PCR with primers for flt3279 or c-kit,300 as described. Primers for IL-15Rα amplification were designed from the published IL-15Rα sequence.301 The forward primer (ACCTTCCACAGGAACCACAG) and reverse primer (AGGTAGCATGCCAGGAGAGA) yield a 213 base pair product. The products were separated on 2% agarose gel containing 0.2 µg/mL ethidium bromide. The integrity of RNA samples was verified by amplification of the β-actin transcript.71

Statistical analysis

Experimental groups were compared by the Student’s t test where indicated, with $P \leq 0.05$ considered significant.
2.3 Results

FL synergizes with IL-15 in the generation of human CD56+ NK cells from CD34+ BM HPCs

The ability of FL, IL-15, or a combination of FL plus IL-15 to promote the development of human NK cells from BM HPCs in 3-week cultures was evaluated. Purified CD34+LIN- HPCs (2 x 10^6/well) cultured in FL (100 ng/ml) alone exhibited a 4.6 ± 0.7-fold increase in total MNC number, but did not give rise to any CD56+ NK cells. Culture of CD34+ HPCs in IL-15 alone (100 ng/ml) for 3 weeks resulted in only a 1.3 ± 0.5-fold increase in total MNC number but 74 ± 6.9% of cells expressed CD56 at high density (CD56^bright), which produced a low absolute number of CD56+ NK cells. However, culture of CD34+ HPCs with FL plus IL-15 resulted in an 8.4 ± 1.7-fold increase in total MNC number with 72 ± 8.6% of cells CD56^bright, which produced a 14.4 ± 6-fold greater absolute number of CD56+CD3- NK cells compared to IL-15 alone (P ≤ 0.025) (Fig. 2.1A-C). The CD56+ cells generated by culture in FL plus IL-15 or IL-15 alone exhibited LGL morphology, an absence of CD3, and minimal surface density expression of CD2 and CD16 (data not shown).

Analysis of NK cell cytotoxic activity, cytokine production, and natural killer cell receptor (NKR) expression

In order to assess the functional characteristics of the CD56+ cells derived from culture of CD34+ HPCs in FL plus IL-15, cytotoxic activity and cytokine production were tested. Following 3-week culture in the presence of FL, MNCs had no CD56 expression and no
significant cytotoxic activity against K562 target cells, which lack MHC class I. In contrast, CD34+ HPCs cultured in FL plus IL-15 or IL-15 alone had high CD56 expression (Fig. 2.1) and consistently exhibited over 70% cytotoxicity against K562 target cells (Fig. 2.2A).

The monocyte-derived cytokines IL-12 and IL-15 are potent costimulators for IFN-γ and MIP-1α production by mature NK cells. Three-week cultures of CD34+ HPCs in FL plus IL-15, or IL-15 alone were harvested and separated by FACS into CD56+CD3− and CD56−CD3− subsets. Equal numbers of each subset (5 x 10^4 per well) were then costimulated with IL-15 plus IL-12 for 48 hours, after which cell-free culture supernatants were harvested and assayed for IFN-γ or MIP-1α protein production by ELISA. CD56+ cells generated from CD34+ HPCs cultured in either FL plus IL-15 or IL-15 alone produced abundant amounts of IFN-γ and MIP-1α after a 48 hour costimulation with IL-15 plus IL-12, while CD56− cells from the same cultures showed little or no production of above cytokines (Fig. 2.2B). There was no spontaneous production of immunomodulatory cytokines, i.e., IFN-γ, MIP-1α, TGF-β, or TNF-α, detected from supernatants in the above culture conditions, or from cells cultured in FL or KL alone for 3 weeks, or following an additional 2-week culture in IL-15 (data not shown).

CD56− and CD56+ BM-derived NK cell progenitors and BM-derived NK cells were evaluated, respectively, for NKR expression. CD34+ HPCs cultured for 3 weeks in FL or KL alone did not express CD56 or NKR (not shown). However, following washout of FL or KL and culture in IL-15 for an additional 2 weeks, NKR expression was observed within the CD56+ fraction of cells. This included modest but distinct expression of Ig-
superfamily killer inhibitory receptor (KIR) antigens on CD56+ NK cells, as well as abundant expression of the C-type lectin NKR on CD56+ cells. CD56 negative cells did not express NKR. These results are summarized for three donors in Table 2.1 and a representative panel of 2-parameter histograms is shown in Fig. 2.2C. Thus, following culture in FL and IL-15 all NK cell phenotypic and functional characteristics were restricted to the CD56+ subset of cells.

Comparison of the effects of FL and KL on NK cell precursor frequency

Mrozek et al. previously reported that KL, a BM stromal factor with strong homology to FL, can synergize with IL-15 to enhance the expansion of CD56+ NK cells from CD34+ HPCs. Therefore, FL and KL were directly compared for their ability to increase NK cell precursor frequency among CD34+ HPCs in a limiting dilution analysis (LDA, Fig. 2.3). NK cell precursor frequency in freshly isolated CD34+ HPCs cultured in IL-15 alone was 1 in 483 ± 16 cells, or approximately 0.21 ± 0.007%. However, when CD34+ HPCs were first cultured for 3 weeks in KL and then analyzed for NK cell precursor frequency in subsequent culture with IL-15 alone, there was a significant (P < 0.005) increase in precursor frequency to 1 in 67 ± 8.3 cells (1.5 ± 0.19%). Likewise, initial 3 week culture in FL increased the subsequent NK cell precursor frequency in IL-15 alone to 1 in 25 ± 2.5 cells (3.9 ± 0.4%, P ≤ 0.002), which was significantly higher than that observed in KL (P ≤ 0.01, Fig. 2.3).
Identification of an NK cell precursor following culture in FL or KL

The significant increase in NK cell precursor frequency following culture of CD34+ HPCs in FL or KL suggested that these factors may be responsible for the generation of an intermediate, IL-15-responsive NK cell precursor. The IL-2/15Rβ chain (CD122) is required for IL-15 signaling.53 Therefore, CD122 expression by flow cytometric analysis on both freshly isolated human CD34+ HPCs, and CD34+ HPCs cultured in FL for 10 days was examined. Fresh CD34+ BM HPCs expressed no detectable CD122 on their surface, consistent with earlier reports.120,302 However, following 10-day culture in FL alone, 20 ± 3% of CD34+ HPCs became CD122+. Interestingly, this fraction was among the cells expressing CD34 with relatively high surface density, i.e. the CD34bright subset (Fig. 2.4A).

To determine the NK cell precursor frequency in these CD34+ HPC subsets following the 10-day culture in FL, FACS sorted CD34brightCD122+ and CD34dim/negCD122− cells were compared using LDA. The NK cell precursor frequency in CD34brightCD122+ cells was between 20- and 60-fold higher than the NK cell precursor frequency among the CD34dim/negCD122− subset, and between 65- and 235-fold higher than the frequency in fresh CD34+ HPCs (Table 2.2). Thus, it appears that culture of human CD34+ HPCs in FL induces IL-2/15Rβ (CD122) expression in CD34bright HPCs, creating a cell that represents a phenotypic and functional intermediate within human NK cell development. CD34brightCD122+ is the phenotype that identifies this subset after culture in FL but prior to culture in IL-15.
The CD34^{bright}CD122^{+} subset is CD38^{+}CD7^{−}CD56^{−} by flow cytometric analysis. Prior to culture in IL-15, the CD34^{bright}CD122^{+} subset does not express any of the NKR shown in Table 2.1 and Fig. 2.2C, nor does it have any cytotoxic activity against NK targets (data not shown). While IL-15Rα protein expression was undetectable by flow cytometric analysis of this subset, we were able to easily detect the expression of IL-15Rα transcript following culture of CD34^{+} HPCs in FL (Fig. 2.4B).

Importantly, KL has similar, albeit less dramatic, effects as FL in the generation of this NK cell intermediate. Following a 10-day culture of CD34^{+} HPCs in KL, 7.5 ± 0.7% of the CD34^{+} HPCs co-expressed CD122, again within the CD34^{bright} fraction (Fig. 2.4A). This was significantly lower than the percentage of CD34^{bright}CD122^{+} cells found following culture with FL (P ≤ 0.01). This is consistent with the significantly lower NK cell precursor frequency seen when CD34^{+} HPCs were cultured for 21 days in KL compared to FL, as shown above in Fig. 2.3. The IL-15Rα transcript was also detected following incubation of CD34^{+} HPCs in KL (Fig. 2.4B).

**The effect of IL-15 on NK cell development**

As described above, there is a striking correlation between the expression of CD56 and the appearance of NK cell function. The mechanism by which CD56^{+} NK cells develop from their CD34^{+}CD56^{−} precursor populations following the addition of IL-15, i.e., differentiation or proliferation, is unknown. Therefore CD34^{+} HPCs were examined after 21 days of culture in FL, and quantitated the MNC number, viability, percent apoptosis, and cell cycle status at 3-day intervals after the addition of IL-15. For all values, similar
results were obtained with 21-day culture in KL (data not shown). Absolute cell number did not change significantly over 14 days following the addition of IL-15, yet CD56\(^+\) cells went from < 1% to over 80%. Cell cycle analysis demonstrated that a constant fraction (14 ± 2.1%) of cells were in G\(_{2}/S\) phase over the subsequent 14-day culture with IL-15, which was approximately equal to the fraction undergoing apoptosis (15 ± 1.4%). When analyzed on days 10 through 12 after addition of IL-15 (i.e., days 31-33), 13 ± 1.5% of the CD56\(^-\) fraction was cycling (G\(_{2}/S\)), whereas only 2.2 ± 0.6% of the CD56\(^+\) fraction was cycling. The absolute increase in CD56\(^+\) cells from day 10 (36,450) to day 12 (81,420) could not be accounted for by proliferation, as virtually every CD56\(^+\) cell on day 31 would have to double once in 48 hours, data not supported by the cell cycle analysis. These data are summarized in Table 2.3, and suggest that the increase in CD56\(^+\) NK cells in the presence of IL-15 is more likely the result of NK precursor cell differentiation to mature NK cells rather than the selective outgrowth of a small fraction of CD56\(^+\) cells. It does not, however, suggest that all CD34\(^+\) HPCs cultured in FL or KL were committed to NK cell development prior to the addition of IL-15. Indeed, when CD34\(^+\) HPCs after 21-day culture in FL or KL, but before culture in IL-15, were exposed to other hematopoietic factors such as G-CSF, GM-CSF, erythropoietin or thrombopoietin, morphologic evidence of differentiation to other lineages was observed (not shown).
Synergistic effect of FL with other cytokines upon CD56* NK cell generation from CD34+ HPCs

Next, it was investigated whether FL could combine with other cytokines that have been shown to act in early hematopoiesis, for the production of CD56* NK cells from CD34+ HPCs. CD34+ HPCs were cultured for 3 weeks with rhIL-2, IL-7, IL-9, IL-12, IL-15, and KL, in the presence of FL. The absolute number of CD56*CD3~ cells generated among total cells in each culture condition is shown in Fig. 2.5A. A direct comparison of IL-2 and IL-15 in combination with FL revealed that these cytokines were not significantly different in their ability to generate CD56*CD3~ NK cells in this culture system. Both IL-7 and IL-9 demonstrated weak synergy with FL in the production of CD56*CD3~ NK cells. However, these factors alone yielded no CD56*CD3~ NK cells (data not shown). IL-12, alone or in combination with FL, did not stimulate production of CD56*CD3~ NK cells in this culture system. The combination of KL and FL did not generate any CD56*CD3~ NK cells, and FL, KL and IL-15 together were no more effective in generating CD56*CD3~ NK cells from CD34+ HPCs than were FL plus IL-15 or KL plus IL-15 (Fig. 2.5B). When CD34+ HPCs were cultured with FL and IL-15 and TNF-α, the generation of CD56*CD3~ NK cells was inhibited by 90 ± 7% (FL plus IL-15 vs. FL plus IL-15 plus TNF-α, P ≤ 0.005). Similarly, the addition of TGF-β to cells being cultured with FL plus IL-15 resulted in a 72 ± 8% reduction in the generation of CD56*CD3~ NK cells (FL plus IL-15 vs. FL plus IL-15 plus TGF-β, P ≤ 0.005). MIP-1α did not significantly inhibit the generation of NK cells from CD34+ HPCs (Fig. 2.5B). Thus, the most potent combinations for the generation of CD56*CD3~ NK cells from
CD34⁺ BM HPCs were FL plus IL-15 or KL plus IL-15, and TNF-α, TGF-β can function as potent negative regulators of NK cell development.

**Expression and functional assessment of flt3 and c-kit transcript during NK cell differentiation.**

CD34⁺ BM HPCs, BM-derived CD56⁺CD3⁻ NK cells, CD56 brigth and CD56 dim blood NK cells, CD3⁺ T cells, and unfractionated PBL were examined by RT-PCR for their expression of flt3 and c-kit transcripts. Amplification of the flt3 transcript (210 bp) was observed in CD34⁺ BM cells from five consecutive normal donors. However, 5 samples of BM-derived CD56⁺CD3⁻ NK cells obtained from a 21-day culture of CD34⁺ HPCs with FL plus IL-15, as well as 5 samples of CD56 brigth, CD56 dim blood NK cells, and CD3⁺ T cells were negative for the flt3 mRNA transcript (Fig. 2.6A). One of 5 unfractionated PBL samples was positive for flt3 mRNA, which may be explained by expression of flt3 in rare circulating CD34⁺ HPCs. The c-kit transcript (1090 bp) was observed in all 5 samples of CD34⁺ BM cells, BM-derived CD56⁺CD3⁻ NK cells obtained from a 21-day culture of CD34⁺ HPCs with FL plus IL-15, as well as CD56 brigth blood NK cells, CD3⁺ T cells, and unfractionated PBL. The CD56 dim blood NK cell subset was negative for c-kit mRNA, consistent with previous functional observations. To confirm the absence or presence of functional gene products in blood NK cell subsets, sorted CD56 brigth cells were cultured in IL-2, FL plus IL-2, or KL plus IL-2 for 96 hours, and assayed for a proliferative response (Fig. 2.6B). CD56 brigth NK cells cultured with FL plus IL-2 demonstrated no enhancement of [³H]-thymidine incorporation over the same cells cultured with IL-2 alone. In contrast, a parallel culture
of CD56\textsuperscript{bright} NK cells in KL plus IL-2 showed a proliferative synergy compared to those cells cultured in IL-2 alone. This is consistent with earlier observations of a functional c-kit receptor on this NK cell subset.\textsuperscript{283} Thus, flt3 and c-kit appear to have distinct patterns of expression during NK cell differentiation.

2.4 Discussion

In this chapter, the role of FL in the regulation of human NK cell development from BM-derived CD34\textsuperscript{+} HPCs was investigated, and compared to that of KL. Neither FL nor KL had any ability to induce NK cell differentiation from CD34\textsuperscript{+} HPCs in the absence of IL-15. However, the ability of both FL and KL to significantly increase NK cell precursor frequency among CD34\textsuperscript{+} HPC prior to the addition of IL-15 strongly suggested that these factors contribute to the survival and/or expansion of an IL-15-responsive NK precursor cell. Indeed, here it was demonstrated that incubation of CD34\textsuperscript{+} HPCs in FL or KL resulted in the generation of a novel human NK cell intermediate characterized by CD34\textsuperscript{bright} and CD122 (IL-2/15R\(\gamma\)) expression. It is unclear if activation of either c-kit or flt3 by their respective ligands induces the IL-15R components on a fraction of CD34\textsuperscript{+} cells, or whether there is a selective expansion of a small CD34\textsuperscript{bright}CD122\textsuperscript{+} subset. The failure to detect CD122 expression by flow cytometric analysis of 10,000 CD34\textsuperscript{+} cells before culture in FL or KL might suggest that at least some component of enhanced CD122 gene expression via activation of these two type III RTKs is likely. However, here and elsewhere\textsuperscript{97} a low frequency of NK precursor cells in fresh CD34\textsuperscript{+} HPCs was detected by LDA, so the IL-2/15R\(\gamma\_c\) chains are likely to be constitutively expressed on some small number of CD34\textsuperscript{+} HPCs at a surface density that is below detection by flow
cYTOMETRY. Indeed, others have failed to detect IL-2/15Rγc on the surface of mature NK cells that are known to functionally respond to IL-2 and IL-15.\(^\text{304}\) The same may be true for IL-15Rα expression on CD34+ HPCs. The preliminary studies reported in this chapter suggest that FL and KL may upregulate this chain as well, although a quantitative analysis of the IL-15Rα gene product has not yet been performed. In addition to these scenarios, our data do not exclude the possibility that FL and/or KL may mediate some of their effects by inducing the expression of additional factors from the starting population of CD34+ HPCs.

The incorporation of the LDA assay for assessment of NK cell precursor frequency provided strong support for the CD34\(^{\text{bright}}\)CD122+ cell as an NK cell intermediate. There was a striking positive correlation of CD56 expression with NKR expression, LGL morphology, cytotoxic activity, and cytokine production, as well as a notable absence of these features in the CD56\(^-\) population. This allowed the use of CD56 expression as a simple and accurate readout for human NK cell outgrowth. A very high NK cell precursor frequency in the CD34\(^{\text{bright}}\)CD122+ subset was detectable, in contrast to the CD34\(^{\text{dim/neg}}\)CD122- subset. Further, we demonstrated that pre-incubation of CD34+ HPCs in either KL or FL significantly increased the NK cell precursor frequency seen with IL-15, compared to fresh CD34+ cells plated in an LDA with IL-15. As neither KL nor FL by themselves induce NK cell differentiation, the mechanism likely involves the generation of the CD34\(^{\text{bright}}\)CD122+ NK cell intermediate that is in turn responsive to IL-15. The observation that the NK cell precursor frequency was significantly higher with
pre-incubation in FL versus KL is consistent with FL inducing over double the percent of
CD34brightCD122+ NK cell intermediates.

IL-15 was also shown to induce NKR and CD56 expression, LGL morphology, cytotoxic
properties as well as prepare the cell for potent cytokine and chemokine production
following monokine stimulation. The CD56+ NK cells generated from human BM
CD34+ HPCs with FL followed by IL-15 expressed both C-type lectin (NKG2A) and Ig-
superfamily (CD158a, CD158b, and p70/CDw159 KIR) NKR. This appears to be in
contrast to NK cell progenitors in the thymus cultured in IL-15 or IL-15 plus KL, where
only C-type lectin NKR were induced. The reason for variation in KIR expression between these two in vitro systems may relate to differences in BM versus
thymic NK precursor cells, accessory cells in the cultures, or culture conditions. Studies
are currently underway to further characterize the factors that may be critical for NKR
induction during human NK cell development.

These findings, along with earlier demonstration of IL-15 protein production within
human bone marrow stroma, provide compelling evidence for an important role for
IL-15 in human NK cell development. Genetic disruption studies provide supporting
evidence for this in the mouse. The interferon response factor-1 (IRF-1) knockout mouse
lacks the ability to express IL-15 transcript in bone marrow stroma, and lacks NK cells.
Incubation of IRF-1+ HPCs in IL-15 or transplantation of IRF-1+ HPCs into lethally
irradiated wild type mice leads to the generation of NK cells, strongly suggesting that IL-
15 is required in the process of NK cell development. Likewise, there is support for
the role of FL in NK cell development, as mice genetically deficient in FL have low NK
cell numbers. This would be consistent with the notion that absence of FL leads to fewer IL-15-responsive NK cell intermediates. The NK deficiency in FL" mice would suggest that KL is not fully redundant with FL in this regard. The Sl^d/Sl^d mouse, which lacks expression of c-kit, has not been noted as NK deficient, but it is unclear if a careful quantitative analysis of NK cells has been undertaken. Finally, Williams et al. have recently demonstrated that, in the mouse, a combination of IL-6, IL-7, KL and FL can induce CD122 on multipotential murine bone marrow HPCs that in turn become responsive to IL-15 for NK cell development.

The data from the current study would suggest that both FL and KL have the capacity to induce CD34^brightCD122^ NK cell precursors from CD34^+ HPCs, but that FL may perform this function more efficiently. The reasons for this are not entirely clear, but may relate to differences in flt3 and c-kit expression on the earliest HPCs. Shah et al have reported that FL was able to induce significantly greater proliferation of quiescent CD34^+CD38^- cells than KL in long term culture. Haylock et al, found that in single cell assays FL, but not KL, was able to recruit an additional subpopulation of HPCs representing approximately 12% of CD34^+CD38^- cells that were rhodamine^dull and 4-hydroperoxycyclophosphamide (4-HC) resistant. Consistent with this, Miller et al have recently shown that NK cell progenitor frequency increases when FL is added to switch cultures of CD34^+CD33^- HPCs in IL-2, IL-7, and KL. In contrast to FL, KL has effects on mature CD56^bright NK cells found in blood, promoting their survival and potentiating their IL-2/15-induced proliferation. KL does not enhance CD56^bright NK cell cytotoxic activity in the presence or absence of IL-2/15, which is consistent with
its inability to induce NK cell differentiation from HPCs. These differences highlight some distinct functional effects of FL and KL on NK cells due to the distribution of their respective receptors.

In the absence of FL or KL, IL-15 induces NK cell differentiation from CD34+ HPCs, but without significant expansion.71,120 This suggests that IL-15 itself promotes NK cell development via differentiation of committed precursors, not via proliferation. To address this possibility, a kinetic analysis of CD56+ cells was performed during NK cell development from CD34+ HPCs in the presence of IL-15 and either FL or KL. Cell cycle analysis during the substantial increase in CD56 expression proved that IL-15's effect on CD56+ NK cell development could not be accounted for by induction of proliferation. Thus, IL-15 appears to be the factor most likely responsible for inducing the expression of genes that characterize NK cells.

Collectively, these data suggest that in humans, both FL and KL participate in the generation of NK cell progenitors from CD34+ HPCs. Both induce an NK cell intermediate from HPCs, and both are likely to enhance their expansion while IL-15 induces NK cell differentiation. In this capacity these two RTK ligands are likely to be functionally redundant.308 The failure of a combination of KL and FL to enhance the absolute number of CD56+ NK cells obtained from CD34+ HPCs cultured with IL-15 plus either FL or KL alone supports this. However, evidence from this chapter and others291,306,307 would suggest that FL might also perform its function on an additional subset of NK precursors, which are not responsive to KL. It was also noted that CD34+ HPCs cultured in FL or KL can subsequently differentiate along different lineages in the
absence of IL-15 and in the presence of other growth factors. Further, factors that negatively regulate human NK cell development in this system were identified. These observations remind us that the process of NK cell development is likely to be substantially more complex in vivo. Continued characterization of growth factors and their receptors on HPCs and their progeny will hopefully provide additional insight into this fascinating biological process.
% of NKR Expression in CD56* Cells

<table>
<thead>
<tr>
<th>NKR</th>
<th>NKR Subfamily</th>
<th>Donor 1</th>
<th>Donor 2</th>
<th>Donor 3</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD158a</td>
<td>KIR2DL1/S1</td>
<td>0.59</td>
<td>1.14</td>
<td>0.56</td>
<td>0.76 ± 0.2</td>
</tr>
<tr>
<td>CD158b</td>
<td>KIR2DL2/L3/S2</td>
<td>7.03</td>
<td>3.10</td>
<td>6.80</td>
<td>5.6 ± 1.0</td>
</tr>
<tr>
<td>p70/CDw159</td>
<td>KIR3DL1/S1</td>
<td>5.1</td>
<td>0.37</td>
<td>0.04</td>
<td>1.8 ± 1.3</td>
</tr>
<tr>
<td>p50.3</td>
<td>KIR2DS4</td>
<td>0.88</td>
<td>0.08</td>
<td>0.10</td>
<td>0.35 ± 0.2</td>
</tr>
<tr>
<td>NKG2A</td>
<td>C-type lectin</td>
<td>80.72</td>
<td>47.8</td>
<td>76.42</td>
<td>68.3 ± 8.4</td>
</tr>
</tbody>
</table>

Table 2.1  NKR Surface Expression by CD56* Cells Derived from CD34+ HPCs Cultured in FL for 21 days, Followed By Culture in IL-15 for 14 days

Purified CD34+ HPCs were cultured in FL (100 ng/ml) for 21 days, washed, and cultured in IL-15 (100 ng/ml) for an additional 14 days. Cells were harvested and stained with anti-NKR mAbs and anti-CD56-PE as described in Material and Methods. The flow cytometry dot-plot for donor 1 is shown in Fig. 2.2C. No detectable NKR or CD56 expression was observed in the 3 week cultures prior to the addition of IL-15. Similar NKR profiles were obtained when KL (100 ng/ml) was substituted for FL in these experiments (not shown). Individual results from 3 donors, as well as the mean ± SEM, are shown.
Table 2.2 Human NK Cell Precursor Frequency of Fresh CD34+ HPCs, or 10-Day Culture of CD34+ HPCs in FL

<table>
<thead>
<tr>
<th>Donor</th>
<th>Fresh CD34+ HPCs</th>
<th>CD34^{bright}\text{CD122}^+</th>
<th>CD34^{dim/neg}\text{CD122}^-</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:458</td>
<td>1:7</td>
<td>1:147</td>
</tr>
<tr>
<td>2</td>
<td>1:471</td>
<td>1:2</td>
<td>1:127</td>
</tr>
<tr>
<td>3</td>
<td>1:527</td>
<td>1:5</td>
<td>1:161</td>
</tr>
</tbody>
</table>

Purified CD34+ HPCs were either assayed immediately for NK cell precursor frequency by LDA, or further cultured in FL (100 ng/ml) for 10 days, sorted by FACS into CD34^{bright}\text{CD122}^+ or CD34^{dim/neg}\text{CD122}^- subsets (>98% pure), and then plated for LDA. In each LDA, cell populations were cultured in IL-15 (100 ng/ml) for an additional 14 days, stained with anti-CD56-PE mAb and analyzed for CD56 expression and NK cell precursor frequency as described in Materials and Methods.
<table>
<thead>
<tr>
<th>Day of Culture</th>
<th>No. of Cells/Well (x1000)</th>
<th>%CD56⁺</th>
<th>%Non-Viable</th>
<th>%Apoptosis</th>
<th>%Cycling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 21</td>
<td>154 ± 22</td>
<td>0.8 ± .05</td>
<td>2.5 ± 0.4</td>
<td>21 ± 8</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>Day 24</td>
<td>171 ± 15</td>
<td>9.5 ± 2.2</td>
<td>2.6 ± 0.5</td>
<td>14 ± 3</td>
<td>12 ± 3</td>
</tr>
<tr>
<td>Day 27</td>
<td>163 ± 21</td>
<td>13 ± 2.8</td>
<td>5.7 ± 1.2</td>
<td>15 ± 2</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>Day 30</td>
<td>135 ± 18</td>
<td>27 ± 2.5</td>
<td>6.3 ± 0.9</td>
<td>12 ± 2</td>
<td>12 ± 4</td>
</tr>
<tr>
<td>Day 33</td>
<td>138 ± 10</td>
<td>59 ± 6.4</td>
<td>9.8 ± 1.5</td>
<td>11 ± 2</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>Day 36</td>
<td>138 ± 11</td>
<td>94 ± 3.1</td>
<td>8.6 ± 1.2</td>
<td>14 ± 1</td>
<td>11 ± 1</td>
</tr>
</tbody>
</table>

Table 2.3  Time Course of Cell Number, CD56 Expression, Viability, Percent Apoptosis, and Cell Cycle Status of CD34⁺ HPCs Cultured in FL for 21 Days, Followed By the Addition of IL-15 for 14 Days

Purified CD34⁺ HPCs were plated at density of 2 x 10⁴ cells per well and firstly cultured in medium containing FL or KL (100 ng/ml) for 21 days. IL-15 was then added to culture in combination with either FL or KL, respectively, for an additional 14 days. Cells were examined at 3-day intervals after addition of IL-15 as described in Materials and Methods.
Fig 2.1 FL enhances IL-15–mediated development of CD56⁺CD3⁻ NK cells from CD34⁺ HPCs in vitro.

A) Flow cytometric analysis of CD56 and CD3 expression on MNCs generated in 21-day cultures of CD34⁺ HPCs under the indicated conditions (right column). Non-reactive MsIg isotype control mAb were used to determine background fluorescence (left column). Results displayed are representative of ten separate experiments. B) Fold increase in the absolute number of MNCs after 21 days of culture of CD34⁺ HPCs in the indicated conditions. C) The absolute number of CD56⁺CD3⁻ cells after a 21-day culture of CD34⁺ HPCs in the indicated conditions. These values were calculated by multiplying the absolute number of viable MNCs by the percentage of cells that were CD56⁺CD3⁻ by flow cytometric analysis. Results shown in B and C represent the mean ± SEM of five separate experiments. The asterisk indicates a value of $P \leq 0.025$ compared with IL-15 alone.
Fig 2.2 Characterization of NK cell cytotoxicity, cytokine production, and NKR expression by MNCs, CD56<sup>+</sup>CD3<sup>+</sup> and CD56<sup>−</sup>CD3<sup>−</sup> cells generated from CD34<sup>+</sup> HPCs in various conditions.

A) Cytotoxicity assay. MNCs were harvested after 21-day culture with indicated cytokines, washed, and tested for cytotoxic activity against the NK sensitive K562 target cell line at an 8:1 E:T ratio. B) Cytokine (IFN-γ) and chemokine (MIP-1α) production. MNCs were harvested after 21-day culture with indicated cytokines, sorted into CD56<sup>−</sup>CD3<sup>−</sup> and CD56<sup>−</sup>CD3<sup>−</sup> fractions, and costimulated with IL-15 plus IL-12 for 48 hours. Cell-free supernatants were harvested and tested for IFN-γ and MIP-1α by ELISA. For both A and B results represent the mean ± SEM of four separate experiments. The asterisk in A denotes a value of $p \leq 0.01$ compared with culture in FL alone. C) NKR expression. NK cells generated in vitro by culture in FL for 3 weeks, and then IL-15 for 2 weeks, were stained with anti-NKR and anti-CD56 mAbs and analyzed by flow cytometry as described in Materials and Methods. Flow cytometry data is representative of 3 separate donors, which are summarized in Table 2.1.
FL and KL increase NK cell precursor frequency in CD34+ HPCs.

Limiting dilution analysis (LDA) of NK cell precursor frequency within freshly isolated CD34+ HPCs (Day 0), or CD34+ HPCs cultured for 21 days in KL (day 21/KL) or FL (Day 21/FL). CD34+ HPCs cultured in KL (*, $P \leq 0.005$) or FL (**, $P \leq 0.002$) had significantly increased NK cell precursor frequency compared to freshly isolated CD34+ HPCs. In addition the 21 day/FL cultures had significantly greater NK cell precursor frequencies when compared to 21 day/KL culture (#, $P \leq 0.01$). The results represent the mean ± SEM of 3 independent experiments.
Fig 2.4  Expression of CD122 (IL-2/15Rβ) protein and IL-15Rα mRNA on CD34+ HPCs after culture in FL or KL.

A) Flow cytometric analysis of CD122 on freshly isolated CD34+ HPCs (day 0) or CD34+ HPCs cultured in FL or KL for 10 days. The percentage of CD34+CD122+ cells (gated population) is indicated for each condition. B) RT-PCR for IL-15Rα mRNA transcript expression. CD34+ HPCs from 2 donors were examined for IL-15Rα on day 0 (lane 1 and 4), day-10 culture in KL (lane 2 and 5), or day-10 culture in FL (lane 3 and 6), and 10-day culture in FL followed by culture in IL-15 for 14 days (lane 7). Lane 8 was H2O control. RT-PCR analysis was performed as described in Materials and Methods.
Fig 2.5  Effect of FL in combination with various cytokines on the generation of CD56⁺CD3⁻ NK cells from CD34⁺ HPCs.

Purified CD34⁺ HPCs were plated in complete RPMI-1640 medium in the presence of the indicated cytokines at the concentrations described in Materials and Methods. Results represent the mean ± SEM of total mononuclear cell number and absolute CD56⁺ NK cell number of five separate experiments. * Indicates $P \leq 0.01$, and ** indicates $P \leq 0.005$ compared to cultures with FL plus IL-15.
Fig 2.6  Analysis of c-kit and flt3 expression and function during NK cell development.

A) RT-PCR analysis of flt3 and c-kit mRNA expression in HPCs and various lymphocyte populations. Lanes 1-3, purified CD34+ HPCs from three normal BM donors; lanes 4-5, CD56+CD3- NK cells generated from CD34+ HPCs after culture in FL plus IL-15 for 3 weeks; lanes 6-7, CD56bright blood NK cells, lanes 8-9, CD56dim blood NK cells from two normal blood donors; lane 10, unfractionated PBL; lane 11, blood CD3+ T cells. Below: Human β-actin control of the identical samples. B) Proliferation of CD56bright NK cells in response to various concentrations of IL-2, IL-2 plus FL (100 ng/ml), and IL-2 plus KL (100 ng/ml). The results are representative of three separate experiments, and indicate the mean cpm ± SEM of triplicate measures.
NK cell progenitors that respond to early acting RTK ligands (FL and KL) differentiate into an IL-15-responsive NK cell precursor, by upregulating the IL-15R complex on their surface. The NK precursor then responds to IL-15 to differentiate into a mature NK cell. Characterization of the resultant CD56\textsuperscript{bright} NK cells produced strongly suggests that other (e.g. stromal) signals are likely required for complete NK cell differentiation. FL\textsuperscript{-/} mice are deficient in NK cells, suggesting that this RTK ligand serves a critical, non-redundant function in NK cell development or expansion in mice in vivo, most likely at the level of the NK cell progenitor.\textsuperscript{293} In addition, RAG2/\gamma\textsubscript{c}\textsuperscript{-/} mice (lack T, B, and NK cells) reconstituted with c-kit\textsuperscript{+} progenitors have defects in NK cell expansion and survival, suggesting that KL serves a critical role in these functions in vivo.\textsuperscript{309} Further, IL-15R\alpha\textsuperscript{-/} and IL-15\textsuperscript{-/} mice lack NK cells, suggesting that IL-15 is critical for differentiation of mature NK cells from an NK cell precursor (see Table 2.1).
CHAPTER 3

IL-15 COSTIMULATES INNATE IMMUNE IFN-γ PRODUCTION
AND THE SHWARTZMAN REACTION IN VIVO

3.1 Introduction

Interleukin (IL)-15 is a pleiotropic cytokine that acts upon both innate and antigen-specific immune cells. IL-15 was identified as a soluble factor produced by the CV-1/EBNA kidney epithelial cell line, and the T cell leukemia line HuT-102, that was able to support growth of IL-2-dependent CTLL-2 cells. Consistent with this, IL-15 shares many functions with IL-2 and utilizes the IL-2/15 receptor (R) β and the common gamma (γc) chain for binding and signaling. However, the IL-2 and IL-15 receptor complexes each contain unique alpha chains that confer specific high affinity binding. As IL-15 is produced by macrophages following stimulation with bacterial components in vitro, it has been proposed to have a role in the early innate pro-inflammatory response to infection.
The generalized Shwartzman reaction is a lethal cytokine-induced shock response elicited by sequential priming and challenge with bacteria or bacterial components (e.g. LPS), originally identified in rabbits and later in mice and humans.\cite{258, 259} IL-12-induced IFN-γ is critical for sensitization of macrophages during LPS priming administered s.c. in the footpad.\cite{260, 261} After initial IFN-γ-dependent priming, a subsequent i.v. LPS challenge 18-24 h later results in cytokine-induced shock and mortality, largely due to the release of tumor necrosis factor (TNF)-α and IL-1.\cite{260, 262} The monokines that may act synergistically with IL-12 to induce the IFN-γ important for LPS priming of the Shwartzman reaction have yet to be fully characterized.

NK cells are a critical component of the innate immune response to infection, commonly through their elaboration of IFN-γ prior to development of an effective adaptive immune response.\cite{11, 13} IL-15, acting through the IL-15Rαβγ, induces human NK cell proliferation, cytotoxicity, and synergizes with IL-12 to stimulate production of IFN-γ, TNF-α, and macrophage inflammatory protein (MIP)-1α in vitro.\cite{33, 138} Co-culture of LPS-activated human macrophages and NK cells results in abundant IFN-γ production that is partially dependent upon IL-15.\cite{67} In this innate immune cytokine loop, LPS-stimulated IL-15 acts in concert with other monokines (e.g., IL-12) to stimulate IFN-γ production by human NK cells in vitro. NK cells have been implicated as important contributors of IFN-γ during priming of the generalized Shwartzman reaction in mice,\cite{310} and contribute to IFN-γ production and lethality in LCMV-infected mice challenged with LPS.\cite{311}
In this chapter, it is shown that neutralization of IL-15 during the priming phase of the generalized Shwartzman reaction provides protection from this lethal process. We also show that endogenous IL-15 is critical for optimal IFN-γ production by LPS-challenged SCID mice in vivo.

3.2 Materials and Methods

Reagents and Mice

Purified cytokines were provided: rmIL-15, Immunex (Seattle, WA); rhIL-2, Hoffman LaRoche (Nutley, NJ); rmIL-12, Genetics Institute (GI, Cambridge, MA). Anti-mIL-2/15Rβ (TM-β1) Ab and rat IgG2b control were purified from hybridoma supernatants. Additional reagents include sheep anti-mIL-12 Ab and sheep IgG control (GI), rabbit anti-mIL-15 antiserum (Immunex) and normal rabbit serum control, LPS from E. coli (Difco, Detroit, MI). Rat anti-mIL-2 Ab and isotype control, anti-mIFN-γ-FITC, anti-DX5 (PAN-NK)-PE, and PE/FITC-conjugated isotype control Abs were purchased from PharMingen (San Diego, CA). C.B.-17 SCID mice (Taconic) and IL-2−/− mice (Jackson) were housed in SPF animal facilities. All experiments were performed under approved OSU ULAR protocols.

Induction of the Generalized Shwartzman Reaction

SCID mice (6-week old females) were given a s.c. 5 μg priming dose of LPS, and 24 h later an i.v. 50 μg LPS challenge dose. These doses were established to induce 80-100% mortality within 24 h following the LPS challenge (data not shown). Mice (n=11/group) were pre-treated (i.p.) with the anti-mIL-15 antiserum or control 1 h prior to the priming
dose of LPS. The specificity of the anti-mIL-15 antiserum was confirmed through neutralization of CTLL-2 proliferation stimulated by rIL-15, but not by rIL-2 (data not shown). All injections were performed in a blinded fashion.

**Isolation and Stimulation of SCID Splenocytes In Vitro**

SCID mouse spleens were harvested, processed into a single cell suspension, and stimulated with medium (RPMI-1640 + 10% FCS) alone, rmIL-15 (1 ng/ml), rmIL-12 (10 ng/ml), or IL-15 + IL-12 (5 x 10^5/well). These concentrations were chosen based upon their ability to synergistically induce IFN-γ production by SCID mouse splenocytes, while inducing little or no IFN-γ when used alone in repeated experiments. Some splenocyte preparations were stimulated with LPS (10 μg/ml) or PBS (control). After 48 h, supernatants were harvested and assayed for mIFN-γ by ELISA (Biosource, sensitivity 10 pg/ml). Some preparations were pre-incubated with anti-mIL-2 Ab or control (50 μg/ml), anti-mIL-2/15Rβ or control (50 μg/ml), anti-mIL-12 or control (12 μg/ml), or anti-mIL-15 or control (1:100 dilution), prior to stimulation.

**Inhibition of LPS-Induced IFN-γ In Vivo**

SCID or IL-2^-^ mice (n=5-6) were injected i.v. with LPS (400 μg/SCID mouse and 600 μg/IL-2^-^ mouse), blood was collected 6 h after challenge, and serum was assayed for IFN-γ by ELISA. In some experiments, groups of mice were injected i.p. with anti-IL-2/15Rβ or control Ab (100 μg/mouse), and anti-IL-15 antiserum or control 1 h prior to LPS challenge. Splenocytes were obtained 6 h after LPS injection, cultured in Brefeldin-A (10 μg/ml, Sigma) and LPS (10 μg/ml) for 4 h to allow IFN-γ protein to accumulate in
the golgi, harvested, stained and analyzed for intracellular IFN-γ as described previously. No IFN-γ was detectable by flow cytometry in splenocytes from PBS-treated mice cultured in Brefeldin-A and LPS for 4-24 h (data not shown), suggesting that differences in IFN-γ observed in LPS-injected mice were due to in vivo activation.

Real-Time PCR Quantification of Cytokine Transcripts In Vivo

Real-time PCR (PE Biosystems, TaqMan technology) is a novel method that allows an accurate and precise quantitation of gene transcripts through measurement of target amplification during (i.e. in real-time) the reaction, utilizing fluorochrome-labeled probes. Groups of SCID mice (n=5/time-point) were injected i.v. with LPS (400 μg/mouse) and spleens were snap-frozen at the indicated time points after LPS challenge. RNA isolation, RT, and real-time PCR assays were performed as described with modifications to specifically quantitate murine cytokine transcripts. Final quantitation is reported as the fold difference relative to a calibrator cDNA (untreated SCID splenocytes) prepared in parallel with the experimental cDNAs.

Statistical Analysis

Experimental groups were compared by the Student’s t test with P < 0.05 considered significant. Survival (Kaplan-Meier) significance was determined by the Log rank test.
3.3 Results

Neutralizing IL-15 Protects Against Lethality in the LPS-Elicited Generalized Shwartzman Reaction

We hypothesized that IL-15 may contribute to IL-12-induced IFN-γ in the Shwartzman reaction, and therefore tested whether administration of an anti-IL-15 antiserum affected mortality during this lethal response. Pre-treatment of SCID mice with anti-IL-15 antiserum prior to LPS priming provided significant protection from lethality after the subsequent i.v. LPS challenge, compared to the control (Fig. 3.1, P = 0.0172). Next, a series of experiments were performed to determine if LPS-induced IL-15 costimulated IFN-γ production.

LPS Induces Endogenous IL-15 that Costimulates IFN-γ Production by SCID Mouse Splenocytes In Vitro

Stimulation of resting SCID mouse splenocytes (macrophages and NK cells) with IL-15 or IL-12 alone induced little or no IFN-γ production, while costimulation with IL-15 + IL-12 resulted in abundant IFN-γ protein (Fig. 3.2A). Next, resting SCID splenocytes were cultured in the presence or absence of LPS to examine whether endogenous IL-15 could costimulate innate immune IFN-γ production. Resting SCID splenocytes produced no IFN-γ, while identical LPS-stimulated cultures produced IFN-γ protein (Fig. 3.2B). To determine if endogenous production of IL-15 contributed to the in vitro IFN-γ, splenocytes were pre-incubated with anti-IL-2/15Rβ, anti-IL-15, anti-IL-12, or anti-IL-2 Abs or appropriate controls, and then stimulated for 48 h with LPS (Fig. 3.2B). Pre-
incubation with controls or the anti-IL-2 Ab had no effect on LPS-induced IFN-γ (n=5). In contrast, pre-incubation with anti-IL-2/15RP Ab (50.7±6.2% decrease, P ≤ 0.04, n=5) or anti-IL-15 antiserum (64.4±9.6% decrease, P ≤ 0.02, n=3) resulted in significantly lower amounts of IFN-γ. The anti-IL-12 Ab also abrogated LPS-induced IFN-γ in vitro (61.3±4.1% decrease, P ≤ 0.01, n=5), confirming previous results demonstrating a role for IL-12 in NK cell IFN-γ production. Pre-incubation with both anti-IL-15 and anti-IL-12 Abs reduced IFN-γ to nearly undetectable levels (P ≤ 0.03, n=3). These data suggest that endogenous IL-15 protein, in combination with IL-12, is produced in vitro by LPS-activated macrophages and contributes to IFN-γ production.

**LPS Induces IL-15 Transcript in SCID Mouse Splenocytes In Vivo**

The expression of IL-15, IL-12 p40, IL-2 and IFN-γ mRNA was quantitated in vivo following LPS injection of SCID mice by real-time RT-PCR. While there was no change in cytokine gene expression following PBS administration to SCID mice, LPS-induced IL-15 transcript in the spleen increased approximately 6-fold with a peak at 3 h. This induction of IL-15 partially overlapped with that of IL-12, which increased 60-fold peaking at 1 h. Importantly, the first increase in IFN-γ mRNA (3 h) follows simultaneous elevation of IL-12 and IL-15 gene expression (Fig. 3.3). IL-2 gene expression was unchanged at all time-points (data not shown). Thus, the time-course of IL-12 and IL-15 expression in vivo supports a role for these monokines during LPS-induced IFN-γ production.
Blockade of the IL-2/15Rβ Reduces LPS-Induced IFN-γ In Vivo

As IL-15 was important for optimal LPS-induced IFN-γ production by SCID mouse splenocytes in vitro, and LPS increased IL-15 transcript in vivo, it was next tested whether endogenous IL-15 was important for IFN-γ production in vivo. First, the IL-2/15Rβ, one signaling component of the IL-15R complex, was targeted with an anti-IL-2/15Rβ Ab that blocks ligand binding. Injection of SCID mice with LPS resulted in measurable serum IFN-γ production that peaked 6 h post-injection, and was unaffected by pre-treatment with PBS (data not shown). However, pre-treatment with anti-IL-2/15Rβ Ab significantly decreased serum IFN-γ measured in response to LPS (54.8±9.4% decrease, P < 0.02), compared to control Ab (Fig. 3.4A). As IL-15 shares the IL-2/15Rβ with IL-2, IL-2−/− mice pre-treated with the anti-IL-2/15Rβ Ab were also examined for an effect upon LPS-induced IFN-γ. A similar decrease in LPS-induced IFN-γ was observed in IL-2−/− mice (Fig. 3.4B, 53.4±8.3% decrease, P < 0.02), indicating that the cytokine binding to the IL-2/15Rβ chain in this system was not IL-2. Importantly, during the time-course of this experiment, administration of the anti-IL-2/15Rβ Ab did not significantly change the percentage of DX5+ NK cells present in the spleen, as assessed by flow cytometry (data not shown). The data presented in Fig. 3.3 suggest that an LPS-induced factor that requires the IL-2/15Rβ for signaling, but not IL-2, contributes to LPS-induced IFN-γ in vivo. Next, experiments were performed to confirm that this factor was IL-15.
LPS-Induced IL-15 Contributes to IFN-γ Production In Vivo

It was tested whether LPS-induced IL-15 contributed to the IFN-γ response in vivo by pre-treating SCID mice with the anti-IL-15 antiserum or control prior to challenge with LPS. Pre-treatment with anti-IL-15 significantly decreased the serum IFN-γ measured following LPS challenge in vivo (66.3±7.0% decrease, \( P \leq 0.004 \), \( n=5 \)), compared to pre-treatment with control (Fig. 3.5A). SCID mouse DX5* splenocytes were also examined after in vivo LPS administration for production of intracellular IFN-γ. Pre-treatment with anti-IL-15 antiserum (\( n=5 \)) significantly decreased the percentage of DX5* splenocytes producing IFN-γ (41.2±8.5% decrease, \( P \leq 0.008 \)), and the DX5* IFN-γ mean fluorescence intensity (MFI) (54.2±8.3% decrease, \( P \leq 0.004 \)) induced by LPS in vivo, compared to mice pre-treated with control (Fig. 3.5B and data not shown).

3.4 Discussion

Several studies have shown that IL-15 can participate in tissue inflammation, and can be produced by macrophages following LPS stimulation in vitro. However, this chapter describes the first in vivo evidence for IL-15’s role during the early innate immune response, represented here by challenge with LPS. These data, together with the earlier in vitro studies, suggest that IL-15 acts in concert with IL-12 and possibly other monokines to induce optimal IFN-γ production in vivo following certain infectious insults. As IFN-γ is the prototypic macrophage activating factor, such NK-derived IFN-γ may feedback to further activate the LPS-stimulated macrophage in vivo. These results clarify how neutralization of IL-15 during LPS priming of the Shwartzman
reaction may reduce IFN-γ production, and ultimately enhance survival to subsequent LPS challenge.

While the data presented in this chapter show that IL-15 is required for optimal IFN-γ production in response to LPS in vivo, it is likely that other LPS-induced monokines (e.g. IL-18 and TNF-α) also contribute to generate IFN-γ in vivo.\textsuperscript{135,314,315,318} It was also examined whether IL-15 may be operating through the induction of IL-18, and found that while LPS challenge induced measurable IL-18 protein in the serum of SCID mice, there was not a significant difference comparing groups of mice pre-treated with the anti-IL-15 antiserum (2449±231 pg/ml) or control (2607±490 pg/ml). One possible explanation for these observations is that IL-15 may be required for IL-12 to efficiently induce IFN-γ, regardless of other operative costimuli (e.g. IL-18). In addition, data presented in this chapter suggests that the host immune response to infection may be augmented through the supply of exogenous IL-15, especially for those pathogens that require IFN-γ for effective clearance. This is supported by a report showing that rIL-15 augments IFN-γ production by SCID splenocytes stimulated with \textit{T. gondii} in vitro.\textsuperscript{319}

In conclusion, in vivo evidence was provided in this chapter that IL-15 participates in the innate immune response leading to IFN-γ production in SCID mice. Consistent with these results, neutralization of IL-15 during LPS-priming of the generalized Shwartzman reaction protected mice against mortality. These results suggest that IL-15 may be considered a pro-inflammatory cytokine produced by macrophages to activate NK cells (Fig. 3.6). Therefore, IL-15 may be a therapeutic target to manipulate the innate immune response, to either augment host defense or diminish excessive immune activation.
Fig 3.1 Neutralizing IL-15 protects against lethality in the LPS-elicited generalized Schwartzman reaction.

SCID mice (n=11/group) were pre-treated in a blinded fashion with anti-IL-15 or control antiserum 1 h prior to a priming dose of LPS. Mice were challenged with an i.v. dose of LPS 24 h later. Results are shown in a Kaplan-Meier survival plot, and there was a significantly higher survival in the anti-IL-15 group, compared to the control group ($P = 0.0172$).
Fig. 3.2 Endogenous IL-15 contributes to LPS-induced IFN-γ production by resting SCID mouse splenocytes in vitro.

Resting splenocytes were stimulated with either (A) IL-15, IL-12, the combination of IL-15 + IL-12, or (B) LPS. Supernatants were harvested after 48 h and assayed for mIFN-γ. Details of in vitro culture, stimulation, and neutralizations are provided in Materials and Methods. Results represent the mean ± SEM of replicate wells, and are representative of at least 3 independent experiments. *See text for P values.
Fig. 3.3  LPS induces IL-12, IL-15 and IFN-γ transcript in the spleen in vivo.

SCID mice (n=5) were injected i.p. with LPS and the spleens were harvested at various time points post-injection. RNA was isolated, reverse transcribed into cDNA and analyzed for IL-12 p40, IL-15 and IFN-γ expression by real-time PCR. Of note, only after simultaneous elevation of IL-12 and IL-15 (arrow) IS IFN-γ transcript elevated.
Fig. 3.4 IL-2/15Rβ, but not IL-2, is involved in LPS-induced serum IFN-γ production in SCID and IL-2+ mice in vivo.

(A) SCID (n=5/group) or (B) IL-2+ mice (n=6/group) were treated with anti-IL-2/15Rβ or control Abs, and 1 h later challenged with LPS. Serum IFN-γ was assayed after 6 h. (*, P < 0.02).
Fig 3.5  Endogenous IL-15 is required in vivo for optimal LPS-induced IFN-γ production in SCID mice.

(A) SCID mice (n=5/group) were injected i.p. with anti-IL-15 or control antiserum, and 1 h later challenged with LPS. Serum IFN-γ was assayed after 6 h (*, P < 0.004). (B) SCID mice (n=5/group) were treated as in (A), and spleens were removed and co-stained for DX5 and IFN-γ. Representative flow cytometric density plots illustrate IFN-γ production after gating on viable splenocytes from mice pre-treated with either control (top) or anti-IL-15 antiserum (bottom). The complete data summary for this set of experiments is presented in the Results section.
Fig. 3.6  IL-15 participates in innate immune cross talk between activated monocyte/macrophages and NK cells.

Following infectious insult, macrophages produce monocyte-derived cytokines (monokines), including IL-15, that bind to constitutively expressed monokine receptors on NK cells. Monokine-stimulated NK cells in turn produce cytokines that activate the macrophage, including IFN-γ, allowing the macrophage to clear the offending pathogen and stimulating further monokine production.
CHAPTER 4

EARLY EXPANSIONS IN NK AND MEMORY-PHENOTYPE CD8⁺ T CELLS IN INTERLEUKIN-15 TRANSGENIC MICE ARE FOLLOWED BY FATAL LEUKEMIA

4.1 Introduction

Interleukin (IL)-15 is a pleiotropic cytokine that is important for both innate and adaptive immune cell homeostasis, as well as peripheral immune functions. IL-15 shares the common γ chain (γc) and IL-2/15RIβ with IL-2 for signaling, but utilizes a private IL-15RIα subunit for high affinity binding. Numerous in vitro and in vivo studies have documented a critical role for IL-15 in the development, survival, and function of the natural killer (NK) cell lineage. Further, IL-15 is required for the normal expansion and/or survival of non-classical T cells and memory-phenotype TCRαβ CD8⁺ T cells, while not being essential for their development. Additional studies have documented a role for IL-15 in peripheral immune functions such as T lymphocyte trafficking, innate immune IFN-γ production, and host defense against infectious pathogens. These studies are consistent with the broad expression of IL-15.
and IL-15Ra by multiple cell types and tissues, suggesting that this ligand/receptor may mediate a wide range of functions in vivo.\textsuperscript{27,54} Indeed, the phenotypes of mice deficient in IL-15/IL-15Ra,\textsuperscript{34,35} compared to mice deficient in IL-2/IL-2Ra,\textsuperscript{106,107} definitively demonstrate a large variety of unique in vivo functions mediated by IL-15.\textsuperscript{321}

Despite an abundance of transcript in multiple tissues and cell types, IL-15 is poorly translated and secreted. Three primary posttranscriptional checkpoints are responsible for this observation: multiple AUGs in the 5' UTR,\textsuperscript{27,30} unusual long (LSP) and short (SSP) signal peptides,\textsuperscript{39,40} and a negative regulator near the C-terminus of the precursor proteins.\textsuperscript{40} Through the systematic elimination of these three checkpoints the synthesis of bioactive human IL-15 protein increased 250-fold in vitro.\textsuperscript{86} Such tight posttranscriptional control of the IL-15 gene product is unusual for most cytokines thus far characterized, suggesting that constitutively abundant IL-15 protein may somehow be deleterious to the host.

Recently, a connection between chronic inflammatory processes and the genesis of cancer has been appreciated.\textsuperscript{322} Inflammation resulting from persistent infections has been linked to malignancies, including \textit{H. pylori} and gastric carcinoma, schistosomiasis and bladder cancer, as well as hepatitis C virus and hepatocellular carcinoma.\textsuperscript{322} The increased production of macrophage migration inhibitory factor (MIF) during \textit{H. pylori} infection, a molecule that down-regulates the p53 tumor suppressor gene during inflammation, provided one direct mechanism whereby the proinflammatory state may result in susceptibility to transforming genetic mutation.\textsuperscript{323} Further, individuals with
polymorphisms at the IL-1β gene, resulting in increased expression of proinflammatory IL-1β during *H. pylori* infection, have a higher risk of developing gastric cancers.\textsuperscript{324}

Leukemia is a complex, heterogeneous disorder with multiple molecular etiologies.\textsuperscript{325} However, direct evidence that mediators of inflammation can, via alterations in proliferation or survival, contribute to leukemogenesis is lacking. We hypothesized that deregulation of IL-15 gene expression, resulting in alterations in lymphocyte homeostasis, could promote malignant transformation in lymphocytes. In this chapter, these hypotheses were tested in vivo by engineering transgenic mice that lack posttranscriptional control of IL-15 gene expression, thereby efficiently translating and secreting murine IL-15 protein. These IL-15 transgenic mice have early expansions of peripheral blood lymphocytes, specifically NK cells and memory-phenotype CD8\textsuperscript{+} T cells. Later, IL-15 transgenic mice develop a striking leukemic expansion, some composed primarily of clonal CD3\textsuperscript{+}TCR\\textgamma\textbeta\textsuperscript{+} T cells, along with progressive alopecia, multi-organ lymphocytic infiltrates, and premature death, not unlike the leukemia of large granular lymphocytes that occurs in patients.\textsuperscript{233}

### 4.2 Materials and Methods

**Reagents**

The following mAbs reactive with murine cells were purchased from PharMingen (San Diego, CA) CD2 (RM2-5), CD3 (145-2C11), CD4 (RM4-5), CD5 (53-7.3), CD8a (53-6.7), CD8b2 (53-5.8) CD19 (1D3), CD25 (7D4), CD44 (IM7), CD69 (H1.2F3), CD122 (TM-\textbeta1), CD62L (MEL-14), Ly6C (AL-21), DX5 (DX5), NK1.1 (PK136), Ly49D (4E5), B220 (RA3-6B2), Mac-1 (M1/70), TCR\\textbeta (H57-597), TCR\\textgamma\textdelta (GL3), V\textbeta2 (B20.6), 106
Vβ11 (RR3-15), IFN-γ (XMG1.2), and utilized as direct conjugates to PE, FITC or PerCP. The following mAb were purchased from PharMingen and used as isotype controls hamster IgG (G235-2356), mouse IgG2b/k (49.2), rat IgG2a/k (R35-95), rat IgG2b/k (A95-1), rat IgM/k (R4-22) and utilized as direct conjugates to PE, FITC, or PerCP. For immunohistochemical analysis CD3 (145-2C11) mAb and anti-hamster IgG HRP-conjugated secondary reagents were used (PharMingen). For western analysis the anti-FLAG M2 and M5 (Sigma) and probe 8 (Santa Cruz) antibodies were used. The following cytokines were kindly provided: rmIL-15 (Immunex), rhIL-2 (Hoffman LaRoche), and rmIL-12 (Genetics Institute).

**IL-15 transgene design and construction**

The IL-15 transgene was designed to eliminate posttranscriptional checkpoints thereby optimizing for the over-expression of an efficiently translated and secreted murine IL-15 mature protein (Fig. 4.1). The transgene was cloned utilizing standard DNA cloning techniques as follows. The murine mIL-2 signal peptide (SP) coding sequence (nt 49-108, GenBank K02292) was amplified from the pmut-1 plasmid (ATCC) with forward primer (5'-GGCATGTACAGCATGCAGCTCG-3') and a reverse primer engineered with a NarI restriction site (underlined) (5'-ATCGGCCTCTGCCTTCTTGTGACAAGGAGC-3'). The murine IL-15 cDNA encoding the mature protein (nt 610-951, GenBank U14332) was PCR amplified from a full-length mIL-15 cDNA with a forward primer engineered with a NarI restriction site (5'-GATGGCGCCAACTGGATAGATGAAGATATG-3') and a reverse primer (5'-GATCGCATCCCTATTTGTCATCGTCGCTTCGTTGATGAGGAC-3').
3') engineered with a BamHI restriction site (underlined) and FLAG epitope tag (italics). Both PCR products were TA cloned into the PCR2.1 vector (Invitrogen, San Diego, CA) and accuracy confirmed by sequencing (ABI 377XL sequencer). The mIL-2SP coding sequence was subcloned into pBluescript II SK (Stratagene, San Diego, CA) utilizing an EcoRI site. Next, the NarI/BamHI fragment of mIL-15 mature protein sequence was ligated into this construct. The NarI restriction sites used to ligate the mIL-2SP and mIL-15 mature protein ensured maintenance of the proper open reading frame. Next the BamHI/NotI fragment of the human growth hormone (hGH) gene\(^{326}\) was ligated out-of-frame into the IL-2SP/IL-15 construct, downstream of the IL-15 cDNA. Then the XhoI/HindIII fragment of mouse MHC class I promoter (D\(^{d}\))\(^{327,328}\) from pGEM4z-2.7 was ligated into the multiple cloning site of pGEM7zf (Promega). Finally, the resultant XhoI/SalI fragment of D\(^{d}\) promoter was ligated into the construct, upstream of the IL-2 signal peptide, resulting in the final transgenic construct. A sequential digest of the construct with XhoI and NotI, which releases the linearized 5.2 kb transgene from pBluescript, was used for microinjections.

**Generation of IL-15 transgenic mice**

The microinjection fragment was isolated from the vector in a TAE buffered 1% agarose gel slice. DNA was purified from the agarose using the Qiaex II purification kit (Qiagen; Hilden, Germany) and eluted in an injection buffer consisting of 10 mM Tris-HCl pH 7.4 and 0.1 mM EDTA. An aliquot of the 5.2 kb microinjection fragment was used to determine the DNA concentration by direct comparison with the High DNA Mass Ladder (Life Technologies, Gaithersburg, MD) on an ethidium bromide stained gel. DNA was
injected into a single pronucleus of FVB/N embryos at a concentration of 2 ng/µL. Injected embryos were transferred to the oviducts of pseudopregnant ICR foster mice. Potential transgenic mice were screened by isolating genomic DNA from tail biopsies and testing for transgenic sequences by Southern hybridization (see below).

**Southern analysis**

High molecular weight mouse DNA was isolated from diced tail clips using a proteinase K, phenol/chloroform extraction as described. For Southern analysis, 10 µg of genomic DNA was digested to completion with SstI and fractionated on a 1% agarose gel followed by alkaline transfer to a positively charged nylon membrane. DNA blots were hybridized with a 32P-labeled 600 bp probe directed against hGH sequence found in the transgene (Fig. 4.1) and analyzed using a Storm 860 PhosphoImager and ImageQuant software (Molecular Dynamics). The expected size of SstI-digested transgene band is 2.6 kb.

**Real-time quantitative RT-PCR**

Tissues from mice were snap-frozen, and RNA isolation, reverse transcription were performed as described. IL-15 transgene mRNA transcripts were quantified by the dual-labeled fluorogenic probe method, using a Prism 7700 thermal cycler and sequence detector (Perkin-Elmer/ABI). Primers used were: IL-15 transgene forward: 5'-CGACGATGACAAATAGGGATCC-3', reverse: 5'-GACGTCCGGGAGCCTGTA-3', probe: 5'-FAM AACTCCCCGAAACCACCTCAGGGTCTC TAMRA-3'; 18S rRNA forward: 5'-CGGCTACCACATCCAAAGGAA-3', reverse: 5'-
GCTGGGAATTACCGCGGCT-3', probe: 5'-VIC TGCTGGCACCAGACTTGCCCTC TAMRA-3'. In parallel with experimental samples, standard curves for the IL-15 transgene and 18S rRNA (reference control) of known concentration were quantitated, and absolute copy numbers were calculated. Final quantitation is reported as the absolute copy number of IL-15 transgene transcripts per 10^6 18S rRNA copies.

Western blot analysis

Splenocyte lysates were loaded directly onto 8-16% gradient gels (Tris-HCL, Bio-Rad, Hercules, CA). Recombinant FLAG protein was used as a positive control (Sigma). Proteins were electrophoresed under denaturing conditions and electroblotted to nitrocellulose membranes at 100V for 1 hour at 4°C. Membranes were blocked overnight with 5% nonfat dry milk in TBS + 0.5% tween-20 (TBST) and then incubated for 1.5 hours with rabbit anti-FLAG antibody (Santa Cruz) diluted 1:400 in TBST plus 2% nonfat dry milk. Membranes were washed with TBST and incubated for 1 hour with HRP conjugated donkey anti-rabbit IgG (Amersham Pharmacia Biotech, Piscataway, NJ) secondary antibody diluted 1:3000 in TBST. Proteins were detected using enhanced chemiluminescence reagents (ECL Plus and ECL hyperfilm, Amersham Pharmacia Biotech).

Murine IL-15 ELISA

Measurement of murine IL-15 in the serum of IL-15tg and wild type mice was performed with a capture ELISA developed in collaboration with Dr. Christine Biron and colleagues (Brown University). This assay consists of a soluble murine IL-15Ra as the
primary capture reagent, polyclonal rabbit anti-IL-15 antiserum as the secondary
detecting reagent, and a tertiary anti-rabbit-HRP. The ELISA specifically detected
rmIL-15 (Immunex) and had a sensitivity of 9-40 pg/ml depending on assay and the
dilution of the serum samples. It did not cross-react with recombinant murine IL-2.

Tissues and blood

For autopsy, mice were anesthetized, sacrificed by cervical dislocation, and weighed.
Peritoneal lavage was performed with 10 mL ice cold PBS. Tissues were removed,
examined grossly, and processed for RNA or protein isolation (snap frozen in LN₂),
histology (fixed in 10% neutral buffered formalin), and immunohistochemistry (frozen in
OCT medium). Body weight and selected organ weights were determined, and relative
body weight ratios were calculated. Spleen, thymus, and lymph nodes were disrupted,
RBC lysed, and strained through 70 µm nylon mesh to obtain single cell leukocyte
suspensions. Liver leukocytes were isolated following collagenase digestion of liver
homogenate and density centrifugation over Lympholyte M (Cedarlane). Bone marrow
mononuclear cells were isolated from two femurs by flushing with ice cold PBS.
Peripheral blood was harvested from the tail or retro-orbital plexus, and blood smears
were prepared. Whole blood was RBC lysed and all leukocyte suspensions were
enumerated electronically (Coulter Z1 Cell Counter) and manually (hemacytometer) in a
blinded fashion.
Histopathology

Fixed tissues were dehydrated with ethanol, transferred to xylene, and embedded in paraffin using standard histology techniques, and 3 µm sections were cut and stained with hematoxylin and eosin. Peripheral blood smears were fixed in ethanol and Wright/Giemsa stained.

Analysis of NK cell function

Fresh peripheral blood leukocytes were utilized as effectors, and YAC-1 tumor cells as targets, in a standard $^{51}$Cr release assay. For NK cell IFN-γ production, murine leukocytes were costimulated with IL-12 (10 ng/ml) and IL-15 (1 ng/ml) for 48 hours, and cell-culture supernatants were then harvested and assayed for IFN-γ protein by ELISA.$^{320}$

Flow cytometry

Staining of leukocyte suspensions with fluorochrome-conjugated mAb reacting with cell surface antigens and intracellular IFN-γ was performed as described.$^{135}$ Forward scatter, side scatter, and fluorescence data were collected on a Coulter XL flow cytometer (Coulter Immunology) and analyzed with the WinMDI software program (Joseph Trotter, Scripps Research Institute, La Jolla, CA). Nonreactive isotype control staining of identical cells was used to set quadrant gates with ≥ 99% of cells located in the negative quadrant.
DNA PCR assessment of TCRβ clonality

Dβ to Jβ rearrangements were analyzed in high molecular weight genomic DNA isolated from fresh peripheral mouse leukocytes using a proteinase K, phenol/chloroform extraction as described. PCR reactions (50 μL) contained 100 ng genomic DNA template, 3 pmol of each primer, 0.2 mM of each dNTP, 2 mM MgCl₂, and 1 U Taq DNA polymerase in 1X PCR buffer as supplied (Perkin Elmer). Reactions were run on a Perkin Elmer 9700 thermocycler under the following conditions: 3 min at 94°C; 32 cycles of 45 s at 94°C, 90 s at 65°C and 150 s at 72°C; and, 10 min at 72°C. PCR primers spanning the Dβ-Jβ region were used as published. Populations that appeared to have clonal or oligoclonal Dβ-Jβ rearrangement were further analyzed by the above method for Vβ usage. Primers located within each of the Vβ regions and the reverse Jβ primers were used as published. Ethidium bromide stained PCR products were run on a 1 % agarose gel and visualized under ultraviolet light. To verify that single Vβ-Jβ bands were indeed indicative of a single TCR, PCR products were cloned and at least 4 different clones from each product sequenced. In all cases, identical sequences were obtained from such Vβ-Jβ products.

Statistical analysis

Experimental groups were compared by the Student’s t test with P < 0.05 considered significant.
4.3 Results

Generation of transgenic mice that over-express an efficiently translated and secreted IL-15 mature protein

Previous reports have identified major posttranscriptional regulatory mechanisms that control human IL-15 translation and secretion.\textsuperscript{29,40,86} We constructed a modified murine IL-15 cDNA that lacked the three primary posttranscriptional checkpoints controlling wild type IL-15, thereby optimizing for IL-15 over-expression (Fig. 4.1). Modifications to the cDNA included removing upstream AUGs that impeded translation, replacing the inefficiently translated and secreted endogenous IL-15 signal peptides with the IL-2 signal peptide, and stabilizing the C-terminus of the mature protein with a FLAG epitope tag. Near global over-expression of this modified IL-15 cDNA was driven by the MHC class I D\textsuperscript{d} promoter,\textsuperscript{327} and the 3' portion of the human growth hormone gene was spliced downstream and out of frame, to maximize transcription, translation, and processing of the transgene in vivo.\textsuperscript{326} Transfection of COS-7 cells with an expression plasmid containing the modified IL-15 cDNA resulted in secretion of bioactive murine IL-15 protein (data not shown), confirming that these modifications resulted in efficient IL-15 protein translation and secretion.

Transgenic mice were created by microinjection of the IL-15 transgene into pronuclear stage FVB/N embryos. Three IL-15 transgenic (IL-15tg) lines were identified by Southern blot analysis of genomic DNA with a human growth hormone cDNA probe (Fig. 4.2a). While all three IL-15tg lines demonstrate a similar phenotype, the severity varied based upon the intensity of Southern banding and expression at the transcript
levels. Here, the phenotype of one representative IL-15tg line is reported (3304 in Fig. 4.2a). IL-15tg mice developed normally as neonates, but grossly manifested progressive alopecia beginning at 5-6 weeks of age (see below). The transgene transcript, now efficiently translated and secreted, was quantified and found to be abundantly expressed in multiple tissues from IL-15tg mice (Fig. 4.2b), in a similar pattern as the endogenous IL-15 transcript\(^\text{27}\) that is poorly translated and secreted. The transgenic protein was detected by immunoblot analysis of the FLAG epitope and was present in multiple tissues (Fig. 4.2c). In addition, IL-15tg mice had measurable serum levels of murine IL-15 (Fig. 4.2d). IL-15 protein was detected (mean ± SEM: 186.7 ± 41.8 pg/ml) in the serum of 9 of 12 IL-15tg mice tested with the lower limit of detection in the ELISA 9-40 pg/ml. In all (n=9) wild type non-transgenic mice IL-15 protein was undetectable using this ELISA. Thus, IL-15tg mice express measurable serum levels of IL-15 protein at 6-20 weeks of age, while wild type mice do not.

**Early lymphocytosis in IL-15tg mice**

Previous studies have documented a role for IL-15 in the homeostasis of several lymphocyte subsets.\(^\text{81}\) Therefore the peripheral blood of IL-15tg (n=71) and non-transgenic littermates controls (n=51) at 6 weeks of age was examined. The IL-15tg mice exhibited a significant increase in leukocyte number (31,694 ± 3,267/μL blood), compared to controls (7,983 ± 503/μL blood, \(P < 10^{-7}\)) (Fig. 4.3a). The lymphocyte counts in IL-15tg mice (21,712 ± 2,718/μL blood) were significantly higher compared to controls (3,648 ± 375/μL blood, \(P < 10^{-8}\)), and the lymphocytosis in the IL-15tg mice was responsible for the elevated leukocyte numbers (Fig. 4.3b). Examination of peripheral
blood smears from IL-15tg mice confirmed the expansion of small and large granular lymphocytes (Fig. 4.3c). This lymphocytosis was observed in IL-15tg mice as early as 3 weeks of age.

Early expansion in NK cell number and function in IL-15tg mice

IL-15, acting through the IL-15R complex, has a requisite role in NK cell lineage development, and provision of exogenous IL-15 expands murine NK cells in vivo. Immunophenotyping the peripheral blood of IL-15tg mice revealed that the major population of expanded lymphocytes was DX5<sup>+</sup>CD3<sup>-</sup> NK cells (Fig. 4.4a). In IL-15tg mice, both the percentage (46 ± 1.5% vs. 4 ± 0.2%, \( P < 10^{-12} \)) and absolute number (10,161 ± 1,220/μL blood vs. 142 ± 15/μL blood, \( P < 10^{-11} \)) of NK cells were consistently and significantly increased, compared to non-transgenic littermate controls (Fig. 4.4b). Further, those DX5<sup>+</sup>CD3<sup>-</sup> cells co-expressed the Ly49D NK receptor at a frequency comparable to wild type NK cells (Fig. 4.4a).

In order to confirm the expanded DX5<sup>+</sup>CD3<sup>-</sup> lymphocytes were functionally NK cells, the natural cytotoxicity of fresh peripheral blood leukocytes from IL-15tg mice and non-transgenic controls were examined for their ability to lyse YAC-1 target cells. Leukocytes from IL-15tg mice efficiently lysed YAC-1 targets at relatively low effector:target cell ratios (Fig 4c), consistent with the increased percentage of NK cells in IL-15tg mice. Of note, fresh leukocytes from IL-15tg mice were able to efficiently lyse YAC-1 tumor targets without additional in vivo or in vitro activation. As a further indicator of NK cell function, leukocytes from IL-15tg mice were tested for their ability to secrete IFN-γ following monokine-costimulation. Abundant IFN-γ protein was
measured following 48 hour incubation of IL-15tg lymphocytes in IL-12 + IL-15 (data not shown), similar to that reported for mature NK cells. Thus, at 6 weeks of age, the peripheral blood of IL-15tg mice contains a major population of functional DX5+CD3-Ly49\textsuperscript{hi} NK cells.

**Early expansion of memory-phenotype CD8\textsuperscript{+} T cells in IL-15tg mice**

IL-15 has also been shown to be critical for memory-phenotype CD8\textsuperscript{+} T cell expansion and/or survival in mice. Analysis of the T cell compartment revealed that the percentage of TCR\textbeta\textsuperscript{+}CD3\textsuperscript{+} cells present in the peripheral blood of IL-15tg mice was reduced, due to the NK cell expansion (Fig. 4.5a). However, the normal ratio of CD4:CD8 T cells (3.26 ± 0.1) in non-transgenic controls was dramatically inverted within IL-15tg mice (0.59 ± .04, \( P < 10^{-32} \)). This inversion was due to an expansion of CD8\textsuperscript{+} T cells (10-fold, \( P < 10^{-4} \)), as the absolute number of CD4\textsuperscript{+} T cells was identical between IL-15tg and non-transgenic littermate controls (Figs. 4.5b, c). Further phenotypic analysis of these CD8\textsuperscript{+} T cells showed that they are CD44\textsuperscript{hi}CD62L\textsuperscript{lo}CD69\textsuperscript{+}Ly6C\textsuperscript{hi}, consistent with a memory-phenotype (Fig. 4.6).

**Later, IL-15tg mice develop fatal lymphocytic leukemia**

Between 12-30 weeks of age, most IL-15tg mice develop a cluster of signs including decreased activity, weight loss, and difficulty breathing. These signs progress for 3-7 days when the mice become acutely moribund and are sacrificed (average 19.6 ± 1.1 weeks of age). In evaluable IL-15tg mice, a massive elevation in peripheral blood leukocyte number, consisting mainly of lymphocytes, was observed (mean 186,582 ±
32,433/uL blood, range 47,000-606,000/uL blood, n=22). Immunophenotyping revealed that while DX5^CD3^- NK cells remained elevated, the major lymphocyte population was consistently CD3^TCRβ^DX5^- T cells. Representative flow cytometric analyses of the blood from several IL-15tg mice that developed such massive lymphocyte outgrowth are shown in Fig. 4.7a. The expanded CD3^+ T cells and NK cells were observed in multiple lymphoid tissues including blood, spleen, and bone marrow (Fig. 4.7b). Flow cytometric analyses of expanded lymphocyte populations isolated from 22 different IL-15tg mice revealed a consistent phenotype of CD3^+TCRβ^+CD2^+CD5^+CD44^+DX5^-CD8^-+. In one additional case, a CD3^-TCRβ^-CD8^-CD4^-DX5^+ lymphocyte population was expanded, likely representing a murine NK cell leukemia (data not shown), and in no case was CD4 expression observed. Concurrently, these mice demonstrated massive splenomegaly (Fig. 4.7c) with grossly enlarged livers. Analysis of peripheral blood smears confirmed the dramatic lymphocyte expansion, some of which had striking blast-like morphological features and prominent nucleoli (Fig. 4.7d).

Next, expanded populations of CD3^+TCRβ^+ lymphocytes from IL-15tg mice were examined for clonality of the TCRβ locus. The analyses were performed by DNA PCR with primers that amplify all possible rearrangements of the Jβ1 and Jβ2 loci, then followed by another DNA PCR assay that amplifies all possible Vβ-Jβ rearrangements.332 A sample expansion of a clonal TCRβ^+ T cell population isolated from a mouse that developed fatal lymphocytic leukemia is shown in Fig. 4.8a. Amplification of the Jβ2 region demonstrated that only a single J segment (Jβ2.7) was utilized by these lymphocytes (Fig. 4.8b). After identifying the Jβ clonal population, PCR amplification
of all possible V-Jβ2 rearrangements identified the rearranged Vβ present (Vβ16) in the genomic DNA (Fig. 4.8c). Further, cloning the amplified Vβ-D-Jβ products, followed by sequencing multiple clones from each product revealed that 100% contained identical rearrangements at the DNA level (n=4 leukemias, data not shown). Flow cytometric studies on TCR Vβ expression also provided evidence that the lymphocyte expansions were indeed clonal (Fig. 8d). Through such experiments, expanded lymphocytes from 9 of 19 (48%) IL-15tg mice were determined to be clonal, consistent with leukemia.

**Progressive alopecia and multi-organ lymphocytic infiltration of leukemic IL-15tg mice**

Animals judged to be in the leukemic phase of their disease by flow cytometric analysis of peripheral blood samples were sacrificed for pathologic analysis. All IL-15tg mice were remarkable for whole-body alopecia (Fig. 4.9), splenomegaly, and lymphadenopathy. Microscopic examination of tissues harvested from these animals showed diffuse lymphocytic infiltrates, most prominent in the peritoneum and surrounding intra-abdominal organs. In addition, all showed marked skin involvement and expansion of splenic white pulp. Effacement of normal architecture by this infiltrate was seen in enlarged lymph nodes. Prominent lung and liver infiltrates were seen in most leukemic animals.

Fig. 4.10 illustrates the skin, lung, and liver pathology found in IL-15tg mice. Skin infiltrates were dense within the dermis with individual lymphoid cells present within the epidermis (Fig. 4.10 b, c). Also remarkable was the presence of mast cells within the dermal infiltrate. Multiple skin ulcerations were evident, and acute inflammatory cells
were present at these foci. In immunohistochemical studies on frozen tissue the invading lymphocytes were CD3 positive (data not shown), while no mouse NK cell marker is available for use on tissue sections. The pattern of cutaneous involvement found in these animals is very similar to that seen in patients with NK/T cell lymphomas\(^{334}\). Other skin changes consistent with long term inflammation include epidermal hyperplasia, hyperkeratosis, and loss of adnexal structures (e.g. hair follicles).

A spectrum of lung involvement was observed. In the least affected mice, dense lymphoid infiltrates were identified around blood vessels. In more affected animals lung involvement was diffuse, involving the walls of alveolar spaces. Fig. 4.10 \(d\) and \(e\) are photomicrographs, taken at 10X and 40X magnification, of a lung section from a transgenic animal. Lymphoid cells are seen surrounding a lymphatic channel, itself containing large lymphocytes as well as marked interstitial involvement. Fig. 4.10 \(h\) and \(i\) demonstrate the common pattern of hepatic disease, with perivascular infiltrates and sinusoidal involvement.

### 4.4 Discussion

IL-15 is a pleiotropic cytokine important in lymphocyte homeostasis and unusual in its tight posttranscriptional control.\(^8\) In this chapter, the phenotype of transgenic mice engineered to efficiently translate and secrete murine IL-15, which is normally abundant as transcript in multiple tissues but poorly translated and secreted, was reported. IL-15\(tg\) mice exhibit peripheral blood lymphocytosis, with significant expansions in both the NK cell and memory-phenotype CD8\(^+\) TCR\(\alpha\beta\) T cell compartments early in life. These
results demonstrate that IL-15 is a growth factor for these cell types in vivo, and are consistent with gene targeting studies demonstrating that IL-15Rα^−/− and IL-15^−/− mice lack NK cells and are severely deficient in memory/activated-phenotype CD8^+ T cells.34,35 Similarly, mice that lack inducible IL-15 gene expression due to disruption of the interferon regulatory factor (IRF)-1 gene,82,83 or are deficient in other IL-15R components,108,109 exhibit NK cell and CD8^+ T cell defects. Continual translation and secretion of the proinflammatory cytokine in the IL-15tg mice over several months eventually led to the manifestation of T-NK lymphocytic leukemia in a significant fraction of mice.

The exogenous provision of high doses of rhIL-15 to wild type or IL-15^−/− mice for one week results in transient NK cell increases.34,128 IL-15 has been shown to drive NK cell differentiation from human71,335 and murine120 IL-2/15Rβ^+ NK cell precursors in the bone marrow, as well as support mature NK cell survival in the absence of serum or other factors.89 This suggests that multiple mechanisms, including increased differentiation and prolonged survival in the periphery, are likely responsible for the accumulation of NK cells observed in IL-15tg mice. It also supports the notion that IL-15 may provide a physiologic set point that normally regulates the total number of NK cells present in vivo. It has been previously shown that IL-15 augments NK cell function33, and consistent with this, fresh NK cells from IL-15tg mice exhibit potent cytolytic activity against YAC-1 tumor target cells.

Similarly, exogenous provision of rhIL-15 to mice for one-week increases memory/activated-phenotype CD8^+ T cell numbers.34,90 Previous studies have suggested
an important role for IL-15 in the antigen-independent maintenance of the memory CD8 T cell pool.\textsuperscript{34,47,90,333} The consistent increase in CD8\textsuperscript{+}CD44\textsuperscript{hi}Ly6C\textsuperscript{+} T cells within IL-15tg mice supports a role for IL-15 as one homeostatic control for this population. Definitive evidence that IL-15 maintains memory-phenotype CD8\textsuperscript{+} T cells would require adoptive transfer experiments into IL-15\textsuperscript{-/-} and wild type mice. In IL-15tg mice, housed within specific pathogen free (SPF) environments, it is unclear what antigens are driving the CD8\textsuperscript{+}CD44\textsuperscript{hi} T cell expansion. We speculate that these expansions could represent an exaggerated response to normally encountered non-pathogenic antigens in the SPF environment. Nishimura et al. recently described transgenic mice that globally over-express the endogenous long signal peptide (LSP) form of IL-15, and observed an increase in functional CD8\textsuperscript{+}CD44\textsuperscript{hi} T cells in lymph nodes and spleen,\textsuperscript{47} yet there was no NK cell expansion or leukemia reported. A recent report of transgenic mice expressing a chimeric hIL-4R/mIL-15R on T cells suggests that there are unique properties of CD8\textsuperscript{+}CD44\textsuperscript{hi} T cells, in addition to high IL-2/15R\(\beta\) expression, that allows them to selectively proliferate in response to IL-15 signals.\textsuperscript{336} It is interesting that IL-15 appears to control the level of both NK and memory-phenotype CD8\textsuperscript{+} T cells as both of these effectors act coordinately for the clearance of many intracellular pathogens.\textsuperscript{12,337}

Collectively, these data point toward potential clinical utility for time-limited provision of low amounts of IL-15 in the expansion of immune effector cells in immunodeficient patients, or as an adjuvant to boost cellular immune responses following vaccination.

IL-15tg mice develop fatal lymphocytic leukemia after several months of chronic proinflammatory cytokine stimulation and lymphocyte expansion. In IL-15tg mice with
leukemia, clinical findings such as weight loss, reduced activity, and respiratory distress occur immediately prior to premature death. The extraordinary lymphocyte elevations, clonality, clinical course, and histopathology are all consistent with the development of leukemia. The striking leukemic manifestation of this disease suggests that the malignant cell is likely derived from blood or bone marrow, as opposed to a peripheral lymphoid tissues. The chronic proliferation and/or extended survival of lymphocytes in IL-15tg mice likely contribute to the accumulation of additional transforming mutations, as has been postulated for other cancers. In support of this, IL-15 has been shown to costimulate stem cell proliferation, and extend the survival of normal NK cells and T cells by preventing apoptosis. Studies to sequentially analyze these cells for such secondary genetic alterations are underway, with the hope of providing new insights into the pathogenesis of lymphocytic leukemia.

IL-15 was originally identified as a 4-α helix bundle cytokine with similar in vitro biological properties as IL-2, consistent with their shared receptor components (IL-2/15Rβγc). Interestingly, Ishida and colleagues generated mice that globally over-express recombinant IL-2 driven by a MHC class I promoter. These IL-2 transgenic mice develop a mild lymphocytic skin infiltrate composed of T cells, but do not demonstrate excessive lymphocyte expansions or malignant transformation such as leukemia. To my knowledge, IL-15tg mice are unique among cytokine transgenics in their induction of leukemia. Collectively, these data strongly support existing data that show little in vivo redundancy when comparing the roles of IL-2 and IL-15 in health and disease.
The chronic lymphocytosis and subsequent leukemia observed in IL-15tg mice share some clinical features and manifestations with the human disease large granular lymphocytic (LGL) leukemia. These include extra-lymphoid involvement, a chronic course evolving to an acute expansion, and the prevalence of T cell subtypes. Further, the histopathology of the lymphocytic infiltrates in IL-15tg mice resembles those observed in NK-T lymphomas. Lymphocytes isolated from patients with LGL leukemia express all three components of the IL-15R complex (αβγ), and increased IL-15 expression was noted in macrophages from these patients. In addition, early in vitro propagation of LGL leukemia cell lines can be maintained in the presence of IL-15. We are currently investigating as to whether increased IL-15 protein expression in the bone marrow or other tissues of LGL leukemia patients may contribute to the initiation or pathophysiology of this disease.
Three primary posttranscriptional checkpoints were eliminated: 5' AUGs, the inefficient IL-15 signal peptide, and a C-terminus retention sequence. Near global over-expression was achieved by the MHC class I promoter, efficient translation and secretion by use of the IL-2 signal peptide, and stabilization of C-terminus by the addition of the FLAG epitope. The 3' portion of the hGH gene is fused out-of-frame for straightforward identification of the transgene by Southern blot and to optimize transgene expression in vivo.
Fig. 4.2 Detection and expression of the IL-15 transgene.

a, Three FVB/N IL-15tg lines positive by Southern blot analysis with hGH gene probe (3284, 3285, 3304), while negative line (3286) is shown for comparison. Triangle denotes the expected 2.6 kb size of the SstI digested transgene. b, Real-time RT-PCR of tissues from a representative IL-15tg mouse. Results show the mean ± SEM of triplicate measurements of IL-15 transgene expression from total cellular RNA, isolated from IL-15tg tissues.
Fig. 4.2  Detection and expression of the IL-15 transgene.

c, Immunoblot analysis of splenocyte lysates for transgenic protein with the FLAG epitope tag. Equal amounts of total cellular protein were loaded as follows: lane 1, spleen cells from non-transgenic WT FVB mouse; lanes 2-4, spleen cells from three different IL-15tg mice.  
d, IL-15 protein levels in IL-15 transgenic mice.  

Serum from IL-15tg mice and non-transgenic wild type controls were analyzed using a specific murine IL-15 ELISA (see text for details).
Fig. 4.3 Early lymphocytosis in IL-15 transgenic mice.

a and b, Total white blood cell and lymphocyte counts in IL-15tg (n=71) and non-transgenic littermate control (n=51) at 6 weeks of age. Graphs show the mean ± SEM, with a significant increase in both the absolute white blood cell (*, $P < 10^{-7}$) and lymphocyte counts (**, $P < 10^{-5}$) in IL-15tg mice. c, Photomicrographs (100X) of expanded large granular lymphocytes from an IL-15tg peripheral blood smear at 6 weeks of age.
Fig. 4.4 Early expansion of NK cells in IL-15tg mice.

a. Flow cytometric analysis of peripheral blood lymphocytes from representative IL-15tg and non-transgenic littermate wild type controls (WT). NK cells are DX5^CD3^-Ly49^+, while T cells are CD3^DX5^-Ly49D-. b, IL-15tg mice (n=71) have a significant increase in the absolute NK cell number, compared to non-transgenic littermate controls (n=51) (*, P < 10^-11). Data shown are the mean ± SEM. c, Cytotoxicity of fresh leukocytes isolated from IL-15tg or non-transgenic wild type littermate controls (WT) against YAC-1 tumor targets, without any additional in vitro or in vivo activation. Data shown are the mean ± SEM of triplicate wells from three representative IL-15tg and two WT control mice.
Fig. 4.5  
Early expansion of CD8+ T cells within IL-15tg mice.

a. Flow cytometric analysis of peripheral blood lymphocytes from representative IL-15tg and non-transgenic littermate wild type controls (WT). Most lymphocytes in wt mice are CD3+TCRβ+ T cells, while the percentage of this population is reduced in IL-15tg mice due to dilution by the expanded NK cells. The CD4:CD8 ratio is significantly inverted in IL-15tg mice. b. The absolute number of CD4+ T cells is identical in IL-15tg and control mice. c. The absolute number of CD8+ T cells is significantly increased in IL-15tg (P < 10^-4), compared to control mice. This increase in CD8+ T cells is responsible for the CD4:CD8 ratio. For b and c data represent the mean CD4 or CD8 counts ± SEM of IL-15tg (n=71) and control (n=51) mice.
Fig. 4.6 Memory-phenotype of expanded CD8⁺ T cells.

Flow cytometric analysis of peripheral blood lymphocytes from a representative IL-15tg mouse at 6 weeks of age, demonstrating the CD44⁺Ly6C⁺CD69⁺CD62L⁻ phenotype. Similar results were obtained in all IL-15tg mice examined (n=10).
Later, IL-15tg mice develop fatal lymphocytic leukemia.

a. Representative flow cytometric analyses of peripheral blood lymphocytes isolated from representative IL-15tg mice immediately prior to death. While the DX5+CD3- NK cell expansion persisted, the major lymphocyte population is CD3⁺TCRβ⁺DX5⁻⁻ T cells.  

b. Similar lymphocyte expansions are evident in multiple lymphoid tissues from leukemic IL-15tg mice (blood, spleen, bone marrow), as illustrated by a representative IL-15tg mouse.
Later, IL-15tg mice develop fatal lymphocytic leukemia.

c, Leukemic IL-15tg mice have gross splenomegaly, as evidenced by increased spleen to body weight ratio. Graph shows the mean spleen/body weight ratio ± SEM of 22 leukemic IL-15tg mice. d, Photomicrographs (100X) illustrating the morphology of the leukemic lymphocytes from peripheral blood smears of four representative IL-15tg mice.
Fig. 4.8  Clonal TCRβ T cell expansion in IL-15tg mice.

a, Schematic of an example rearranged TCRβ gene that was observed in an IL-15tg mouse that died of fatal lymphocytic leukemia. b, DNA PCR gel showing a monoclonal Jβ2.7 usage in the expanded lymphocytes from an IL-15tg mouse (tg), compared to polyclonal control (C). c, DNA PCR gel showing a monoclonal Vβ16-Dβ2-Jβ2.7 usage in the expanded lymphocytes from an IL-15tg mouse. A faint band is visible with the prominently used Vβ4, and is present on most gels examining the FVB/N TCRβ.
Fig. 4.8 Clonal TCRβ T cell expansion in IL-15tg mice.

An example of clonal TCR Vβ6+ T cell population in an IL-15tg mouse utilizing flow cytometry. Blood cells were gated on CD3+ lymphocytes and assessed for expression of the Vβ6 TCR. A significant clonal population was detected as 53% of blood T cells expressed this single TCR Vβ chain.
Fig. 4.9 Progressive alopecia in IL-15tg mice.

Photograph of a representative 18-week-old IL-15tg mouse, illustrating the progressive alopecia that involves 50-90% of the skin surface area. The alopecia typically begins at 4-6 weeks of age initially involving the head and proximal limbs. A sex and age-matched WT FVB mouse is shown below for comparison.
Fig. 4.10 Multi-organ lymphocytic infiltration in leukemic IL-15tg mice.

Histology sections of skin (a-c), lung (d-f) and liver (g-i) stained with hematoxylin and eosin. Low power (10X) micrographs contrast WT (a, d, g) and IL-15tg (b, e, h) tissues. High power (40X) micrographs demonstrate the lymphocytic morphology of the infiltrating cells in the IL-15tg mice (c, f, i). See text for detailed description of the pathology.
CHAPTER 5

CONCLUSIONS

5.1 Future Experiments

5.1.1 NK Cell Development

In chapter 2, a model of human NK cell development (Fig. 2.7) is described. This model includes an early phase dependent upon the class III receptor tyrosine kinase ligands KL and FL for differentiation of an NK progenitor (NK$_{pre}$) to the IL-2/15R$\beta^+$CD56$^-$ NK precursor (NK$_{pre}$), and a late phase dependent upon IL-15 for further differentiation of the NK$_{pre}$ to a mature CD56$^+$CD3$^+$IL-2/15R$\beta^+$ NK cell. Based upon this novel in vitro culture system, several experiments may now be performed examining the process of differentiation of NK$_{pre}$ to mature NK cells at the molecular level. Planned experiments include utilizing cDNA microarray analyses to assess global gene and restriction landmark genomic scanning (RLGS)$^{339-341}$ to examine the methylation status of genes as NK$_{pre}$ differentiate into NK cells in the presence of IL-15. Such interrogation of the NK cell genetic program will likely identity transcription factors that function as major switches controlling NK-lineage specific genes. Further, as transcription factors...
controlling cell cycle and survival of lymphocytes are often targets during leukemogenesis, such analyses may then lead to genes involved in the malignant transformation of NK cells.

The regulatory mechanisms orchestrating the acquisition of NK cell receptors during human NK cell development is not currently known. Moreover, as the MHC class I recognizing receptors in mice and humans are different, understanding this process during human NK cell development must be performed using human cells in vitro. The culture system described in Chapter 2 would allow the cell sorting of NKpro and NKpre and evaluation of their NKR acquisition during NK cell differentiation, both in the presence and absence of autologous bone marrow stromal layers. Such information would identify if stromal cell contact or MHC class I ligand interactions regulate the acquisition of NK cell receptors, thereby further refining our understanding of the cellular events governing NK cell ontogeny.

5.1.2 IL-15 as a costimulator of IFN-γ production

In chapter 3 it is demonstrated in vivo that IL-15 plays a role in LPS-induced innate immune IFN-γ production. While simulated gram negative infection with LPS is a useful model system, it will now be interesting to perform similar in vivo experiments in model infections that depend upon IFN-γ for clearance such as *Toxoplasma gondii*. Preliminary evidence also suggests that IFN-γ induced by injection of exogenous recombinant IL-12 depends on the presence of IL-15 (Fig. 5.1). Additional experiments will examine whether 1) small amounts of constitutively produced IL-15 or 2) IL-15
induced in the cytokine cascade following IL-12 injection is the mechanism responsible for such IL-15-dependency, as well as the tissue compartments involved.

5.1.3 IL-15 transgenic mice as a model of lymphocytic leukemia

Chapter 4 describes the characterization of IL-15 transgenic mice including an early lymphocytosis and later, lymphocytic leukemia. While the leukemia is well documented, with clonal T cell populations, multi-organ infiltration and premature death, additional experiments will be performed to better characterize this malignant process. For example, several questions remain to be addressed including are these malignant lymphocytes transplantable? Is the “soil” of the IL-15 transgenic mouse or the “seed” of transgenic stem cells adequate for the development of leukemia? Do lymphocytes accumulate early due to prolonged survival or enhanced proliferation? The hypothesis that during a period of prolonged stimulation with IL-15, resulting in enhanced proliferation or survival, lymphocytes have increased susceptibility to transforming mutations is put forth in Chapter 4. In this case, additional heterogenous mutations that result in transformation will be present in the leukemias that develop within IL-15tg mice. These mice could therefore be used as a tool for identifying genetic changes associated with the malignant transformation of lymphocytes. Indeed, experiments are planned to compare the global gene expression (cDNA microarrays) in the benignly expanded lymphocytes, and later the malignant leukemic cells from the same IL-15tg mouse. Gene products identified as being up- or down-regulated in the leukemic cells, compared to benign lymphocytes could then be evaluated for transforming ability and presence in human leukemia patients.
5.2 Interleukin-15: Therapeutic Promise?

A remarkable amount of progress has been made in our understanding of IL-15 biology, its role in the normal host immune response, and its potential for participation in the pathogenesis of disease since its discovery in 1994. As IL-15 has pleiotropic activity that ultimately results in immunoregulatory cross talk between natural and specific immune cells, it may now be considered a cytokine that bridges the innate and adaptive immune systems (Fig. 1.4).

Out of the multitude of basic studies on the biology of IL-15 and pre-clinical models of human disease reviewed in Chapter 1, it is important to identify those lines of investigation that point towards potential clinical benefit. Two major directions of immediate interest are 1) immune augmentation through exogenous provision of IL-15 and 2) immune down-regulation through elimination of improperly expressed IL-15. Additional clinical applications of IL-15 are emerging from basic and pre-clinical studies on this cytokine (e.g. vaccine adjuvant) but require further development.

5.2.1 Cytokine therapy with IL-15

Numerous cytokines produced through recombinant DNA technology have now been used successfully to expand or activate immune cells or their progenitors in patients including erythropoietin (EPO), granulocyte colony stimulating factor (G-CSF), macrophage (M)-CSF, granulocyte-macrophage (GM)-CSF, thrombopoietin (TPO), IL-11, IFN-α, and IL-2. One direction with strong rationale from Chapters 2 and 4 and studies reviewed in Chapter 1 is the use of exogenous IL-15 to expand lymphocyte
subsets in patients with cancer and/or immunodeficiency. Can IL-15 be used in vivo to increase those cell types that require it for development, expansion, and/or survival (e.g. NK cells, NK-T cells, CD8+ memory T cells) without significant toxicity to patients? Obvious parallels can be made between immunotherapy with IL-2 and IL-15 due to their similar in vitro biologic effects and shared receptor components. As IL-2 has been FDA-approved since 1992, and is effective for the increase of lymphocyte subsets at low doses without significant toxicities, \textsuperscript{101-103} why pursue therapeutic pathways with IL-15? IL-2 and IL-15 utilize unique high affinity receptor complexes that have dramatically different expression profiles in humans. Because of this, low concentrations of exogenous IL-15 may have the ability to modulate other immune cell compartments without significant toxicity. This is supported by the fact that IL-15 and IL-2 have very different, non-redundant roles in vivo in mice. \textsuperscript{35,82,83,106,107,110} In addition, IL-15 and IL-2 appear to have different biodistribution following administration in vivo, with IL-15 more localized in the BM. \textsuperscript{343} In murine models comparing treatment with IL-15 to IL-2 for efficacy against experimental lung metastases (MCA-205), doses of IL-15 six times higher than IL-2 were required to induce pulmonary vascular leak, ultimately resulting in a higher therapeutic index for IL-15.\textsuperscript{128} The first step towards evaluating IL-15 for cytokine therapy would be pre-clinical animal trials examining biological parameters and toxicity, and if warranted, phase I and II studies determining maximal tolerated doses and biological modulation when IL-15 is administered to humans.
5.2.2 Blockade of improperly expressed IL-15

Agents that selectively block the action of improperly regulated cytokines have also been successfully used in the clinic, one prime example being soluble TNF receptor for inflammatory conditions.\textsuperscript{344,345} Several studies in murine models have identified agents that can block the action of murine IL-15 in vivo, including a soluble IL-15Rα\textsuperscript{202} an antagonist IL-15mutant/Fcγ2a fusion protein,\textsuperscript{316} and a polyclonal neutralizing antiserum (Chapter 3). Strong evidence (described in Chapter 1) now exists for the role of IL-15 in rheumatoid arthritis, and the blockade of IL-15 is of potential clinical benefit.\textsuperscript{346} Thus, with recent evidence in animal models demonstrating that blockade of IL-15/IL-15R in vivo provides amelioration from collagen-induced arthritis,\textsuperscript{202} and Ag-specific delayed-type hypersensitivity responses,\textsuperscript{316} performance of additional pre-clinical studies, and early clinical trials appear warranted. As there is now strong pre-clinical and animal models suggesting IL-15 may be involved in leukemias of lymphocytes, such as LGL leukemia, these patients may also benefit from blockade of the IL-15/IL-15R pathway (Chapter 4). Thus, further development of effective human IL-15 blocking agents (e.g. high affinity soluble IL-15Rα, humanized anti-IL-15 mAb) with in vivo blocking activity could facilitate rapid translation to the clinic.

5.3 Closing Remarks

As with most aspects of nature, our understanding of IL-15 remains far from complete. The studies presented here have sought to ask important, appropriate, and useful questions about the biology of IL-15 and its place within the innate immune system.
While this endeavor has been fruitful, resulting in novel observations that extend our knowledge of IL-15 and its role in NK cell development, as a regulator of innate immune cytokine production, and importance in lymphocyte homeostasis, new questions have arisen that are waiting to be addressed. Such new directions were briefly described above (Section 5.1), and it is my sincere hope that the new tools are utilized to yet further advance this topic of Immunology, ultimately leading toward IL-15 as an agent or target to ameliorate human disease.
Fig. 5.1  IL-15 is required for rIL-12-induced IFN-γ in vivo.

C57Bl/6 SCID mice were treated with anti-IL-15 antiserum (Immunex) or control, and twelve hours later injected with rIL-12 (1 ug, i.p.). Twenty-four hours after the rIL-12 infection, serum was collected and assayed for mIFN-γ using an ELISA (Biosource).


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