INTERLEUKIN-15-MEDIATED IMMUNOTOXICITY AND EXACERBATION OF SEPSIS:
ROLE OF NATURAL KILLER CELLS AND INTERFERON γ

By

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Dissertation

Submitted to the Faculty of the
Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Microbiology and Immunology

December 2016

Nashville, Tennessee

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ACKNOWLEDGEMENTS

I would like to thank my thesis mentor, Dr. Edward Sherwood for his tremendous instruction and support during my entire Ph.D. training course. I joined Dr. Sherwood’s lab in the department of Microbiology and Immunology of University of Texas Medical Branch (UTMB) at Galveston in 2011. I appreciated the offer Dr. Sherwood provided to move to Vanderbilt University together. During the new lab setup period, he gave me a lot of encouragement about going out to meet students and professors at the Pathology, Microbiology and Immunology Department. From making new friends in our department, I started to know a lot of useful information and resources about how to adapt to new life in Nashville and how to prepare for the qualifying exam. Dr. Sherwood is a highly responsible and enthusiastic mentor although he has busy medical practice in the operating room and needs to write many grants. I have to say, he is the most diligent person I have seen in the world. It is amazing to me that he never seems to be exhausted or desensitized with reviewing our papers, reading papers, writing grants, and discussing data and new ideas. But at the same time, he never passes his stress to us and allows us to work in our hours at our own pace. He is also open-minded as he allows us to explore new ideas, techniques or models in our research.

I feel very fortunate to have great colleagues in Dr. Sherwood’s lab. Julia Bohannon is a great friend and instructor that extensively contribute to my Ph.D. training. She teaches me many basic lab techniques and offers me good suggestions from being a great scientist to being a great mom. I would like to thank Liming Luan, a wonderful helper with my experiments. With his generous help, I am able to fasten my graduation by generating a lot of data from big
experiments. I would like to thank Jingbin Wang for helping me breed IL-15 KO mice during my maternity leave and check mice at night sometimes. I thank Naeem Patil and Antonio Hernandez for their great help with my experiments and paper review. I also thank Benjamin Fensterheim as he is the greatest teacher for my academic writing and speaking. He also tells me many interesting things in music, politics, movies and living styles in the USA.

Special thanks to my thesis committee who have been continuously supportive throughout my graduate training. I thank Dr. Luc Van Kaer for chairing my committee and providing useful scientific knowledge and suggestions. I felt very grateful when he agreed to chair my committee, even if he never taught me or even knew me, as a transfer student by that time. I thank Dr. Lorraine Ware for providing a detailed review of my proposal for the qualifying exam and supporting my thesis project and new parental life. I thank Dr. Stokes Peebles for lending me his immunology textbook, asking insightful scientific questions and taking the time to meet me out of his busy schedule. Finally, I thank Dr. Daniel Moore for providing great suggestions for my project, offering me IL-15 knockout mice from the colony in his lab and allowing me to work with Blair Stocks, an MSTP student in his lab for human IL-15 superagonist project.

I also would like to thank other people in our department. Whitney Rabacal is my great friend in Dr. Eric Sabzda’s lab. She taught me how to isolate leukocytes from livers and how to do immunohistochemistry staining on my slides. We also encourage and support each other when we meet with difficulties for our research or life. I thank Dr. Lan Wu for providing a lot of good suggestions about my science career and the balance between work and family. I also
want to thank Pavlo Gilchuk for his generous offer of flow antibodies for my experiments. Special thanks to my classmates and professors at UTMB who have offered great help during my first year in the USA.

At last, I would like to thank my family. My parents and parents in law have always been supportive of my graduate training. They helped me take care of Amy, my daughter so I can have sufficient time to devote myself to scientific research. I also thank my husband, Xiang Zhang, for conferring endless support and care and encouraging me to take exercise after work. Finally, I would like to thank my daughter Amy, who is the best thing that ever happened to me. She gave me a lot of inspirations for everyday life and makes me a stronger and a greater person.

Overall, I would like to thank Vanderbilt University for providing a friendly and multi-cultural environment for international students. I feel grateful to meet friends from other countries, which introduces me to a broader world and become open-minded.
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<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cells</td>
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<tr>
<td>APC</td>
<td>Activated protein C</td>
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<td>NK</td>
<td>Natural killer</td>
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<td>NKT</td>
<td>Natural killer T</td>
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<td>mCD8⁺T</td>
<td>Memory phenotype CD8⁺ T</td>
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<td>IEL</td>
<td>Intraepithelial lymphocytes</td>
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<td>IL-15 SA</td>
<td>IL-15 superagonist</td>
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<tr>
<td>R</td>
<td>receptor</td>
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<tr>
<td>JAK</td>
<td>Janus kinase</td>
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<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription proteins</td>
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<td>DC</td>
<td>Dendritic cells</td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor</td>
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<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>G-CSF</td>
<td>granulocyte colony-stimulating factor</td>
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<td>MOF</td>
<td>Multiple organ failure</td>
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<td>PAMP</td>
<td>Pathogen-associated molecular patterns</td>
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<td>PPR</td>
<td>Pattern recognition receptors</td>
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<td>TLR</td>
<td>Toll-like receptor</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<td>Cecal ligation and puncture</td>
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<td>Lipopolysaccharide</td>
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<td>ALT</td>
<td>Alanine Aminotransferase</td>
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<tr>
<td>AST</td>
<td>Aspartate Aminotransferase</td>
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<tr>
<td>BUN</td>
<td>Blood urea nitrogen</td>
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<td>LCMV</td>
<td>Lymphocytic choriomeningitis virus</td>
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<td>SOFA</td>
<td>Sepsis-related organ failure assessment</td>
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<td>MAP</td>
<td>Mean arterial pressure</td>
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CHAPTER I
INTRODUCTION

Thesis overview

This thesis provides insight into the immunobiology of the cytokine Interleukin-15 (IL-15) and the receptor-ligand complex known as IL-15 superagonist (IL-15 SA, IL-15/IL-15 Rα complex), the immunotoxicity of IL-15 SA and the role of endogenous and exogenous IL-15 in the pathogenesis of sepsis. In Chapter I, I provide an overview of current concepts in immunotherapy, IL-15 application in immunotherapy, the discovery of IL-15, its trans-presentation, immunological functions, toxicity and role in diseases. I also review the generation of IL-15 SA, its anti-tumor effect and clinical drug development. I then shift focus to provide an overview of sepsis, its epidemiology and pathogenesis, experimental models of sepsis, and sepsis therapies. I emphasize the immunological alterations caused by sepsis and summarize the current literature about the role of IL-15 and IL-15-dependent cell populations in the pathobiology of sepsis. In Chapter II, I present my studies that define the mechanisms by which IL-15 SA causes dose- and time-dependent immune-toxicity in mice, a constellation characterized by hypothermia, weight loss, liver injury and mortality. This is the first report to address the toxic effects induced by IL-15 SA, although there is substantial interest in applying IL-15 SA in cancer immunotherapy. My findings provide a detailed analysis of NK, NKT and CD8+ T cell subsets, particularly in regard to the importance of IL-15 for their maintenance and function. My studies further indicate that the systemic toxicity of IL-15 SA is mediated by hyperproliferation of activated NK cells and production of the pro-inflammatory cytokine IFNγ. In Chapter III, I examine the pathogenic role of IL-15 SA in the context of sepsis. IL-15 is
currently promoted as an immunotherapeutic to treat sepsis-associated immunosuppression. However, my studies show that sustained administration of IL-15 SA to wild type mice or acute administration at the onset of sepsis will exacerbate sepsis-associated pathobiology by expanding or activating NK cells. My studies further show that IFNγ contributes significantly to NK cell-mediated inflammation and injury during septic shock as well as in IL-15 SA-induced exacerbation of sepsis-associated pathobiology. In Chapter IV I evaluate the role of endogenous IL-15 in the pathogenesis of sepsis. I fully assess the response of IL-15 knockout (IL-15 KO) mice to septic shock induced by lethal cecal ligation and puncture (CLP) or lipopolysaccharide (LPS) challenge. My study shows that IL-15 KO mice have marked depletion of NK cells and mCD8^+ T cells and are resistant to sepsis-induced mortality, organ injury and physiologic dysfunction. The restoration of NK cells, but not mCD8^+ T cells, by treatment with exogenous IL-15 restores the susceptibility of IL-15 KO mice to sepsis. Acute neutralization of IL-15 prior to the onset of sepsis is not protective whereas prolonged treatment with anti-IL-15 causes NK cell depletion and renders wild type mice resistant to septic shock. Thus, I conclude that endogenous IL-15 doesn’t play a direct role in the pathogenesis of septic shock, but is able to contribute to septic mortality by maintaining the integrity of NK cells, which are considered to be a detrimental mediator during septic shock. In Chapter V, I summarize my thesis findings and address future directions that will provide more useful information regarding the immunobiology of IL-15, IL-15 SA and sepsis.

Immunotherapy background

Immunotherapy is a treatment approach that modulates our body’s immune response to fight cancer, infections and inflammatory diseases (1-3). Compared to conventional
chemotherapy, immunotherapy elicits less toxic, more antigen-specific and long-lasting memory responses against target cells like tumor cells (4, 5). Over the past decades, immunotherapy has been a rapidly evolving field of basic and clinical research. Recent advances show that the de-inhibition of cytotoxic lymphocytes by antibodies that block co-inhibitory receptors such as programmed death-1 (PD-1) and cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) on T lymphocytes are effective in treating advanced cancers. These promising findings have recently led to fast track approval of the monoclonal antibodies pembrolizumab (anti-PD-1) and ipilimumab (anti-CTLA-4) for the treatment of metastatic melanoma (6, 7).

Emerging evidence shows cytokine-based treatments also hold promise for the treatment of cancer. As small proteins secreted by a broad range of cells in the host, cytokines play a critical role in preventing tumor development and progression by directly stimulating cytotoxic effector cells to recognize and kill tumor cells (8). The cytokine IL-2 was the first effective cytokine immunotherapy for the treatment of renal cancer and metastatic melanoma. The administration of high-dose IL-2 in vivo enhances anti-tumor activity by stimulating cytotoxic CD8$^+$ T and NK cell growth (9). However, IL-2 also exerts an immunoregulatory effect by maintaining regulatory T cells and inducing activation-induced cell death (10, 11). Bolus IL-2 injection has been reported to cause a significant capillary leak, most notably in the lungs, in cancer patients, limiting its application in the treatment of human cancers (12). Ex vivo stimulation of lymphocytes by IL-2 in culture resulted in the development of lymphokine-activated killer (LAK) cells that are capable of killing cancer cells in patients along with the administration of high-dose IL-2 (13). However, like IL-2 therapy, LAK cells/IL-2 treatment resulted in significant toxicity in cancer patients, including a severe capillary leak syndrome (14).
Other lymphocyte-activating cytokines such as interferon gamma (IFNγ), IL-12, IL-15, IL-18 and IL-21, are currently undergoing clinical trials to test their efficacy for treatment of patients with advanced cancer (8).

**IL-15: a promising candidate in immunotherapy**

In my thesis, I focused on the cytokine IL-15, a 14-15 kDa cytokine that is essential for the development, proliferation and biological activities of natural killer (NK), natural killer T (NKT), memory phenotype (m) CD8+ T lymphocytes and intraepithelial cells (IELs) (15). Like IL-2, IL-15 utilizes receptor β and the common γ chain to induce lymphocyte activation. However, unlike IL-2, IL-15 does not maintain regulatory T cells or mediate apoptosis of activated effector cells, nor induce a toxic capillary leak syndrome (11, 15). Thus, IL-15 may be superior to IL-2 as an agent to enhance anti-tumor or anti-viral immunity in humans (16). As such, IL-15 has been used to augment the efficacy of human immunodeficiency virus (HIV) vaccines by generating long-lasting cellular immunity (17). Treatment with IL-15 alone, or as an adjuvant in anti-tumor vaccines, has shown effectiveness in mice with melanoma (18). Engineered expression of IL-15 by antigen-presenting cells enhances the efficacy of tumor-infiltrating lymphocytes isolated from patients with melanoma (19). A recent paper by Conlon et al reported the first-in-human trial of recombinant human IL-15 in cancer patients (20). IL-15 induced clearance of pulmonary lesions in patients with metastatic melanoma and renal cancer. The administration of IL-15 also prompts bone marrow reconstitution after allogeneic bone marrow transplantation (21). In addition, IL-15 holds promise for treatment of sepsis via restoration of immune cell dysfunction.
in septic patients who are immunosuppressed and at a high risk of developing secondary infections (22).

The recent development of IL-15 superagonist (IL-15 SA, IL-15/IL-15 Rα complex) advances the potential of IL-15-based therapy. IL-15 SA has more powerful and long-lasting biological activities than native IL-15 (23). A more thorough introduction of IL-15 SA will be described later in this chapter. One of Altar's lead products, ALT-803, an IL-15/IL-15Rα fusion protein, possesses enhanced anti-tumor efficacy in experimental tumor models compared to IL-15 alone (24-26). ALT-803 is currently in Phase I/II clinical trials for the treatment of patients with relapsed or refractory multiple myeloma (27).

**IL-15 discovery, trans-presentation and expression**

IL-15 is a member of four-α-helix bundle family of cytokines that have 3-dimensional structure with four-helix “up-up-down-down” bundles (other cytokines include IL-2, IL-4, IL-7 and IL-9) (28). IL-15 was first identified in 1994 by Kenneth et al as a T lymphocyte growth factor (29). IL-15 is unique among four-helix bundle cytokines in that it utilizes a unique mechanism of action referred to as trans-presentation (30). The unique high-affinity receptor alpha (IL-15Rα), which is expressed by IL-15-producing cells, such as macrophages and dendritic cells, chaperones IL-15 from inside the cell and shuttles it to the cell surface for delivery to neighboring NK, NKT and memory CD8⁺ T cells expressing IL-15 receptor β (also known as IL-2 receptor β) and the common γ chain (shared with other cytokines including IL-2, IL-4, IL-7, IL-9 and IL-21) (31, 32) (**Figure 1**). Mouse IL-15 shares 70% amino acid sequence identity with
human IL-15. Both human and mouse IL-15 exhibit similar properties of trans-presentation, signal transduction and biological activities (33, 34).

IL-15 mRNA is constitutively expressed by a wide variety of cells including dendritic cells (DCs), monocytes, macrophages, bone marrow stromal cells, and intestinal epithelial cells (35, 36). IL-15 can be further induced by stimulation with the gram negative bacterial product lipopolysaccharide (LPS), type I (IFNα/β) and type II (IFNγ) interferons (IFN), double-stranded RNA, and infection with viruses (37, 38). Transpresentation of IL-15 is required for development and homeostasis of IL-15-dependent cell lineages and regulation of distinct biological events in the body (30, 39, 40).

![Figure 1: IL-15 transpresentation and biological functions.](image)

Unlike most cytokines, which are secreted in soluble form, IL-15 is expressed in association with its high affinity IL-15 Rα on the surface of IL-15-producing cells and delivers signals to target cells that express IL-2 Rβ/yc receptor subunits. IL-15 stimulates proliferation and activation of NK, NKT and CD8\(^+\) T cells, especially memory phenotype CD8\(^+\) T cells, leading to increased cytotoxicity and production of IFN-γ and IFN-α. In addition, IL-15 inhibits apoptosis of immune cells by increasing expression of anti-apoptotic proteins and decreasing production of pro-apoptotic proteins. Adapted from (32).
**IL-15 signaling**

Trans-presented IL-15/IL-15 Rα signals through β and γ chains expressed on responding cells, leading to the recruitment and activation of Janus kinase 1 and 3 (JAK1 and JAK3) (40). Activated JAK1 and JAK3 further phosphorylate signal transducer and activator of transcription proteins 3 and 5 (STAT3 and STAT5), which prompts the transcription of IL-15-modulated genes in effector cells (Figure 2) (40). IL-15 alone can also bind to the intermediate-affinity β and γ receptor complex in the absence of the high affinity receptor α, resulting in the activation of other tyrosine kinases such as Lck, Fyn, Lyn, Syk and cross talk with the PI3K and MAPK pathways (34).

**Figure 2: IL-15 signaling pathway.** IL-15 is transpresented in association of IL-15 Rα to the IL-2 receptor β and γc subunits, leading to phosphorylation and activation of Janus kinase 1 (Jak1) and Jak3, respectively. Activated Jak1 and Jak3 result in phosphorylation and activation of signal transducer and activator of transcription 3 (STAT3) and STAT5. Adapted from *Wikipedia* [https://en.wikipedia.org/wiki/Interleukin_15](https://en.wikipedia.org/wiki/Interleukin_15)
**IL-15 superagonist (IL-15 SA)**

In light of this unique transpresentation of IL-15, combination of IL-15 with soluble IL-15 receptor α in solution generates a novel form of IL-15 with similar but more potent and prolonged actions than native IL-15 (23). Thus, the resulting IL-15/IL-15Rα complex has been termed IL-15 superagonist (IL-15 SA). Multiple lines of evidence have demonstrated that IL-15 SA is more effective than IL-15 in causing regression of established melanoma and pancreatic cancer in mice (41, 42). IL-15 SA appears to be superior to IL-15 as a promising anti-cancer agent. However, little is known about the toxicity profile of IL-15 SA as it advances through drug development. In Chapter II, I address the adverse effects of IL-15 SA in vivo and provide mechanistic insight into the immune-toxicity of IL-15 SA. IL-15 SA has also been promoted as a promising immunostimulatory agent to reverse sepsis-associated immunosuppression. In Chapter III, I examine whether sustained treatment or acute administration of IL-15 SA alters sepsis-associated pathogenesis.

**Impact of IL-15 on innate immune cells**

**Natural Killer (NK) Cells**

Natural killer (NK) cells are bone marrow-derived large granular lymphocytes possessing a “natural” ability to kill tumors without prior priming (43). Although NK cells share the same lineage origin with T and B lymphocytes of the adaptive immune system, NK cells lack the expression of germ-line rearranged antigen receptors that recognize specific antigens. However, NK cells possess the capability of mounting a rapid, non-specific innate immune response against cancer cells and cells infected with intracellular pathogens (44). Activation of NK cells is governed by the balance between signaling mediated through inhibitory and activating
receptors (45). Inhibitory receptors include Ly49A/C/I/P in mice and KLR2DL1/2/3/5 in humans that deliver inhibitory signals to NK cells upon engagement with mouse H-2 and human leukocyte antigen (HLA) class I (46). During infections, NK cells recognize and destroy cells that downregulate expression of the class I major histocompatibility complex (MHC-I) such as some virus-infected cells (“missing self” hypothesis) (47). However, under some situations, NK cells are still capable of killing cells that express normal levels of MHC-I as long as their activating receptors are activated by interaction with the target cell (48, 49). NK cell activating receptors include NKG2B/C/D/E and NKp30/44/46 which bind to stress-induced ligands or viral antigens (46). NK cells can also be activated by cytokines such as type I IFNs, IL-2, IL-12, IL-15 and IL-18, which are released by infected cells and activated antigen-presenting cells (APCs) (50-52).

Distinct from other activating cytokines, IL-15 plays an essential role in the development, differentiation and survival of NK cells (53-55). The development of NK cells in the bone marrow arises from committed NK precursors that express IL-15 signaling chain β (CD122) before the initiation of expression of other characteristic markers like NK1.1 or DX5 (56). The importance of IL-15 in the development of NK cells is further evidenced by the NK cell deficiency observed in IL-15-deficient, IL-15Rα-deficient, and IL-2/IL-15β-deficient mice (53-55).

As IL-15 is expressed by a wide array of cell types, the relative importance of cell-specific IL-15 expression for maintaining NK cells was examined by genetic depletion of IL-15 or IL-15Rα. Specific depletion of IL-15Rα in DCs (via CD11c-Cre/Flox-IL-15Rα) or macrophages (via LysM-Cre/Flox-IL-15Rα) causes the loss of one half of all peripheral NK cells, whereas NK cells in bone marrow are not affected (57). Another study showed that overexpression of IL-15Rα by DCs in
IL-15Rα−/− mice partially rescued peripheral NK cells with normal cytotoxicity and cytokine production (58). These findings indicate that the maintenance of mature NK cells in the periphery is mainly attributed to IL-15 trans-presented by DCs and macrophages, while the genesis of NK cells in bone marrow is dependent on trans-presented IL-15 on non-hematopoietic cells, such as bone marrow stromal cells (59, 60).

Studies from Chapter II of this dissertation and others show that murine IL-15, especially IL-15 SA, prompts the differentiation of NK cells, which progress from immature precursors (CD11blow CD27low) and the proliferative subset (CD11blow CD27high) to mature inflammatory (CD11bhigh CD27high) and the cytotoxic (CD11bhigh CD27low) subsets (58, 61). In addition, IL-15 and IL-15 SA potently activate peripheral NK cells as indicated by up-regulated early activation marker CD69 expression and increased production of effector cytokines IFN-γ and TNF-α (61, 62). IL-15 also increases the cytotoxicity of NK cells towards tumor cell lines by elevating production of the cytotoxic effector molecules perforin and granzyme B (62, 63). Mature NK cells in the periphery are exclusively dependent on the availability of IL-15 in the host as antibody-mediated neutralization of IL-15 in wild type mice causes rapid systemic depletion of NK cells (64). IL-15 is also required for the homeostatic proliferation of NK cells when they are adoptively transferred to an NK cell deficient environment (65).

In humans, IL-15 plays a critical role in the generation of mature CD56+ NK cells from precursors in the bone marrow (66). In a humanized animal model in which Rag2−/−γc−/− mice were engrafted with human hematopoietic stem cells, human IL-15 showed little effect but IL-15 coupled to IL-15 Rα robustly regenerated human NK cells (67). In addition, IL-15 is required...
to prompt the differentiation of the reconstituted NK cells from pro-inflammatory CD56\textsuperscript{high}CD16\textsuperscript{−}KIR\textsuperscript{−} to cytotoxic CD56\textsuperscript{low}CD16\textsuperscript{+}KIR\textsuperscript{−}, and finally to the most mature CD56\textsuperscript{low}CD16\textsuperscript{+}KIR\textsuperscript{+} phenotype (67).

**Natural Killer T (NKT) Cells and Intraepithelial Lymphocytes (IEL)**

NKT cells share properties of both T and NK cells and play an essential role in immune defense against pathogens by recognizing lipid antigens presented by CD1d (68). IELs are lymphocytes residing in the epithelial layer of the intestines and play a protective role in safeguarding the integrity of intestinal epithelium (69). The importance of IL-15 in the development of NKT cells and TCR-αβ and TCR-γδ expressing intestinal IEL that co-express CD8-αα homodimers has been demonstrated by the deficiency of these cells in mice genetically deficient in IL-15 or IL-15 receptor subunits such as IL-15 receptor α or receptor β (53).

**Impact of IL-15 on adaptive immune cells**

**CD8\textsuperscript{+} T lymphocytes**

The requirement of IL-15 for the development of CD8\textsuperscript{+} T cells, especially memory phenotype CD8\textsuperscript{+} T cells, has been demonstrated in IL-15- and IL-15 Rα-deficient mouse strains, both of which exhibit a marked reduction in the number of CD44\textsuperscript{high} memory CD8\textsuperscript{+} T lymphocytes (53, 55). IL-15 Rα expression by DCs is essential for the development of central memory CD8\textsuperscript{+} T lymphocytes, while IL-15Ra expression on macrophages supports both central and effector memory CD8\textsuperscript{+} T cells (57). Residual memory CD8\textsuperscript{+} T cells that survive in IL-15- or IL-15 Rα-deficient mice appear to be mainly dependent on IL-7, another hematopoietic cytokine in the common γ chain family (70, 71). Characterization of the IL-15 Rα knockout mice demonstrates
that both peripheral naïve CD44\textsubscript{low} CD8\textsuperscript{+} T cells and memory CD44\textsubscript{high} CD8\textsuperscript{+} T cells exhibit increased apoptosis due to lack of IL-15 signaling (72). However, exogenous IL-15 treatment reverses the deficiency of CD8\textsuperscript{+} T cells by prompting proliferation and preventing apoptosis (53, 73). IL-15 induced production of the anti-apoptotic protein Bcl-2 in naïve and both Bcl-2 and Bcl-x\textsubscript{L} in memory CD8\textsuperscript{+} T cells (73). Compared to naïve CD8\textsuperscript{+} T cells, memory CD8\textsuperscript{+} T cells in the periphery express higher levels of CD122 (IL-15 Rβ), reflecting greatly enhanced responsiveness to IL-15 (74). Several lines of evidence have shown that IL-15 is essential for rescuing antigen-specific CD8\textsuperscript{+} T cells that are usually destined to die during contraction of primary infection, which promotes their transition to the long-term memory subset (75). IL-15 is also required for the homeostatic proliferation of antigen-independent memory CD8\textsuperscript{+} T cells in lymphopenic environments (71). Stimulation of memory phenotype CD8\textsuperscript{+} T cells \textit{in vitro} by IL-15 increased synthesis of effector molecules such as IFN-γ, TNF-β, granzyme B and perforin and enhanced cytotoxicity induction (76).

\textbf{CD4\textsuperscript{+} T lymphocytes}

Compared to memory phenotype CD8\textsuperscript{+} T cells, the development and survival of memory phenotype CD4\textsuperscript{+} T cells are less dependent on IL-15 signaling (77). IL-15- and IL-15 Rα-deficient mouse strains exhibit neither decline in the number (53, 55), nor increase in apoptosis of resting CD4\textsuperscript{+} T cells including both naïve and memory subsets (my unpublished data). Treatment of wild type mice with IL-15 does not induce proliferation or activation of memory CD4\textsuperscript{+} T cells, a phenomenon associated with the levels of IL-15 Rβ expression by these cells (23, 61, 77). Memory CD4\textsuperscript{+} T cells produce much less of the IL-15 Rβ signaling chains than memory
CD8⁺ T cells (77). In addition, IL-15 is not required for the homeostatic proliferation of memory phenotype CD4⁺ T cells in T cell-deficient environment (78). However, in rhesus macaques infected with simian immunodeficiency virus in the setting of anti-retroviral therapy, IL-15 reserves the defect in the T lymphocyte reservoir by inducing the proliferation of memory but not naïve CD4⁺ and CD8⁺ T lymphocytes (79). In another model of humanized mice, IL-15 triggered improved development of both human CD4⁺ and CD8⁺ T lymphocytes with naïve and memory phenotypes (80).

**B lymphocytes**

Multiple studies indicated that IL-15 has no role or a limited role in resting B cell development, survival and proliferation (53, 55). However, *in vitro* studies show that IL-15 promotes proliferation of activated B cells and stimulates IgM, IgG1, and IgA secretion (81). Chan-Sik Park *et al* showed that human follicular DCs produce IL-15 to enhance germinal center B cell proliferation through transpresentation (82). They further showed that IL-15 increases human follicular DC proliferation and cytokine secretion, which is essential for a protective germinal center reaction against viral infections (83). However, a very recent study indicated that IL-15 *in vivo* decreases the number of B cells through NK cell-derived IFNγ (84).

**IL-15 knockout (IL-15 KO) mice**

In Chapter IV, I examined the role of IL-15 in the pathogenesis of sepsis by using IL-15 KO mice. Germline deletion of IL-15 in mice causes a deficiency in IL-15-dependent NK, NKT, memory CD8⁺ T cells and IELs (53). However, cellular defects observed in IL-15 KO mice are reversible as IL-15-dependent cells repopulate upon treatment of IL-15 KO mice with IL-15 (53),
or IL-15 in complex with soluble IL-15Rα (my data). The ability of IL-15 KO mice to mediate viral clearance and tumor surveillance has been evaluated. IL-15 KO mice exhibit increased susceptibility to viral infections such as vaccinia virus (53). In response to infection with lymphocytic choriomeningitis virus (LCMV), long-term maintenance of virus-specific memory CD8⁺ T cells is weakened due to lack of IL-15 signals in IL-15 KO mice, although IL-15-deficient mice are able to generate effective viral clearance and potent primary effector CD8⁺ and antigen-specific memory CD8⁺ T cell responses at the early phase (85). In addition, dysregulated anti-tumor responses have been observed in IL-15 KO mice. IL-15-deficient mice that were injected with breast cancer cells or crossed with Polyoma Middle T-expressing mice that form spontaneous breast cancer show accelerated tumor formation, early metastasis and increased mortality (86, 87).

**IL-15 toxicity**

Although IL-15 is a novel and potent candidate for tumor immunotherapy, IL-15 administration may also have untoward consequences. Genetic overexpression of IL-15 in mice leads to the early genesis of lymphocytic leukemia with a T-NK phenotype (88). Another study confirmed that chronic exposure of IL-15 induces transformation of large granular lymphocyte leukemia *in vitro* (89). The administration of IL-15 also increases the risk of graft-versus-host disease after allogeneic bone marrow transplantation (21). In nonhuman primates, IL-15 is known to cause considerable toxicity such as grade 3/4 transient neutropenia, weight loss and skin rash at higher doses (90). Most recently, the toxicity profile for IL-15 in cancer patients was defined. Conlon and colleagues reported grade 3 hypotension, thrombocytopenia, liver injury, fever and rigors in patients with metastatic malignancies during treatment with human
recombinant IL-15 (20). Thus, as IL-15 moves through drug development, it is important to understand its toxicity profile. Furthermore, it is important to understand the mechanisms underlying IL-15 immunotoxicity, which are defined in this dissertation.

The role of IL-15 in autoimmune and chronic inflammatory diseases

In the setting of autoimmune and chronic inflammatory diseases, IL-15 appears to be a dangerous pro-inflammatory mediator that prevents IL-2-induced activation-induced cell death, leading to impaired maintenance of peripheral self-tolerance (11). Also, IL-15 is an important activator of cells that secrete IFN-γ, IL-1β and TNF-α, which are critical pro-inflammatory mediators in inflammatory autoimmune disease processes (33, 61). McInnes *et al* suggested that IL-15 play a detrimental role in the pathogenesis of rheumatoid arthritis by inducing lymphocytic inflammatory infiltrate into synovial fluid (91-93). IL-15, which is produced by endothelial cells in rheumatoid arthritis, activates recruited T cells on site and the activated T cells further induce TNF-α expression by macrophages (94). Agostini *et al* showed that IL-15, which is produced by alveolar macrophages, facilitates pathogenesis of pulmonary sarcoidosis (95). In addition, disordered IL-15 expression significantly correlates with the severity of many other chronic inflammatory diseases, including inflammatory bowel disease, multiple sclerosis and chronic allograft rejection (96-98).

Sepsis

Definition of sepsis

The term “sepsis” has been used for centuries to describe a critical illness caused by infections. It was derived from the ancient Greek word sipsi meaning rotten flesh and
putrefaction (99). The origins of this term can be traced back to Hippocrates, who claimed that sepsis was a process “when continuing fever is present, it is dangerous if the outer parts are cold, but the inner parts are burning hot” (100). When the germ theory was widely accepted due to efforts from Semmelweis, Pasteur, and others (101), sepsis was interpreted as a blood poisoning as a result of invading pathogens spreading in the bloodstream (102). However, the germ theory could not explain the pathobiology of sepsis as antibiotic treatment in modern medicine failed to save many patients with sepsis even if microbes in the host were completely eliminated (103). Indeed, blood cultures are positive in only one third of patients with sepsis, and up to one third of patients are negative in cultures from all sites of the body (104, 105). Thus, scientists suggest that it is the host response that leads to deleterious outcomes during sepsis (103). William Osler mentioned in the Evolution of Modern Medicine that, “Except on a few occasions, the patient appears to die from the body's response to infection rather than from it.” (106)

A 1991 consensus conference by the American College of Chest Physicians and Society of Critical Care Medicine developed the initial clinical definition of sepsis (107). The group concluded that sepsis is a medical condition that stems from the systemic inflammatory response syndrome (SIRS) in host response to infections (107). The criteria for SIRS are defined as 2 or more of the following: body temperature < 36°C or > 38°C, Heart rate > 90 beats / Min, respiratory rate > 20 breaths / Min (or hyperventilation with a PaCO2 < 32mmHg), white blood count > 12,000 cells / mm³, or immature neutrophils > 10% (107). Thus, sepsis is mainly defined under the SIRS criteria. Furthermore, sepsis that is complicated by the development of organ dysfunction is termed severe sepsis. Severe sepsis can further progress to septic shock, in which
hypotension persists regardless of adequate fluid resuscitation (107). Since then, the definition of sepsis, severe sepsis and septic shock have remained unchanged in most parts over 20 years with few alternations by the second consensus conference in 2003 (108). However, this guideline for diagnosis of sepsis proved to be too sensitive and lack specificity, thus not recognizing the complex nature of the sepsis syndrome. The early definition of sepsis also potentially prevented targeted treatments according to patients’ specific features (109). In 2016, the Society of Critical Care Medicine and the European Society of Intensive Care Medicine arranged a task force to develop new recommendations for definitions and clinical criteria of sepsis (110). They denoted that sepsis should be defined as “life-threatening organ dysfunction caused by a dysregulated host response to infection”. Organ dysfunctions can be recognized by “an increase in the Sequential (sepsis-related) Organ Failure Assessment (SOFA) score of 2 points or more, which is associated with in-hospital mortality greater than 10%.” The task force also developed a new bedside clinical score termed quickSOFA (qSOFA) to rapidly identify adult patients with suspected infections at a greater risk of developing sepsis in out-of-hospital or emergency department settings. The defined criteria are a respiratory rate of 22/min or greater, altered mentation, or systolic blood pressure of 100 mm Hg or less (110). Septic shock should be “a subset of sepsis in which particularly profound circulatory, cellular, and metabolic abnormalities are associated with a greater risk of mortality than with sepsis alone”. Clinically, patients with septic shock can be identified by the necessity of vasopressor administration to maintain a mean arterial pressure of 65 mm Hg or greater and serum lactate level greater than 2 mmol/L (>18 mg/dL) in the absence of hypovolemia, which are associated with hospital mortality rates greater than 40% (110). Under the current guideline for the definition of sepsis,
the term of severe sepsis is eliminated as it is redundant and can be exchangeable with the
term of sepsis. Also, cessation of using “severe sepsis” will break the misleading understanding
of sepsis as a continuum through severe sepsis to septic shock. However, there is still no
diagnostic panel of biological markers in the guideline and the mechanisms underlying the
pathogenesis of sepsis remain elusive.

Epidemiology of sepsis

Sepsis remains one of the leading causes of death in critically ill patients worldwide. In
the United States, approximately 750,000 people develop sepsis with 30% mortality annually
(111). Sepsis also imposes an increasing healthcare burden from approximately $15.4 billion in
2003 to $24.3 billion in 2007 (111). Between 1979 and 2000, the incidence of sepsis increased
by 8.7% per year, although the mortality of sepsis has significantly decreased by 9.9% on
average per year (112). Sepsis is more common among men than among women and has a
higher incidence among nonwhite people than among white people. Black men have the
highest incidence and mortality of sepsis (112). Furthermore, organ failure had an additive
effect on mortality as patients with three or more failing organs exhibited higher mortality
(112). The increase in the incidence of sepsis is associated with risk factors including aging of
the population, transplantation, chemotherapy, increased use of immunosuppressive drugs and
invasive procedures, increased number of people infected with HIV and antibiotic resistance
(113).
Etiology of sepsis

Sepsis is initiated by infections with bacteria, fungi, viruses or parasites. The type of microorganisms that cause sepsis is one of the determinants of septic outcomes. A study by Martin and colleagues reported gram-negative bacteria were the predominant causative pathogen of sepsis between 1979 and 1987 in the USA (112). But infection with gram-positive bacteria increased by an average of 26.3% per year and became as common as gram-negative infection in causing sepsis after 1987 (112). A 2009 study by the European Prevalence of Infection in Intensive Care (EPIC II) reported more gram-negative than gram-positive infections (62.2% vs. 46.8%) that are responsible for sepsis, although the difference is relatively small (113, 114). Fungal infections are also a common cause of sepsis as the rate of fungal sepsis increased by 207%, from 1979 to 2000 (112). *Staphylococcus aureus*, *Streptococcus pneumonia*, *Pseudomonas* species, *E. coli*, *Klebsiella* species and *Candida* are the predominant causative organisms of sepsis. *Pseudomonas* infection has been reported to significantly correlate with hospital mortality (114). With respect to routes of infections, pneumonia is the most common cause of sepsis, representing nearly half of all cases and is followed by other causes such intraabdominal and urinary tract infections (114).

Treatments of sepsis

In 2012, the third update of clinical guidelines for the management of severe sepsis and septic shock was released by the Surviving Sepsis Campaign, a joint collaboration of the Society of Critical Care Medicine and the European Society of Intensive Care Medicine (115). The key recommendations include two bundles. Within 3 hours of recognition of sepsis, the initial bundle requires the completion of lactate level measurement, acquisition of blood culture
prior to antibiotic use, administration of broad-spectrum antibiotics and quantitative fluid resuscitation with intravenous crystalloids (115). Then, within 6 hours of patients’ presentation, the subsequent bundle requires the accomplishment of clinical management, including vasopressor administration, reassessment of volume status and tissue perfusion if hypotension persists despite adequate fluid resuscitation and re-measurement of lactate levels (115). The purpose of the first-6-hour fluid resuscitation is to prevent sepsis-induced tissue hypoperfusion and suspicion of hypovolemia and the goal of it is to maintain the central venous pressure (8-12 mm Hg), mean arterial pressure [MAP] ≥65 mm Hg and urine output ≥0.5mL/kg/hour (115). After the first 6 hours, further infection source control and supportive therapies including mechanical ventilation, glucose control, deep vein thrombosis prophylaxis and stress ulcer prophylaxis should be considered to support organ functions and avoid complications (115). Studies investigating the effectiveness of the SSC guidelines showed that the increased compliance with the entire management bundles was associated with the decreased absolute mortality odds ratio (116).

Clinical trials of sepsis

There have been more than 100 Phase II and Phase III clinical trials that were designed to modify the SIRS during sepsis by targeting specific and non-specific mediators (117). However, none of the therapeutic candidates that show efficacy in animal models have demonstrated the same effectiveness in human sepsis, except activated protein C as one short-lived treatment (118). Strategies that have been used include selective neutralization of microbial product LPS or blockade of pro-inflammatory mediators such as tumor necrosis factor (TNF), interleukin-1 (IL-1), platelet-activating factor, and nitric oxide, non-selective modulation
of systemic inflammation by corticosteroids or ibuprofen, stimulation of the immunosuppressive aspect of sepsis by administration of granulocyte colony-stimulating factor (G-CSF) and IFNγ, and prevention of coagulopathy by administration of activated protein C (APC), antithrombin, thrombomodulin, or heparin (117). The failure of targeted interventions in septic patients could be due to the complex and interdependent network of pro-inflammatory responses during sepsis (119). Also, it might be associated with the non-specific clinical criteria of sepsis, as patients with sepsis that were recruited to studies exhibited considerable heterogeneity in genetic background, the nature of infecting microorganisms, site of infections, the magnitude of pro-inflammatory responses and co-morbidities (120, 121). Thus, it is not surprising that one single intervention cannot be uniformly effective in all patients. To address these problems, we need to identify plausible new targets for intervention and better group people by variables that may impact the efficacy of treatment (121). In addition, we have to admit the species difference between mice and humans and also study more of the immune responses of elderly mice to sepsis as elderly patients with co-morbidities are the main population developing sepsis in the clinical setting (122, 123).

**Animal models of sepsis**

Experimental models of sepsis are useful tools to investigate important host-derived mediators that contribute to the pathobiology of sepsis in animals. The preclinical studies on animals play a critical role in prompting the development of novel septic therapeutics in human sepsis. Thus, animal models of sepsis should be designed to mirror major clinical features and disease-associated pathogenesis of patients with sepsis (124, 125). However, currently, none of the experimental models of sepsis have faithfully reproduced all the essential pathogenic
features observed in septic patients, causing a big obstacle in translating lab findings to clinical application. Generally, animal models of sepsis that are most commonly used can be divided into three categories: administration of a microbial toxin (like lipopolysaccharide (LPS), also called endotoxin); instillation or infusion with a vial microorganism (such as bacteria, fungi); alternation of the host protective barrier (such as cecal ligation and puncture (CLP) allowing intestinal bacteria leaking into the peritoneal cavity) (124, 125) (Table 1).

In my thesis studies, I examined the role of IL-15 in the pathogenesis of sepsis induced by LPS or CLP. In LPS-induced endotoxin shock, a bolus injection of LPS in mice caused physiological dysfunctions, organ injuries and acute systemic inflammation which resemble most clinical characteristics of patients with sepsis (126, 127). For example, LPS administration causes hypothermia, elevated alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN) and creatinine concentrations in plasma, indicating acute liver and kidney injury and increased concentrations of pro-inflammatory cytokines such as TNF-α, IL-1β, and interleukin-6 (IL-6) in plasma (128-130). Also, the sepsis model of LPS is highly reproducible and consistent given that the dose of LPS can be controlled to cause similar severity of the disease. LPS challenge triggers a more rapid and higher peak in pro-inflammatory cytokine levels as compared to that observed in clinical sepsis (125).

CLP is a widely used and clinically relevant experimental model of sepsis as it mimics the diseases of ruptured appendicitis or perforated diverticulitis in hospitals (124, 125). The procedure involves the ligation of the cecum to cause ischemic necrosis and needle puncture of the ligated cecum to release fecal matter (131) (Figure 3). Through the protocol, CLP causes
polymicrobial intra-abdominal sepsis as the mixed bacterial flora elicit systemic infection by disseminating from the peritoneal cavity to the blood stream and initiate inflammatory responses along with the necrotic intestinal tissue (131). CLP recreates similar systemic cytokine production, hemodynamic and metabolic phases of human sepsis (132-134). In addition, the CLP procedure reproduces the immunosuppressive phase of sepsis by exhibiting immune cell apoptosis and dysfunctions (135, 136). However, CLP-induced severity of sepsis can be affected by surgical variations including the ligation length of the cecum, needle size, and the number of punctures (124). The severity is also associated with mouse strains and the administration of supportive therapies such as fluid resuscitation and antibiotic management (124, 131).

**Figure 3: The CLP model of sepsis.** Cecal ligation is performed below the ileocaecal valve to prevent bowel obstruction by using a 3-0 silk tie. The ligated cecum is punctured through-and-through by a needle, allowing fecal material to leak into the normally sterile peritoneal cavity. Adapted from (124).
Table 1: Summary of animal models of sepsis [adapted from (125)]

<table>
<thead>
<tr>
<th>Animal model of sepsis</th>
<th>Advantage</th>
<th>Disadvantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipopolysaccharide (LPS) injection</td>
<td>Simple, sterile, some similarities with human sepsis pathophysiology</td>
<td>Early and transient increase in inflammatory mediators; more intense than in human sepsis; no active infection</td>
</tr>
<tr>
<td>Cecal ligation and puncture (CLP)</td>
<td>Moderate and delayed peak of mediators; multiple bacterial flora; peritonitis is a common cause of sepsis</td>
<td>Age and strain variability; variability related to experimental technique and differences in resident microflora; adds a component of bowel ischemia; fails to reproduce acute lung or kidney injury</td>
</tr>
<tr>
<td>Cecal slurry</td>
<td>Bacterial peritonitis caused by multiple bacterial flora that mimics features of clinical peritonitis; can be more reproducible than CLP with attention to detail</td>
<td>Not associated with bowel injury, so does not completely reproduce clinical scenarios; there can be significant variability in results among different labs</td>
</tr>
<tr>
<td>Infusion or instillation of exogenous bacteria</td>
<td>Representative of clinical infections (depending on model) caused by common pathogens</td>
<td>Acute, severe models do not mimic usual clinical scenarios</td>
</tr>
</tbody>
</table>

Sepsis timeline

The traditional paradigm indicates that sepsis is hallmarked by the initial hyper-inflammatory responses at the acute phase followed by compensatory anti-inflammatory responses with the impaired ability to clear secondary pathogens at the late phase (137, 138). However, this paradigm has been widely questioned due to its limitation to reflect the complex nature of sepsis (139, 140). The current hypothesis suggests that both pro- and anti-inflammatory responses occur simultaneously, but exhibit varying magnitude among patients with sepsis (139, 140). The new clinical definition of sepsis is no longer using SIRS as the criteria.
for recognizing sepsis as it lacks sufficient specificity in human sepsis (110). However, acute inflammation remains the essential component of the multifaceted process of sepsis and has been extensively studied.

**Pathophysiology of sepsis**

Sepsis is a complicated and multifactorial course that affects almost all the systems in the host (Figure 4). Here, I will highlight the characteristic pathophysiological alternations during sepsis, including inflammation, microvascular damage and failure, multiple organ dysfunctions and coagulopathy.

**Figure 4: Pathophysiology of sepsis.** Upon sensing pathogens, innate immune cells, including neutrophils, macrophages and endothelial cells, are activated to produce large amounts of pro-inflammatory cytokines and chemokines and to upregulate adhesion molecules on endothelial cells and neutrophils, leading to enhanced neutrophil function and mobilization to sites of infection to kill pathogens. However, as a result of excessive pro-inflammatory responses, microcirculatory failure and coagulopathy, tissue damage and organ failure occur, resulting in early death. At the later (hyporeactive) phase of sepsis, multiple immune cells exhibit immunosuppressive phenotype, leading to increased susceptibility toward secondary infections. Adapted from (141).
Inflammation

Inflammation is an adaptive response that evolves in the host to ensure a rapid protective response to invading pathogens (142). It is initiated by the host recognition of conserved motifs associated with a group of pathogens called pathogen-associated molecular patterns (PAMPs) (143). Common PAMPs include bacteria-associated peptidoglycan, lipopolysaccharide, DNA with CpG motifs, flagellin, and virus-derived single- or double-stranded RNA as well as glucans on the fungal wall (142). PAMPs can directly activate innate cells (neutrophils, monocytes, dendritic cells, macrophages and endothelial cells) that express specific receptors called pattern recognition receptors (PRRs) (144). PRRs like toll-like receptors (TLRs) play a critical role in mediating protective biological responses to clear harmful microorganisms (145). Acute inflammation exhibits classical manifestations of calor, dolor, rubor, tumor (heat, pain, redness and swelling) and loss of function (146). However, intrinsically it is a complex and interdependent process associated with vasodilation and increased permeability, rapid extravasation of leukocytes (mainly myeloid granulocytes) to the site of infection, and activation of multiple cascade systems in the circulation (142, 143). During this process, a wide array of biochemical mediators is released from activated cells, such as pro-inflammatory cytokines, chemokines, prostaglandins, leukotrienes, histamine, nitric oxide, thromboxanes, platelet-activating factor, and complement (147-149). However, dysregulated inflammatory responses on the site of infections will directly cause tissue damage (150). Furthermore, a systemic inflammatory cascade will lead to hemodynamic abnormality, cardiovascular collapse, coagulopathy, physiological dysfunctions and multiple end organ failure, which is characteristic of sepsis (134, 136).
Cytokines have long been implicated in the pathogenesis of the hyper-inflammatory state of sepsis. TNF-α, produced mainly by activated macrophages, plays a critical role in establishing host defense against pathogens by prompting acute-phase reactions, catabolism, insulin resistance and phagocytosis by macrophages (151, 152). However, an excessive amount of TNF-α alone could reproduce many of the pathophysiological features of sepsis such as high fever, hypotension, metabolic acidosis, coagulation pathway activation, hypoglycemia, and acute hepatocellular and renal dysfunction (153, 154). IL-1β, another pro-inflammatory cytokine produced by activated monocytes and endothelium, has been detected in the plasma of up to 90% of patients with sepsis (154, 155). Similar to TNF-α, IL-1β injection alone triggers sepsis-like syndromes, including hypotension, leukopenia, thrombocytopenia, hemorrhage, and pulmonary edema (156). Compared to TNF-α and IL-1β, IL-6, a pro-inflammatory mediator, serves as a more reliable marker for the severity of sepsis in adult patients (157). In addition, plasma levels of IL-6 >160 pg/ml were 100% sensitive for the diagnosis of neonatal sepsis (158). IL-2, IL-8, IL-12, IL-18, IFN-γ are also among cytokines that actively participate in the propagation of hyper-inflammatory cascades during acute sepsis (159-163).

**Microvascular dysfunction**

Microcirculation is the circulation of blood in the smallest blood vessels that are embedded within organ tissues (164). It is comprised of arterioles, capillaries and venules and plays a critical role in delivering oxygen and nutrients to and removing carbon dioxide from the tissues (164). Alterations in microvascular blood flow and oxygenation is a common and serious issue in septic patients (165). Several preclinical studies observed slow capillary blood flow during sepsis due to decreased perfusion pressure as a result of the presence of systemic
hypotension (166). In a septic model using CLP in rats, reduced perfused capillary density in striated muscles and intestinal mucosa indicates that the microcirculation is shut off early in severe sepsis or septic shock (167). During sepsis, large quantities of white blood cells adhere to the endothelium for moving out of the circulatory system and towards the site of infection (168). Increased adherence of immune cells results in elevated resistance to the blood flow and even obstruction (168). Activated leukocytes, particularly neutrophils result in considerable damage to endothelial lining, causing increased capillary leakage and exposure of tissue factors (169). The coagulation pathways are activated in large part through the extrinsic pathway (tissue factor-factor VIIa) during sepsis, leading to a hyper-coagulable state with the excessive formation of thrombi in the microvasculature (170). Excessive amounts of pro-inflammatory mediators (IL-6, TNF-α and IL-1β) in the blood stimulate activation of coagulation cascades by inducing tissue factors (168, 170). A systemic activation of the coagulation pathways results in the development of a lethal consequence of sepsis referred to as disseminated intravascular coagulation (DIC), which is manifested as widespread microthrombi formation in the majority of the small blood vessels throughout the body (171). As a consumptive coagulopathy, DIC remarkably increases the risk of severe bleeding and serves as a prognostic marker for the mortality of septic patients (172). Development of endothelial damage, capillary leakage, systemic thrombus formation and leukocyte accumulation in the microcirculation cause compromise of tissue blood flow and lead to poor tissue perfusion. These alterations contribute to end-organ failure and tissue necrosis (173). Activated protein C (APC) functions to deactivate coagulation factors Va and VIIIa as an anticoagulant (174). It also exhibits anti-inflammatory properties indicated by a decrease in pro-inflammatory cytokine production and
a decline in neutrophil adherence to the endothelium (175, 176). In 2001-2002, APC was approved in the USA and Europe for use in patients with high risk for death in sepsis, as it significantly reduced 28-day mortality of sepsis from a large clinical trial called PROWESS (the Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis) (176). However, as the only approved drug on the market for treatment of sepsis for decades, APC was withdrawn from the worldwide market in 2011 after a subsequent trial called ‘PROWESS Shock’ trial failed to demonstrate an improved outcome by APC (176).

**Multiple organ dysfunction**

The new recommendations for definitions of sepsis highlight the clinical significance of organ dysfunction as a hallmark of sepsis. Organ dysfunctions may be the first clinical sign of sepsis as a result of the septic insult itself (usually bacteria or bacterial components) or may develop as a consequence of the inflammatory response of the body to infections (177). Poor tissue perfusion due to microcirculatory failure causes cell hypoxia, loss of function and ultimately necrosis in individual organs (74). Excessive leukocytes, which are recruited to infected organs increased release of pro-inflammatory mediators and lysosomal enzymes, disrupting the normal functions of individual organs (168, 170). In the host, no organ system can be immune to the malignant systemic inflammatory cascades of sepsis (177). Alteration in organ function can vary from a mild damage to entirely irreversible organ failure (178). Multiple organ failure (MOF) is the major cause of morbidity and mortality of critically ill patients with sepsis (112, 179). Observation studies indicate that patients with three or more failing organs experienced significantly increased mortality (112). Hemodynamic collapse is typical of sepsis (180). Circulatory insufficiency induced by sepsis includes vasodilation, hypotension in big blood
vessels and decreased blood flow in microcirculation embedded in end organs (168). Cardiac
dysfunction is mainly featured by diastolic dysfunction and myocardial depression (181). TNF-α
and IL-1β act as myocardial depressant factors (182), in synergy with other pro-inflammatory
mediators such as nitric oxide, lysozyme, leukotrienes and prostaglandins (183). Sepsis is the
most common cause of acute lung injury and acute respiratory distress syndrome (ARDS) (184).
Endothelial injury in the pulmonary vasculature leads to capillary leakage and ultimately
interstitial and alveolar edema (185). Gas exchange in the alveoli is further impaired by
activated neutrophils, which are entrapped in the pulmonary capillary and intra-alveolar space
(186). Iatrogenic damage to the lung is also an emerging concern as endotracheal intubation
and mechanical ventilation have been widely utilized in ICU patients with sepsis (187). These
interventions also increase the risk of secondary respiratory infections, particularly ventilator-
associated pneumonia, leading to aggravated mortality of patients (187). Acute kidney injury
(AKI) is another classic sign of sepsis, characteristic of a significant decrease in glomerular
filtration rate and elevation in serum blood urea nitrogen and creatinine (188). The mechanism
of sepsis-induced AKI is complex and appears to be multi-factorial. Leukocyte infiltration and
release of inflammatory mediators in renal tissue, microcirculatory failure, renal
vasoconstriction and systemic hypotension all contribute to renal injury (181). The use of
norepinephrine to treat hemodynamic derangements during sepsis will further decrease blood
flow to the kidney and exacerbate renal failure, indicating the difficulties in the clinical
management of sepsis (189, 190). Liver failure resulting from sepsis is manifested by elevation
of liver enzymes and bilirubin in the serum, abnormal synthesis of pro-inflammatory mediators,
and lack of ability to metabolize toxic products such as ammonia (191). Gastrointestinal and
central nervous system dysfunctions can also be observed in patients with sepsis (192, 193). Thus, sepsis is a severe medical condition that affects almost all organ systems, which increases the difficulties of using interventions targeted on one single system to achieve an improved global effect.

**Sepsis-associated immunosuppression**

Increasing evidence indicates a central role for immunosuppression in sepsis (22, 194). With improved intensive care management and supportive treatment, early mortality from hyper-inflammatory responses has decreased (22, 194). However, prolonged sepsis has been shown to promote innate and adaptive immune cell dysfunctions including anergy, enduring inflammation or decreased cytokine production, increased apoptosis, and reduced proliferation and effector function (195). Patients that have “recovered” from acute sepsis are more likely to develop recurrent infections, viral reactivation, opportunistic infections and late death (Figure 5) (195). In particular, low HLA-DR expression on monocytes and DCs has been shown to correlate with poor outcomes of sepsis (196, 197). Blood monocytes isolated from septic patients exhibit reduced ability to secrete pro-inflammatory cytokines like TNF-α, IL-1, IL-6 and IL-12 (198). T lymphocyte defects are also observed in patients with prolonged sepsis. CD4⁺ T cells exhibit increased apoptotic cell death and have impaired ability to secrete both type I (Th1) and type II T helper (Th2) cytokines (199). Increased regulatory T cell ratios have been observed in septic patients and correlates with the septic lethality (200). Currently, sepsis immunotherapy has been promoted as an effective means to reverse sepsis-associated immunosuppression (22, 194). Potential immunotherapeutic agents include G-CSF, IFNγ, IL-7 and IL-15 (22).
Figure 5: Timeline of sepsis. In severe cases, sepsis is characterized by an enduring inflammatory state and a dysfunctional host response to infection that may culminate in persistent organ injury and death of the patient. In parallel, some patients with sepsis develop suppression of innate and adaptive immunity, which impairs their ability to respond to the primary infection and may increase susceptibility to secondary infection with nosocomial pathogens. There is high one year mortality in patients that survive the acute episode of sepsis. Persistent derangements in innate and adaptive immune system functions may also contribute to long-term mortality by increasing the susceptibility of sepsis survivors to future infections. Adapted from (22)

IL-15 and sepsis

Although IL-15 is well studied as a deleterious mediator in the pathogenesis of autoimmune and chronic inflammatory diseases, there are few studies examining the role of IL-15 during acute inflammation, such as sepsis.

Evidence suggests that IL-15 is an essential pro-inflammatory mediator during sepsis. Akifumi reported that serum IL-15 concentration is highly correlated with the severity of organ dysfunction and outcomes of septic patients (201). Zane Orinska reported that IL-15 KO mice exhibit improved survival during experimental septic shock induced by cecal ligation and puncture (CLP) (202, 203). They showed that mast cell-specific IL-15, which is retained inside
the cell, acts to inhibit mast cell chymase activity. Deletion of IL-15 releases the inhibition of mast cell chymase activity, which enhances the ability of IL-15 KO mice to cleave neutrophil-attracting chemokines and augment bacterial clearance at the early phase of septic shock (at 3 hours after CLP). Another study by Toshiaki Ohteki showed that DC-derived IL-15 is an essential mediator of inflammatory responses in vivo as IL-15 KO mice are resistant to P. acnes- or zymosan-primed endotoxin shock, but could become susceptible to the disease when transferred with wild type but not IL-15 KO DCs. A recent paper from our laboratory showed that IL-15 SA is effective in expanding NK, NKT and mCD8+ T cells in burned mice but does not improve survival in a model of Pseudomonas burn wound infection (204). In contrast, Shigeaki et al reported that treatment with exogenous IL-15 attenuates sepsis-induced apoptosis, reverses associated immune dysfunction and improves survival during sepsis (205). The differences observed among the studies could be due to the use of different mouse strains, the dosage of IL-15 SA, delivery routes of IL-15 SA as well as different severity of the models of sepsis.

**IL-15-dependent cells and sepsis**

In addition to an essential role in tumor surveillance and elimination of virus-infected cells (206, 207), NK cells also actively participate in the propagation of acute inflammation caused by bacterial infections (208, 209). Our lab previously showed that NK cells quickly migrate to sites of infection within 4-6 hours after the initiation of intraabdominal sepsis, in a manner regulated by the chemokines CXCL9 and CXCL10 (210, 211). Recruited NK cells exhibit an activated phenotype and increase production of the pro-inflammatory cytokines TNF-α and IFN-γ, which are known to potently activate macrophages, dendritic cells and other antigen-
presenting cell populations (212). In turn, the activated macrophages further activate NK cells by increasing expression of effector cytokines IL-15, IL-18 and IL-12 (213). The amplification of pro-inflammatory responses via positive feedback may induce excessive systemic inflammation, which is characterized by unbridled cytokine storm during septic shock (40). As shown in previous studies from our lab and others, selective NK cell depletion attenuates systemic inflammation and improves survival in experimental models of polymicrobial peritonitis (44-46), endotoxin shock (214), pneumococcal pneumonia (215), systemic *Escherichia coli* and *Streptococcus pyogenes* infection (216, 217), and polytrauma (218).

Similar to NK cells, memory CD8⁺ T cells have been shown to exhibit properties that are characteristic of innate effector cells such as rapid migration to sites of infection and elimination of pathogens independent of cognate antigen recognition (218). Memory CD8⁺ T cells are also considered to be an important cellular source of IFN-γ. However, the contribution of memory CD8⁺ T cell subset to the pathogenesis of acute inflammatory diseases, such as septic shock, have not been well characterized since it is currently impossible to selectively deplete them. Previously studies from our lab reported that mice depleted of total CD8⁺ T cells show improved survival during intraabdominal sepsis induced by CLP (44-46). Wesche-Soldato et al showed that CD8⁺ T cells facilitate acute liver injury after CLP (219). However, there is some controversy about the role of CD8⁺ T cells in the pathogenesis of septic shock. Emoto et al reported that β₂-microglobin KO mice, which lack CD8⁺ T cells, are more susceptible to LPS-induced endotoxin shock, although these mice have been shown to be protected from CLP (55).
NKT cells may also play a pathogenic role during septic shock. Caroline et al reported NKT-deficient Jα18 KO mice are resistant to CLP-induced septic shock (220). However, the role of NKT cells in the pathogenesis of sepsis remains controversial. Another two groups showed that the lack of invariant NKT cells in CD1d KO mice and wild type mice treated with anti-CD1d antibody fails to confer protection against septic shock induced by CLP. Currently there are no proper antibodies available that can selectively deplete NKT cells in IL-15 SA-treated IL-15 KO mice, so our exploration of the role of NKT cells in this setting is limited. In addition, we did not note a significant decrease in NKT cell numbers in IL-15 KO mice. Thus, loss of NKT cells does not appear to contribute to the resistance of IL-15 KO mice to septic shock.

The role IELs play during sepsis has been shown to be either immunoregulatory or pro-inflammatory. Chun-Shiang Chung et al showed that CD8+ and CD4+ double-positive and double-negative IELs exhibit increased apoptosis during sepsis. A further study from the same group demonstrated that deficiency in γδ T cells, which comprise a large part of the IEL population, augments Th1 cytokine production and exacerbates mortality of sepsis. However, a study by Nüssler observed IELs from septic mice exhibit an activated phenotype with increased proliferation, IFN-γ production and cytolytic activity and may play an active role in facilitating pro-inflammatory responses in the mucosal system during sepsis.

Objectives of this dissertation

My thesis study is focused on the cytokine IL-15, especially a more potent form of IL-15 called IL-15 superagonist (IL-15 SA, IL-15/IL-15 Rα). Throughout my dissertation, I mainly answered three questions about IL-15 or IL-15 SA. Is IL-15 SA toxic in intact normal mice? What is the effect of IL-15 SA treatment on the pathogenesis of experimental sepsis? What is the
intrinsic role of IL-15 in the pathogenesis of experimental sepsis? My thesis is correspondingly divided into three main parts to address each of these questions.

In Chapter II, I will explore the toxicity of IL-15 SA in mice and further determine the cellular and molecular mediators that cause toxicity of IL-15 SA.

Rationale, hypothesis and significance: cytokine therapy is showing promise in the preclinical and clinical studies of cancer. IL-2 has been approved as the first effective immunotherapeutic cytokine for human cancers. Emerging evidence indicates that IL-15 is superior to IL-2 as IL-15 retains all immunostimulatory properties, but not the adverse responses of IL-2. IL-15 coupled to its soluble IL-15 Rα generates a compound possessing greater and prolonged biological actions than IL-15 alone and is termed IL-15 superagonist (IL-15 SA). Multiple preclinical studies show greater efficacy of IL-15 SA over IL-15 in clinical relevant models of tumor. However, it remains unknown whether IL-15 SA will cause immunotoxicity in vivo and what the underlying mechanisms are. Thus my overarching hypothesis is that IL-15 SA causes systemic immune-toxicity in vivo in a time- and dose-dependent manner by hyperproliferation of NK cells. In studies using mice, I am able to fully characterize the physiological and immunological alterations by IL-15 SA and its toxicity profile. These studies will provide new insights into the mechanisms of IL-15 SA-mediated immunotoxicity that will be important to consider as IL-15 SA progresses through pre-clinical and clinical cancer drug development.

In Chapter III, I study the effect of IL-15 SA on the pathobiology of sepsis.
Rationale, hypothesis and significance: Sepsis remains one of the leading causes of death in critically ill patients worldwide. Increasing evidence shows that patients who “recover” from acute sepsis may develop persistent immune cell dysfunctions which are associated with increased susceptibility to secondary infections and late death. IL-15 SA, one of the immunostimulatory agents, has been shown to reverse sepsis-induced apoptosis of immune cells and improve survival in CLP-induced septic shock. Thus, IL-15 SA is currently being promoted as a therapy to reverse sepsis-associated immunosuppression. However, little is known whether IL-15 SA will exacerbate sepsis-induced pathobiology, as IL-15 SA enhances the activity of NK cells and mCD8+ T lymphocytes. Previous studies from my lab show that NK cells and CD8+ T lymphocytes contribute to the pathogenesis of sepsis. Therefore, my overarching hypothesis is that treatment with IL-15 SA during the acute phase of sepsis increases septic lethality by activating NK and mCD8+ T cells. This study will divulge the pro-inflammatory role of IL-15 SA in the context of sepsis and provides important information that IL-15 SA should be used with caution as an immunomodulatory agent for human sepsis.

In Chapter IV, I further study the intrinsic role of IL-15 in the pathobiology of sepsis.

Rationale, hypothesis and significance: Previous studies from my lab and others show that NK cells and CD8+ T lymphocytes facilitate physiological dysfunction and systemic inflammation during sepsis. However, little is known about the factors that regulate the functions of NK and CD8+ T lymphocytes in the context of sepsis. IL-15 is a cytokine that plays a unique role in maintaining NK and memory phenotype CD8+ T lymphocyte number and functions. Therefore, my overarching hypothesis is that endogenous IL-15 plays a critical role
in the pathogenesis of sepsis by maintaining NK and mCD8^+ T lymphocyte number and function. By taking advantage of IL-15-deficient mice and IL-15 neutralizing antibody, I will fully characterize the role of IL-15 in physiological dysfunctions, cytokine secretion, organ injuries, bacterial clearance and mortality during septic shock. This study will expand our current knowledge regarding the pro-inflammatory role of endogenous IL-15 in facilitating septic lethality through maintaining NK and mCD8^+ T lymphocytes. NK and mCD8^+ T cells may represent new therapeutic targets for sepsis.
INTERLEUKIN-15 SUPERAGONIST CAUSES SYSTEMIC IMMUNO-TOXICITY BY INDUCING
HYPERPROLIFERATION OF ACTIVATED NATURAL KILLER CELLS AND PRODUCTION OF
INTERFERON-γ

Introduction

IL-15 is a pluripotent cytokine that facilitates the generation, proliferation and function of NK, NKT, memory CD8⁺ T cells and IELs (221, 222). Administration of exogenous IL-15 facilitates the expansion of NK and CD8⁺ T cell populations, both of which play important roles in anti-cancer and anti-viral immunosurveillance (222-225). The target cell specificity of IL-15 provides the possibility of it being superior to other cytokines as an agent to enhance anti-tumor and anti-viral immunity (223, 225, 226). As such, IL-15 has been used to augment the efficacy of HIV vaccines and as an anti-cancer agent (223, 227, 228). Treatment with IL-15 alone, or as an adjuvant in anti-tumor vaccines, has shown efficacy in several experimental cancer models (229-232). In cancer clinical trials, IL-15 has been administered alone and in combination with tumor-infiltrating lymphocytes (233). A recent first-in-human trial of recombinant human IL-15 in cancer patients showed clearance of lung lesions in patients with malignant melanoma (20). The toxicity profile for IL-15 was also defined and included fever, grade 3 hypotension and liver injury. The authors reported expansion of peripheral blood natural killer cell numbers and a spike in plasma interferon gamma (IFNγ) concentrations in patients receiving IL-15 treatment. However, the mechanisms by which IL-15 mediates toxicity were not provided and are difficult to determine in human models.
In light of the unique delivery of IL-15 via trans-presentation, Rubinstein and colleagues combined IL-15 with the IL-15 receptor α subunit in solution. The resulting compound displayed longer half-life and greater biological activity than native IL-15 and thus has been termed IL-15 superagonist (IL-15 SA) (234). Recent pre-clinical studies showed that IL-15 SA is more effective than IL-15 in causing regression of established melanoma and pancreatic cancer in mice (41, 42). IL-15/IL-15Rα fusion proteins and IL-15 SA-expressing adenovirus expression systems have also been efficacious in experimental tumor models (235-237). These promising pre-clinical studies have generated robust interest in the application of IL-15 SA as an anti-cancer agent. However, little is known about the in vivo toxicity and dose limitations of IL-15 SA.

In Chapter II, I explored the hypothesis that IL-15 SA causes systemic toxicity by causing hyperproliferation of activated NK cells and production of IFNγ. Findings in Chapter II directly addressed the toxicity profile of IL-15 SA in a time- and dose-dependent manner. Specific endpoints include core body temperature, weight loss, organ injury and mortality. In addition, I assessed the impact of IL-15 SA on immunological responses by examining the expansion, subsets, activation and cytokine production by NK, NKT and mCD8+ T cells. Cell-depletion, cell-deficient mice and cell adoptive transfer strategies were employed to determine whether expanded NK, NKT or mCD8+ T cells contribute to the immune-toxicity induced by IL-15 SA. Furthermore, these studies demonstrate whether the toxicity of IL-15 SA is mediated by production of IFNγ, TNF-α or perforin. IFNγ, TNF-α, and perforin are pro-inflammatory or cytotoxic mediators that are produced by NK and cytotoxic T cells and are known to mediate immune-toxicity. Taken together, these studies provide new insights into the mechanisms of IL-
15 SA-mediated immunotoxicity that will be important to consider as IL-15 SA progresses through pre-clinical and clinical cancer drug development.

**Results**

**Treatment with 2 μg IL-15 SA for 4 days causes toxicity in mice.**

To determine the dose of IL-15 SA that causes systemic toxicity *in vivo*, C57BL/6 mice were treated with escalating doses of IL-15 SA at 0, 0.5, 1 or 2 μg for 4 consecutive days (day 0-day 3). Body temperature was measured daily during the IL-15 treatment period and also at 24 hours after the last IL-15 SA injection. Significant hypothermia was observed on days 3 and 4 in mice that received 2 μg of IL-15 SA but not in mice that received the 0, 0.5 or 1 μg doses (*Figure 1A*). Further characterization of IL-15 SA toxicity was performed by looking at mortality, cachexia, injuries of vital organs, and size change in spleens and livers. Treatment with 2 μg of IL-15 SA for 4 days induced 20% mortality (8/10 surviving) on day 4. There was significant weight loss in mice treated with 2 μg of IL-15 SA, beginning on day 2 of treatment and progressing out to day 4 (*Figure 1B*). Elevation of liver enzymes (ALT and AST) in serum was also observed in the treated mice, indicative of acute hepatocellular injury (*Figure 1C*). However, IL-15 SA treatment did not trigger significant acute kidney injury or lung injury, since plasma BUN and creatinine concentrations and blood PaO₂/FiO₂ ratios were not altered in IL-15 SA-treated mice (data not shown). Spleen weight more than doubled in IL-15 SA-treated mice compared to control (*Figure 1D*). However, liver weight was not different between groups (data not shown).

To examine the duration of toxicity that was induced by IL-15 SA, body temperature was measured in mice that survived the initial toxicity. Interestingly, body temperature recovered to
normal levels within 2 days after stopping treatment (day 5) (Figure 1E). None of the mice died thereafter.
**Figure 1: IL-15 SA treatment causes systemic toxicity: Study of kinetics of IL-15 SA-induced toxicity.** (A) In dose escalation studies, wild type C57Bl/6 mice (WT) received intraperitoneal injections of 0, 0.5, 1 and 2 μg IL-15 SA for 4 consecutive days (day 0-day 3). Body temperature and mortality were recorded daily on days 0-4. (B) To assess the temporal effect of IL-15 SA, WT mice received daily administration of 2 μg IL-15 SA for 4 days. Untreated WT mice served as control. Body weight was measured daily from day 0 to day 4. Values represent body weight changes compared to the initial day 0 measurements. (C) Serum AST (aspartate transaminase) and ALT (alanine transaminase) concentrations were measured at 24 hours after the fourth intraperitoneal injection of 2 μg IL-15 SA. Vehicle-treated mice served as control. (D) Upon 4 days of 2 μg IL-15 SA treatment, spleens were harvested from WT mice. Untreated WT mice were included as control. Spleen weights were measured in the two groups. (E) To study the kinetics of IL-15 SA toxicity, WT mice were treated with 2 μg IL-15 SA treatment for 4 days. Body temperature was measured at 24 and 48 hours as well as 1, 2, 3, 4 and 7 weeks after the last treatment of IL-15 SA. * p < 0.05 compared to designated control. n=8-10 mice per group. Data are representative of two to four separate experiments.

**IL-15 SA treatment expands NK, NKT and mCD8⁺ T cells in spleen and liver.**

To determine the impact of IL-15 SA treatment on lymphocyte numbers and phenotype, I examined IL-15 SA-mediated lymphocyte expansion via flow cytometry. After 4 days of 2 μg IL-15 SA treatment, splenic NK cells (CD3⁻ NK1.1⁺) increased ~16.3-fold; NKT cells (CD3⁺ NK1.1⁺) expanded ~13.1-fold; mCD8⁺ T cells (CD8⁺CD44<sup>high</sup>) were increased ~11.5-fold, respectively, compared to vehicle-treated mice (Figure 2A-C). However, naïve CD8⁺ T (CD8⁺CD44<sup>low</sup>), CD4⁺ T and B cells in spleen showed a decrease in percentage and no change in total numbers following IL-15 SA treatment (Figure 2B-C). A similar expansion of hepatic NK, NKT and mCD8⁺ T cells was observed in IL-15 SA-treated mice (Figure 2D-F).

To get a better understanding of cellular changes induced by IL-15 SA within spleens and livers, I performed H&E and immunohistochemical (IHC) staining of spleens and livers from vehicle- and IL-15 SA-treated mice. H&E staining of spleen sections showed that the red pulp was filled with leukocytes in response to IL-15 SA treatment (Figure 2G). Immunohistochemical (IHC) staining identified the majority of leukocytes in red pulp expressed NKp46, which is a
surface marker found on NK and NKT cells (Figure 2G). In the liver, increased numbers of leukocytes were present in liver sinusoids and especially accumulated around the central vein in IL-15 SA-treated mice (Figure 2H). IHC staining of NK (NKp46+ only), NKT (NKp46+TCRβ+) and T (TCRβ+ only) lymphocytes showed a substantial number of NK (brown), NKT (brown and pink) and T (pink) cells were located in the liver sinusoids and around the central vein in response to treatment with IL-15 SA (Figure 2H). Of note, NK and NKT cell numbers decreased to the baseline level by 7 days after cessation of IL-15 SA treatment (Figure 2I). Memory CD8+ T (mCD8+) cells also declined after stopping IL-15 SA treatment but remained elevated at 7 weeks after cessation of treatment (Figure 2J).

A

Spleen

B

% Splenic Lymphocytes

C

Total Splenic Lymphocytes

D

Liver

E

% Hepatic Lymphocytes

F

Total Hepatic Lymphocytes
Figure 2. IL-15 SA treatment elicits expansion of NK, NKT and memory phenotype CD8\(^+\) T lymphocytes in vivo. WT mice were treated with vehicle or 2 µg IL-15 SA for 4 days. At 24 hours following the last treatment, splenic and liver NK (CD3\(^-\)NK1.1\(^+\)), NKT (CD3\(^-\)NK1.1\(^+\)) and memory CD8\(^+\) T (CD8\(^+\)CD44\(^{high}\)) lymphocyte numbers were determined using flow cytometry. Dot plots (A, D) are representative of results obtained from 9-15 mice per group. The bar graphs show the relative (B, E) and total numbers (C, F) of splenic and hepatic NK, NKT and memory CD8 T as well as naïve CD8\(^+\) T (CD8\(^+\)CD44\(^{low}\)), CD4 (CD4\(^+\)) and B (CD3\(^-\)CD19\(^+\)) lymphocytes in IL-15 SA-treated (black bars) and vehicle-treated groups (white bars). Data are representative of three to four separate experiments. * p < 0.05 compared to vehicle control. n = 9-15 mice per group. WT mice received daily administration of vehicle or 2 µg IL-15 SA for 4 days. Spleens (G) and livers (H) were then harvested on the next day for H&E (top, 100X magnification in spleen sections and 400X magnification in liver sections) and immunohistochemical (IHC, bottom, 100X magnification in spleen sections and 400X magnification in liver sections) staining. In IHC stained sections of spleens, NK and NKT cells were identified by Nkp46 expression and are stained brown; macrophages in the marginal zone stained with anti-MOMA-1 and are shown in pink. In an IHC staining of liver sections, NK (Nkp46 positive, brown), T (TCR-\(\beta\) positive, pink) and NKT (Nkp46 and TCR-\(\beta\) double positive, brown and pink) cells are shown, respectively. Olympus BX43 microscope and Olympus digital color camera DP73 were used for the acquisition of the images. (I) To determine the kinetics of leukocyte expansion post IL-15 SA treatment. WT mice were treated with 2 µg IL-15 SA treatment for 4 days. NK, NKT and mCD8+ T lymphocytes in the spleen were enumerated at the designated time points. The dashed line shown in the figure represents the baseline level of mCD8+ T lymphocytes in untreated control mice. n=4-10 mice per group. Data are representative of two to four separate experiments.
IL-15 SA treatment promotes NK and NKT subset expansion.

To determine the immunological effects of IL-15 SA on different subsets of NK and NKT cells, I further characterized NK and NKT cells after treatment with IL-15 SA. Generally, NK cell subsets were defined based on CD11b and CD27 expression and classified as immature (I, CD11$^{\text{low}}$CD27$^{\text{high}}$), pro-inflammatory (II, CD11$^{\text{high}}$CD27$^{\text{high}}$) or cytotoxic (III, CD11$^{\text{high}}$CD27$^{\text{low}}$) (238). Both stage II and III NK subsets are mature and distinct in their preference to secrete pro-inflammatory cytokines or mediate cytotoxicity. IL-15 SA treatment promoted expansion of all subsets in total number but preferentially expanded the pro-inflammatory CD11$^{\text{high}}$CD27$^{\text{high}}$ subset in both percentage and absolute number (~23.9-fold increase in spleen and ~115-fold increase in liver) (Figure 3A-F). This data suggests that IL-15 promotes all NK subset growth but favors the development of pro-inflammatory subset which exhibits greater ability to secrete pro-inflammatory cytokines such as IFN-γ and TNF-α. In the liver, there are two recently identified NK populations, classical NK cells (CD3$^-$NK1.1$^+$DX5$^+$CD49a$^-$Trail$^-$), resembling those in the spleen, and tissue-resident NK cells (CD3$^-$NK1.1$^+$DX5$^-$CD49a$^+$Trail$^+$) with a “memory-like” phenotype (239). Both hepatic NK subsets were expanded by IL-15 SA treatment, but the classical hepatic NK cell population showed greater expansion than tissue-resident NK cells (Figure 3G-I).

NKT cells are also heterogeneous and comprised of two major subsets: type I (invariant TCR, CD1d/α-Galcer reactive) and type II (variant TCR, not reactive to CD1d/α-Galcer) (240). Both type I (CD3$^+$NK1.1$^{+/+}$ α-Galcer-CD1d tetramer$^+$) and type II NKT subsets (CD3$^+$NK1.1$^{+-}$ α-Galcer-CD1d tetramer$^-$) were examined by flow cytometry. Upon IL-15 SA treatment, the relative number of type I NKT cell subset was lower than vehicle control and type II NKT cells...
remained the same in percentage. However, the absolute number of two NKT subsets was significantly increased by the treatment with IL-15 SA in spleen and liver (Figure 3J-O).
Figure 3. Characterization of NK and NKT subsets following IL-15 SA treatment. Upon treatment with vehicle or 2 µg IL-15 SA for 4 days, spleens and livers were harvested on day 5. In the representative histograms, splenic (A) and hepatic (D) NK cells are divided into four subpopulations based on CD11b and CD27 expression, namely precursor (CD11b<sub>low</sub>CD27<sub>low</sub>), immature (I, CD11b<sub>low</sub>CD27<sub>high</sub>), mature pro-inflammatory (II, CD11b<sub>high</sub>CD27<sub>high</sub>) and mature cytotoxic (III, CD11b<sub>high</sub>CD27<sub>low</sub>) NK cells. The percentage and total numbers of NK cell subsets in the spleen and liver are shown graphically in B, C, E and F. n= 6-10 mice per group. In the liver, NK cells are specifically classified into two separate subsets, namely classical (NK1.1<sup>+</sup>DX5<sup>+</sup>CD3<sup>-</sup>CD49a<sup>-</sup>TRAIL<sup>-</sup>) NK cells that resemble those in the spleen and tissue-resident (NK1.1<sup>+</sup>DX5<sup>-</sup>CD3<sup>-</sup>CD49a<sup>+</sup>TRAIL<sup>+</sup>) NK cells that mediate antigen-specific memory-like responses. The representative dot plot, percentage and total numbers of NK cell subsets in the liver are shown in G, H and I. In representative dot plots, type I (CD3<sup>+</sup>NK1.1<sup>±</sup>α-Galcer-CD1d tetramer<sup>+</sup>) and type II NKT subsets (CD3<sup>+</sup>NK1.1<sup>+</sup>α-Galcer-CD1d tetramer<sup>+</sup>) in spleen (J) and liver (M) are shown in the vehicle- and IL-15 SA-treated groups. The percentage (K, N) and total numbers (L, O) of each subset are shown in bar graphs. n= 5 mice per group. * p < 0.05 compared to vehicle control. Data are representative of two to three separate experiments.
IL-15 SA treatment activates NK cells, but not NKT and mCD8⁺ T cells.

To determine the impact of IL-15 SA treatment on NK cell activation, cytokine production and cytotoxicity, I examined the expression of the early surface activation marker CD69 and production of IFNγ, granzyme B and perforin in NK cells (Figure 4A). Treatment with IL-15 SA induced increased expression of CD69 and activating receptors NKG2D and NKp46 (Figure 4A) on splenic NK cells. IL-15 SA treatment also elicited IFNγ, granzyme B and perforin production by NK cells (Figure 4A). These results indicate that NK cells are fully activated with enhanced ability to secrete pro-inflammatory cytokines and perform cytotoxicity.

However, IL-15 SA did not upregulate CD69 expression or IFNγ production by NKT cells (Figure 4B). There is no augmented CD69 expression and slight increase in IFNγ production by mCD8⁺ T cells upon IL-15 SA treatment (Figure 4C). These results indicate that NKT and mCD8⁺ T cells showed minimal activation, if any, after treatment with IL-15 SA.
Figure 4. Characterization of NK, NKT and memory CD8\(^+\) T cell activation following IL-15 SA treatment. Representative histograms show the percentage of splenic NK cells (A) expressing CD69, NKG2D and Nkp46 on the surface as well as production of intracellular IFNγ, granzyme B and perforin from vehicle- (blue) and IL-15 SA- (red) treated mice. The gates were drawn based on isotype control staining (green). The bar graphs shown on the right quantify representative data with 9-10 mice per group. The activation status of NKT and memory CD8\(^+\) T cell upon IL-15 SA treatment was also analyzed. The representative histograms show expression of CD69 by splenic NKT (CD3\(^+\)NK1.1\(^+\)) (B) and mCD8\(^+\) (CD8\(^+\)CD44\(^{\text{high}}\)) T cells (C) from vehicle- (blue) and IL-15 SA- (red) treated mice. IFNγ expression by NKT and mCD8\(^+\) T cells is also shown. Specific isotype staining (green) was determined as control. The percentage of CD69\(^+\) and IFNγ\(^+\) cells in each group is shown in the figure. V = vehicle, SA = IL-15 SA. The bar graphs shown on the right quantify representative data with 8-10 mice per group. * p<0.05 compared to vehicle. Data are representative of two to three separate experiments.

Depletion of NK cells reverses IL-15 SA-mediated toxicity in mice.

To determine whether these expanded cells contribute to the toxicity of IL-15 SA, I treated mice with specific cell-depleting antibodies prior to IL-15 SA treatment and monitored body temperature, body weight and ALT, AST levels as signs of toxicity. Depletion of NK and NKT cells by treatment with anti-asialoGM1 or anti-NK1.1 prior to IL-15 SA treatment prevented development of hypothermia (Figure 5A) and weight loss (data not shown). However, mice
depleted of NK and NKT cells did not modify the modest increase in liver enzymes ALT and AST observed in wild type mice (Figure 5B). Here, I weighed body temperature more than ALT and AST levels for indicating the toxicity caused by IL-15 SA, since the loss of ability to maintain core temperature highly correlated with mortality in my experiments. Thus, these results indicate that NK and/or NKT cells play a pathogenic role in IL-15 SA-induced toxicity.

To further identify it is NK or NKT cells that contributed to the toxicity of IL-15 SA, I used invariant NKT cell-deficient CD1d KO mice that were generously provided by Lan Wu in Luc Van Kaer’s lab and treated them with 2 µg of IL-15 SA for 4 days. However, CD1d KO mice were not protected from IL-15 SA-induced hypothermia (Figure 5C) and showed significant weight loss (data not known). The results above show that NK but not NKT cells are culprits of IL-15 SA-mediated toxicity indicated by hypothermia and weight loss.

Additional studies were undertaken to determine the effect of CD8+ T cell depletion on IL-15 SA-induced toxicity. Total CD8+ T cells were depleted since it is not currently possible to selectively deplete mCD8+ T cells. Thus, my study is limited to explore the exact contribution of mCD8+ T cells to IL-15 SA toxicity. In contrast to NK cell-depleted mice, mice depleted of CD8+ T cells by treatment with anti-CD8α showed significantly more hypothermia in response to treatment with IL-15 SA compared to mice treated with non-specific IgG (Figure 5D). Likewise, CD8 KO mice showed significantly more hypothermia after IL-15 SA treatment than wild type control mice (Figure 5D). Upon IL-15 SA treatment, AST levels in WT mice treated with anti-CD8α showed no difference to untreated mice and mice treated with non-specific IgG. However, these anti-CD8α-treated mice showed an elevation of ALT levels in responses to IL-15 SA treatment which was comparable to mice treated with IgG (Figure 5E). Several studies show
that compared to AST, ALT is a more specific indicator of acute liver inflammation and injury, as AST levels can also be elevated under other conditions such as cardiac and muscular injuries. Thus, ALT data suggests that anti-CD8α treatment didn’t exacerbate liver injury caused by IL-15 SA. However, as I mentioned above, mice deficient in CD8⁺ T cells exhibited exacerbated hypothermia which is a more important sign of toxicity associated with IL-15 SA. This set of data implies that instead of being toxic, CD8⁺ T cells are even protective in preventing IL-15 SA-mediated toxicity.

To determine whether NK cells that were expanded in CD8 KO mice contributed to IL-15 SA toxicity, I treated CD8 KO mice with anti-asialoGM1 prior to IL-15 SA treatment and body temperature was recorded. Interestingly, IL-15SA-induced hypothermia was reversed in these treated CD8 KO mice compared to IgG control (Figure 5F). Thus, this finding further confirms that NK cells are the main mediator of IL-15 SA-induced toxicity.

Analysis of splenic lymphocyte populations showed that treatment with anti-asialoGM1 or anti-NK1.1 nearly ablated IL-15 SA-induced NK and NKT cell expansion, whereas mCD8 T cell expansion was unaffected (anti-asialoGM1) or markedly enhanced (anti-NK1.1) (Figure 5G-I). Treatment with anti-CD8α greatly attenuated IL-15 SA-induced expansion of CD8⁺ T cells and CD8⁺ T cells were absent in CD8 KO mice (Figure 5L). Expansion of NK or NKT cells was not affected by anti-CD8α treatment and in CD8 KO mice (Figure 5J, K). These results along with my previous observations provide evidence about the order of priority of NK and mCD8⁺ T cells competing for IL-15 in the local environment. Apparently, NK cells exclusively depend on IL-15 as in IL-15-deficient mice, NK cells are undetectable while mCD8⁺ T cells are still present but with a markedly reduced level (53). Also, in IL-15 KO mice, NK cells regenerate more rapidly
with a higher number than mCD8⁺ T cells in response to IL-15 SA treatment. In WT mice, NK cells drop back to the baseline level more quickly than mCD8⁺ T cells after stopping IL-15 SA treatment. Here, CD8⁺ T cell depletion in WT mice doesn’t affect NK cell growth, while at least anti-NK1.1-treated WT mice expand more CD8⁺ T cells in response to IL-15 SA treatment. Taken together, my data suggests that NK cells may have the priority to respond to IL-15 compared to mCD8⁺ T cells. Mechanisms are still unknown yet. It might be due to a stronger interaction between NK cells and antigen presenting cells that bear IL-15 on the cell surface or the availability of NK cells to directly respond to soluble IL-15 in the circulation.
Figure 5. Effect of lymphocyte depletion on IL-15 SA-induced toxicity. Mice were treated with 2 µg of IL-15 SA for 4 days; rectal temperature was measured on day 5. (A) Mice were treated with anti-asialoGM1 or anti-NK1.1 IgG to deplete NK and NKT cells one day before initiation of 2 µg IL-15 SA treatment. (B) Serum AST (aspartate transaminase) and ALT (alanine transaminase) concentrations in mice treated with anti-NK1.1 or non-specific IgG were measured at 24 hours after the fourth intraperitoneal injection of 2 µg IL-15 SA. Vehicle-treated mice served as control. (C) CD1d KO mice were treated with 2 µg IL-15 SA. (D) CD8 T cells were depleted by treatment with anti-CD8α IgG at 24 hours prior to IL-15 SA treatment or CD8 KO mice were treated with IL-15 SA. (E) Serum AST and ALT levels in mice treated with anti-CD8α IgG or non-specific IgG were measured at 24 hours after the last injection of IL-15 SA. (F) To deplete NK and NKT cells in CD8 KO mice, CD8 KO mice were treated with anti-asialoGM1 one day before IL-15 SA treatment. Isotype specific or non-specific IgG served as control in antibody-induced leukocyte depletion experiments. WT mice served as control for experiments using genetically altered mice. NK (G,J), NKT (H, K) and mCD8+ T cells (I, L) from spleens of WT mice were characterized in mice treated with anti-asialoGM1, anti-NK1.1 or anti-CD8α IgG as well as in CD8 KO mice. Untreated WT mice or WT mice treated with isotype matched IgG served as controls. *p < 0.05 compared to untreated WT control. +p < 0.05 compared to IgG group or WT control. n= 6-10 mice per group. Data are representative of two to three separate experiments.
**Adoptive transfer of NK cells into Rag2<sup>−/−</sup> γc<sup>−/−</sup> mice re-establishes IL-15 SA toxicity.**

The contribution of NK cells to IL-15 SA-mediated toxicity was further demonstrated by adoptive transfer of NK cells from wild type mice into Rag2<sup>−/−</sup> γc<sup>−/−</sup> mice (Figure 6). Rag2<sup>−/−</sup> γc<sup>−/−</sup> mice lack NK, T and B lymphocytes (44, 241). Adoptive transfer of lymphocytes to lymphopenic Rag2<sup>−/−</sup> γc<sup>−/−</sup> mice provides a valuable tool for assessing the role of particular lymphocyte population in various disease models. Treatment of Rag2<sup>−/−</sup> γc<sup>−/−</sup> mice with 2 ug IL-15 SA for 7 days after adoptive transfer of wild type NK cells elicited significant hypothermia and weight loss and showed 22% mortality (7/9 surviving) (Figure 6A, B). Rag2<sup>−/−</sup> γc<sup>−/−</sup> mice that were treated with IL-15 SA but did not receive NK cell adoptive transfer did not develop hypothermia or lose weight (Figure 6A, B). Likewise, Rag2<sup>−/−</sup> γc<sup>−/−</sup> mice that received wild type NK cell adoptive transfer and were treated with vehicle did not develop hypothermia or weight loss (Figure 6A, B).

Livers from Rag2<sup>−/−</sup> γc<sup>−/−</sup> mice that received adoptive transfer of NK cells and IL-15 SA treatment exhibited altered gross morphology and histology (Figure 6C). The livers of Rag2<sup>−/−</sup> γc<sup>−/−</sup> mice that were treated with IL-15 SA but did not receive NK cell adoptive transfer as well as Rag2<sup>−/−</sup> γc<sup>−/−</sup> mice that received NK cell transfer and vehicle treatment had normal hepatic architecture (Figure 6C). IL-15 SA-treated Rag2<sup>−/−</sup> γc<sup>−/−</sup> mice that were engrafted with NK cells exhibited hepatic inflammation and H&E-stained liver sections showed accumulation of leukocytes and destruction of normal liver sinusoidal architecture (Figure 6C). Hepatocellular injury was present in those mice as evidenced by elevation of serum ALT and AST levels (Figure 6D).
The number of NK cells in spleens and livers of engrafted Rag2\(^{−/−}\) \(γc^{−/−}\) mice significantly increased upon IL-15 SA treatment when compared to the other two control groups, and at a level comparable to that of NK cells that expanded and caused toxicity of IL-15 SA-treated WT mice (Figure 6E, H). IL-15 SA treatment induced activation of engrafted NK cells as indicated by increased CD69 expression (Figure 6F, G, I, J).
Figure 6. Toxicity of IL-15 SA in Rag2⁻/⁻γc⁻/⁻ mice receiving adoptive transfer of wild type NK cells. WT NK cells (1.0 × 10⁶ cells/mouse) were injected intravenously into Rag2⁻/⁻γc⁻/⁻ mice 3 hours before the initiation of IL-15 SA (2 µg) treatment. Rag2⁻/⁻γc⁻/⁻ mice that received NK cell transfer and vehicle treatment or IL-15 SA treatment without NK cell transfer served as control. (A) Body temperature was measured at 8 days after NK cell transfer and IL-15 SA treatment. (B) Body weight was measured at the indicated time points. (C) Liver gross morphology and H&E stained liver sections (400X magnification) at 8 days after treatment. Olympus BX43 microscope and Olympus digital color camera DP73 were used for acquisition of the HE stained images. (D) ALT and AST concentrations in serum at 8 days after adoptive transfer and IL-15 SA treatment. Splenocytes and hepatic leukocytes were harvested at 8 days after NK cell transfer and IL-15 SA treatment. The numbers of splenic and hepatic NK cells (E, H) and CD69 expression by NK cells (F, G, I, J) was determined using flow cytometry. *p < 0.05 compared to mice that did not receive NK cell transfer and were treated with IL-15 SA. +p < 0.05 compared to mice that received NK cell transfer but not IL-15 SA treatment. n= 6-9 mice per group. Data are representative of two separate experiments.
Ablation of IFNγ, but not TNFα or perforin, reverses IL-15 SA-induced immunotoxicity.

To determine the functional importance of IFNγ, TNFα and perforin/granzyme in the pathogenesis of IL-15 SA-induced immunotoxicity, IL-15 SA-induced hypothermia was examined in IFNγ-, TNFα- and perforin-deficient mice (Figure 7). IFNγ KO mice did not develop significant hypothermia in response to IL-15 SA treatment compared to wild type control mice (Figure 7A). Treatment of TNFα and Pfn KO mice with IL-15 SA induced hypothermia at a level that was not significantly different from WT mice (Figure 7B, C).

Compared to WT control mice, there was no difference in NK cell number in the spleens of IFNγ and TNFα KO but more NK cells in Pfn KO mice upon IL-15 SA treatment (Figure 7D). However, NK cells failed to be activated by IL-15 SA stimulation in IFNγ KO mice as indicated by the absence of increased CD69 expression (Figure 7E, F). However, NK cell CD69 expression by NK cells from TNF-α and Pfn KO mice treated with IL-15 SA was increased and not different from WT treated with IL-15 SA (Figure 7E, F).

NKT cell numbers were higher in IFNγ and TNFα KO mice and not different in Pfn KO mice in response to IL-15 SA treatment compared to WT mice. Upon IL-15 SA treatment, mCD8+ T cell numbers were higher in IFNγ KO mice and not different in TNFα and Pfn KO mice in comparison to WT mice (Figure 8A, D).

NKT and mCD8 T cells in IFNγ KO mice failed to be activated by IL-15 SA stimulation as indicated by lack of CD69 upregulation whereas activation of NKT and mCD8 T cells from TNFα and perforin-deficient mice was similar to that observed in WT mice after IL-15 SA treatment (Figure 8B, C, E, F).
Figure 7. Effect of IFNγ, TNFα or perforin deficiency on IL-15 SA-mediated immunotoxicity.
IFNγ KO, TNFα KO, Pfn KO mice and WT controls received 2 µg IL-15 SA treatment for 4 days. (A, B, C) Body temperature was measured at 24 hours after the last IL-15 SA treatment. Untreated WT mice were included as control. Splenic NK numbers (D) and CD69 expression (E, F) were measured using flow cytometry. *p < 0.05 compared to untreated WT control. +p < 0.05 compared to IL-15 SA-treated WT mice. n= 9-16 mice per group. Data are representative of two to three separate experiments.
Figure 8. Effect of IFNγ, TNF-α or perforin deficiency on IL-15 SA induced NKT and mCD8+ T lymphocyte expansion and activation. After 4-day treatment with IL-15 SA, the total number of NKT (A) and memory CD8+ T lymphocytes (D) from spleens of IFNγ, TNF-α, Pfn KO mice and WT control mice were measured. Untreated WT mice served as control. The percentage of cells expressing CD69 (B, E) and CD69 MFI (C, F) are also shown. *p < 0.05 compared to untreated WT control. +p < 0.05 compared to IL-15 SA-treated WT mice. n= 9-16 mice per group. Data are representative of two to three separate experiments.
Differential effects of IL-15 SA at escalating doses on lymphocyte number and activation in the blood and spleen.

As shown in Figure 1A, significant hypothermia was observed in mice that received 2 μg of IL-15 SA for 4 days but not in mice that received the 0, 0.5 or 1 μg doses. Here, I further characterized total white blood cell (WBC) and lymphocyte counts in the blood and lymphocyte number and activation in spleens of the treated mice. Total WBC and lymphocyte counts were significantly increased in the blood after treatment with 1 and 2 μg of IL-15 SA compared to untreated control. 1 μg of IL-15 SA induced the highest number of WBCs and lymphocytes among all the doses (Figure 9A, B). Similarly, mice that received the 0.5, 1 or 2 μg doses of IL-15 SA exhibited increased number of NK and mCD8$^+$ T cells in spleens. The highest numbers of NK and mCD8$^+$ T cells were observed in 1 μg IL-15 SA –treated mice (Figure 9C, E). In addition, NK cell were activated by all doses of IL-15 SA as indicated by increased CD69 expression. The highest activation of NK cells was observed in mice that received 2 μg of IL-15 SA (Figure 9D). The expression of CD69 was not increased in mCD8$^+$ T cells by all doses of IL-15 SA (Figure 9F).
Figure 9. Differential effects of IL-15 SA at escalating doses on lymphocyte number and activation in the blood and spleen. Wild type mice received 0, 0.5, 1 or 2 μg doses of IL-15 SA for 4 days. At 24 hours after the last treatment, blood was harvested for measurement of white blood cell and lymphocyte numbers (A, B). Spleens were also harvested for measurement of NK and mCD8^+ T cell number and CD69 expression (C-F). *p < 0.05, **p < 0.01. n= 5-12 mice per group. Data are representative of two separate experiments.
Discussion

Cancer immunotherapy is a rapidly evolving field of basic and clinical research. Recent advances show that de-inhibition of lymphocytes by blockade of co-inhibitory receptors such as programmed death-1 (PD-1) is effective in treating advanced cancers, which recently resulted in fast track approval of pembrolizumab for the treatment of metastatic melanoma (242-244). Cytokine-based treatments also hold promise for the treatment of cancer and viral diseases. IL-15 is an attractive drug candidate due to its selective actions on NK and mCD8⁺T cells, both of which mediate anti-cancer and anti-viral immunity (225, 245). Several papers have demonstrated the efficacy of IL-15 in experimental models of melanoma, pancreatic cancer and multiple myeloma (236, 246). The recent paper by Conlon et al reported clearance of pulmonary lesions in patients with malignant melanoma that were treated with IL-15 (20).

However, IL-15 administration may also have untoward consequences. It has been implicated in the pathogenesis of acute lymphoblastic leukemia (247) and large granular lymphocyte leukemia (89). The administration of IL-15 also exacerbates graft-versus-host disease after allogeneic bone marrow transplantation (21). Thus, as IL-15 moves through drug development, it is important to understand its toxicity profile. IL-15 is known to cause considerable toxicity in nonhuman primates at higher doses. Adverse side effects included weight loss and skin rash (15, 90, 248). Most recently, Conlon and colleagues reported hypotension, thrombocytopenia, liver injury, fever and rigors in cancer patients receiving IL-15 (20). Unlike IL-2, IL-15 did not cause vascular leak syndrome (12). In the present study, IL-15 SA treatment caused marked hypothermia and weight loss and a modest increase in liver enzymes in wild type mice. Thus, the toxicity profile of IL-15 family immunobiologials is similar among species. However, the
cellular and molecular mechanisms by which IL-15 and its analogs mediate systemic toxicity had not been previously defined. Work in this Chapter advances current knowledge by showing that NK cells and IFNγ are central to IL-15 SA-mediated immunotoxicity.

Conlon and colleagues reported expansion of NK and mCD8+ T cells in blood and markedly increased plasma IFNγ concentrations in cancer patient receiving IL-15 treatment (20). However, a cause and effect relationship between increased NK cell numbers and IFNγ production with IL-15-associated toxicity was not established. The present study provides a direct link between NK cell expansion and IFNγ production with the adverse responses caused by IL-15 SA in mice. Of note, the dose of IL-15 SA applied to mice in our study is higher than the highest dose of IL-15 administered to patients in the cancer treatment trial (19). However, Conlon and colleagues administered IL-15 to patients for 12 consecutive days whereas mice were treated for a period of 4 days in our study. Thus, we administered a higher dose but over a shorter period of time. Nevertheless, IL-15 SA induced physiologic dysfunction, weight loss and liver injury in mice, a toxicity profile that was similar to that reported in humans. In addition, the toxicity observed in mice was more severe and characterized by 20% mortality. Thus, although species differences in sensitivity to IL-15 SA-induced toxicity may exist, we were able to provide mechanistic insights into IL-15 SA-mediated immunotoxicity that cannot be addressed in human studies.

Rubinstein and colleagues (25) utilized a dose of IL-15 SA (1.5 μg) in mice that was similar to the dose employed in our study and was administered for 2 days rather than 4. Rubinstein et al observed NK and mCD8 T cell expansion that was similar to that reported in our study but did not mention toxic signs in mice under their treatment regimen. In our dose escalation study, we did not observe toxicity when administering IL-15 SA at 1 μg per day but
observed toxicity at the 2 μg dose. Thus, it is likely that Rubinstein et al were employing a dose that was slightly less than the toxic dose observed in our study and was administered over a shorter period of time (2 vs 4 days). These observations indicate that IL-15 SA-induced immunotoxicity is dose and time dependent.

IL-15 SA preferentially expanded the pro-inflammatory (CD11b<sup>high</sup>CD27<sup>high</sup>) NK subset, which produces large amounts of cytokines, such as IFNγ and TNF-α, and also possesses significant cytotoxic functions (249). NK cell expansion and activation appears to contribute to IL-15 SA-induced immunotoxicity since NK cell depletion ablated IL-15 SA-induced hypothermia and weight loss but did not modify the modest increase in liver enzymes observed in wild type mice. Interestingly, adoptive transfer of NK cells into Rag2γc KO mice followed by treatment with IL-15 SA caused significant hepatotoxicity characterized by gross pathology, marked hepatic leukocyte infiltration and liver enzyme elevations. The more profound liver injury observed in that model may reflect loss of the attenuating influence of CD8<sup>+</sup> T cells, and possibly other lymphocyte populations, that are absent in Rag2γC KO mice. Our results show that CD8-deficient mice have an increased sensitivity to IL-15 SA-induced hypothermia, which may reflect lack of a small subset of CD8<sup>+</sup> regulatory T cells (CD8<sup>+</sup>CD44<sup>+</sup>CD122<sup>+</sup>Ly49<sup>+</sup>) in CD8-deficient mice. These CD8<sup>+</sup> regulatory T cells are dependent on IL-15 and essential for maintenance of self tolerance and inhibition of the development of untoward inflammatory responses (250).

IFNγ-deficient, but not TNFα- and perforin-deficient, mice were resistant to IL-15 SA-induced hypothermia and weight loss. Moreover, IFNγ played an essential role in IL-15 SA-mediated NK cell activation as indicated by attenuation of NK cell activation in IFNγ-deficient
mice. Although TNFα is also an important pro-inflammatory cytokine that is secreted by NK cells, it does not appear to play an important role in the immunotoxicity caused by IL-15 SA. That contention is further supported by our observation that IL-15 SA-mediated NK cell activation is independent of the presence of TNFα. Taken together; our results suggest that NK cell-derived IFNγ is an important mediator of IL-15 SA-induced immunotoxicity. IL-15, in conjunction with the monokines IL-12 and IL-18, is a potent inducer of IFNγ secretion by NK cells (251). Previous studies have described a toxic role for IFNγ during endotoxin-induced shock and polymicrobial sepsis (252, 253). NK cells facilitate the activation of macrophages, dendritic cells and other antigen presenting cell populations to amplify pro-inflammatory responses through positive feedback mechanisms (254). The enhanced inflammatory response results in heightened cytokine secretion, leukocyte recruitment and cytotoxic activity. However, the cellular interactions underlying IL-15 SA-induced toxicity remain to be fully defined. Of note, I have not observed increased production of pro-inflammatory cytokines including IFNγ in the plasma of wild type mice at 24 hours after the last injection of IL-15 SA (data not shown). One possibility is that I have missed the spike in cytokines by the time I harvested samples. The other possibility is that IFNγ and other mediators primarily induce local responses, causing damage to kidney, liver and other systems.

The recent discovery of IL-15 SA advances the potential of IL-15-based therapy. IL-15 SA has more potent and prolonged action than native IL-15 and preliminary studies suggest enhanced efficacy in experimental models of cancer (234, 235). The mechanisms of IL-15 SA-mediated immunotoxicity and anti-tumor efficacy may be mediated through different cellular mechanisms. Although IL-15 SA-induced toxicity is mediated through expansion and activation
of NK cells, the anti-tumor efficacy is reportedly dependent on expansion and activation of tumor resident mCD8+ T cells. Cheng et al reported that the efficacy of IL-15 SA in models of liver cancer is mediated through activation of tumor specific CD8+ T cell responses (255). Xu and colleagues demonstrated the importance of mCD8+ T cells in the anti-tumor efficacy of an IL-15/IL-15Rα fusion protein in a murine model of multiple myeloma (235). Chang and colleagues delivered IL-15 SA-expressing adenovirus in a model of hepatocellular carcinoma (236). However, the authors in this paper concluded that the anti-tumor effect of the IL-15 SA adenovirus construct was mediated by NK cells. Thus, there is some controversy in the field. Lastly, the toxicity of IL-15 SA is transient. NK and NKT cell numbers declined rapidly after conclusion of IL-15 SA treatment and returned to baseline value by 1 week after IL-15 SA treatment. Although mCD8 T cell numbers also declined after cessation of IL-15 SA treatment, levels of mCD8 T cells remained above baseline at 7 weeks after completion of IL-15 SA treatment. Taken together, these findings provide new information regarding the cellular and molecular mechanisms underlying IL-15 SA-induced toxicity in vivo.

In conclusion, the present study demonstrates time- and dose-dependent toxicity of IL-15 SA in mice. Toxicity is characterized by systemic physiologic dysfunction reflected by the development of hypothermia and weight loss. Acute liver injury was also observed in IL-15 SA-treated mice. The toxic effects of IL-15 SA appear to be mediated primarily by expansion and activation of NK cells and are dependent on the presence of IFNγ. Thus, the present study provides significant characterization of specific systemic, cellular and molecular alterations caused by IL-15 SA treatment.
CHAPTER III
INTERLEUKIN-15 SUPERAGONIST EXACERBATES THE PATHOGENESIS OF SEPTIC SHOCK
BY EXPANDING AND ACTIVATING NATURAL KILLER CELLS

Introduction

Sepsis is characterized by organ dysfunctions due to dysregulated pro-inflammatory immune responses to invading pathogens (110). Patients with acute sepsis may develop multiple organ failure and early mortality (256). After the acute events of sepsis have resolved, septic patients might exhibit persistent immune cell dysfunctions which are associated with increased susceptibility to secondary infections and late mortality (194). Recent studies have been focused on immunomodulatory therapy for reversing immunosuppression of sepsis (32). IL-15 SA (IL-15/IL-15Rα complex) is a complex with greater and prolonged activity than native IL-15 (32). IL-15 SA has been shown to prevent sepsis-induced apoptosis of NK cells, DCs, CD8\(^+\) and CD4\(^+\) T cells and improved outcomes in experimental models of sepsis (205). Due to its ability to rescue innate and adaptive immune cell dysfunctions, IL-15 SA is currently considered as a promising immunostimulatory agent to target the immunosuppressed state in critically ill patients (32).

However, my results in Chapter II and other groups show that IL-15 SA is a potent inducer of NK and mCD8\(^+\) T cell proliferation and activation (61). Both NK and CD8\(^+\) T cells have been implicated in the pathogenesis of acute sepsis (212, 214-217, 257). Moreover, patients with sepsis exhibit an increase in IL-15 concentration in the plasma which highly correlates with the severity of septic outcomes (201). Thus, exogenous IL-15 SA treatment may have an
additive effect with endogenous IL-15 on exacerbating the pathobiology of sepsis. My hypothesis in Chapter III is that treatment with IL-15 SA at the acute phase of sepsis increases septic lethality by activating NK and mCD8\(^+\) T cells.

Studies in Chapter III examined the effect of IL-15 SA treatment on the mortality of wild type mice to CLP- or LPS-induced sepsis. Sepsis-relevant endpoints included core temperature, systemic cytokine production, and organ injury markers upon LPS challenge. The present studies used 2 μg of IL-15 SA which can cause immuno-toxicity upon four injections. But here, wild type mice were treated with two injections of IL-15 SA which didn’t cause any signs of sickness in the absence of LPS challenge. Further studies examined the effect of IL-15 SA on lymphocyte numbers and activation during sepsis. Moreover, studies determined whether depletion of NK and/or mCD8\(^+\) T cells or neutralization of IFN\(\gamma\) altered the response of IL-15 SA-treated wild type mice to sepsis. The effect of sustained treatment with low-dose IL-15 SA on the host response to septic shock was also examined in the current study. Taken together, my findings illuminate the detrimental effect of IL-15 SA treatment at the hyper-inflammatory phase of sepsis. Thus, IL-15 SA should be used with caution as it advances through drug development for human sepsis. NK and mCD8\(^+\) T cells may represent new therapeutic targets for acute sepsis.
Results

Acute administration of IL-15 SA to wild type mice exacerbates sepsis-induced mortality and hypothermia.

As shown in Chapter II, systemic administration of IL-15 SA at 2 μg for 4 consecutive days caused immuno-toxicity characterized by hypothermia, weight loss, liver injury, and mortality in wild type mice (61). Here, studies were performed to examine the effect of 2 μg IL-15 SA on the response of wild type mice to septic shock induced by CLP or LPS. If given 30 minutes prior to and 24 hours after CLP or LPS, IL-15 SA (2 μg) worsened sepsis-induced mortality and hypothermia compared to vehicle control, while treatment with IL-15 SA alone didn’t cause any signs of toxicity or mortality (Figure 1A-C).
Figure 1. Effect of IL-15 SA treatment on survival and core temperature of wild type mice during septic shock. Wild type mice received vehicle or IL-15 SA at 2 μg 30 minutes prior to and 24 hours after CLP or a sublethal dose of LPS (100 μg) and survival studies were followed (A, B). Core temperature was measured at 24 hours after LPS challenge (C). * p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.0001 compared to vehicle wild type control. n=5-20 mice per group. Data are representative of two to three separate experiments.
Acute administration of IL-15 SA to wild type mice exacerbates sepsis-induced organ injuries and systemic cytokine production.

To get a better understanding of the impact of IL-15 SA on the pathophysiology of wild type mice during sepsis, I further examined sepsis-related endpoints including markers of liver and kidney injury and systemic pro-inflammatory cytokine production. Higher AST and BUN concentrations were also observed in the plasma of wild type mice upon IL-15 SA treatment at 24 hours after LPS, indicating the development of acute liver and kidney injury (Figure 2A, B). In addition, IL-15 prompted wild type mice to produce higher concentrations of pro-inflammatory cytokines IFNγ, IL-6, IL-12 and IL-1β as compared to vehicle control, at 6 and/or 24 hours after LPS challenge (Figure 2C-F). TNF-α and IL-10 concentrations in the plasma were not different when comparing IL-15 SA and vehicle treatment groups after LPS challenge (Figure 2G, H).

![Organ Injuries Diagram](image)
Figure 2. Effect of IL-15 SA treatment on organ injuries and cytokine production of wild type mice during septic shock. Wild type mice received vehicle or IL-15 SA at 2 μg 30 minutes prior to and 24 hours after a sublethal dose of LPS (100 μg). AST and BUN levels (A, B), and IFNγ, IL-6, IL-1β, IL-12, TNF-α and IL-10 concentrations (C-H) in the plasma were measured at 24 hours after LPS challenge. * p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.0001 compared to vehicle wild type control. n=4-12 mice per group. Data are representative of two separate experiments.

Acute administration of IL-15 SA potentiates NK and mCD8+ T cell activation during septic shock.

To determine the effect of IL-15 SA on lymphocyte numbers and activation during sepsis, I examined NK, mCD8+ T, naïve CD8+ T and CD4+ T in spleens of wild type mice treated with IL-15 SA at 0 and 1 hour after LPS challenge. IL-15 SA facilitated the rapid recruitment of NK cells
from spleens at 1 hour following LPS challenge. IL-15 also promoted the activation of NK and mCD8+ T cells as indicated by the upregulation in CD69 expression as early as 1 hour after LPS challenge (Figure 3A-D). However, naïve CD8+ T and CD4+ T numbers were not different when comparing wild type mice treated with IL-15 SA and vehicle during sepsis (Figure 3E, G). CD69 expression was modestly increased on naïve CD8+ T cells and was not altered on CD4+ T cells upon IL-15 SA treatment at 1 hour after LPS challenge (Figure 3F, H).
Figure 3. Effect of IL-15 SA on lymphocyte numbers and activation after LPS challenge. Wild type mice were treated with 2 μg of IL-15 SA 30 minutes prior to LPS (100 μg) challenge. Spleens were harvested at 0 and 1 hours after LPS challenge for measurement of NK, mCD8⁺ T, naïve CD8⁺ T and CD4⁺ T cell total number and CD69 expression (A-H). * p < 0.05, *** p < 0.001, compared to vehicle wild type control. n=4-8 mice per group. Data are representative of two to three separate experiments.

NK and CD8⁺ T cells mediate IL-15 SA-induced exacerbation of sepsis-associated pathobiology.

To determine whether activated NK and mCD8⁺ T cells exacerbated septic mortality of IL-15 SA-treated wild type mice, I depleted NK and/or CD8⁺ T cells in these mice and observed their response to LPS challenge. When anti-asialoGM1 and/or anti-CD8α was given to wild type mice 24 hours prior to the initiation of IL-15 SA, it depleted NK and/or CD8⁺ T cells and also
significantly attenuated mortality of wild type mice treated with IL-15 SA during sepsis (Figure 4A). NK cell depletion provided greater survival benefit than CD8⁺ T cell depletion only during endotoxin shock (Figure 4A). Moreover, NK cell depletion by anti-asialoGM1 caused attenuated hypothermia and concentration of ALT in the plasma of wild type mice treated with IL-15 SA (Figure 4B, C). Concentrations of pro-inflammatory cytokines IFNγ, IL-6, IL-1β were decreased and anti-inflammatory cytokine IL-10 was increased in wild type mice treated with anti-asialoGM1 and IL-15 SA as compared to those treated with IgG and IL-15 SA during septic shock (Figure 4D, E, F, I). IL-12 and TNF-α concentrations in IL-15 SA-treated mice were not altered by anti-asialoGM1 treatment (Figure 4G, H).
Figure 4. Effect of anti-asialoGM1 and/or anti-CD8α treatment on the response of IL-15 SA-treated wild type mice to LPS challenge. Wild type mice received anti-asialoGM1 and/or anti-CD8α i.p. at 24 hours prior to IL-15 SA treatment and then were challenged with a sublethal dose of LPS with 100 μg and a second dose of IL-15 SA at 24 hours thereafter. Survival rate was monitored over 7 days (A). Isotype-specific IgG serve as control. Core temperature and the concentration of ALT in the plasma were measured at 24 hours after LPS challenge (B, C). Concentrations of IFNγ, IL-6, IL-1β, IL-12, TNF-α and IL-10 concentrations in the plasma were measured at 6 and 24 hours after LPS (D-I). * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, compared to IgG wild type mice that were treated with 2 μg IL-15 SA. n=5-10 mice per group. Data are representative of two to three separate experiments.
Ablation of IFNγ reverses IL-15 SA-induced exacerbation of sepsis-associated pathobiology.

IFNγ is a pro-inflammatory cytokine that is mainly secreted by activated NK cells and plays a critical role in the pathogenesis of sepsis. Additional studies were performed to examine the functional importance of IFNγ in the response of IL-15 SA-treated wild type mice to septic shock. IFNγ KO mice displayed improved survival and attenuated hypothermia during endotoxin shock (Figure 5A, B). The level of BUN in the plasma of IFNγ KO was decreased compared to wild type control after LPS challenge, indicative of less kidney injury (Figure 5C). At 6 and/or 24 hours after LPS challenge, IFNγ was not detected in the plasma of IFNγ KO mice, while concentrations of IL-6, IL-1β, IL-12, TNF-α were lower and IL-10 was higher in IFNγ KO mice as compared to wild type control (Figure 5D-I).
Figure 5. Effect of IL-15 SA on the response of IFNγ KO mice to LPS challenge. IFNγ KO mice received intraperitoneal injection with 2 μg of IL-15 SA 30 minutes prior to and 24 hours after LPS (100 μg) challenge. A survival study was performed over 7 days (A). Core temperature and the concentration of BUN in the plasma were measured at 24 hours after LPS challenge (B, C). IFNγ, IL-6, IL-1β, IL-12, TNF-α and IL-10 concentrations in the plasma were measured at 6 and 24 hours after LPS (D-I). * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 compared to wild type control treated with IL-15 SA. n=5-10 mice per group. Data are representative of two separate experiments.
Sustained IL-15 SA pretreatment increases the sensitivity of wild type mice to septic shock.

Wild type mice received 0.1 μg IL-15 SA for consecutive 4 days and were challenged with CLP or LPS at 24 hours after the last IL-15 SA injection. Low-dose IL-15 SA treatment increased the sensitivity of wild type mice to septic shock as indicated by exacerbated mortality and hypothermia (Figure 6A, B, C). Examination of lymphocyte count and activation shows that both NK and mCD8⁺ T cells in the spleen expanded but failed to be activated upon sustained low-dose IL-15 SA treatment (Figure 6 D, E). The effect of 0.1 μg of IL-15 SA on NK and mCD8⁺ T cell expansion was minimal as compared to that of 2 μg of IL-15 SA.
Figure 6. The effect of sustained IL-15 SA pretreatment on the host response to septic shock. Wild type mice received vehicle or 0.1 μg IL-15 SA for 4 days. At 24 hours after the last injection, mice were subject to LPS or CLP challenge for survival studies (A, C). Body temperature was measured at 6 and 24 hours after LPS challenge (B). Lymphocyte number and activation in spleens were analyzed at 24 hours after LPS challenge (D, E). ** p < 0.01, *** p < 0.001 compared to vehicle-treated wild type control. n=3-12 mice per group. Data are representative of two separate experiments.
Discussion

Immuno-stimulatory therapy offers promise for the treatment of septic patients who are at a risk of immunosuppression and increased susceptibility to secondary or nosocomial infections. Recent studies reported the potential therapeutic effect of IL-7, IL-3, IFNγ as well as blockade of co-inhibitory receptor PD-1 on rejuvenating innate and adaptive immune functions during sepsis (32). Although still in preclinical studies, IL-15 is a promising candidate for the treatment of human sepsis as it prevents sepsis-induced apoptosis, and induces the proliferation and activation of NK, NKT and CD8⁺ T cells (205). However, little is known about whether the administration of IL-15 (or IL-15 SA) would worsen physiological dysfunctions and systemic inflammation during sepsis, as IL-15-dependent NK and CD8⁺ T cells are implicated in the pathogenesis of sepsis.

In this Chapter, I found that IL-15 SA treatment played a detrimental role in the pathogenesis of acute sepsis. Administration of IL-15 SA at the acute phase of sepsis increased mortality and worsened hypothermia as compared to vehicle control. Wild type mice treated with IL-15 SA elevated concentration of pro-inflammatory cytokines in the plasma as compared to vehicle control. IL-15 SA also augmented AST and BUN levels in the plasma, indicating acute liver and kidney injury. Examination of lymphocyte activation shows that acute treatment with IL-15 SA preferentially prompted the activation of NK and mCD8⁺ T cells as early as 1 hour after LPS challenge. Wild type mice depleted of NK cells, mCD8⁺ T cells, or both, prior to IL-15 SA treatment, attenuated pathobiology of sepsis. NK cell depletion provided better survival benefit than mCD8⁺ T cell depletion. It indicates that IL-15 SA facilitates the pathobiology of sepsis, primarily by promoting activation of NK cells. IFNγ neutralization protects IL-15 SA-treated wild
type mice against sepsis. In addition, sustained treatment with low-dose IL-15 SA expands NK cells and increased the host sensitivity to septic shock as indicated by exacerbated mortality and hypothermia. Taken together, the present study demonstrated that NK cells and IFNγ are central to IL-15 SA-regulated pathogenesis of acute sepsis.

In this chapter, I mainly evaluated the detrimental role of IL-15 during acute sepsis induced by CLP or LPS. Another recent paper from our laboratory showed that IL-15 SA is effective in expanding NK, NKT and mCD8+ T cells in burned mice but does not improve survival in a model of Pseudomonas burn wound infection (204). In contrast, the original paper showing the protective effect of IL-15 SA during sepsis reported IL-15 SA attenuates sepsis-induced apoptosis, reverses associated immune dysfunction and improves survival during sepsis (205). The differences observed between the studies could be due to the use of different mouse strains, dosage of IL-15 SA, delivery routes of IL-15 SA as well as different severity of our models of sepsis. Nevertheless, our current study indicates that IL-15 should be used with caution as an immunomodulator in subjects with acute sepsis due to its ability to augment systemic inflammatory responses.

NK cells are large granular innate lymphoid cells that play an essential role in tumor surveillance and elimination of virus-infected cells (206, 207). NK cells also actively participate in the propagation of acute inflammation caused by bacterial infections (208, 209). Our lab previously showed that NK cells quickly migrate to sites of infection within 4-6 hours after the initiation of intraabdominal sepsis, in a manner regulated by the chemokines CXCL9 and CXCL10 (209-211, 258). Recruited NK cells exhibit an activated phenotype and increase production of the pro-inflammatory cytokine IFN-γ, which is known to potently activate macrophages,
dendritic cells and other antigen presenting cell populations during inflammation (212, 259). In turn, the activated macrophages further activate NK cells by increasing expression of effector cytokines IL-15, IL-18 and IL-12 (213). The amplification of pro-inflammatory responses via positive feedback may induce excessive systemic inflammation, which is characterized by unbridled cytokine storm during septic shock (110). As shown in previous studies from our lab and others, selective NK cell depletion attenuates systemic inflammation and improves survival in experimental models of polymicrobial peritonitis (209, 212, 258, 260), endotoxin shock (214), pneumococcal pneumonia (215), systemic *Escherichia coli* and *Streptococcus pyogenes* infection (216, 217), and polytrauma (218).

Similar to NK cells, memory CD8$^{+}$ T cells have been shown to exhibit properties that are characteristic of innate effector cells such as rapid migration to sites of infection and elimination of pathogens, independent of cognate antigen recognition (218). Memory CD8$^{+}$ T cells are also considered to be an important cellular source of IFN-γ. However, the contribution of memory CD8$^{+}$ T cell subset to the pathogenesis of septic shock has not been well characterized, since it is currently impossible to selectively deplete these cells. Previously studies from our lab reported that mice depleted of total CD8$^{+}$ T cells show improved survival during intraabdominal sepsis induced by CLP (260, 261). Wesche-Soldato *et al* showed that CD8$^{+}$ T cells facilitate acute liver injury after CLP (219). However, there is some controversy about the role of CD8$^{+}$ T cells in the pathogenesis of septic shock. Emoto *et al* reported that β$_2$-microglobulin KO mice, which lack CD8$^{+}$ T cells, are more susceptible to LPS-induced endotoxin shock, although these mice have been shown to be protected from CLP (257). The findings in Chapter IV further showed antibody-mediated depletion of CD8$^{+}$ T cells in wild type mice fails to
confer protection from endotoxin shock. Thus, CD8⁺ T cells plays a minimal role in the setting of acute sepsis induced by LPS.

The present study also demonstrated the critical role of IFNγ in IL-15 SA-regulated pathophysiology of sepsis as IL-15 SA failed to facilitate physiological dysfunction and systemic inflammation in IFNγ KO mice. IFNγ is an important proinflammatory mediator that is secreted by activated NK and CD8⁺ T cells and is elevated during sepsis (160, 262, 263). Previous studies have described IFNγ is detrimental during LPS-induced shock and polymicrobial sepsis (160, 264). Here, I found concentration of IFNγ was further augmented by IL-15 SA treatment at the early phase of sepsis. IFNγ also plays a critical role in IL-15 SA-mediated NK cell activation as indicated by attenuation of NK cell activation in IFNγ-deficient mice, as shown in Chapter II. It is possibly mediated by the positive feedback mechanisms as described in the last paragraph.

TNF-α is another important proinflammatory cytokine that is secreted by NK cells (265). The concentration of TNF-α is not increased in the plasma of wild type mice treated IL-15 SA during sepsis and IL-15 SA-mediated NK cell activation is independent of the presence of TNFα (61). In addition, TNF-α does not appear to contribute to IL-15 SA-mediated immune-toxicity, as shown in Chapter II (61). Taken together, it appears that IFNγ, but not TNF-α, participates in the adverse events facilitated by IL-15 SA during LPS-induced septic shock.

In conclusion, the present study demonstrates the adverse effect of IL-15 SA on facilitating systemic inflammation, organ dysfunctions and mortality during sepsis. Acute administration of IL-15 SA at the onset of sepsis potently activates NK cells and pretreatment with low-dose IL-15 SA expands NK cells, both of which causes wild mice to be more susceptible to endotoxin shock. Results of this study and others indicate that IFNγ contributes significantly
to NK cell-mediated inflammation and injury during septic shock and also plays a critical role in 
IL-15-induced exacerbation of sepsis-associated pathobiology. Therefore, IL-15 SA potentiates 
the pathobiology of sepsis and should be used with caution as an immune-stimulatory agent for 
reversing immunosuppression phase of sepsis.
CHAPTER IV

ENDOGENOUS INTERLEUKIN-15 FACILITATES THE PATHOGENESIS OF SEPTIC SHOCK

BY MAINTAINING NATURAL KILLER CELL NUMBERS AND INTEGRITY

Introduction

Interleukin (IL)-15 is a cytokine that is essential for the homeostasis and effector functions of natural killer (NK) and memory CD8\(^+\) (mCD8\(^+\)) T lymphocytes. IL-15 prompts the generation of mature NK cells in the bone marrow (60); it potently expands and activates peripheral NK cells to perform cytotoxic functions and facilitate cytokine secretion during viral and bacterial infections (67, 266); IL-15 also plays a pivotal role in the generation, cytotoxicity and survival of CD8\(^+\) T lymphocytes, especially the mCD8\(^+\) subset (74, 267); and is essential for survival of natural killer T (NKT) and intestinal intraepithelial lymphocytes (IELs) (55, 268). Germline deletion of IL-15 in mice causes deficiency in NK, mCD8\(^+\) T, NKT cells and intraepithelial lymphocytes (53). IL-15 is constitutively expressed by multiple types of cells including monocytes, macrophages, dendritic cells (DCs), fibroblasts and epithelial cells (35, 36). Its expression is induced by cytokines such as type I (IFN\(\alpha/\beta\)) and type II (IFN\(\gamma\)) interferons (IFN) as well as microbial products such as lipopolysaccharide, poly I:C, and viruses (37, 38). IL-15 is primarily presented in association with its unique high-affinity receptor IL-15 R\(\alpha\) on the surface of IL-15 producing cells and delivers signals to target cells that express the IL-2R \(\beta\) and \(\gamma\) receptor subunits, a process called trans-presentation. The IL-15/IL-15R\(\alpha\) complex can also be released in a soluble form after cleavage of the transmembrane domain of the receptor \(\alpha\) (58,
This combined form generated in vitro possesses longer half-life and greater biological activity than naïve IL-15 and is thus termed IL-15 superagonist (IL-15 SA) (23).

NK and CD8⁺ T lymphocytes have been shown to facilitate physiological dysfunction and systemic inflammation during sepsis (260, 261, 271). However, little is known about the factors that regulate the functions of NK and CD8⁺ T lymphocytes in the context of sepsis. IL-15 appears to be an essential pro-inflammatory mediator during sepsis, as IL-15 KO mice show resistance to sepsis (202). However, the underlying mechanisms by which IL-15 facilitates the pathogenesis of sepsis have not been well characterized. In addition, IL-15 KO mice are not only deficient in IL-15, but have markedly decreased numbers of NK and CD8⁺ T cells, which are implicated in the pathogenesis of sepsis (260, 261, 271). Therefore, it is unclear if lack of IL-15 alone or lack of IL-15-dependent NK and mCD8⁺ T cells in IL-15 KO mice confers protection against septic shock.

In Chapter IV, we investigated the role of endogenous and exogenous IL-15 in the pathogenesis of sepsis and its regulatory effect on NK and mCD8⁺ T cell viability and activity during sepsis. The studies were designed to address the hypotheses that IL-15 KO mice are resistant to septic shock due to an intrinsic deficiency of NK and mCD8⁺ T cells. We fully examined the response of IL-15-deficient mice to sepsis caused by CLP or lipopolysaccharide (LPS) challenge. Specific endpoints included survival, organ injury, systemic cytokine production, bacterial clearance and indices of acute organ injury. We further assessed whether regeneration of NK and mCD8⁺ T cells by treatment with IL-15 SA would alter the response of IL-15-deficient mice to sepsis. Additional experiments examined whether regenerated NK or CD8⁺ T cells results in the restored septic mortality of IL-15 KO mice treated with IL-15 SA. At last, I
compared the differential effects of acute and sustained neutralization of IL-15 on the response of wild type mice to septic shock.

**Results**

**IL-15 KO mice are resistant to CLP-induced septic shock.**

IL-15 null mice have previously been reported to be deficient of NK, NKT and mCD8+ T cells (272). This was examined in our colony. NK and mCD8+ T cells were significantly decreased in the spleens and livers of IL-15 KO mice whereas NKT and B cell numbers were not significantly different in either tissue as compared to wild type controls. Naïve CD8+ T cells were slightly decreased and CD4+ T cells were increased in the spleen of IL-15 KO mice as compared to wild type control (Figure 1 A, B).

![Figure 1. Lymphocyte counts in spleens and livers of wild type and IL-15 KO mice.](image)

Figure 1. Lymphocyte counts in spleens and livers of wild type and IL-15 KO mice. Spleens (A) and livers (B) were harvested from wild type and IL-15 KO mice for measurement of NK, NKT, memory and naïve CD8+ T, CD4+ T and B lymphocyte numbers. * p < 0.05, ** p < 0.01, **** p < 0.0001, compared to wild type mice. n=3-9 mice per group. Data are representative of two separate experiments.
To examine whether IL-15 KO mice were resistant to septic shock, a survival study was undertaken to assess mortality in IL-15 KO and wild type mice during septic shock induced by a lethal model of CLP. A significant survival advantage was observed in IL-15 KO mice, which exhibited 50% long-term survival and a 120-hour median survival time as compared to 0% survival and 36-hour median survival time in wild type mice (Figure 2A). Both wild type and IL-15 KO mice developed sepsis-induced hypothermia (Figure 2B). However, core body temperature was significantly higher in IL-15 KO mice at 6 and 18 hours after CLP as compared to wild type controls (Figure 2B).

![Figure 2. Survival and core temperature of wild type and IL-15 knockout (KO) mice during septic shock induced by CLP.](image)

Figure 2. Survival and core temperature of wild type and IL-15 knockout (KO) mice during septic shock induced by CLP. Wild type and IL-15 KO mice were subject to 1.0-cm CLP procedure and were monitored for 7-day survival (A). Body temperature (B) was measured at 6 h or 18 hours after CLP. The median value is designated in Figure 1B. * p < 0.05, *** p < 0.001, compared to CLP wild type mice. n=11-14 mice per group. Data are representative of two to three separate experiments.
To get a better understanding of attenuated pathobiology in IL-15 KO mice, I further assessed the effect of IL-15 deficiency on pro-inflammatory cytokine production and bacterial clearance after CLP. At 6 and 18 hours after CLP, IL-15 was elevated in the plasma of wild type mice but was not detectable in IL-15 KO mice (Figure 3A). Concentrations of several pro-inflammatory cytokines, including IL-6, TNFα, IL-1β, IFNγ and IL-12, were significantly lower in the plasma of IL-15 KO mice compared to wild type controls (Figure 3B-F). Meanwhile, neutrophil recruitment into the peritoneal cavity at 6 hours after CLP was not different between IL-15 KO and wild type mice (Figure 3G). The colony forming units of bacteria were lower in peritoneal lavage fluid of IL-15 KO mice than wild type control mice at 6 hours after CLP (Figure 3H) but showed no significant difference between groups in blood or peritoneal cavity at 18 hours after CLP (Figure 3I, J).
Figure 3. Concentrations of pro-inflammatory cytokines, neutrophil recruitment and bacterial clearance in wild type and IL-15 KO mice during CLP-induced septic shock. Blood was harvested at 6 and 18 hours after CLP challenge for measurement of pro-inflammatory cytokines IL-15, IL-6, IL-1β, TNF-α, IL-12p70, IFN-γ (A-F). Neutrophil recruitment to peritoneal cavity (G) and bacterial colony forming units in blood and peritoneal fluid (H, I and J) were also measured at designated time points in wild type and IL-15 KO mice. * p < 0.05, ** p < 0.01, *** p < 0.001 compared to wild type mice at designated time points. n=6-8 mice per group. Data are representative of two separate experiments.
IL-15 KO mice are resistant to endotoxin-induced shock.

To determine the dose of LPS that can induce septic shock, IL-15 KO and wild type mice were intraperitoneally injected with escalating doses of LPS (100 or 150 μg) and monitored for sepsis-induced mortality over 7 days. Wild type mice showed dose-dependent mortality with 43% survival after 100 μg LPS and 0% after 150 μg LPS, while IL-15 KO mice showed improved survival at both doses of LPS (100% survival after 100 μg LPS and 67% after 150 μg LPS) (Figure 4A, C). IL-15 KO mice developed significantly less hypothermia than wild type controls at 24 hours after LPS challenge (Figure 4B, D). LPS at the dose of 150 μg elicited more severe septic shock than at 100 μg and thus was chosen to induce lethal endotoxin shock in subsequent experiments, which were performed to assess organ injury and pro-inflammatory cytokine production after LPS challenge.
Figure 4. Survival and core temperature of wild type and IL-15 KO mice during septic shock induced by LPS. In a dose escalation study, IL-15 KO and wild type mice were injected i.p with 100 or 150 ug LPS and observed for 7-day survival (A, C) and body temperature at 24 hours after LPS injection (B, D). ** p < 0.01, *** p < 0.001, **** p < 0.0001 when compared to wild type mice; n=8-23 mice per group. Data are representative of two to four separate experiments.

I further examined the cytokine production and organ injuries of wild type and IL-15 KO mice during endotoxin shock. IL-15 concentration in the plasma was augmented in wild type mice but not in IL-15 KO mice, at 6 and 24 hours after LPS challenge (Figure 5A). Meanwhile, lower concentrations of plasma pro-inflammatory cytokines were observed in IL-15 KO mice than in wild type mice (Figure 5B-F). Also, lower concentrations of AST, BUN and creatinine were observed in the plasma of IL-15 KO mice than in wild type controls at 24 hours after LPS challenge, indicative of reduced acute hepatocellular and kidney injury during septic shock (Figure 5G-I).
Figure 5. Plasma pro-inflammatory cytokines, AST, BUN and creatinine in wild type and IL-15 KO mice after LPS challenge. Blood were harvested at 6 h and 24 h after LPS for measurement of proinflammatory cytokines IL-15, IL-6, IL-1β, TNF-α, IL-12p70, IFN-γ in plasma (A-F). AST (aspartate transaminase), BUN (blood urea nitrogen) and creatinine (G-I) concentrations in plasma were also measured at 24 hours post LPS. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 when compared to wild type mice. n=8 mice per group. Data are representative of two separate experiments.
Treatment with IL-15 SA for 4 days regenerates NK and mCD8\(^+\) T cells in IL-15 KO mice.

To define whether lack of NK and mCD8\(^+\) T cells in IL-15 KO mice conferred resistance to septic shock, I regenerated NK and mCD8\(^+\) T cells in IL-15 KO mice to a level comparable to the wild type baseline before the initiation of septic challenges. IL-15 KO mice were treated with IL-15 superagonist (IL-15 SA, 0.125 μg/day) for 4 days and lymphocyte numbers in spleen and liver were examined. Total NK cell (CD3\(^-\)NK1.1\(^+\)) numbers in spleen and liver were significantly higher in IL-15 SA-treated IL-15 KO mice than in vehicle-treated IL-15 KO mice and were ~3.2-fold higher in spleen and ~7.4-fold higher in liver compared to wild type mice (Figure 6A, B).

After IL-15 SA treatment, splenic mCD8\(^+\) T cells (CD8\(^+\)CD44\(^{\text{high}}\)) increased to equal numbers and hepatic mCD8\(^+\) T cells to ~3.2-fold higher as compared to wild type mice (Figure 6A, B). NKT (CD3\(^-\)NK1.1\(^+\)) cell numbers were increased in liver, but not in spleen, in IL-15 KO mice treated with IL-15 SA compared to vehicle-treated controls (Figure 6A, B). Naïve CD8\(^+\) T (CD8\(^+\)CD44\(^{\text{low}}\)), CD4\(^+\) T and B cells were not significantly different when comparing IL-15 KO mice treated with IL-15 SA or vehicle (Figure 6A, B).

To define the impact of low-dose IL-15 SA treatment on regenerated NK and mCD8\(^+\) T cell activation and function, additional experiments examined expression of the early surface activation marker CD69 and production of IFN\(\gamma\) by the cells. Expression of CD69 on splenic NK cells in IL-15 KO mice treated with IL-15 SA was not different compared to vehicle-treated IL-15 KO mice or wild type mice (Figure 6C). The relative number of mCD8\(^+\) T cells expressing CD69 was increased by IL-15 SA treatment of IL-15 KO mice compared to vehicle IL-15 KO control but exhibited no difference when compared to wild type control (Figure 6E). PMA/ionomycin-induced IFN\(\gamma\) was significantly higher in IL-15 KO mice treated with IL-15 SA as compared to
those treated with vehicle (Figure 6D, F). The effect of IL-15 SA on regenerated NK cells was further analyzed. NK cell subsets were characterized based on CD11b and CD27 expression and classified as immature (I, CD11b^{low}CD27^{high}), pro-inflammatory (II, CD11b^{high}CD27^{high}) or cytotoxic (III, CD11b^{high}CD27^{low}) (238) (Figure 6G). IL-15 SA treatment increased the relative numbers of mature stage II NK cells in IL-15 KO mice to a level comparable to the wild type baseline but did not increase that of the stage III NK subset (Figure 6H, I).
IL-15 SA treatment reestablishes sepsis-induced mortality of IL-15 KO mice.

To determine the response of IL-15 KO mice treated with IL-15 SA to septic shock, studies were undertaken to assess CLP- or LPS-induced mortality of IL-15 KO mice that received IL-15 SA for 4 days prior to septic challenge. IL-15 SA treatment was continued throughout the experimental period to maintain regenerated NK and mCD8+ T cell populations. During septic shock elicited by CLP or LPS, IL-15 SA reestablished mortality in IL-15 KO mice to a level comparable to wild type control and significantly higher than IL-15 KO mice treated with vehicle (Figure 7A, B). Pretreatment with IL-15 SA for 4 days only did not cause a decrease in core temperature or increase in cytokine concentrations prior to the initiation of sepsis (data not shown). However, IL-15 KO mice treated with IL-15 SA showed a marked decrease in core body temperature after LPS challenge compared to vehicle-treated IL-15 KO mice (Figure 7C). Elevated production of pro-inflammatory cytokines, including IL-6 and IL-1β, was observed in the plasma of IL-15 SA-treated IL-15 KO mice, at 24 hours after LPS, compared to vehicle-treated IL-15 KO mice (Figure 7D, E). Also, IL-15 SA treatment prompted the development of
acute kidney injury as indicated by elevated BUN and creatinine production, after LPS challenge
(Figure 7F, G).
Figure 7. Survival, core temperature, pro-inflammatory cytokines and organ injuries of wild type and IL-15 KO mice upon pretreatment with low-dosed IL-15 SA during septic shock. 0.125 μg of IL-15 SA was initiated 4 days prior to septic challenge and continued throughout the experimental period to maintain regenerated NK and mCD8^+ T cell populations. Mice were monitored for survival rate over 7 days (A and B). Core temperature was measured at 24 hours after LPS challenge (C). Vehicle-treated wild type and IL-15 KO mice served as control. To assess the effect of IL-15 SA on sepsis-induced systemic inflammation and organ injuries in IL-15 KO mice, blood was drawn from IL-15 KO mice at 24 hours after LPS challenge for measurement of IL-6 and IL-1β (D and E) as well as ALT, AST, BUN and creatnine (F and G) concentrations in plasma. * p < 0.05, ** p < 0.01, **** p < 0.0001 compared to designated groups. n=7-14 mice per group. Data are representative of two to four separate experiments.

*Regeneration of NK, but not CD8^+ T cells, restores septic mortality of IL-15 KO mice treated with IL-15 SA.*

Studies were performed to determine the role of NK and mCD8^+ T cells in the pathogenesis of LPS-induced endotoxin shock. Depletion of NK cells by treatment with anti-asialoGM1 improved survival of wild type mice during endotoxin shock (Figure 8A). In further experiments, total CD8^+ T cells were depleted by treatment with anti-CD8α as it is not currently possible to selectively deplete mCD8^+ T cells (Figure 8A). As opposed to depletion of NK cells, CD8^+ T cell depletion in wild type mice failed to provide protection during endotoxin shock (Figure 8A).

To determine whether regenerated NK and mCD8^+ T cells restored septic mortality in IL-15 KO mice treated with IL-15 SA, I proposed to prevent NK or mCD8^+ T cell regeneration in these mice and observed their response to endotoxin shock. If anti-asialoGM1 was given to IL-15 KO mice prior to the initiation of IL-15 SA treatment, it prevented NK cell regeneration (Figure 8B) and also significantly reversed septic lethality in IL-15 SA-treated IL-15 KO mice.
(Figure 8C). However, preventing CD8$^+$ T cell regeneration by treatment with anti-CD8α (Figure 8D) failed to rescue IL-15 KO mice treated with IL-15 SA during endotoxin shock (Figure 8E).
Figure 8. Survival of wild type and IL-15 SA-treated IL-15 KO mice after treatment with anti-asialoGM1 or anti-CD8α. To deplete NK or CD8⁺ T lymphocytes, wild type mice were treated with anti-asialoGM1 or anti-CD8α i.p. at 24 hours prior to LPS challenge and monitored for survival over 7 days (A). In further experiments, IL-15 KO mice received anti-asialoGM1 or anti-CD8α at 24 hours prior to the initiation of IL-15 SA (0.125 μg) and NK and memory CD8⁺ T lymphocyte counts were measured at 24 hours after stopping IL-15 SA treatment. Dotted lines represent the baseline levels of NK and memory CD8⁺ T cells in wild type controls (B and D). A survival study was also undertaken to assess the effect of anti-asialoGM1 or anti-CD8α on the survival of IL-15 SA-treated IL-15 KO mice (C and E). Isotype-specific IgG served as control. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 compared to designated groups. n=5-15 mice per group. Data are representative of two to four separate experiments.
**Short-term neutralization of IL-15 fails to protect mice against septic shock.**

Generally, IL-15 is retained inside the cell until it is chaperoned with the high-affinity receptor α and shuttled to the cell surface for delivering signaling to target cells, a process called trans-presentation. But the IL-15/IL-15Rα complex in a soluble form is also present in plasma *in vivo* after proteolytic cleavage of the transmembrane domain of IL-15Rα from the cell surface. Analysis of the secreted form of IL-15/IL-15Rα complex by ELISA showed that concentration of IL-15/IL-15Rα complex was elevated in the plasma of wild type mice during septic shock induced by CLP or LPS, but was significantly neutralized by M96, an anti-IL-15 neutralizing antibody, when given to WT mice 2 hours prior to CLP or LPS (**Figure 9**). However, short-term neutralization of IL-15 by M96 failed to confer survival benefit to wild type mice compared to IgG control during CLP and LPS challenges (**Figure 10A, B**). Examination of cytokine expression profile shows that the acute neutralization of IL-15 didn’t alter concentrations of other plasma pro-inflammatory cytokines except IFN-γ, which was lower compared to IgG control at 6 and 24 hours after LPS challenge (**Figure 10C-H**). Under the current treatment regimen, M96 didn’t alter the number of NK and mCD8+ T cells nor their activation as indicated by CD69 expression during septic shock as compared to IgG control (**Figure 10I-L**).
Figure 9. Plasma level of IL-15 in wild type mice treated with IgG or M96, an IL-15 neutralizing antibody post LPS. Wild type mice received 20 μg of M96 or IgG i.p. at 2 hours prior to CLP or LPS challenge. Blood was harvested at 0, 6 and 24 hours after LPS for measurement of IL-15 in the plasma. ** p < 0.01 compared to IgG control at designated time points. n=4-5 mice per group. Data are representative of two separate experiments.
20 ug M96/IgG  Harvest samples from LPS group  

CLP or LPS  Survival study  

A  M96 - 2 h Prior CLP  

B  M96 - 2 h Prior LPS  

Pro-inflammatory Cytokines

C  IFN-γ  

D  IL-6  

E  IL-1β  

F  IL-12  

G  TNF-α  

H  IL-10  

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Figure 10. Survival and lymphocyte characterization of wild type mice with short-term IL-15 neutralization during septic shock. Wild type mice received 20 μg of M96, an IL-15 neutralizing antibody i.p. at 2 hours prior to CLP or LPS challenge and a survival study was followed (A and B). Specific IgG serve as control. Concentrations of IFNγ, IL-6, IL-1β, IL-12, TNF-α and IL-10 in the plasma were measured at 6 and 24 hours after LPS challenge (C-H). Splenic NK and mCD8+ T cell number and activation were measured at 0, 6 and 24 hours after LPS challenge (I-L). * p < 0.05, ** p < 0.01 compared to IgG control. n=5-10 mice per group Data are representative of two to three separate experiments.
Long-term neutralization of IL-15 depletes NK cells and provides survival benefit during septic shock.

Studies were undertaken to assess CLP- or LPS-induced mortality of WT mice upon pretreatment with M96. M96 treatment was initiated 4 days prior to septic challenge and a second administration was given at the time of CLP or LPS. As opposed to acute neutralization of IL-15, long-term IL-15 neutralization significantly reversed septic mortality of wild type mice upon both CLP and LPS challenges (Figure 11A, B). Analysis of splenic and hepatic lymphocyte populations showed M96 depleted 80.8% of splenic and 57.5% hepatic NK cells when it was administered 4 days prior (Figure 11C). However, mCD8+, NKT, naïve CD8+, CD4+ and B lymphocyte number was not altered by pretreatment with M96 (data not shown).
Figure 11. Survival and lymphocyte number of wild type mice with long-term IL-15 neutralization during septic shock. Wild type mice received 20 μg of IL-15 neutralizing antibody M96 or IgG i.p. at 4 days prior to (day -4) and at the time of CLP or LPS challenge (day 0). A survival study was undertaken over 7 days (A and B). Splenic and hepatic NK cell number was measured at day 0 without LPS challenge (C). ** p < 0.01, **** p < 0.0001, compared to IgG control. n=6-10 mice per group. Data are representative of two to three separate experiments.
Discussion

Although previous studies have shown that IL-15 exhibits toxicity (61) and aggravates chronic inflammatory disorders (32-38), there are few studies examining the role of endogenous IL-15 in the pathogenesis of acute inflammation, which is typical of sepsis (110). We report that mice genetically deficient in IL-15 show a significant survival benefit over wild type mice during CLP- and LPS-induced septic shock. IL-15 KO mice display markedly decreased NK and mCD8+ T cell numbers and attenuated sepsis-induced hypothermia, hepatocellular and kidney injuries and systemic pro-inflammatory cytokine production compared to wild type mice. Similarly, neutralization of IL-15 over a 4-day period causes NK cell depletion and provides protection from septic shock. Treatment of IL-15 KO mice with low-dose IL-15 SA regenerates NK cells and reestablishes susceptibility to septic shock, whereas acute neutralization of IL-15 in wild type mice does not deplete NK cells and fails to provide protection. Thus, we conclude that IL-15 contributes to the pathogenesis of acute septic shock by maintaining NK and, possibly, mCD8+ T cells. Combined with my findings from Chapter III, these cells facilitate systemic inflammation and organ injury during septic shock through a mechanism that is dependent on the production of IFNγ.

Orinska et al previously showed the genetic deletion of IL-15 results in improved survival and better bacterial clearance at the early phase during septic shock induced by CLP (42). The present study also demonstrates improved survival in IL-15 KO mice and a small, but significant, improvement in bacterial clearance at 6 hours after CLP. However, we didn’t observe changes in bacterial clearance in blood or peritoneal cavity among groups at 18 hours after CLP. In another experimental model of septic shock elicited by injection with LPS, IL-15 KO mice also
display protection, although no live bacterial infection is present. Thus, our studies suggest that the improved septic outcomes in IL-15 KO mice are associated with attenuated inflammation as evidenced by decreased systemic cytokine production in both the CLP and LPS models rather than alterations in bacterial clearance mechanisms.

As I discussed in Chapter III, NK cells have been implicated in the pathogenesis of experimental models of polymicrobial peritonitis (209, 212, 258, 260), endotoxin shock (214), pneumococcal pneumonia (215), systemic *Escherichia coli* and *Streptococcus pyogenes* infection (216, 217), and polytrauma (218). In the current study, we show that loss of NK cells is a major factor by which IL-15 KO mice are protected from septic shock. Thus, using a unique model, we provide further evidence that NK cells play an important role in augmenting acute inflammation and contributing to the pathogenesis of organ injury and physiological dysfunction during septic shock.

The contribution of memory CD8$^+$ T cell subset to the pathogenesis of septic shock has not been well characterized, since it is currently impossible to selectively deplete memory CD8$^+$ T cells. CD8$^+$ T cells have been reported to facilitate acute liver injury and mortality of intraabdominal sepsis induced by CLP (260, 261), (219). β2-microglobin KO mice, which lack CD8$^+$ T cells, are more susceptible to endotoxin shock induced by LPS, although these mice have been shown to be protected from CLP (257). The present study further showed antibody-mediated depletion of CD8$^+$ T cells in wild type mice fails to confer protection from endotoxin shock. Preventing CD8$^+$ T cell regeneration fails to attenuate restore septic mortality in IL-15 SA-treated IL-15 KO mice. Thus, we conclude that lack of CD8$^+$ T cells doesn’t contribute significantly to the resistance of IL-15 KO mice to septic shock. NKT cells are increased in IL-15
KO mice after treatment with low-dose IL-15 SA. It is possible that NKT cells also play a pathogenic role during septic shock as NKT-deficient mice are shown to be resistant to CLP-induced septic shock (220). However, currently there are no antibodies available that can selectively deplete NKT cells in IL-15 SA-treated IL-15 KO mice so our exploration of the role of NKT cells in this setting is limited. Nevertheless, we did not note a significant decrease in NKT cell numbers in IL-15 KO mice. Thus, loss of NKT cells does not appear to contribute to the resistance of IL-15 KO mice to septic shock.

IL-15 KO mice lack not only NK cells but also the cytokine itself. It is unclear whether IL-15 alone plays an acute role in the pathobiology of sepsis. Here, we showed that concentration of soluble IL-15/IL-15Rα was elevated in the plasma of septic mice compared to non-septic mice. Recent studies correlate elevated serum IL-15 concentration with the development of organ dysfunction and mortality in patients with septic shock (201). Thus, it is possible that IL-15 alone plays a detrimental role in the pathogenesis of septic shock. The current study shows that acute neutralization of IL-15 at the onset of septic shock failed to confer protection, while long-term neutralization of IL-15 prior to septic shock conferred protection. Further studies showed that acute neutralization of IL-15 neither depleted NK cells nor blocks NK cell activation at the early phase of septic shock. In contrast, long-term neutralization of IL-15 depleted 80.8% of splenic NK cells. Therefore, our studies suggest that endogenous IL-15 does not play an acute role in the pathogenesis of septic shock but contributes to septic mortality by maintaining NK cells which aggravate systemic inflammation.

In conclusion, the present study demonstrates the role of IL-15 in the pathogenesis of septic shock. Endogenous IL-15 doesn’t play a direct role in aggravating septic mortality but is
able to maintain NK cells, which facilitates sepsis-induced systemic inflammation, hypothermia, acute organ injuries and death. Combined with my findings from Chapter III, both endogenous and exogenous IL-15 appear to act on the same cell type but exhibit different actions in the context of septic shock. NK cells may represent effective targets for treatment of septic shock.
CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

Overview

Immunotherapy that modulates various aspects of the host immune system has offered promise for the treatment of human diseases including autoimmunity, cancer or sepsis. IL-15 SA is one of the promising immunomodulatory therapies and is currently undergoing clinical trials to test the efficacy for treatment of advanced cancer. My thesis study provides a full characterization of IL-15 SA-induced immune-toxicity and the role of endogenous and exogenous IL-15 in the pathogenesis of sepsis. In Chapter V, I will discuss the potential application of my findings, unanswered or new questions related to my findings and future directions for my project. Summaries of my findings, schematic diagrams of my findings and future directions are presented in Figure 1. Identification of the adverse effects of IL-15 SA in intact or septic mice and the associated causative factors will provide important safety information and rational approaches to prevent side effects when IL-15 SA advances through drug development. The clear understanding of the role of endogenous IL-15 during sepsis provides conceptual support of using the IL-15 neutralizing antibody to prevent patients at high risk of developing sepsis.
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<th>Model Figures</th>
<th>Future Directions</th>
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<td><img src="image1.png" alt="Model Figure" /></td>
<td><strong>Chapter II</strong></td>
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<tr>
<td>• IL-15 SA causes time- and dose-dependent immunotoxicity in wild type mice</td>
<td><strong>Model Figure</strong></td>
<td>• Mouse models with humanized immune systems to evaluate human IL-15 SA toxicity</td>
</tr>
<tr>
<td>• NK cells and IFNγ mediate the immuno-toxicity of IL-15 SA</td>
<td></td>
<td>• NK cell-specific IFNγ depleted mice (Ncr1-Cre +/- IFNγ flox/flox mice) to determine the role of NK cell-specific IFNγ in IL-15 SA-mediated toxicity</td>
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<tr>
<td><strong>Chapter III</strong></td>
<td><img src="image2.png" alt="Model Figure" /></td>
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<tr>
<td>• Acute administration of IL-15 SA exacerbates the pathogenesis of septic shock in wild type mice</td>
<td></td>
<td>• NK cell-specific IFNγ depleted mice (Ncr1-Cre +/- IFNγ flox/flox mice) to determine the role of NK cell-specific IFNγ in the pathogenesis of sepsis</td>
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<td><strong>Chapter IV</strong></td>
<td><img src="image4.png" alt="Model Figure" /></td>
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<td>• Endogenous IL-15 does not play an acute role in the pathogenesis of septic shock</td>
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</tr>
<tr>
<td>• But endogenous IL-15 facilitates the pathogenesis of septic shock by maintaining NK cell integrity and functions</td>
<td><img src="image5.png" alt="Model Figure" /></td>
<td>• To identify whether IL-15 KO mice are immunosuppressive during sepsis</td>
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**Figure 1.** Summaries of thesis findings, schematic diagrams of the findings and future directions

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The application of IL-15 SA in human patients: safety evaluation

In Chapter II, I found the time- and dose-dependent adverse effects of IL-15 SA in mice. The toxicity profile includes hypothermia, weight loss, acute liver damage, splenomegaly and mortality, which is similar to the observations from a recent first-in-human trial of recombinant human IL-15. Conlon and colleagues reported grade 3 hypotension, thrombocytopenia, liver injury, fever and rigors in patients with metastatic malignancies by human recombinant IL-15 treatment (20). As shown in my study and that paper, IL-15 toxicity is transient as adverse effects disappear after stopping IL-15 treatment.

Although the toxicity of IL-15 has been reported in human patients, my studies still provide a few novel findings. First, the focus of my thesis is IL-15 SA, a more potent form of IL-15. The alternative treatment with IL-15 SA with lower doses may limit toxicity but retain sufficient efficacy of IL-15 for human patients. However, it is currently unknown whether IL-15 SA is toxic in vivo, which may slow down the clinical drug development of IL-15 SA. My thesis is the first to fully evaluate the adverse effects of IL-15 SA and provide mechanistic insights by using mice. Although immunological alterations are in large part different between mice and human, IL-15 SA may act like IL-15 which induces similar immunological responses in both human and mice. In addition, humanized mice could be used to shorten the gap between mice and human for evaluating the toxicity of IL-15 SA. Specifics will be described in my future directions.

Second, my studies performed a full analysis of NK and NKT subsets by IL-15 SA treatment, which was not examined in the first-in-human trial of human recombinant IL-15. I
found IL-15 SA increased the number of all NK and NKT subsets as IL-15 SA expands the total population of the two cell types. But IL-15 SA preferentially expands, mature CD11b^{high}CD27^{high} NK subset, which exhibits the enhanced ability to secrete pro-inflammatory cytokines such as IFN\(\gamma\) (273). IFN\(\gamma\) is known to induce inflammatory responses that cause damage to the host (160, 264, 274). Thus, it is possible that IL-15 exhibits a pro-inflammatory property by activating NK cells and prompting effector cytokine IFN\(\gamma\) production in toxic mice. Further mechanistic studies confirm this hypothesis. Moreover, my findings show that IL-15 SA appears to have a minimal effect on the proliferation of liver tissue-resident NK cells, but markedly expands conventional NK cells in both spleen and liver. Liver tissue-resident NK cells (NK1.1^{+}CD3^{-}DX5^{-}CD49a^{+}Trail^{+}) are a newly identified NK subset that possesses a distinct lineage from conventional, thymic and uterine tissue-resident NK cells and requires IL-15 for their development (239). My observation may expand the current knowledge about liver tissue-resident NK cells by showing these cells are less dependent on IL-15 for their proliferation compared to other NK cell subpopulations.

Additional studies evaluated the effect of IL-15 SA on the expansion of NKT subsets. Interestingly, IL-15 SA preferentially increases the percentage and total number of type II (CD3^{+}NK1.1^{+}α-Galcer-CD1d tetramer^{−}) NKT subsets in the spleen and liver, at a level greater than type I NKT subset. Compared to type I NKT cells that express invariant T cell receptor α chain, type II NKT cells express diverse non-V\(\alpha\)14 T cell receptors but are similarly restricted by CD1d (240). Type II NKT cells possess immune-regulatory phenotype and have been shown to suppress tumor immunosurveillance (275). Thus, it is possible that the expanded type II NKT cells regulate immune-toxicity but compromise anti-tumor efficacy of IL-15 SA. Taken together,
the comprehensive analysis of NK and NKT subsets may help us better interpret the toxic and therapeutic effects of IL-15 SA by pinpointing the associated cellular and molecular factors.

Third, I evaluated the dose response of IL-15 SA on lymphocyte number and activation, which is shown in Figure 9 at Chapter II. Compared to lower doses, IL-15 SA at 2 μg induced greater activation of NK cells. Over-activation of NK cells by 2 μg of IL-15 SA treatment contributes to the development of immuno-toxicity in treated mice. In contrast, 1 μg of IL-15 SA does not cause any toxicity and is able to promote the greatest expansion of NK and mCD8⁺ T cells among all doses, which I considered to be the optimal dose. Thus, my studies imply the toxic dose and the optimal dose of IL-15 SA are different. It is important to find such optimal dose of IL-15 SA for safety and maximal efficacy in human patients.

Finally, my findings are the first to provide a direct link between NK cell expansion and IFNγ production with the adverse responses caused by IL-15 SA in mice, which cannot be directly addressed in human patients. Therefore, my findings may provide rational approaches to prevent severe side effects of IL-15 SA by transiently neutralizing IFNγ or depleting NK cells.

**How do NK cells and IFNγ mediate the immune-toxicity of IL-15 SA?**

I first sought to examine concentrations of pro-inflammatory cytokines in the plasma of wild type mice following IL-15 SA treatment. However, I did not see a spike in IFNγ, IL-6 or TNF-α in the plasma of IL-15 SA-treated wild type mice at 24 hours after the last IL-15 SA injection. It is possible that I have missed the time point to look at the cytokine peak by IL-15 SA treatment. In addition to the potential systemic inflammation, I hypothesize that IL-15 SA also causes
inflammation-associated injury at the tissue level. In my studies from Chapter II, H&E staining of liver sections shows increased numbers of NK, NKT and CD8+ T lymphocytes in liver sinusoids, especially accumulating around the central vein in IL-15 SA-treated mice. It is possible that the hyperproliferation of IL-15-dependent lymphocytes in the liver will contribute to acute hepatocellular injury. Indeed, multiple lines of evidence show that NK, NKT and CD8+ T lymphocytes contribute to acute liver injury in different experimental models (276-278). Of note, my additional study shows that single depletion of NK or CD8+ T cells fails to attenuate the increase in ALT and AST levels in the serum of wild type mice treated with IL-15 SA. However, it is possible that NK, NKT and CD8+ T lymphocytes play a synergistic role in causing liver injury and combined depletion of all three of them is needed to abrogate IL-15 SA-induced hepatocellular pathology. Furthermore, treatment of mice with toxic doses of IL-15 SA causes a sepsis-like syndrome including hypothermia and weight loss, which could be attributed to dysregulated function of central nervous system. One possibility is that increased concentrations of pro-inflammatory cytokines like IL-1β, TNF-α, IFNγ and IL-6 in the circulation could induce hypothermia and cachexia in mice (279, 280). In addition, NK cells might infiltrate into the hypothalamus, causing neuroinflammation on site (281).

**The application of IL-15 SA to patients with sepsis: friend or foe?**

In Chapter III, I addressed the detrimental role of IL-15 SA in the pathogenesis of acute sepsis when administered to wild type mice at the onset of sepsis. In particular, IL-15 SA facilitates sepsis-induced hypothermia, systemic cytokine production, acute liver and kidney injury through activation of NK cells and promoting IFNγ production. However, IL-15 SA has
been previously shown to prevent sepsis-induced apoptosis of innate and adaptive immune cells and improved outcomes in experimental models of sepsis (205). Due to its ability to reverse immune cell dysfunctions, IL-15 SA is currently considered as a promising immunostimulatory agent to target the immunosuppressed state in patients with sepsis (32). Therefore, my results could convey important information that IL-15 SA should be used with caution as it advances through drug development. In addition, my findings suggest the importance of identifying the immunopathological stages of septic patients to be hyperinflammatory or immunosuppressive. Compared to IL-15 SA, IL-7 might be a better therapy as IL-7 has little effect on peripheral mature NK cells that are pathogenic and is able to promote T and B lymphocyte viability and functionality (282-284). Administration of IL-7 to wild type mice right after the onset of sepsis improves survival in intraabdominal sepsis (285). My lab is currently conducting a phase II-a clinical trial of IL-7 in septic patients at the intensive care units with the corporation with Dr. Hotchkiss’s lab at Washington University School of Medicine.

**NK cells: a new target for treatment of acute sepsis?**

My lab has consistently shown the pathogenic role of NK cells during experimental sepsis by using a wide array of experimental approaches. Deficiency of NK cells is achieved by using IL-15 KO mice, wild type mice treated with anti-asialoGM1, anti-NK1.1 or anti-IL-15 (212, 257, 260, 261). All these mice exhibit resistance to acute sepsis induced by CLP and/or LPS. In addition, treatment of wild type mice with anti-CXCR3 after the onset of intraabdominal sepsis blocks NK cells trafficking to the site of infection and confers protection with a survival advantage (210, 258). As shown in previous studies, selective NK cell depletion attenuates
systemic inflammation and improves survival in experimental models of sepsis induced by pneumococcal pneumonia (215), systemic *Escherichia coli* and *Streptococcus pyogenes* infection (216, 217), and polytrauma (218). Taken together, all these preclinical studies imply NK cells play a detrimental role during the early acute phase of sepsis. Modulation of NK cell viability and effector functions appears to be a promising immunotherapeutic approach for the treatment of septic patients during the hyper-inflammatory phase. However, this approach should be used with caution as the interruption of NK cell activity may exacerbate sepsis-associated immunosuppression and increase the risk of viral infections or reactivation.

**Future Directions**

**To identify molecular mechanisms by which NK cells mediate toxicity of IL-15 SA**

In Chapter II, I demonstrate that NK cells and IFNγ mediate IL-15 SA-induced immune-toxicity as NK cell-depleted or IFNγ-deficient mice exhibit resistance to IL-15 SA toxicity. However, IFNγ KO mice show IFNγ deficiency in all cell types. Little is known about the role of NK cell-specific IFNγ in the in vivo toxicity of IL-15 SA. Ncr1-Cre mice generated by Eva Eckelhart would be a valuable mouse model to study specific functions of mediators by NK cells (286). In this model, the Cre recombinase is expressed under the control of the Ncr1 (NKp46) promoter which dominantly exists in NK cells (286). Although not present yet, theoretically, background crossing of IFNγ flox/flox mice with Ncr1-Cre mice would generate NK cell-specific IFNγ depleted mice, which provide a valuable in vivo platform to evaluate the precise functions of IFNγ by NK cells in experimental studies. Using the same concept, Ncr1-Cre mice could be crossed with other
effector molecules flanked with loxP sites to determine the specific functions of effector molecules by NK cells.

**To identify mechanisms by which NK cells are detrimental during sepsis**

My thesis study demonstrates the critical role of NK cells in the pathogenesis of sepsis. However, the exact mechanisms by which NK cells facilitate the pathobiology of sepsis are currently unclear. Previous studies from my lab have shown that IFNγ, which is mainly produced by NK cells, exacerbates physiological abnormalities, systemic inflammation and mortality during CLP-induced sepsis (160). However, IFNγ concentration has been shown to be very low in the plasma of septic mice as compared to other pro-inflammatory cytokines like IL-6 and IL-1β. Moreover, mice with global depletion of IFNγ used by previous studies fail to examine the role of NK cell-specific IFNγ during sepsis (160).

Therefore, to identify what mediators are induced by NK cells during sepsis and to what extent, my lab colleagues performed a full analysis of gene expression profiles of purified mouse NK cells in response to LPS stimulation (Table 1). NK cells surprisingly produce the highest amount of CXCL10 (~ 74 fold higher) in the LPS-stimulated group compared to vehicle control. TNF-α, IFNγ, IL-15, and IL-1β are also increasingly produced by NK cells upon LPS, but at a level markedly lower than CXCL10. CXCL10 is mainly produced by monocytes, fibroblasts and endothelial cells (287). It is a chemokine that attracts NK cells, DCs and macrophages that express the chemokine receptor CXCR3 (287). It is unknown why activated NK cells express such high amount of CXCL10 at the transcription level by themselves. My lab colleagues show that CXCR3-deficient mice exhibit survival advantage during sepsis by blocking NK cell trafficking to
sites of infections. However, a more recent lab paper shows CXCL10 deficiency does not impair NK cell trafficking during sepsis but is able to attenuate sepsis-induced pathogenesis and mortality (211). CXCL10-deficient NK cells exhibit attenuated activation indicated by the decrease in CD69 expression during sepsis (211). Thus, all these findings suggest that CXCL10 not only attracts effector cells to sites of infection but also may regulate functions of NK cells in an autocrine pattern and contribute to the pathogenesis of sepsis. In future experiments, I would like to investigate the protein expression level of CXCL10 by NK cells and its role in regulating activation, phenotype and effector functions of NK and other cells that express the CXCL10 receptor.

<table>
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<tr>
<th>Gene Symbol</th>
<th>Fold changes in gene expression of LPS-stimulated murine NK cells/vehicle-treated murine NK cells</th>
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<tbody>
<tr>
<td>Ccl3</td>
<td>1.58</td>
</tr>
<tr>
<td>Ccl4</td>
<td>1.92</td>
</tr>
<tr>
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</tr>
<tr>
<td>Il1b</td>
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</tr>
<tr>
<td>Il2rg</td>
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</tr>
<tr>
<td>Ltb</td>
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<tr>
<td>Tnf</td>
<td>3.10</td>
</tr>
<tr>
<td>Xcl1</td>
<td>2.62</td>
</tr>
</tbody>
</table>

Table 1. Fold changes in gene expression of vehicle- and LPS-stimulated murine NK cells. C57BL/6J mice were injected with LPS (100 μg) and NK cells were isolated from isolated spleens using both negative and positive selection columns at 2 hours after LPS injection.
Total RNA was isolated from $2 \times 10^6$ NK cells and mRNA expression was assessed using a chemokine/cytokine Oligo GEArray (SABiosciences, Frederick, MD).

To identify additional mechanisms leading to the resistance of IL-15 KO mice to sepsis.

In Chapter IV, my studies show that the resistance of IL-15 KO mice to sepsis is partially attributable to lack of NK cells. Other cells or host-derived biochemical mediators might also be involved. Orinska et al previously showed genetic deletion of IL-15 results in improved survival during intraabdominal septic shock induced by cecal ligation and puncture (CLP) (202). They reported that mast cell-specific IL-15, which is retained inside the cell, acts to inhibit mast cell chymase activity. Deletion of IL-15 releases the inhibition of mast cell chymase activity, which enhances its ability to cleave neutrophil-attracting chemokines and augment bacterial clearance at the early phase of septic shock (at 3 hours after CLP). In addition, my studies show a dispensable role of CD8$^+$ T lymphocytes in the pathogenesis of sepsis modulated by IL-15, as preventing CD8$^+$ T lymphocyte regeneration does not attenuate septic lethality in IL-15 SA-treated IL-15 KO mice. However, I observed increased numbers of NK cells in IL-15 SA-treated IL-15 KO mice when CD8$^+$ T lymphocyte regeneration is prevented. This may explain why mice are not protected in this setting. In addition, previous studies from my lab reported a synergistic effect of NK and CD8$^+$ T cells in the pathobiology of CLP-elicited sepsis (212, 257, 260, 261). NK and CD8$^+$ T cells share cellular functions including direct cellular cytotoxicity and pro-inflammatory cytokine production (288). They can activate each other indirectly by enhancing the ability of macrophages to produce NK/CD8$^+$ T cells-activating cytokines during inflammation.
Thus, it is interesting to perform a cell depletion assay for both NK and CD8+ T cells in IL-15 SA-treated IL-15 KO mice and examine their response to sepsis.

IL-15 not only plays a unique role in the development and survival of NK, mCD8+ T lymphocytes and IELs but also exerts general anti-apoptotic effects on both the innate and adaptive immune systems (53, 73). IL-15 potently increases intracellular expression of anti-apoptotic proteins Bcl-2 and Bcl-xL, and decreases expression of pro-apoptotic Bim and PUMA (205). Shigeaki’s study reported attenuated sepsis-induced apoptosis of NK, CD8+, CD4+ T cells and DCs in wild type mice treated with IL-15 (205). Consistently, my preliminary data shows that IL-15 deficiency results in augmented apoptotic death of splenic NK, NKT, naïve and memory CD8+ and CD4+, and B cells by annexin V expression in response to LPS challenge (Figure 2A-F). Although I have not examined the effect of IL-15 deletion on the apoptosis of macrophages and dendritic cells, it is possible that the upregulated apoptotic death of cells in both innate and adaptive immune systems will attenuate systemic inflammatory responses and improves survival of IL-15-deficient mice during acute sepsis. To test this hypothesis, I will evaluate total numbers and apoptosis of innate and adaptive cells and evaluate their ability to secrete pro-inflammatory cytokines at the acute phase of sepsis.
Figure 2. The effect of IL-15 deficiency on lymphocyte apoptosis after LPS challenge. Wild type and IL-15 KO mice received intraperitoneal injections with vehicle or 150 μg LPS. Spleens were harvested at 24 hours after vehicle or LPS injection for measurement of Annexin\(^+\) 7-AAD\(^+\)/NK, B, naïve and memory phenotype CD8\(^+\) and CD4\(^+\) T lymphocytes. Annexin\(^+\)7-AAD\(^+\) cells represent early apoptotic cells and Annexin\(^+\)7-AAD\(^-\) cells represent late apoptotic or necrotic cells. * p < 0.05, *** p < 0.001, **** p < 0.0001, compared to vehicle wild type control. n=3-6 mice per group. Data are representative of two separate experiments.
To identify whether IL-15 KO mice are immunosuppressed during sepsis

My study in Chapter IV shows that the number of bacterial counts at the site of infection is actually lower in IL-15 KO mice than wild type control at 6 hours and has no difference between groups at 24 hours after CLP. Consistently with Orinska’s finding (202), it indicates that IL-15 KO mice, at least, display no impaired anti-microbial ability at the acute phase of sepsis. It would be interesting to have a dynamic measurement of bacterial loads in IL-15 KO mice over an extended period of time (maybe 20 days) to fully assess the ability of IL-15 KO mice to kill bacteria. In additional studies, I will determine whether IL-15-deficient mice are immunosuppressed and more likely to contract secondary infections as their immune system might be weakened by increased cell apoptosis during sepsis. I will use a two-hit model in which IL-15 KO mice will be subjected to a sublethal CLP procedure and then challenged with Pseudomonas aeruginosa infection to mimic secondary infections in the hospital. I will measure survival, bacterial counts and apoptosis of innate and adaptive immune cells in IL-15 KO and wild type mice.


Chapter VI

MATERIALS AND METHODS

Mice

Female and male, 8- to 12-week-old C57BL/6Tac and Rag2/IL-2rg double knockout mice (Rag2<sup>-/-</sup>γc<sup>-/-</sup>) were purchased from Taconic Farms (Hudson, NY). Female, 8- to 12-week-old homozygous CD8 null mice (B6. 129S2-Cd8<sup>α<sub>a</sub>tm1Mak</sup>/J, CD8KO), homozygous IFN-gamma null mice (B6.129S7-Ifng<sup>tm1Ts</sup>/J, IFNγKO), homozygous CD1d null mice (B6.129S6-Cd1d1/Cd1d2<sup>tm1Spb</sup>/J, CD1d KO), homozygous Perforin null mice (C57BL/6-Prf1<sup>tm1Sdz</sup>/J, PfKO), homozygous TNFα null mice (B6.129S6-Tnf<sup>tm1Gkl</sup>/J) and wild type C57BL/6J (WT) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Wild type C57BL/6J mice served as controls in experiments using the knockout strains. Breeding pairs of homozygous IL-15 null mice (C57BL/6NTac-IL15<sup>tm1Imx</sup>N5, IL-15KO) were purchased from Taconic and genotype of offspring was verified by PCR analysis performed by Transnetyx. Age and gender matched IL-15 knockout and C57BL/6Tac control mice were used in experiments. NK and NKT cells were depleted in mice by intraperitoneal injection with anti-asialoGM1 IgG (50 μg/mouse, Cedarlane Laboratories, Hornby, Ontario, Canada) or anti-NK1.1 IgG (clone PK136, 50 μg/mouse; eBioscience, San Diego, CA) at 24 hours prior to initiation of IL-15SA treatment. CD8 T cells were depleted by treatment with anti-CD8α IgG (clone 53-6.7, 50 μg/mouse, eBioscience) at 24 hours prior to IL-15 SA treatment. Isotype-specific or non-specific IgG served as control in all antibody-induced leukocyte depletion experiments. All studies were approved by the Institutional Animal Care
and Use Committee at Vanderbilt University and complied with the National Institutes of Health Guide for the Care and Use of Experimental Animals.

**IL-15 Superagonist (IL-15 SA) Preparation**

Recombinant mouse IL-15 was purchased from eBioscience (San Diego, CA, Cat. no. 34-8151-85). Mouse IL-15 Rα subunit Fc chimera (IL-15 Ra) was purchased from R&D Systems (Minneapolis, MN, Cat. no. 551-MR-100). For preparation of IL-15 SA, 20 µg IL-15 and 90 µg IL-15Ra were incubated in 400 µl of sterile phosphate buffered saline (PBS) at 37 °C for 20 min to form the IL-15/IL-15 Ra complex. The complex was then diluted with sterile PBS to reach a concentration of 2 µg IL-15/ml, then aliquoted and frozen. The IL-15 SA doses reported in the study are based on the amount of IL-15 present in the IL-15 SA complex.

**IL-15 SA Treatment Protocols**

In Chapter II, in dose finding studies, mice received intraperitoneal injections of IL-15 SA at doses of 0, 0.5, 1 or 2 µg for 4 days (day 0-day 3). Rectal temperature and body weight were measured at 24 hours after the fourth injection. In time course studies, mice were treated with 2 µg of IL-15 SA for 4 days. Rectal temperature and body weight were measured daily throughout the treatment period. On day 4, spleen and liver were harvested for measurement of leukocyte numbers and activation.

In Chapter III, wild type mice received intraperitoneal injections with a high- dose (2 µg) of IL-15 SA at 30 minutes prior to and 24 hours after challenge with CLP or 100 µg of LPS. Lymphocyte characterization was measured at 0 and 1 hour after LPS challenge. Survival study on wild type mice treated with IL-15 SA but not LPS was also assessed. In a separate set of experiments, wild
type mice were treated with 0.1 μg of IL-15 SA for 4 days and were subject to CLP or LPS challenge at 24 hours after the last IL-15 SA injection.

In Chapter IV, to regenerate NK and mCD8^+ T cells, IL-15 KO mice received intraperitoneal injections of 0.125 μg IL-15 SA in 0.2 ml PBS for 4 days (day 0-3). IL-15 KO mice that were treated with vehicle served as control. On day 4, spleens and livers were harvested for measurement of leukocyte numbers and activation. In additional experiments, after the same treatment regimen of IL-15 SA, IL-15 KO mice were subjected to CLP or LPS (150 μg) on day 4. In survival studies, IL-15 SA (0.125 μg) injection was continued daily to maintain the regenerated NK and mCD8^+ T cell population.

**M96 treatment protocol**

In Chapter IV, IL-15 neutralizing antibody M96 was generously provided by Amgen (Thousand Oaks, CA). Wild type mice received intraperitoneal injection with 20 μg M96 either 2 hours or 4 days prior to CLP or LPS challenge. A survival study was followed for 7 days after the initiation of septic shock. When M96 was given 4 days prior to CLP or LPS, a second administration of M96 at the same dose was given at the time of septic insults to maintain the effect of M96 on depletion of NK cells.

**Cecal ligation and puncture model**

In Chapter III and IV, I performed cecal ligation and puncture procedure on mice. The protocol was described previously (257). In brief, mice were anesthetized with 2% isoflurane in oxygen. A 1- to 2-cm midline incision was made through the abdominal wall. The cecum was identified and ligated 1.0 cm from the tip using a 3-0 silk tie. A double puncture of the ligated cecum was
performed using a 20-gauge needle. The incision was closed using autoclips. Buprenorphine (0.1 mg/kg) was administered subcutaneously for analgesia 30 minutes before CLP and twice daily thereafter. All mice received fluid resuscitation (Lactated Ringers solution, 1 ml, intraperitoneal) immediately after injury and twice daily thereafter to mimic clinical management of sepsis.

**Endotoxin shock model**

In Chapter III and IV, ultrapure LPS-EB (from E. coli 0111:B4) from InvivoGen (San Diego, CA) was administered at a dose of 100 or 150 μg/mouse via intraperitoneal injection. Measurements of rectal temperature, pro-inflammatory cytokine concentrations and indices of acute organ injury were performed at 6 and/or 24 hours after LPS challenge. Survival study was also followed for 7 days after LPS challenge.

**Cytokine measurement**

In Chapter III and IV, concentrations of IL-6, IFN-γ, IL-12p70, IL-18, TNF-α, IL-1β and IL-10 in plasma were measured using a Bio-Plex Multiplex Assay through Magpix Multiplex Reader (Bio-Rad, Hercules, CA). Results were analyzed with Bio-Plex Manager Software 6.1. The concentration of soluble IL-15/IL-15Rα complex in plasma was measured using Mouse IL-15/IL-15R Complex ELISA Ready-Set-Go (eBioscience, San Diego, CA).

**ALT, AST, BUN and creatinine measurement**

Blood was obtained in heparinized syringes after carotid artery laceration in anesthetized mice breathing 2% isoflurane in oxygen by nose cone. Plasma was obtained from heparinized whole blood after centrifugation (2000g x 10 minutes). Plasma alanine aminotransferase (ALT) and
aspartate aminotransferase (AST) concentrations were measured as indices of acute liver injury. Blood urea nitrogen (BUN) and creatinine concentrations were measured as indices of renal injury. ALT, AST, BUN, and creatinine concentrations were measured in the Translational Pathology Core Laboratory at Vanderbilt University using an ACE Alera Chemistry Analyzer (Alfa Wassermann, Inc. West Caldwell, NJ).

**Microbiology**

In Chapter IV, bacterial counts were performed on aseptically harvested blood and peritoneal fluid. Blood was harvested via carotid laceration. Peritoneal lavage fluid was obtained by injection and aspiration of 2 ml sterile PBS into the peritoneal cavity. Samples were serially diluted in sterile PBS and cultured on tryptic soy agar plates. Plates were incubated at 37°C for 24 hours and bacterial colonies were counted.

**Flow Cytometry**

Splenocytes and hepatic leukocytes were isolated as previously described. Briefly, spleens were harvested, placed in 35 mm dishes containing RPMI-1640 media with 10% fetal bovine serum (FBS), and homogenized by smashing with the plunger from a 10-ml syringe. The homogenate was passed through a 70-μm cell strainer and erythrocytes were lysed with Red Blood Cell Lysis Buffer (Sigma Life Sciences, St Louis, MO). The remaining cells were counted using TC20™ Automated Cell Counter (Bio-Rad, Hercules, CA), centrifuged (300g x 5 minutes) and the cell pellet was resuspended in PBS. Livers were harvested after perfusion, which was achieved by cutting of the hepatic portal vein, insertion of a 25 G needle into the left ventricle of the heart and perfusion with 10 ml PBS. Harvested livers were smashed with the plunger from a 10-ml syringe and passed through a 70-μm cell strainer. The hepatic homogenate was washed,
resuspended with 10 ml of 37.5% Percoll Plus (GE Healthcare Life Sciences) and centrifuged (680g x 12 minutes at room temperature), the supernatant containing hepatocytes was discarded, erythrocytes were lysed, and the resulting mononuclear cells were counted using TC20™ Automated Cell Counter (Bio-Rad, Hercules, CA).

For surface marker staining, in Chapter II, III and IV, cells were suspended in PBS (1 x 10⁷ cells/ml) and incubated with anti-mouse CD16/32 (eBioscience, 1 μl/ml) for 5 minutes to block nonspecific Fc receptor–mediated antibody binding. One million cells were then transferred into polystyrene tubes. Fluorochrome-conjugated antibodies or isotype controls (0.5 μg/tube) were added, incubated (4°C) for 30 min, and washed with 2 ml of cold PBS. After centrifugation (300g x 5 minutes), cell pellets were fixed with 250 μl of 1% paraformaldehyde. Antibodies used for surface marker labeling included CD3-Alex Fluor 488, NK1.1-PE–Cy7, CD8-FITC, CD4-FITC, CD44-PE–Cy5, CD19–PE, CD69–PE, NKG2D–PE, NKp46–PE, CD11b–PE and CD27–APC (eBioscience, San Diego, BD Biosciences, San Diego). CD1d/α–Galcer tetramers were provided by the NIH Tetramer Core Facility (Emory University, Atlanta, GA). Appropriate isotype-specific antibodies were used as controls.

For intracellular staining, in Chapter II, spleens were harvested at 4 hours after the last injection of IL-15 SA. In Chapter IV, 1 x 10⁶ splenocytes were incubated with 4 ul PMA and ionomycin (cell stimulation cocktail, eBioScience, San Diego) in 1 ml of RPMI-1640 media with 10% FBS at 37°C for 5 hours. After one hour, the protein transport inhibitors brefeldin A and monensin (2 ul/ml, eBioScience, San Diego) were added into cell cultures for the rest of 4 hours. After incubation, cell suspensions were labeled with fluorochrome-conjugated antibodies to surface markers as described above. Cells were then fixed and permeabilized with Cytofix/Cytoperm Plus (BD
Biosciences, 250 μl /tube) for 20 minutes at 4°C. After washing with BD Perm/Wash solution, anti-IFNγ-PE (clone XMG1.2, eBioscience) was used to detect intracellular IFNγ production. Fluorochrome-conjugated isotype-specific IgGs served as controls. All samples were analyzed using an Accuri C6 flow cytometer (BD Biosciences, San Diego, CA). Data were analyzed using Accuri C6 software.

**Immunohistochemistry**

In Chapter II, immunohistochemical staining of NK, NKT and T lymphocytes as well as macrophages in the marginal zone of the spleen was performed on 5 μM cryosections of tissue. Primary antibodies included polyclonal goat anti-mouse NKp46 (Cat. no. AF2225, R&D Systems, Minneapolis, MN), biotinylated rat anti-metallophilic macrophage antibody (Clone: MOMA-1, Cat. no. CL89149B Cedarlane Laboratories, Hornby, Ontario, Canada) and biotinylated hamster anti-TCRβ antibody (Clone: H57-597, Cat. no. 553169, BD Biosciences, San Diego). Isotype IgG antibodies or donkey serum was used as controls to stain serial frozen sections. For primary biotinylated antibody staining, avidin-biotin complex alkaline phosphatase (ABC-AP Complex, Cat. no. AK-5000, Vector labs, Burlingame, CA) was added for 1 hour and then developed with Vector Red Kit containing Alkaline Phosphatase Substrate (Cat. no. SK-5100, Vector labs, Burlingame, CA). For goat anti-mouse NKp46 staining, the secondary Donkey anti-goat-biotin was added, followed by incubation with avidin-biotin complex peroxidase (ABC-Standard Complex, Cat. no. PK-6100, Vector labs, Burlingame, CA) and development with DAB-Solution (SK-4100, Vector lab, Burlingame, CA). Sections were counterstained in Meyer’s hematoxylin and slides were mounted with Permount.
Adoptive cell transfer

In Chapter II, splenic leukocytes were harvested as described previously. Splenic NK cells were isolated using mouse NK isolation kit II (Miltenyi Biotec). Briefly, splenocytes were incubated with a biotin-antibody cocktail that binds macrophages/monocytes, dendritic cells, erythrocytes, B cells, and CD3$^+$ T cells. Anti-biotin magnetic microbeads were added to bind antibody-bound cells. The suspension was passed through columns in a magnetic field which bind iron microbead-antibody-laden cells and prevent their passage. NK cells, which do not bind the specified antibodies, were washed through columns and collected. The enriched cell preparation was resuspended in sterile PBS followed by retro-orbital injection (1.0 × 10$^6$ cell/mouse) into Rag2$^{-/-}$γc$^{-/-}$ mice.

Statistics

All data were analyzed using GraphPad Prism software. All values are presented as the mean ± SEM, except for body temperature, ALT and AST, for which median values are designated. A Student’s $t$ test was used to examine the difference between paired vehicle and IL-15 SA groups. Data from multiple group experiments were analyzed using one-way ANOVA followed by a post hoc Tukey’s test to compare groups. Survival data were analyzed using the log rank test. A value of $p < 0.05$ was considered statistically significant for all experiments.
REFERENCES


77. Gil M, Park SJ, Chung YS, Park CS. Interleukin-15 enhances proliferation and chemokine secretion of human follicular dendritic cells. *Immunology* 2010; 130: 536-544.


146. AC C. De medicina. Self published A.D. 25.
164. Granger DN, Sencenkovka E. Inflammation and the Microcirculation. San Rafael (CA); 2010.


244. PD-1 Inhibitor Approved for Melanoma. *Cancer Discov* 2014; 4: 1249.


260. Sherwood ER, Enoh VT, Murphey ED, Lin CY. Mice depleted of CD8+ T and NK cells are resistant to injury caused by cecal ligation and puncture. *Laboratory investigation; a journal of technical methods and pathology* 2004; 84: 1655-1665.


284. Corfe SA, Paige CJ. The many roles of IL-7 in B cell development; mediator of survival, proliferation and differentiation. *Semin Immunol* 2012; 24: 198-208.


