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# Determining The Mechanisms Generating Soluble Il-15 Complexes

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# DETERMINING THE MECHANISMS GENERATING SOLUBLE IL-15 COMPLEXES

A

DISSERTATION

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by

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Dedication

To my incredible wife Claudia,

you are amazing and I am so lucky to have you in my life.

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## Abstract

### Determining the Mechanisms Generating Soluble IL-15 Complexes

A diverse assortment of infectious pathogens and TLR agonists enhance the expression of Interleukin (IL)-15. Additionally, inducing lymphopenia enhances anti-tumor responses in an IL-15-dependent manner. Paradoxically, despite the limited expression of IL-15 during homeostasis, the role of IL-15 during the steady state is well-known, while its roles during inflammation and infections remain largely undefined. IL-15 uses a unique method of production and presentation to support the development and homeostasis of NK and CD8 T cells. IL-15 is produced with its high affinity IL-15Rα and this IL-15Rα/IL-15 complex is shuttled to the cell surface where it is presented in-trans or cleaved into soluble cytokine/receptor complexes. Unfortunately, little is known about the mechanisms generating soluble IL-15 complexes. I set out to elucidate the mechanisms responsible for inducing sIL-15 complexes to test my hypothesis that Type I IFN signaling and ADAM17 are necessary for generating sIL-15 complexes. In Bone marrow dendritic cells, Type I Interferon (IFN) directly regulates the cleavage of sIL-15 complexes using the metalloprotease ADAM17. Mice with the conditional deletion of ADAM17 or a deficiency in IFN signaling led to the surprising discovery that neither Type I IFN signaling nor ADAM17 expression are required *in vivo* for inducing sIL-15 complexes. Interestingly, VSV infection enhances sIL-15 complexes in the absence of both IFN and CD40 signaling pathways, indicating multiple redundant mechanisms generate sIL-15 complexes. I discovered a shared mechanism of enhanced IL-15 transpresentation common to all types of lymphopenia, while only forms of lymphopenia associated with inflammation display increases in sIL-15 complexes. The optimal production of sIL-15 complexes in response to total-body irradiation (TBI) required the activation of both the IFN and STING pathways, indicating a dominant role for inflammatory cell death. Utilizing an adoptive transfer model, I identified a novel role for lymphopenia-induced inflammatory IL-15 on the proliferation of CD8 memory T cells. Overall, I've demonstrated a diverse assortment of stimuli utilize a variety of pathways and cell types to induce the generation

of sIL-15 complexes. These findings lead me to conclude that the increase in sIL-15 complexes is a common event during conditions involving inflammation and immune activation likely contributing to memory CD8 T cell responses.

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**List of abbreviations**

| | |
|---|---|
| PRR | Pattern Recognition Receptor |
| PAMP | Pathogen Associated Molecular Patterns, ligands for PRRs |
| DAMP | Damage-Associated Molecular Patterns |
| TLR | Toll-like receptor; specific family of pattern recognition receptors |
| ADAM | A Disintegrin And Metalloprotease, cell-surface bound protease |
| APC | Professional Antigen Presenting Cell |
| TCR | T cell receptor |
| MHC | major histocompatibility complex |
| DC | dendritic cell |
| TLR | Toll-like receptor |
| γC | common γ chain, CD132 |
| IFN | interferon |
| IFNAR | Type I Interferon Receptor |
| Tcm | central memory |
| Tem | effector memory |
| Trm | resident memory |
| Tg | transgenic |
| VSV | vesicular stomatitis virus |
| SD | standard deviation |
| SEM | standard error of the mean |
| Wt | wild type |
| LN | lymph node |
| mLN | mesenteric lymph node |
| CFSE | carboxyfluorescein succinimidyl ester |
| Ab | antibody |

| | |
|---|---|
| IL-15 complex | IL-15Rα/IL-15 receptor-cytokine complex |
| s | Soluble form |
| DC | Dendritic Cell |
| GMCSF | Granulocyte macrophage colony-stimulating factor (GM-CSF) |
| BMDC | GMCSF-cultured bone marrow dendritic cells |
| Flt3-DC | Flt3L-cultured bone marrow dendritic cells |
| IP | intra-peritoneal |
| IV | intra-venous |
| Poly I:C | poly inosinic:polycytidylic acid; TLR3 ligand |
| LPS | Lipopolysaccharide; TLR4 ligand |
| CpG | CpG oligodeoxynucleotides; TLR9 ligand |
| RAG | recombination activating gene |
| RAD | measure of absorbed radiation |
| CTX | Cyclophosphamide chemotherapy |
| TBI | Total Body Irradiation |
| r | recombinant |
| ACT | Adoptive T Cell Therapy; form of cancer therapy |
| STING | Stimulator of Interferon genes |
| PFU | Plaque forming units; unit quantifying number of infectious virions |
| c-di-GMP | Cyclic diguanylate monophosphate; STING ligand |
| $ER^{T2}$-Cre | Estrogen-Receptor Cre; tamoxifen inducible Cre system |
| BATF3 | Basic Leucine Zipper Transcriptional Factor ATF-like 3 |
| Tmem173 | STING |
| pORF | Empty vector control plasmid |

| | |
|---|---|
| pORF-IFNα | IFNα-encoding plasmid |
| hrs | Hours |
| fl | Allele is flanked by Flox recognition signal sequence for Cre |
| PMA | Phorbol 12-myristate 13-acetate; activator of Protein Kinase C |
| Cre+ | Expression of a single copy of the Cre Tg |
| Cre+/+ | Expression of two copies of the Cre Tg |
| LysM | Lysozyme M; expressed by monocytes and macrophages |
| CD11c | Intergin Subunit; expressed by DCs |
| Em-GFP | Emerald Green fluorescent protein; surrogate for IL-15 expression |
| Pmel-1 | Tg CD8 T cells; specific for Gp100 (25-33) in the context of H2-$D^b$ |
| NFκB | Nuclear factor kappa-B |

## CHAPTER 1: Background

### 1.1 Innate and Adaptive Immune Responses

The immune system is a sophisticated and diverse network of cells whose primary responsibility is to facilitate the elimination of pathogens while maintaining tolerance to self. Active infections with a bacterial, viral or protozoan pathogen induces the activation of innate immune cells termed professional antigen presenting cells (APCs); these include monocytes, macrophages and dendritic cells (DCs). These APCs are characterized by their inherent ability to process and present antigens to T cells. APCs exhibit high expression of a diverse assortment of specialized sensors whose function is to sense pathogen associated molecular patterns (PAMPs). These PAMPs are recognized by receptors that consist of multiple types of germline encoded sensors; together they are termed Pattern Recognition Receptors (PRRs) (1). The primary function of PRRs is to induce an innate immune response, which is by definition, relatively short-lived and non-specific. These PRRs are expressed in several cellular membranes, including intracellular endosomes and cytosolic compartments thereby allowing detection of PAMPs at their various locations of expression (1).

When the innate immune system alone does not eliminate the pathogen, the persistence of infectious insults induces APCs to also serve as a bridge for the initiation of an adaptive immune response, involving the activation of T and B lymphocytes. This can be ascribed to the APCs ability to sense the environment for molecular perturbations, and migrate to a local draining Lymph Node (LN) upon activation. APCs then process and present antigens in the form of small strings of amino acids called peptides to responding T cells in the context of Major Histocompatibility Complexes (MHC). These peptide-MHC-complexes are recognized by a CD4 or CD8 T cell expressing a unique T cell receptor (TCR) which identifies a specific pattern of peptides in the context of MHC class II or I respectively. The presentation of intracellular proteins

is normally accomplished by MHC I, which is expressed by all nucleated cells, while MHC II is only expressed on APCs and serves to present extracellular antigens from the surrounding environment. The recognition of a TCR's cognate antigen in the context of the correct MHC is termed signal 1 and does not alone induce activation of the T cell (2). Instead, the addition of a co-stimulatory signal (signal 2) is required for optimal T cell activation (3). These co-stimulatory molecules are up-regulated on APCs in response to PRR activation and serve to ensure the full activation of a T cell only occurs in the presence of an inflammatory state (1).

Although PAMPs induce the activation of PRRs, they are not the only molecules capable of inducing immune activation. Early evidence indicated that inflammation can be induced in the absence of infection, termed sterile inflammation. In response to these observations Dr. Matzinger developed the danger theory that postulated that the immune system should also be endowed with the ability to sense specific aspects attributed to an infection without the need to directly recognize PAMPs (4). These theories have emerged into a large and ever-growing number of relatively inert structures normally sequestered in specific intracellular compartments that are capable of inducing an immune response when located outside of the cell (5). These endogenous ligands capable of activating PRRs, termed damage-associated molecular patterns (DAMPs) occur during pathogen infections and during other events in which massive numbers of cells are simultaneously undergoing cell death (5). These events are especially important in the induction of natural innate and adaptive immune responses against various types of cancer (6-8). As the cancer cells themselves almost completely consist of self-antigens in the absence of PAMPs, the conundrum of naturally-occurring immune responses against tumor antigens has only been recently explained by the stimulation of DAMPs in concert with the presence of mutated tumor antigens. Although they do play significant roles during infections,DAMPs are fully capable of inducing an immune response in the absence of infection (9,10) as these DAMP-sensing

pathways have been recently discovered to be an integral component of anti-tumor immune responses.

One globally-induced effector molecule produced in response to PRR stimulation is the Type I interferon (IFN) family of proteins. This family consists of 14 IFNα subtypes and a single IFNβ protein. While nearly all cells are capable of producing IFNβ, IFNα is produced primarily by hematopoietic cells, specifically the plasmacytoid DC subset, which is responsible for the majority of IFNα produced *in vivo* (11). The induction of IFN protein expression requires an early burst of production, which acts in autocrine manner leading to a more significant and prolonged production of Type I IFN (9). These soluble proteins are secreted and act in an autocrine or paracrine fashion to induce global changes in cellular function, including a halt in transcription and protein synthesis of many cells while simultaneously activating immune cells. The interferon alpha receptor (IFNAR) 1 and 2 are ubiquitously expressed on the surface of all nucleated cells and both serve as shared receptors for the signaling of IFNα and IFNβ proteins. The importance of Type I IFN signaling is readily apparent as many pathogen infections cleared in a normal host result in fatalities in hosts deficient in Type I IFN signaling (12). In addition, many pathogens contain proteins that function to halt the production or signaling of Type I IFNs. In APCs, Type I IFNs act to enhance the expression of MHC molecules and upregulate the cell surface expression of T cell co-stimulatory molecules (9). Type I IFN signaling in T cells directly augments CD8 T cell proliferation and effector functions. In addition to being a critical anti-pathogen factor, Type I IFNs are a critical factor in naturally-occurring and inducible anti-tumor immune responses (6-8).

One key newly-described PRR pathway involves the Stimulator of Interferon genes (STING) (13,14). This protein serves as an important recognition and adaptor of bacterial and viral DNAs leading to the induction of Type I IFNs (13,14). In addition to its roles in pathogen-

specific immunity, several recent publications have discovered a critical role for STING activation in naturally occurring and induced adaptive anti-tumor immune responses (6,7). These responses involved the specific sensing of tumor-DNA by tumor-infiltrating DCs (6,7). The exact mechanism of how the tumor-DNA makes its way to intracellular compartments in DCs in currently unknown, although clearly this event is occurring *in vivo*. Recent data indicate that tumor-derived products are responsible for endogenous and lymphopenia-augmented anti-tumor responses. This requires the activation of DCs in a non-TLR manner, which was subsequently determined to rely on the activation of the ER membrane bound protein STING and Type I IFN signaling specifically on the CD8+ DCs (6,7). As CD8+ DCs are not dominant producers of Type I IFN and do not express STING, these studies implicate an additional subset as the Type I IFN producer (8,13). As Type I IFN signaling directly induces IL-15 expression, and a STING-Type I IFN dependent pathway is required for the induction of anti-tumor responses, the role of the STING pathway in regulating IL-15 was not clear. **In Chapter 4, I demonstrate that a STING agonist directly induces sIL-15 complexes. Additionally, the optimal generation of sIL-15 complexes in response to TBI requires the activation of the STING and Type I IFN signaling pathways.**

During an adaptive immune response, properly stimulated antigen-specific CD8 T cells undergo massive proliferation in a short period of time. In a normal acute immune response, the massive influx of adaptive immune cells leads to elimination of the pathogen, thereby also eliminating the underlying PRR signaling, inflammation, and the loss of the antigen for which the CD8 T cell recognizes. This clearance of antigen results in the death of the majority of these effector CD8 T cells; however, a small portion of these T cells survive and become long-lived memory CD8 T cells. CD8 T cell memory is characterized by the ability to respond much more quickly upon activation than a naïve T cells while also maintaining the ability to persist in the absence of self-MHC-I or cognate antigen (15). This persistence is instead dependent upon a low level of proliferation termed homeostatic proliferation mediated by the cytokine IL-15 and an

enhanced ability to survive in response to IL-7 and IL-15 (15-17). Loss of either of these cytokines leads to a significant erosion of the CD8 T cell memory population, while loss of both results in a profound loss and inability to generate CD8 memory T cells (18,19).

Memory CD8 T cells can be subcategorized by the differential expression of several surface markers, leading to these cells undergoing preferential migration to different locations. To date these include effector memory (Tem), central memory (Tcm) and resident memory (Trm) CD8 T cells (20,21). Tcm cells have high expression of the selectin CD62L that enables them to migrate into lymphoid tissues through high endothelial venules and thus are found circulating and in lymphoid tissues, Tem cells have low expression of CD62L and instead reside primarily in non-lymphoid tissues, but are also found in circulation (19). Trm cells are characterized by the inability to migrate through the circulation and have high expression of the cell surface markers CD69 and CD103, which impart these cell's the ability to be retained in mucosal and peripheral tissues (21,22). The generation or maintenance of all of these subtypes is at least partially dependent upon the expression of IL-15 , although the required cell types presenting IL-15 were determined to differ between the Tem and Tcm subsets, while the IL-15 presenting cell types required for Trm are currently unknown (23,24).

**1.2 IL-15 discovery and methods of presentation**

In response to TCR signaling and co-stimulation, T cells themselves produce a cytokine which promotes their own proliferation. This cytokine, named IL-2 was determined to selectively induce the growth and survival of T cells (25). Upon activation, T cells quickly express both IL-2 mRNA and protein, indicating that the regulation of IL-2 occurs predominantly at the level of transcription (3). IL-2 was determined to signal through a trimeric receptor on the cell surface of activated T cells, consisting of the common gamma chain gp130 ($\gamma_C$), an IL-2Rβ chain (IL-2/15Rβ)

and the IL-2Rα (26). Later, an additional cytokine, first named IL-T which also exhibited a strong capacity to induce the proliferation of T cells, reminiscent of IL-2 was discovered in HTLV-infected lymphocytes (27,28). Later, IL-T was renamed IL-15. Early studies characterizing the promoter region of IL-15 found it contains functional binding domains for NFκB, IFN regulatory elements, AP-1, IFNγ activation site, and a binding site for Interferon Regulatory Factors (29). A disconnect was observed between the mRNA and protein expression of IL-15 as mRNA was abundantly expressed in nearly all cells, yet the expression of the IL-15 protein was lacking in most tissues and cell types. Later studies provided insight into this divide as it was determined that IL-15 translation is heavily regulated in the form of multiple start cites and an unusually long signal peptide, indicating that unlike IL-2, the majority of the regulation in IL-15 protein expression is post-transcriptional (30,31). No defects were found in the stability of IL-15 mRNA or protein, although an early study noticed that the IL-15 protein intracellular did traffic through cells at a much slower rate than IL-2 (32). To determine the role of the IL-15 regulatory elements, chimeric constructs were generated, the upstream and promoter regions of IL-2 were used to drive IL-15 expression and this increased IL-15 protein expression by nearly 20 fold (32). In addition, altering the 3' end coding sequence also increased the production of IL-15 protein, with the combination of both chimeric constructs resulting in a 250 fold enhancement of IL-15 protein expression (32). Conversely, when the IL-2 upstream region was switched for that of IL-15, the production of IL-2 protein was strongly inhibited (32). These data indicated the presence of strong negative regulatory regions in the upstream, promoter and 3' terminal regions of the IL-15 gene, assumingly to strictly limit the expression of IL-15 at the protein level.

IL-2 and IL-15 were determined to share multiple receptor subunits for signaling, including the $\gamma_C$ and the IL-2/15Rβ, however blocking antibodies to the high affinity IL-2Rα chain had no effect on the ability for IL-15 to induce proliferation in a responding human T cell line (27,28,33).

A follow-up study identified a specific high affinity IL-15Rα chain that was enriched on a highly IL-15-responsive immortalized murine T cell line (34). This IL-15Rα chain exhibited a high affinity for IL-15 alone (kD~1x10$^{-11}$M) equivalent to that of the intact combined IL-2 receptor complex (34). The affinity of IL-15Rα for IL-15 was not further enhanced by the addition of the common gamma and IL-2Rβ chains (34). As both the IL-15Rα and IL-2Rα lack intracellular signaling pathways in responding T cells, IL-2 and IL-15 essentially utilize a single identical signaling pathway through the $\gamma_C$ and the IL-2/15Rβ (35). Further studies examining the signaling pathways for IL-2 and IL-15 confirmed that these pathways are nearly identical, but striking differences were observed between mice lacking either cytokine. IL-2 mice had pronounced global inflammation (36), while mice deficient in either IL-15 or IL-15Rα were healthy, but had a dramatic reduction in several lymphocyte subsets (37,38). These differential effects are partially due to the lack of inhibitory regulatory T cells in the absence of IL-2. These effects are also due to the combination of differential cell types producing these cytokines, the duration and strength of the respective interactions and due to the unique method of delivery utilized by IL-15.

Although T cells express abundant levels of IL-15Rα, surprisingly lymphocytes lacking IL-15Rα expression responded normally to recombinant (r) IL-15 (39). To add further confusion, the adoptive transfer of these IL-15Rα-/- T cells into a Wild type (Wt) IL-15Rα sufficient mouse completely rescued their survival (39,40). This conundrum was solved when it was found that the IL-15 and IL-15Rα proteins were produced by the same cell, termed a cytokine presenting cell, and shuttled to the cell surface as a receptor-protein complex (41,42). This novel process was termed transpresentation and required the presentation of cell-surface retained IL-15 complexes to a responding lymphocyte expressing the IL-2/15Rβ and $\gamma_C$ via a cell contact dependent mechanism (40-42). In addition to transpresentation, a second proposed form of IL-15 delivery predicts the cell surface bound IL-15 complexes can signal in Cis to IL-2/15Rβ and $\gamma_C$ complexes;

however, whether this normally occurs *in vivo* is uncertain. Lastly, membrane-bound IL-15Rα/IL-15 complexes are capable of undergoing proteolytic cleavage into a soluble form (sIL-15 complexes). These sIL-15 complexes are believed to act on responding cells in a paracrine manner (43). These endogenously-produced sIL-15 complexes are induced by PRR activation by Toll-Like Receptor (TLR) agonists, although to relatively low levels compared with other soluble cytokines (43). Importantly, recombinant forms of sIL-15 complexes have demonstrated 50-100 fold greater potency than recombinant IL-15 alone in inducing proliferation in responding CD8 T cells (44-46). To date the role of endogenous generated sIL-15 complexes in mediating IL-15 dependent responses is currently unknown, this is at least partially due the lack of a deep understanding in the mechanisms of sIL-15 complex generation.

The proteolytic cleavage of cell surface substrates is performed by an abundant number of natural proteases. One protease known to cleave an abundant number of proteins is A Disintegrin and Metalloprotease 17 (ADAM17). ADAM17 was initially discovered to be responsible for the cleavage of the membrane bound cytokine Tumor Necrosis Factor α to a soluble form (47). Later studies identified that ADAM17 has a large number of wide ranging substrates, including transforming growth factor-alpha (TGF-α), CD62L and multiple others (48). The ADAM17 protein is expressed nearly ubiquitously in all types of tissue and its expression is required for many normal cellular processes including functions in development, trafficking, and signaling. Since ADAM17 is responsible for so many cellular functions, it is not surprising that a complete lack of ADAM17 resulted in embryonic lethality (47,49). The expression of ADAM17 is predominantly regulated post-transcriptionally, where a pro-form of the protease is cleaved by furin to an enzymatically active protein (49,50). ADAM17 is subsequently shuttled to the cell surface by transporter proteins named iRhoms, of which iRhom2 has been found to be absolutely required for the transport of ADAM17 to the cell surface in hematopoietic cells (51).

ADAM17 is known to cleave its vast number of substrates at the cell surface from membrane-bound into soluble forms (47,49). Cell culture supernatants from epithelial cells co-transfected with IL-15Rα and ADAM17 resulted in the generation of sIL-15Rα and this generation was abrogated with the addition of a pan-metalloprotease inhibitor (52). An additional analysis determined that Granulocyte macrophage colony-stimulating factor (GM-CSF)-induced bone-marrow dendritic cells (BMDCs) stimulated with the TLR3 or TLR4 agonists Poly I:C or Lipopolysaccharide (LPS) resulted in the generation of sIL-15 complexes (43). In addition, LPS is known as a potent activator of ADAM17 activity (51). Due to these results, ADAM17 is currently believed to be the protease responsible for the cleavage of cell-surface bound IL-15Rα/IL-15 complexes into a soluble form. However, definitive evidence demonstrating the role of ADAM17 in the generation of sIL-15 complexes is currently lacking. **I demonstrate in Chapter 3 that the activation of ADAM17 is sufficient and required for the generation of sIL-15 complexes in BMDCs; however, its activity is not required for the generation of the majority of sIL-15 complexes *in vivo*. This indirectly implicates the existence of additional proteases capable of generating sIL-15 complexes.**

### 1.3 Inducers and cellular sources of IL-15 expression

IL-15 protein is expressed at undetectable levels during homeostasis; however, it can be induced in DCs, macrophages, and inflammatory monocytes. In addition, parenchymal cells including fibroblasts and thymic and intestinal epithelial cells have also been shown indirectly to express IL-15 protein (53-57). In general, the expression of IL-15 is primarily studied in the aforementioned myeloid cells. Through cell specific ablation of IL-15Rα, it was determined that DCs and macrophages are dominant cellular sources of IL-15 during homeostasis to CD8 T cells and NK cells *in vivo* (23). In addition, selective IL-15Rα expression in the DC compartment alone

resulted in the partial recovery of the CD8 memory T cell compartment (54). Recent analyses in multiple IL-15 reporter models have implicated the CD8+ DC subset as a major producer of IL-15 *in vivo* during homeostasis and viral infections (58,59). An additional IL-15 reporter model determined that non-hematopoietic epithelial, endothelial and stromal cells also express IL-15 transcripts *in vivo* during the steady state which are increased in response to LPS stimulation (60). In summary, IL-15 is expressed during homeostasis by a diverse number of hematopoietic and parenchymal cells.

In addition to directly enhancing CD8 T cell responses, Type I IFNs also induce IL-15 expression at the mRNA and protein levels (61,62). The TLR agonists Poly I:C, LPS, and CpG which stimulate TLRs 3, 4 and 9 respectively (63) are known to directly induce Type I IFN. These TLR agonists are also well-described stimuli that induce IL-15 mRNA and protein expression *in vivo* and in isolated monocytes and DCs (61,62,64). Type I IFNs are able to induce the transcription of IL-15 via the IRF binding site located in the IL-15 promoter (29). As injection of IFN$\alpha$-encoding plasmids enhance IL-15R$\alpha$ expression on splenic DCs, Type I IFN also augments IL-15 transpresentation that likely contributes to enhanced IL-15-dependent responses (64). In addition, Type I IFN signaling is required for the induction of IL-15 transcription in response to vesicular stomatitis virus (VSV) and Rhinovirus infections (58) (65). Clearly Type I IFN signaling is a key IL-15 regulating pathway; however, the requirement for Type I IFN signaling in the generation of sIL-15 complexes is currently unknown (Figure 1B).

In addition to IFN signaling, stimulation of the co-stimulatory protein CD40 also induces IL-15 mRNA expression (66). *In vivo* injection with an agonistic antibody to CD40 enhances IL-15R$\alpha$ protein expression in DCs (67). Stimulation of the CD40 co-stimulatory pathway in tumor-bearing mice resulted in T cell dependent tumor regression (68,69). Further investigation

determined that this pathway induced IL-15 dependent responses *in vivo* and CD40 signaling was found to enhance the expression of IL-15 and IL-15Rα on splenic DCs (66,69). CD40 is a receptor expressed in B cells, DCs, T cells, macrophages, and endothelial cells (70). The intracellular signaling of CD40 dominantly utilizes non-canonical NFκB signaling as opposed to most TLRs that use canonical NFκB signaling to mediate their downstream effects (71,72). In addition to IL-15 production, CD40 signaling on DCs also promotes the strong induction of IL-12 (73). Among IL-15 producing APCs, baseline CD40 expression is highest on the CD8+ DC subset (74). These CD8+ DCs are localized in the T cell zone of the spleen and secondary lymphoid organs (75) and are specialized in the cross-presentation of exogenous antigen on MHC-I to responding CD8 T cells (75,76). The CD8+ DC subset is highly enriched with the receptors for the specialized uptake and processing of apoptotic and necrotic cells, these exogenous antigens are then cross-presented via MHC-I to CD8 T cells (5,76). Unlike viruses, which actively infect DCs and whose antigens are capable of being presented by MHC I under normal conditions, tumor cells do not readily enter the cytosol of DCs. Therefore, for CD8 T cells to recognize tumor antigens, the exogenous tumor antigens must be cross-presented on MHC I. The CD8+ DC subset is crucial for cross-presentation and the generation of anti-tumor CD8 T cells responses in mouse models (8,76).

Collectively, the literature indicates that IL-15 is produced at low levels during the steady state and IL-15 expression is up-regulated in both the hematopoietic and non-hematopoietic compartments upon TLR stimulation or during viral infections. It should also be noted that this expression is heterogeneous, as many cell types had little to no protein expression of IL-15 while other cell types, such as CD8+ DCs and MHC class II+ thymic epithelial cells display strong IL-15 expression (58,60,77). **In Chapter 3, I will show evidence that DCs and macrophages are the dominant cell types producing sIL-15 complexes upon infection with VSV and CD40 stimulation respectively. In addition, I have determined that although Type I IFN is a**

**significant inducer of sIL-15 complexes, the generation of sIL-15 complexes in response to VSV infection and CD40 stimulation does not require Type I IFN signaling.**

### 1.4 IL-15-mediated functions in the immune system

Attempts to examine IL-15 protein expression at homeostasis have been unsuccessful and past reports have concluded that the expression of IL-15 is extremely limited in the steady state but readily up-regulated upon activation via the various mechanisms previously discussed (77). Nevertheless, mice lacking expression of either IL-15 or the IL-15Rα have a profound reduction in the number of lymphocytes, including CD8 T cells, NK, NKT, γδ and intestinal epithelial cells (IELs) (16,37,38) due to its critical roles in the development of these immune cells. In addition to its roles in lymphocyte development, IL-15 expression is also required for the long term persistence of mature NK cells and antigen-specific memory CD8 T cells (16,42). As DCs and macrophages are producers of IL-15, it is not surprising that loss of IL-15 expression by either subset results in a significant loss of memory phenotype CD8 T cells (23). Interestingly, these cell specific knockout mice exhibit differential effects as loss of IL-15 expression in DCs or macrophages resulted in the preferential loss of Tcm or Tem cells, respectively (23). This reason for this differential affect is currently unknown, but may be due to divergent localization of the T cell subsets and various IL-15 expressing cells (23). Although the overall role of IL-15 in Trm is not well established, one study determined that lL-15 was required for the optimal generation of Trm in the skin (24).

Memory phenotype CD8 T cells stimulated by high doses of recombinant IL-15 undergo proliferation at levels nearly equivalent to TCR-mediated stimulation (78). It is currently debated if IL-15 contributes to the proliferation during early infections, as naïve CD8 T cells specific for the dominant epitopes of several viruses display normal expansion in the absence of IL-15 expression

(17,79). It should be noted that CD8 T cells recognizing minor epitopes of several viruses did exhibit defects in expansion in IL-15 deficient mice (16,17). Overall, these results suggest that IL-15 may not be required for the expansion of the dominant pathogen-specific CD8 T cells but may affect the expansion of CD8 T cells with lower affinity TCR signals. Further support for this comes from the fact that IL-15 signaling augments low affinity T cell responses in the context of autoimmune and anti-tumor responses as high levels of IL-15 are capable of overriding tolerance in numerous animal tumor models (80-83). A recent study determined that TLR agonists or viral infections induced the proliferation of memory CD8 T cells and this proliferation was completely dependent upon enhanced IL-15 expression (84).

Enhanced expression of IL-15 is correlated with increased numbers of activated CD8 T cells in many autoimmune diseases, including Rheumatoid arthritis, Eczema, Multiple sclerosis, Type I diabetes, Systemic lupus erythematosus, Celiac and Inflammatory bowel diseases (85-91). In addition, in human colorectal cancer, IL-15 expression is strongly correlated with an increased number of tumor-infiltrating CD8 T cells and the absence of IL-15 expression alone is a significant prognostic indicator of enhanced risk of disease recurrance (92). IL-15 is clearly a factor associated with autoimmunity and involved in anti-tumor CD8 T cell responses. In addition to proliferation, a dominant function of IL-15 is to promote the survival of CD8 T cells, which is at least partially attributed to its ability to directly enhance the expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL while restraining the expression of the pro-apoptotic protein Bax (93-95).

IL-15 is well established in its roles to facilitate proliferation and survival during lymphocyte development and the homeostatic proliferation of a number of different lymphocytes; however, IL-15 can directly alter effector functions of T cells. Stimulation of CD8 T cells with recombinant IL-15 significantly up-regulates numerous effector molecules, including IFNγ, TNF$\alpha$, Perforin and

Granzyme B (78,96,97). IL-15 also affects T cell trafficking, by directly up-regulating the production of numerous chemokine and chemokine receptors on T cells (98,99). These effects are more prominent on memory CD8 T cells, likely due to their higherexpression of CD122 relative to naïve T cells. Memory CD8 T cells are known to migrate to inflamed tissues and IL-15 has been shown to be required for the optimal accumulation of antigen-specific CD8 T cells in the Broncho alveolar fluid (BAL) of influenza infected mice (100). Interestingly, this migration absolutely requires IL-15, but occurs independently of the presence of cognate antigen (101). This effect was dependent upon IL-15-induced expression of the T cell selectin ligand O-glycan which binds to activated endothelial cells expressing P- and E- selectins. Interestingly, local treatment with rIL-15 complexes alone induces the migration of CD8 T cells (100). Overall, IL-15 clearly plays a dominant role in the activation, survival, proliferation and trafficking of memory CD8 T cells which likely are involved during inflammatory responses.

**1.5 Lymphopenia**

IL-15 dependent responses by CD8 T cells and NK cells can be significantly enhanced by transient depletion of these endogenous lymphocytes via total body irradiation (TBI); these responses are thought to be mediated as a result of the generation of a sink of homeostatic cytokines (19,102). Lymphopenia induced by high dose chemotherapy (CTX) or TBI also significantly enhances the availability of the homeostatic cytokines IL-7 and IL-15 (103,104). Chemotherapy and irradiation treatment therapies have long been used in an attempt to directly kill cancer cells. In many cases, these relatively non-specific therapies lead to tumor regression and long term survival that has been largely believed to be mediated by direct tumor killing. It has only been a recent development that we have been able to fully dissect and appreciate the roles in which the immune system plays in tumor regressions in response to irradiation and chemotherapy treatment regimens. Early studies examining the ability of T cells to augment anti-

tumor responses in murine tumor models demonstrated that the adoptive transfer of tumor-specific T cells alone provided no protection; however, when lymphodepletion conditioning regimens were used prior to T cell transfer, anti-tumor responses were drastically enhanced (105,106). These responses were later shown to be require IL-15 expression for optimal antitumor responses (102). As such, lymphodepletion therapies have been incorporated into anti-tumor clinical trials. Furthermore, in a side by side comparison, more severe lymphodepletion generated with the combination of chemotherapy and TBI or higher dose TBI led to a higher objective response rate in advanced melanomas than each regimen alone (107). Until recently, the treatment with the greatest efficacy in treating human advanced stage melanoma, adoptive T cell therapy (ACT), has been entirely dependent upon lymphodepletion conditioning prior to T cell infusion (107). A study by Bergamaschi et al. demonstrated that lymphodepletion induced by CTX or TBI significantly enhanced the circulating levels of sIL-15 complexes in mice and human melanoma patients (104). In addition, this study further demonstrated that in response to these treatments all soluble IL-15 was associated with the high affinity IL-15R$\alpha$, indicating that free IL-15 alone may not exist (104). Despite its obvious importance, studies into the mechanisms whereby lymphopenia augments IL-15 dependent responses have been largely limited to its relative ability to enhance antitumor responses, while studies examining the upstream factors and cell types required for lymphopenia induced IL-15 expression have been largely ignored. **The cell types required and the molecular mechanism for the generation of sIL-15 complexes following lymphopenia have not been defined. I will demonstrate in Chapter 4 that DCs and macrophages are major sources of TBI-induced IL-15.**

Lymphopenia significantly augments anti-tumor CD8 T cell responses, which are at least partially dependent on IL-15 expression. **I show in Chapter 4 that T cell depletion by $\alpha$Thy1 treatment induces the proliferation of memory CD8 T cells which is entirely dependent**

**upon the induction of inflammatory IL-15.** Although numerous previous studies have determined a critical role for IL-15 in T cell proliferation and survival, and during anti-tumor immune responses; inflammatory lymphopenia-induced IL-15 may have additional direct effects on T cells that contribute to enhanced anti-tumor responses that unfortunately the preceding studies were unable to address.

**Figure 1: Potential inducers and pathways of IL-15 expression**

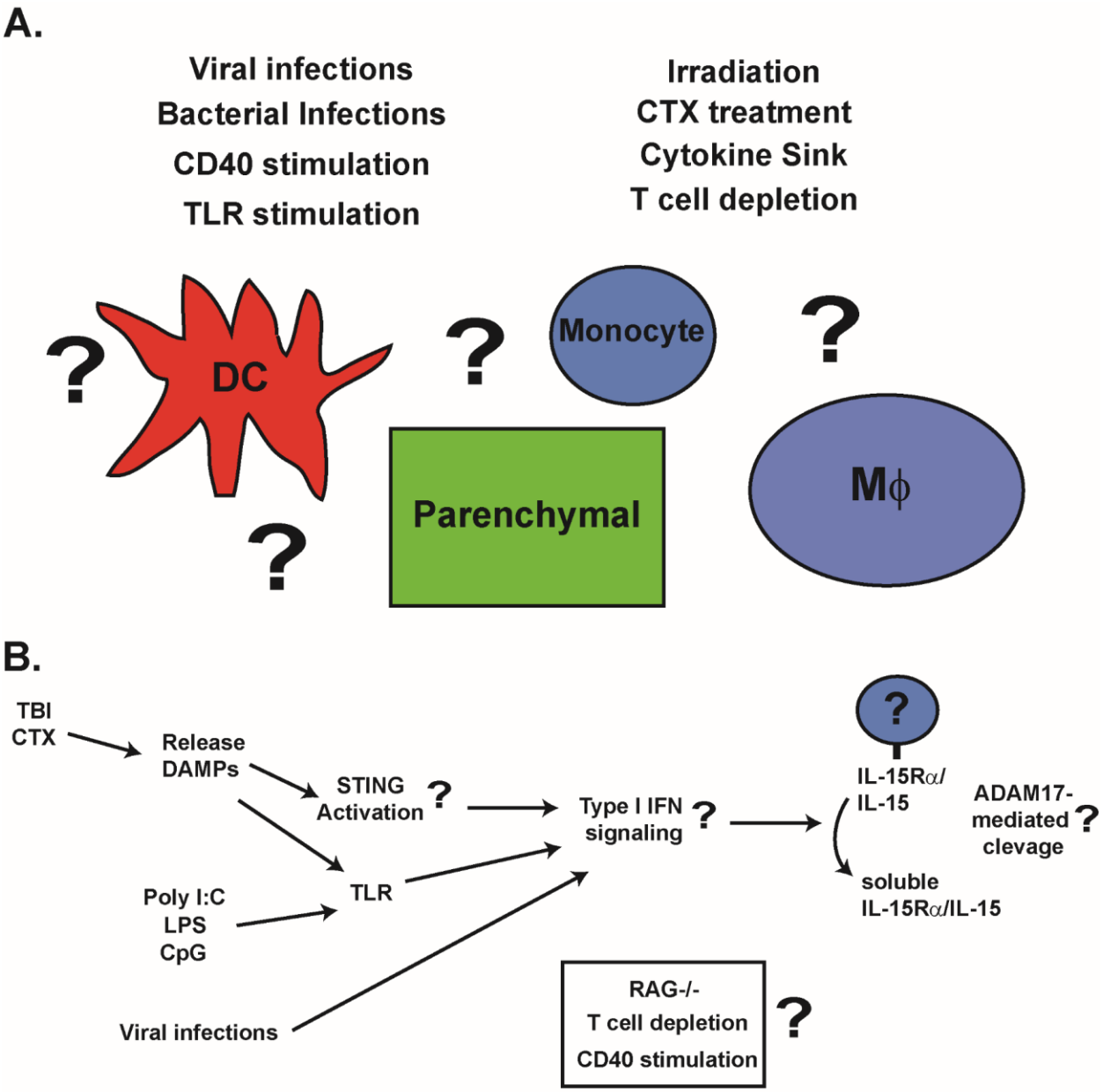

**Figure 1: Potential inducers and pathways of IL-15 expression.** IL-15 is enhanced by infections with bacteria or viruses, or in response to TLR or CD40 stimulations. Additionally, many lymphopenic models also induce IL-15 expression. Recent studies indicate that sIL-15 complexes are also produced in response to infections with rhinovirus and influenza or upon TBI or CTX-induced lymphopenia. Yet, little is known about the mechanisms generating these soluble IL-15 complexes, including the cell types required for this induction (A) or the pathways responsible for mediating these effects *in vivo* (B). In addition, ADAM17 cleaves IL-15Rα from the surface of cells *in vitro;* however, its direct role in the cleavage of IL-15Rα/IL-15 complexes into a soluble form is unknown. Although CD40 stimulation, T cell depletion or lymphopenic RAG-/- mice express elevated IL-15, it is also unknown if these conditions induce sIL-15 complexes.

## Chapter 2: Specific Aims

### 2.1 Determine the mechanisms generating soluble Interleukin-15 complexes

Numerous infectious pathogens and TLR agonists enhance the expression of IL-15 and sIL-15 complexes, yet the mechanisms and cell types required for generating these sIL-15 complexes are not well established. Type I IFN is a common signal induced by these stimuli and ADAM17 was implicated in the cleavage of IL-15Rα in transfected cells; therefore, **we hypothesize that Type I IFN signaling and ADAM17 are required for generating sIL-15 complexes**. We will investigate if IFN directly signals to induce sIL-15 complexes. In addition, multiple known IL-15 inducing stimuli will likewise be investigated in their abilities to induce sIL-15 complexes in the presence or absence of Type I IFN signaling. Through the use of an inducible ADAM17 knockout mouse model, we will directly determine the role of in ADAM17 in the generation of sIL-15 complexes. To determine the cell types responsible for generating sIL-15 complexes, mice with conditional deletion of IL-15 in the macrophage or DC lineages will be investigated.

### 2.2 Examine the regulation of IL-15 expression during lymphopenia

Lymphodepletion regimens enhance anti-tumor responses in a manner dependent upon IL-15 expression. Lymphodepletion regimens also directly induce a transient but systemic induction of sIL-15 complexes in mice and humans; however, the signals mediating this induction and the cell types responsible for the generation of lymphopenia-induced IL-15 are largely unknown. **We hypothesize that lymphopenia requires Type I IFN signaling for the generation of sIL-15 complexes.** Using multiple mouse models of lymphopenia, we will investigate the factors and cell types required for the generation of lymphopenia-induced IL-15 and sIL-15 complexes. Mice lacking key inflammatory signaling pathways will be subjected to TBI to determine their roles in TBI-induced sIL-15 complexes. We will additionally use our conditional IL-15 knockout mice to determine the relative contribution of macrophages or DCs to lymphopenia-induced IL-15 expression.

**Chapter 3:**

This chapter is based upon and reproduced from the following journal article:

Anthony SM, Howard ME, Hailemichael Y, Overwijk WW, and Schluns KS. Soluble interleukin-15 complexes are generated *in vivo* by type I interferon dependent and independent pathways. PLoS One. 2015 Mar 10; 10(3):e0120274. As per PLoS policy, no permission is required to reprint.

### 3.1 Introduction

IL-15 is co-expressed with the high affinity IL-15Rα chain, becomes associated with IL-15Rα in the endoplasmic reticulum, and is subsequently shuttled to the cell surface as a complex (41). This cell surface complex of IL-15 and IL-15Rα is capable of stimulating neighboring cells expressing the IL-2/15Rβ and γC complex during a cell-cell interaction via a mechanism called transpresentation (41). Alternatively, this cell surface IL-15Rα/IL-15 can also be cleaved to form soluble IL-15Rα/IL-15 complexes (sIL-15 complexes) (43,104). *In vitro* and *in vivo* production of sIL-15 complexes have been observed after stimulation with Toll-like receptor (TLR)3 and TLR4 agonists, Poly I:C and Lipopolysaccharide (LPS), respectively (43). Since sIL-15 complexes have agonist properties and exhibit an approximate 50-100 fold greater proliferative effect on responding CD8 T cells over recombinant IL-15 alone (45,108), the generation of sIL-15 complexes is likely an important, yet un-delineated mechanism regulating IL-15 responses. Unfortunately, few studies have examined regulatory mechanisms generating sIL-15 complexes. In addition, transcription of IL-15 and IL-15Rα is increased during numerous types of viral and bacterial infections (58,109,110). Regulation of IL-15 through stimulation of specific immune pathways is evident as TLR ligands, Type I IFNs and agonistic anti-CD40 antibody increase IL-15 expression as well as cell surface IL-15 expression (43,61,62,64,66,111,112). Moreover, enhanced IL-15 responses have also been reported upon stimulation with Poly I:C, IFN-α, or anti-CD40 Ab (43,64,69,113). While elevated IL-15 and IL-15Rα expression are clearly associated with immune activation and inflammation, whether sIL-15 complexes are generated during these situations is not clear. **Hence, the overall goal of this aim was to determine the importance of type I IFNs and identify the immune stimuli and cell types required for the generation of sIL-15 complexes.**

### 3.2 Results.

#### 3.2.1 IFN-α induces the generation of sIL-15 complexes

Previous studies have shown that Type I IFNs increase IL-15 mRNA and protein expression (58,61,62). Moreover, our past studies showed that IFN-α increases surface IL-15 expression on DCs (64,77); however, whether there are concomitant effects on the production of sIL-15 complexes has not been determined. As such, we investigated if Type I IFNs affect the generation of sIL-15 complexes. To induce IFN-α production *in situ*, mice were given plasmid encoding IFN-α (pORF-IFN-α) or control plasmid (pORF) via hydrodynamic injections. In mice given pORF-IFN-α, levels of circulating IFN-α were significantly increased in the serum 48 hrs post injection (Figure 2A). This induction of IFN-α was only induced by the pORF-IFN-α plasmid, as the empty vector control did not induce serum IFN-α protein expression above levels observed in untreated mice (Figure 2A). pORF-IFN-α treatment also significantly increased sIL-15 complexes in the serum of mice 48hrs post injection (Figure 2B) demonstrating that IFN-α alone induces the generation of sIL-15 complexes. This procedure promotes long term production of IFN-α *in vivo* (64). For as much as 21 days after injection of pORF-IFN-α, levels of sIL-15 complexes remained high (Figure 2C) demonstrating that continual Type I IFN-signaling *in vivo* is sufficient for sustaining production of sIL-15 complexes.

To determine if IFN-α acts directly on GMCSF-induced Bone Marrow-derived DCs (BMDC), BMDCs generated from Wt and IFNAR-/- mice were treated with rIFN-α (300U/mL) or Poly I:C (50µg/mL). Both Poly I:C and rIFN-α increased the levels of sIL-15 complexes in supernatants from Wt BMDCs but not IFNAR-/- DCs showing that rIFN-α directly induces production of sIL-15 complexes (Figure 2D).

**Figure 2: IFN-α induces the generation of sIL-15 complexes**

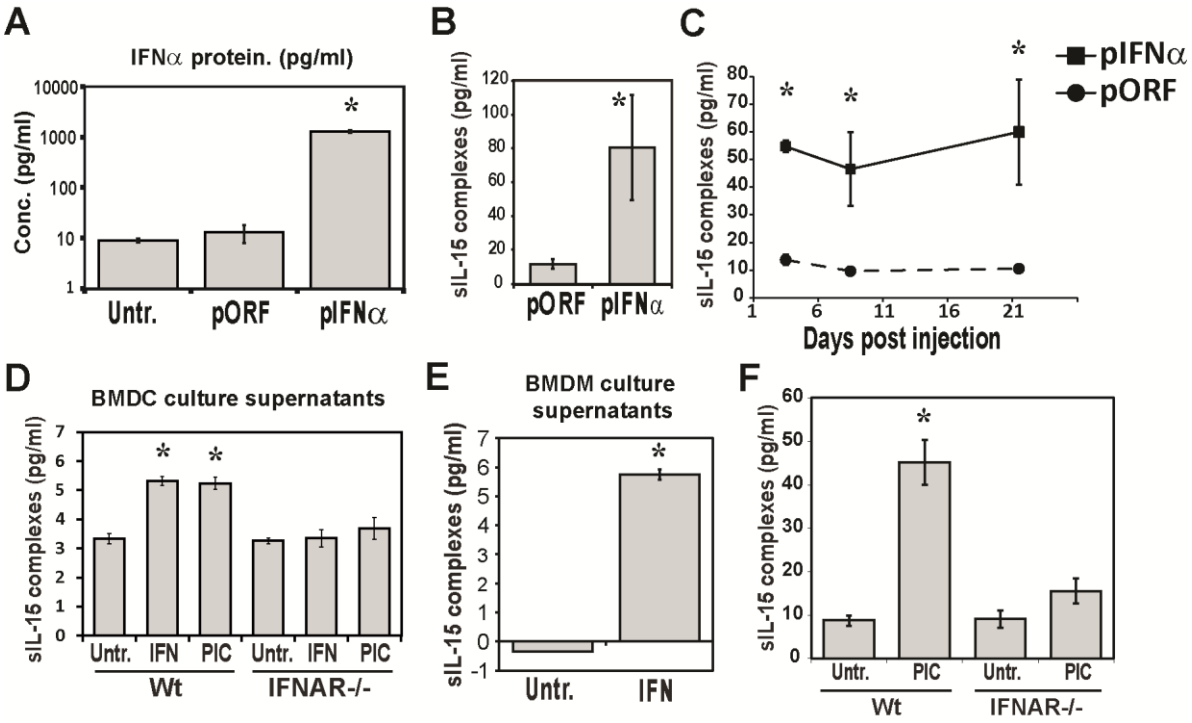

**Figure 2: IFN-α induces the generation of sIL-15 complexes.** (A, B) A plasmid encoding IFN-α, (pIFNα) or an empty vector control plasmid (pORF) were injected i.v. by hydrodynamic injection. Serum was isolated 48 hrs post injection and after various times post plasmid injection (C). Levels of IFN-α protein (A) and sIL-15 complexes (B, C) in serum were measured using IFN-α and sIL-15 complex ELISAs respectively. n=2-3 mice/group. Data is one representative of three experiments. Culture supernatants were collected from Wt and IFNAR1-/- BMDC treated with rIFN-α (300 U/mL) or Poly I:C (50μg/mL) (D) or MCSF-generated BM-Macrophages (E) stimulated with rIFN-α (300 U/mL) for 24 hrs. Levels of sIL-15 complexes in culture supernatants were measured using ELISA. Data is one representative of three experiments. F) Levels of sIL-15 complexes in serum from Wt and IFNAR1-/- mice 24 hours after treatment with Poly I:C (150 μg, i.p.) were measured using ELISA. n=3 mice/group. Data is one representative of three experiments. All error bars represent SD. * indicates $p<0.05$.

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rIFN-α stimulation of Bone Marrow Derived Macrophages (BMDM) also induced sIL-15 complexes in culture supernatants (Figure 2E). To examine the requirement for Type I IFNR signaling *in vivo*, IFNAR-/- mice were analyzed after treatment with Poly I:C, which induces IFN-α as well as sIL-15 complexes (43). sIL-15 complexes were not efficiently induced by Poly I:C in IFNAR-/- mice (Figure 2F) indicating that Type I IFNR signaling is required for the production of sIL-15 complexes in response to TLR3 stimulation. Altogether, these results provide evidence that Type I IFNs directly induce the generation of sIL-15 complexes.

### 3.2.2: VSV-induced inflammatory IL-15 expression

VSV is known to strongly induce abundant levels of Type I IFNs, VSV also enhances the mRNA and protein expression of IL-15 *in vitro* and *in vivo* (58,62,64,73). Colpitts et al. demonstrated a significant induction of IL-15 in CD8$\alpha$+ DCs in response to VSV-infection in an IL-15 transcriptional reporter mouse, although the expression of IL-15 transcripts is not fully representative of IL-15 protein expression, as many nucleated cells express IL-15 mRNA but very few of these cells actually express the cytokine in protein form (58,114). It is currently unknown what cell types are directly responsible for the generation of transpresented IL-15 in response to VSV infection. Therefore, our next goal was to investigate the regulation of IL-15 protein expression during VSV infection.

Splenocytes were isolated from untreated mice, mice 24 hours post VSV-infection or mice treated with Poly I:C as a positive control. Splenic monocytes, macrophages and conventional DCs were gated based on established lineage specific cell surface markers and cell-surface IL-15 expression was analyzed based on staining with a biotinylated anti-IL-15 antibody (Gray histograms, Peprotech) or a Biotin-Ig control antibody (Black Histograms). Untreated splenocytes had a uniform lack of IL-15 cell surface staining in all cells examined (Figure 3A), which is consistent with a previous study (77). VSV infection resulted in a significant induction of IL-15 expression on Macrophages (Figure 3A) at or above the amount induced by *in vivo* Poly I:C stimulation. VSV also induced significant IL-15 cell-surface expression in Monocytes and DCs (Figure 3B), although this induction was not as prominent as that observed in Macrophages. These results highlight that VSV induces significant but differential cell surface IL-15 responses in splenic monocytes, DCs and most prominently in macrophages.

**Figure 3: VSV induces cell surface IL-15 expression**

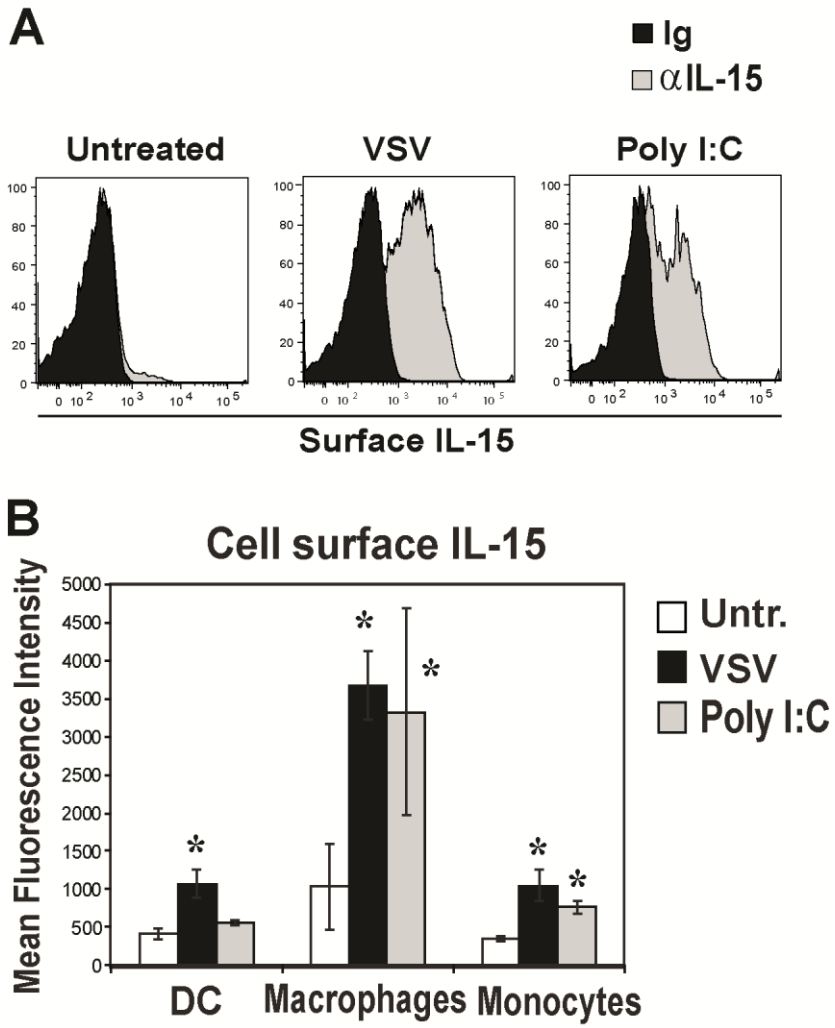


**Figure 3:** **VSV induces cell surface IL-15 expression.** A,B) Cell surface IL-15 expression in splenic DCs, Macrophages and Monocytes by flow cytometry one day after VSV infection ($1x10^6$ PFU/mouse, i.v.) or Poly I:C injection (150 µg/mouse, i.p.) as determined by immunofluorescence staining and flow cytometry. Histograms depict representative staining with control Ig (black histogram) and anti-IL-15 Ab (grey histograms) in macrophages (lineage-CD11b+CD11c-F480+). Graph shows average MFI of IL-15 expression in DCs (lineage-CD11b+/-CD11c+F480-), macrophages, and monocytes (lineage-CD11b+CD11c-F480-) of indicated mice. n=3 mice/group, one representative of three experiments shown. All error bars represent SD. * indicates $p<0.05$. This chapter is based upon and reproduced from the following journal article: Anthony SM, Howard ME, Hailemichael Y, Overwijk WW, and Schluns KS. Soluble interleukin-15 complexes are generated *in vivo* by type I interferon dependent and independent pathways. PLoS One. 2015 Mar 10; 10(3):e0120274. As per PLoS policy, no permission is required to reprint.

We next asked if VSV infection induces the generation of sIL-15 complexes. One day after VSV infection, levels of sIL-15 complexes in serum were transiently increased, returning to baseline two days post infection (Figure 4A) showing that similar to Poly I:C stimulation, increases in cell surface IL-15 coincide with the appearance of sIL-15 complexes. Unexpectedly, VSV efficiently induced sIL-15 complexes in IFNAR-/- mice (Figure 4B) indicating other pathways are involved in VSV-mediated induction of sIL-15 complexes.

As VSV infection up-regulates the expression of CD40 (115), and CD40 is a known inducer of IL-15 expression (68) we examined whether the VSV-induced CD40 signaling was compensating for the lack of Type I IFN signaling in the generation of VSV-induced sIL-15 complexes. Remarkably, the addition of an established CD40L blocking antibody (MR1, 200µg i.p.) had no significant effect on VSV-induced sIL-15 complex generation in IFNAR-/- mice across 2 separate experiments (Figure 4C). Overall, these data show for the first time that sIL-15 complexes are transiently increased early in a VSV infection in a manner independent of Type I IFN signaling. This also indicates that an active VSV infection induces alternative signaling pathways in addition to CD40 and Type I IFNs, for the induction of sIL-15 complexes.

**Figure 4. Viral infection transiently induces sIL-15 complexes *in vivo* independent of IFN or CD40 signaling.**

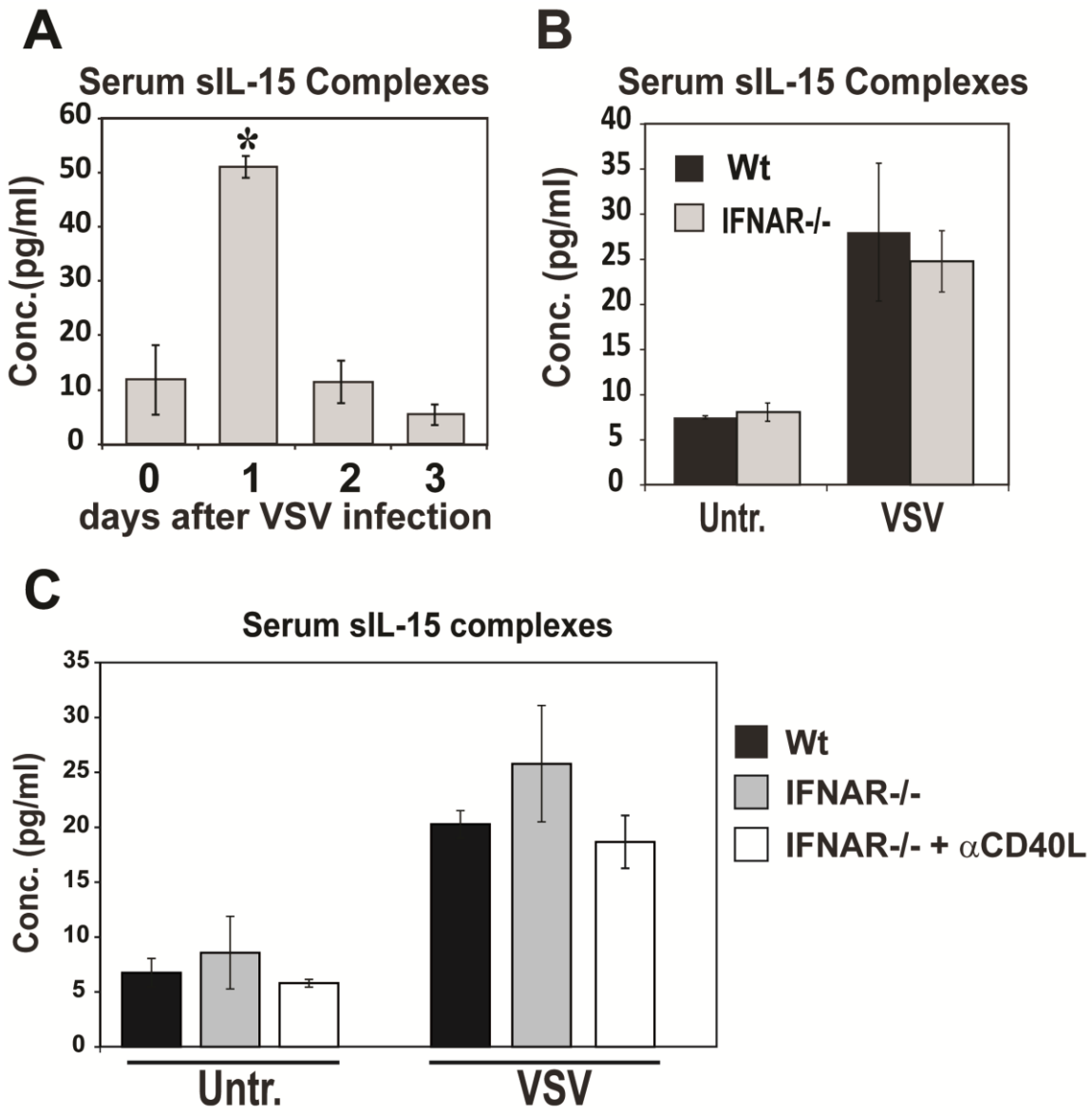


**Figure 4. Viral infection transiently induces sIL-15 complexes *in vivo* independent of IFN signaling.** A) Serum levels of sIL-15 complexes in Wt mice, at indicated times post VSV infection ($1x10^6$ PFU/ mouse, i.v.) (n=2-5 mice/group), one representative experiment of five total is shown. Error bars represent SD. B) Levels of sIL-15 complexes in serum from Wt and IFNAR-/- mice one day post VSV infection. n=3-4 mice/group; data is one representative of three experiments. Error bars represent SD. * indicates $p<0.05$. C) Serum was collected from all mice prior to VSV infection. One group of IFNAR-/- mice was treated with $\alpha$CD40L blocking antibody (clone MR1, 200ug, i.p.) 30 minutes prior to infection. All mice were then infected with VSV ($1x10^6$ PFU, i.v.), sacrificed 24 hrs later and serum was isolated. Levels of sIL-15 complexes in serum were measured using ELISA. N=2-4 mice/group. Data is representative of two experiments. Error bars represent SEM. This chapter is based upon and reproduced from the following journal article: Anthony SM, Howard ME, Hailemichael Y, Overwijk WW, and Schluns KS. Soluble interleukin-15 complexes are generated *in vivo* by type I interferon dependent and independent pathways. PLoS One. 2015 Mar 10;10(3):e0120274. As per PLoS policy, no permission is required to reprint.

### 3.2.3: sIL-15 complexes are transiently increased by CD40 stimulation independent of Type I IFN signaling.

Our results suggest that DC stimulation leads to the induction of sIL-15 complexes, which under some circumstances is mediated by Type I IFN signaling. CD40 stimulation has been reported to increase surface IL-15 and IL-15Rα on DCs and promote IL-15 responses *in vivo* (66,69) but is not reported as a major inducer of type IFNs. To determine if CD40 stimulation generates sIL-15 complexes, mice were given agonistic anti-CD40 Ab (FGK4.5, 200 µg/mouse, i.p.) and levels of sIL-15 complexes in the serum were measured. As with VSV infection, anti-CD40 treatment increased sIL-15 complexes to a maximum level one day after treatment (Figure 5A). Two days after treatment, the levels of sIL-15 complexes were still elevated (Figure 5A). Similar levels of sIL-15 complexes were induced with lower doses of anti-CD40 Ab (50 µg/mouse) during *in vivo* titration experiments (Figure 5B), while levels of sIL-15 complexes in serum returned to baseline two days post injection with lower dose CD40 stimulation, strikingly similar to the transient induction observed during VSV infection (Figure 4A).

To determine if CD40 stimulation acts directly on DCs, BMDCs were generated from Wt mice and were treated with rIFN-α (300U/mL) or αCD40 (50µg/mL). Surprisingly, CD40 stimulation did not induce sIL-15 complexes from BMDCs (Figure 5C). The GMCSF-induced DCs are functionally and phenotypically similar to *in vivo* inflammatory DCs, while the differentiation of bone marrow cells with Flt3L-supplemented cultures are recognized to generate multiple DCs subsets (CD8α+, CD103+, B220+ pDCs) representative of the endogenous splenocyte DC subsets *in vivo* (116). Therefore Flt3L-induced BMDCs were treated with rIFN-α (300U/mL) or αCD40 (30µg/mL).

**Figure 5: sIL-15 complexes are transiently increased by CD40 stimulation independent of Type I IFN signaling.**

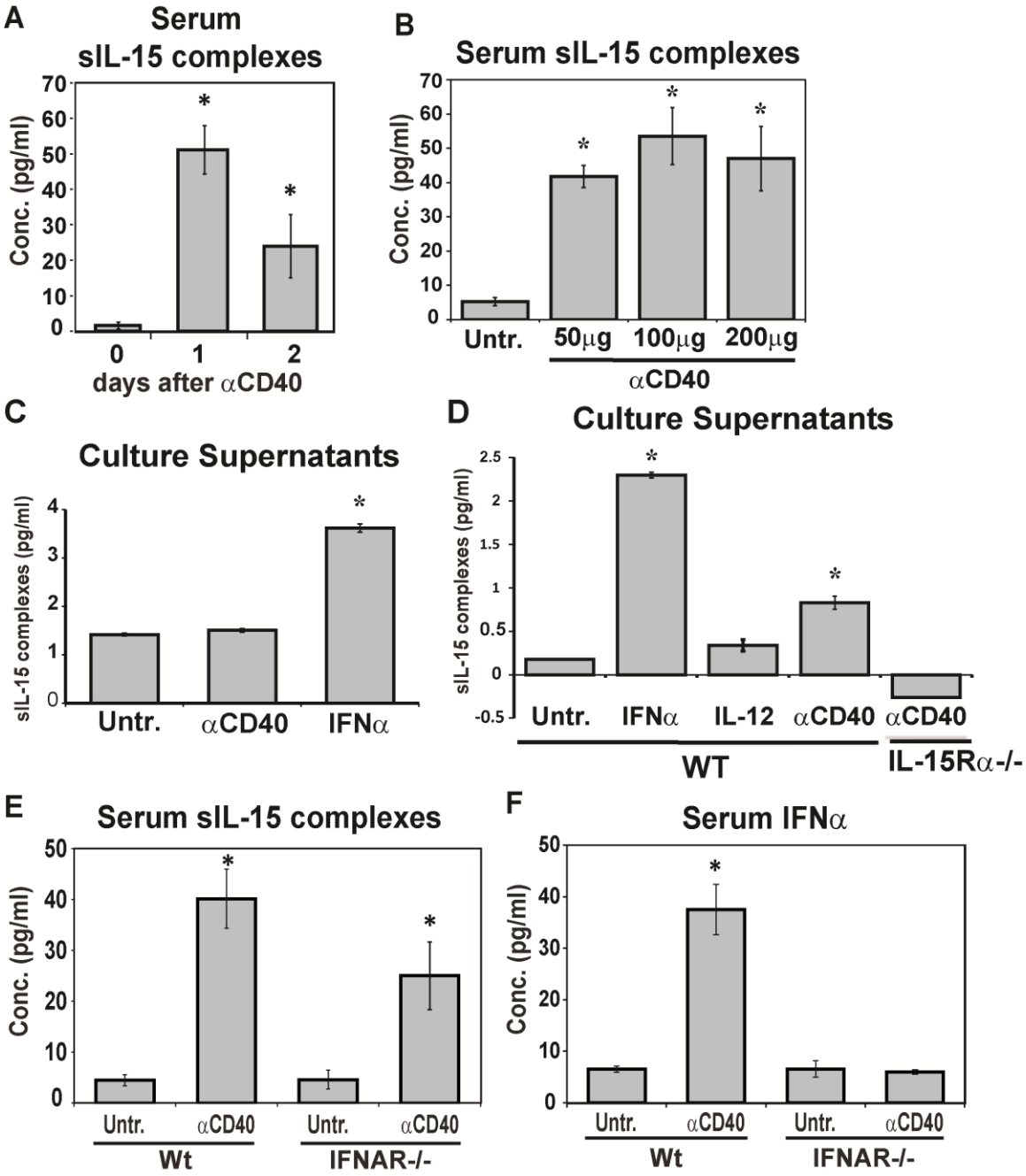


**Figure 5: sIL-15 complexes are transiently increased by CD40 stimulation independent of type I IFN signaling.** A) Serum was isolated from Wt mice 0, 1, and 2 days after treatment with anti-CD40 Ab (200 µg i.p.) mAb. n=3-4 mice/group. B) Serum was isolated 24 hours after treatment with the indicated dose of anti-CD40 mAb. n=2-6 mice/group. Levels of sIL-15 complexes in serum were measured using ELISA. Data is representative of two experiments. C) Culture supernatants were collected from Wt GMCSF BMDCs treated with rIFN-$\alpha$ (300 U/mL), or $\alpha$CD40 (30µg/mL) for 24 hr. D) Wt or IL-15R$\alpha$-/- Flt3L-BMDCs were treated with rIFN-$\alpha$ (300 U/mL), IL-12 (50ng/mL) or $\alpha$CD40 (30µg/mL) for 24 hrs. Levels of sIL-15 complexes in culture supernatants were measured using ELISA. n=2-4 wells/group. One representative experiment of two is shown. Error bars represent SD. E, F) Wt and IFNAR-/- mice were treated with anti-CD40 Ab (50 µg i.p.). Serum was isolated 24 hours later and analyzed for levels of sIL-15 complexes (E) and IFN-$\alpha$ (F) via ELISA. Graph shows average cytokine levels from 3 experiments. N=12 mice/group. Error bars represent SEM. * indicates $p<0.05$. This chapter is based upon and reproduced from the following journal article: Anthony SM, Howard ME, Hailemichael Y, Overwijk WW, and Schluns KS. Soluble interleukin-15 complexes are generated *in vivo* by type I interferon dependent and independent pathways. PLoS One. 2015 Mar 10; 10(3):e0120274. As per PLoS policy, no permission is required to reprint.

Both rIFN-α and, to a lesser degree αCD40 increased the levels of sIL-15 complexes in supernatants from Flt3L DCs (Figure 5D). Direct stimulation of Flt3L-DCs by IL-12 (100ng/mL; 24 hrs) exhibited no induction of sIL-15 complexes (Figure 5D). This demonstrates that CD40 stimulation, but not IL-12, directly induces production of sIL-15 complexes in Flt3L-DCs (Figure 5D). To determine whether CD40-induced sIL-15 complexes could be generated in the absence of Type I IFN signaling, similar experiments were conducted using IFNAR-/- mice. Treatment with anti-CD40 Ab (50 µg/mouse) significantly increased sIL-15 complexes in the absence of IFNAR, but at a reduced level, corresponding to an approximate 40% reduction compared to that induced in Wt mice (Figure 5E). Because CD40-mediated increases in sIL-15 complexes were partially reduced in IFNAR-/- mice, we examined if IFN-α is produced after CD40 stimulation. Interestingly, IFN-α protein was present in the serum of CD40 stimulated Wt mice but not IFNAR-/- mice (Figure 4F). These data indicate IFN signaling is only partially required for CD40 mediated increases in sIL-15 complexes. Therefore, CD40 stimulation provides a model where sIL-15 complexes are increased by both Type I IFN and non-IFN pathways.

### 3.2.4: Role of ADAM17 in the generation of sIL-15 complexes

Since A Disintegrin And Metalloprotease (ADAM) 17 can cleave IL-15Rα from the surface of cells *in vitro* (52,117), ADAM17 is currently suspected to cleave IL-15Rα/IL-15 complexes into a soluble form. Translocation of ADAM17 to the cell surface is indicative of ADAM17 activity (51), therefore cell surface ADAM17 expression by BMDCs was measured using flow cytometry as a means to assess the ability of Type I IFNs to regulate ADAM17 activity. Stimulation with IFNα for 24 hrs strongly up-regulated the cell-surface expression of ADAM17 in Wt BMDCs but exhibited no effect on cell-surface ADAM17 expression in IFNAR-/- BMDCs (Figure 6A). To examine the requirement for ADAM17 activity in IFN-α-induced sIL-15 complex generation, ADAM17 was deleted in BMDC. BMDC were generated from ADAM17$^{flox/flox}$ (ADAM17$^{fl/fl}$) mice in the absence or presence of TAT-Cre to mediate *in vitro* recombination and deletion of ADAM17. While IFN-α increased sIL-15 complexes in non-transduced BMDCs, there was a complete lack of IFN-α-induced sIL-15 complexes in ADAM17-deficient BMDCs (Figure 6B), showing IFN-α-induced sIL-15 complex generation in BMDC requires ADAM17 activity. Overall, these data provide evidence that type I IFNs induce cleavage of IL-15 complexes which requires expression of ADAM17.

We sought to determine the requirement for ADAM17 activity in the generation of sIL-15 complexes *in vivo*. ADAM17-/- mice die *in utero*, hence to examine this question we bred commercially available ADAM17 floxed mice (ADAM17$^{fl/fl}$) with mice expressing an inducible Estrogen–Receptor Cre (ER$^{T2}$-Cre), thereby generating a tamoxifen-inducible ADAM17 knockout strain of mice (ADAM17$^{fl/fl}$ x ER$^{T2}$-Cre+) (118-120).

**Figure 6. IFN-α-induced generation of sIL-15 complexes is dependent on ADAM17 in BMDCs.**

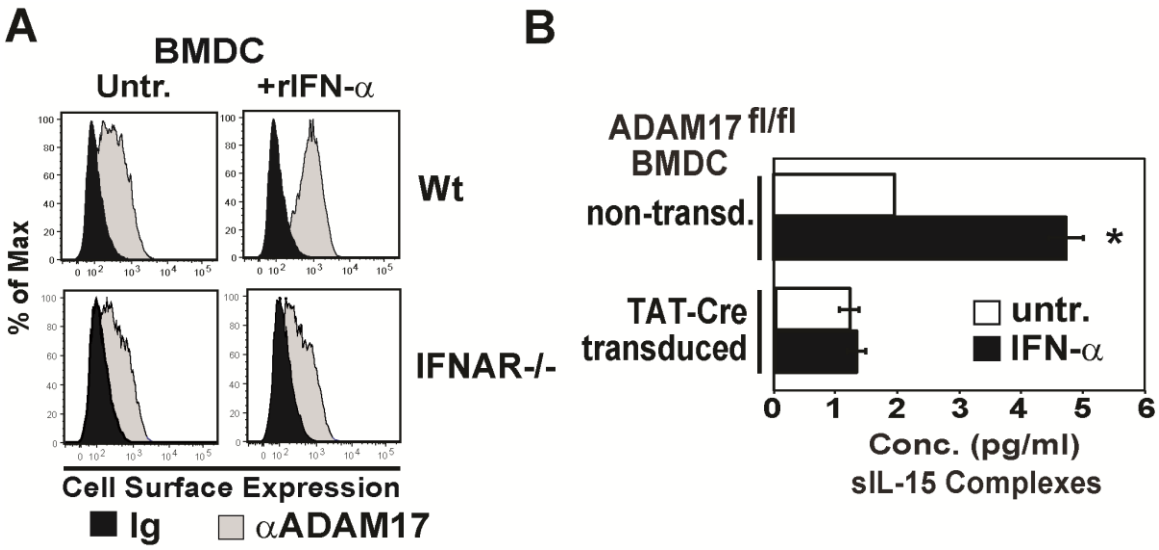
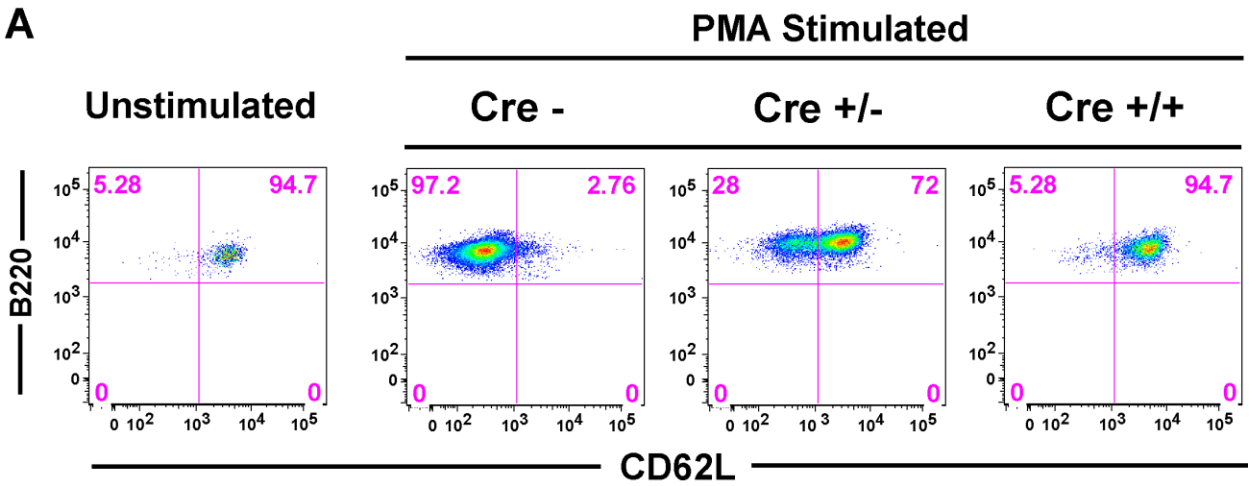



**Figure 6. IFN-α-induced generation of sIL-15 complexes is dependent on ADAM17 in BMDCs.** A) Cell-surface ADAM17 expression in Wt and IFNAR-/- BMDCs was detected by indirect immunofluorescence staining and flow cytometric analysis in untreated cells or after stimulation with rIFN-α (300U/mL) for 24 hrs. B) Non-transduced and TAT-Cre transduced BMDCs generated from ADAM17$^{fl/fl}$ mice were left untreated (open bars) or stimulated with rIFN-α (300U/mL) (filled bars). Culture supernatants were collected 24hrs later and analyzed for sIL-15 complexes. n=1-3 wells/group. Data is representative of two experiments. Error bars represent SD. * indicates $p<0.05$. This chapter is based upon and reproduced from the following journal article: Anthony SM, Howard ME, Hailemichael Y, Overwijk WW, and Schluns KS. Soluble interleukin-15 complexes are generated *in vivo* by type I interferon dependent and independent pathways. PLoS One. 2015 Mar 10; 10(3):e0120274. As per PLoS policy, no permission is required to reprint.

Recombination was induced in 6-12 week old ADAM17$^{fl/fl}$ x ER$^{T2}$- Cre+ mice by a tamoxifen treatment regimen; ADAM17$^{fl/fl}$ x ER$^{T2}$-Cre- littermates were also treated with tamoxifen and served as controls throughout these studies. To define the effectiveness in the loss of ADAM17 functional activity, an *ex vivo* stimulation protocol was designed. ADAM17 activity has been previously shown to be induced by stimulation with the Protein Kinase C activator Phorbol 12-myristate 13-acetate (PMA), and the cell-surface protein L-selectin (CD62L) is a known ligand of ADAM17-mediated cleavage (118). Therefore, after tamoxifen treatment, mice were screened for loss of ADAM17 inducible activity by PMA stimulation of peripheral blood lymphocytes before proceeding to *in vivo* experiments. Unstimulated circulating B cells were uniformly positive for CD62L expression in all samples analyzed irrespective of treatment or Cre expression (Figure 7A). Upon PMA stimulation, B cells isolated from Cre- mice had a nearly complete loss in CD62L expression (Figure 7A, B), as observed in a previous study with ADAM17 deficient B cells (121). However, PMA stimulation of Cre+ B cells resulted in the lack of CD62L cleavage in 70-75% of cells (Figure 7A), indicating an incomplete loss of ADAM17 functional activity in these mice. Therefore, we further generated mice containing 2 copies of the ER$^{T2}$-Cre transgene (ADAM17$^{fl/fl}$ x ER$^{T2}$-Cre+/+) and utilized the same PMA screening assay to screen for ADAM17 functional activity. PMA stimulation of B cells from tamoxifen-treated Cre+/+ mice had no effect on cleavage, as these cells retained uniform high expression of CD62L (Figure 7A, 7B), analogous to levels observed on un-stimulated B cells. This indicates ADAM17 functional activity is lost in the cells isolated from these treated ER$^{T2}$-Cre +/+ mice.

Utilizing our ADAM17$^{fl/fl}$ x ER$^{T2}$-Cre+/+ mice, we investigated the *in vivo* requirement for ADAM17 in the generation of sIL-15 complexes in response to Poly I:C stimulation. Surprisingly, we found no significant impairment in the ability for Poly I:C to induce sIL-15 complexes in the Cre+ or Cre+/+ groups in comparison to ADAM17-sufficient controls (Figure 8A).

**Figure 7: Deletion of ADAM17 functional activity in Tamoxifen-treated ER$^{T2}$-Cre x ADAM17$^{fl/fl}$ mice.**



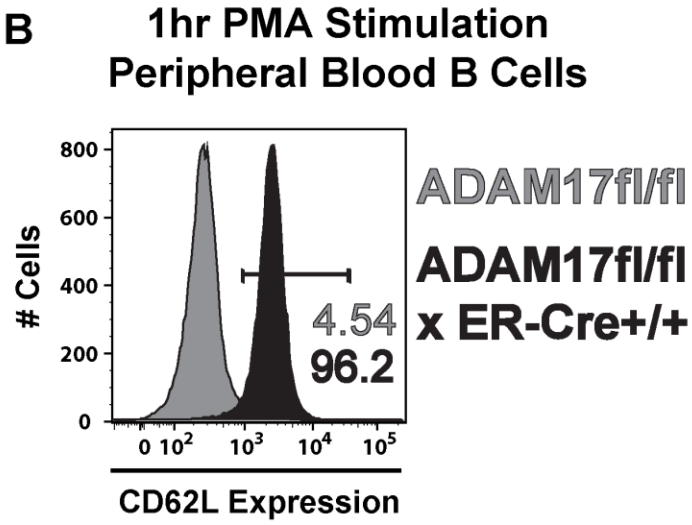



**Figure 7. Deletion of ADAM17 functional activity in Tamoxifen-treated $ER^{T2}$-Cre x $ADAM17^{fl/fl}$ mice** A) Tamoxifen-treated $ADAM17^{fl/fl}$ x $ER^{T2}$-Cre mice were analyzed for inducible ADAM17 activity by an *ex vivo* PMA screening assay. Briefly, peripheral blood was isolated, RBCs lysed and cells were incubated with RPMI alone (Unstimulated) or with PMA (25ng/mL) in RPMI for 1 hour at 37°C. Cultured cells were then washed and subjected to cell-surface antibody staining. The percentage and MFI of the ADAM17-dependent ligand CD62L was analyzed on circulating B cells due to their abundant numbers in peripheral blood. B) Representative histogram overlay of CD62L expression on PMA-stimulated peripheral B cells (CD19+, B220+) in $ADAM17^{fl/fl}$ mice (Grey) and $ADAM17^{fl/fl}$ x $ER^{T2}$-Cre+/+ mice (Black) n=3-4 mice/group. Data is representative of five experiments.

**Figure 8: ADAM17 is contributing to IFNAR-dependent Poly I:C-induced generation of sIL-15 complexes.**

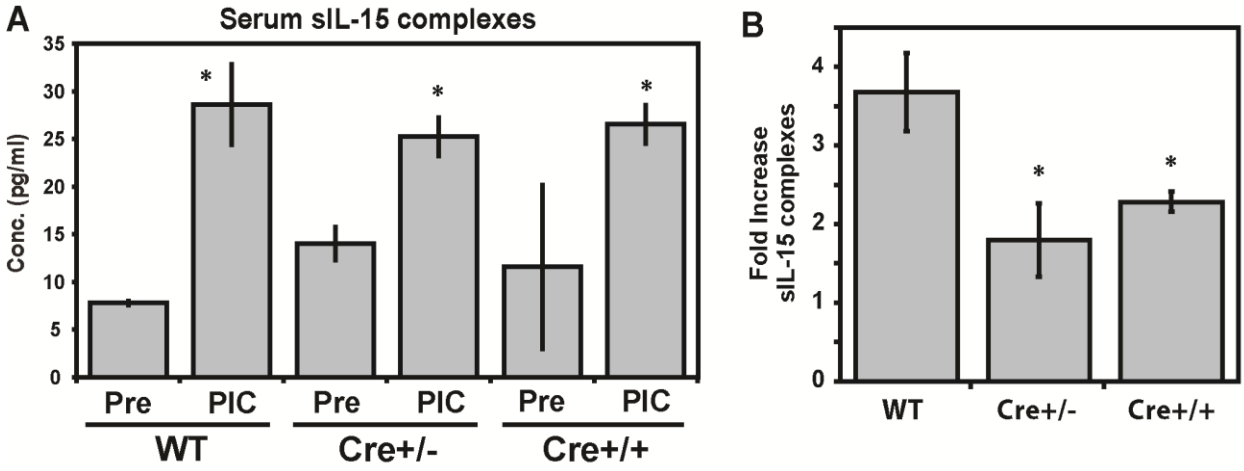



**Figure 8: ADAM17 is contributing to IFNAR-dependent Poly I:C-induced generation of sIL-15 complexes.**

A) Serum levels of sIL-15 complexes in tamoxifen-treated ADAM17$^{fl/fl}$, ADAM17$^{fl/fl}$ x ER$^{T2}$-Cre+/-, and ADAM17$^{fl/fl}$ x ER$^{T2}$-Cre+/+ mice, before stimulation (pre-bleed) and at 24 hours after Poly I:C stimulation (150μg / mouse, i.p.). One representative of two experiments is shown n=3-5 mice/group, B) Serum was collected from all mice prior to Poly I:C injection. Levels of sIL-15 complexes in pre-bleeds were compared to levels after Poly I:C stimulation and the average fold induction in sIL-15 complexes by Poly I:C was calculated for each of the three groups (levels of sIL-15 complexes with Poly I:C stimulation / pre-bleed). Levels of sIL-15 complexes in serum were measured using ELISA. n=3-4 mice/group; data is one representative of two experiments. All error bars represent SD. * indicates $p<0.05$ between sIL-15 complex levels observed in pre-beeds compared to serum post Poly I:C treatment .

However, the baseline levels of sIL-15 complexes were significantly elevated in tamoxifen-treated Cre+ and Cre+/+ mice (Figure 7A). Taking into consideration the differential baseline levels of sIL-15 complexes, we observed a significant loss in the fold induction of sIL-15 complexes (Figure 7B).

Since we determined that αCD40 stimulation induces sIL-15 complexes *in vivo*, we investigated the role of ADAM17 in the generation of CD40-induced sIL-15 complexes *in vivo*. Despite the lack of ADAM17-functional activity in Cre+/+ mice, we again found equivalent levels of sIL-15 complexes produced in these mice compared to untreated controls (Figure 9A). Unlike the Poly I:C-treated groups, the baseline levels of sIL-15 complexes were equivalent between groups irrespective of the absence of ADAM17 functional activity and we observed a comparable response to CD40 stimulation in the fold induction of sIL-15 complexes in ADAM17-deficient mice (Figure 9B). This demonstrates that ADAM17 is not required for the generation of sIL-15 complexes in response to CD40-stimulation *in vivo*. Overall, these data indicate that the role of ADAM17 in the generation of sIL-15 complexes *in vivo* differs substantially from its role observed during *in vitro* stimulations of BMDCs. ADAM17 partially contributes to IFNAR-dependent Poly I:C induction, but is not required for the Type I IFNAR-independent αCD40-induced generation of sIL-15 complexes *in vivo*.

**Figure 9: ADAM17 is not required for CD40-induced generation of sIL-15 complexes.**

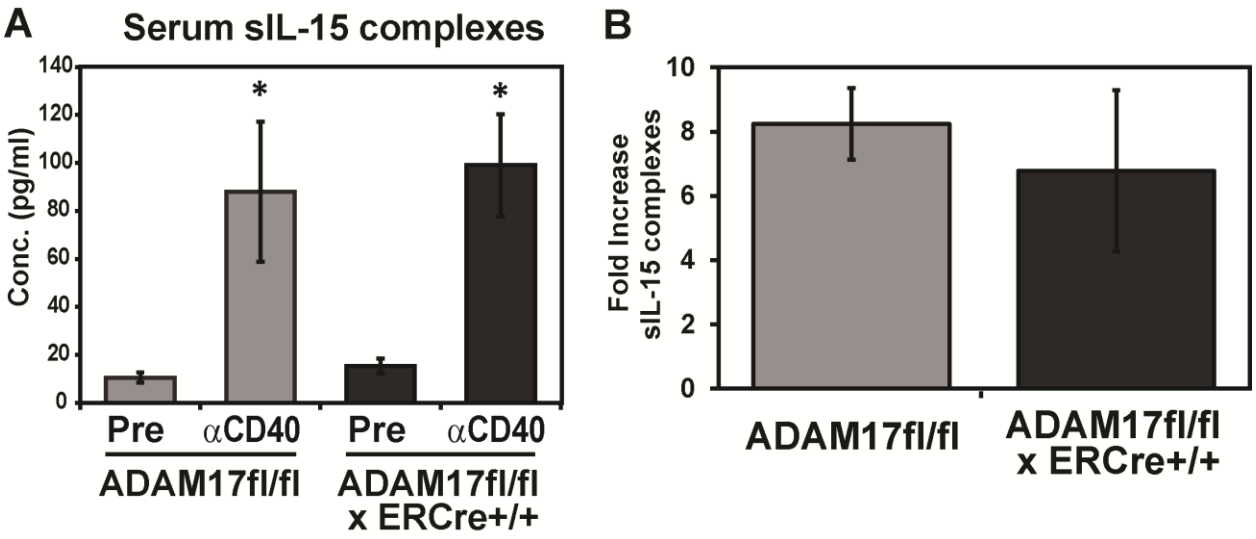



**Figure 9: ADAM17 is not required for CD40-induced generation of sIL-15 complexes.**

A) Serum levels of sIL-15 complexes in tamoxifen-treated ADAM17$^{fl/fl}$ and ADAM17$^{fl/fl}$ x ER$^{T2}$-Cre+/+ mice, before stimulation (pre-bleed) and 24 hours after αCD40 stimulation (FGK4.5, 50 µg / mouse, i.p.) (n=2-5 mice/group), one representative experiment of two is shown. B) Levels of sIL-15 complexes in pre-bleeds were compared to levels post αCD40 stimulation and the average fold induction (αCD40 / Pre) of sIL-15 complexes by αCD40 was calculated for each group. Levels of sIL-15 complexes in serum were measured using ELISA. n=2-5 mice/group; data is representative of two experiments. All error bars represent SD. * indicates $p<0.05$.

### 3.2.5: Cellular sources of sIL-15 complexes *in vivo*

IL-15 expression is tightly regulated at multiple levels throughout expression and intracellular trafficking of IL-15. In the absence of IL-15Rα expression, IL-15 protein remains sequestered in the cell (23). To define the hematopoietic cell types responsible for the generation of sIL-15 complexes in response to VSV infection, we bred commercially available IL-15Rα-floxed mice (IL-15Rα$^{fl/fl}$) to mice expressing Cre under the control of LysM- and CD11c-promoters, resulting in the elimination of IL-15Rα, and thereby IL-15 expression, in the Monocyte/Macrophage lineages or the Dendritic Cell lineages, respectively (23,122,123). These mice were previously generated and observed to have defects in IL-15-dependent lymphocyte populations (1379). IL-15Rα$^{fl/fl}$ only mice, IL-15Rα$^{fl/fl}$ x CD11cCre+/+ and IL-15Rα$^{fl/fl}$ x LysMCre+/+ mice were pre-bled for serum and the levels of circulating soluble IL-15 complexes were measured 24 hours post VSV infection. IL-15Rα$^{fl/fl}$ mice exhibited a significant up-regulation in sIL-15 complexes equivalent to levels observed in Wt B6 mice (Figure 10, Figure 4A). VSV-infection-induced sIL-15 complexes in IL-15Rα$^{fl/fl}$ x CD11cCre+/+ mice to a level equivalent to IL-15Rα$^{fl/fl}$ only mice (Figure 10), suggesting that DCs are not a dominant source of sIL-15 complexes in response to VSV infection. VSV also induced a significant increase in the amount of sIL-15 complexes in the IL-15Rα$^{fl/fl}$ x LysMCre+/+ mice (Figure 10), suggesting that cells other than DCs, monocytes and macrophages are contributing to VSV-induced sIL-15 complexes. In contrast, this induction in the IL-15Rα$^{fl/fl}$ x LysMCre+/+ mice was substantially less than the induction in the IL-15Rα$^{fl/fl}$ mice (Figure 10). This indicates that macrophages and monocytes are the dominant producers of sIL-15 complexes in response to VSV-infection.

**Figure 10: Macrophages and Monocytes are the dominant producers of VSV-induced sIL-15 complexes**

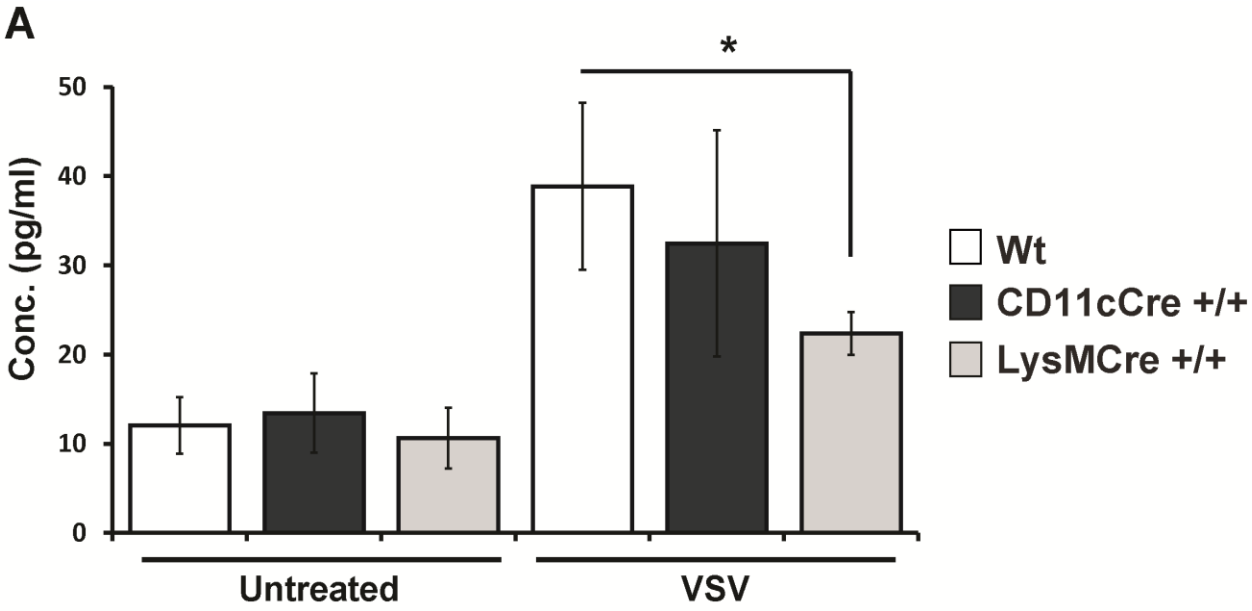



**Figure 10. Macrophages and Monocytes are the dominant producers of VSV-induced sIL-15 complexes** Serum levels of sIL-15 complexes in Wt IL-15R$\alpha^{fl/fl}$ (White), IL-15R$\alpha^{fl/fl}$ x CD11cCre+/+ (Black) and IL-15R$\alpha^{fl/fl}$ x LysMCre+/+ (Grey) mice were isolated from peripheral blood before treatment (Untreated) and 24 hours post VSV infection (VSV) ($1x10^6$ PFU/ mouse, i.v.) (n=2-5 mice/group), one representative of two experiments is shown. Levels of sIL-15 complexes in serum were measured using ELISA. All error bars represent SD. * indicates $p<0.05$.

Since we determined that CD40 stimulation directly induces the production of sIL-15 complexes in Flt3L DCs (Figure 5C), we sought to confirm the dependence of DCs in response to *in vivo* CD40-stimulation using our conditional DC IL-15Rα knockout mice (IL-15R$\alpha^{fl/-}$ x CD11cCre+). Loss of IL-15 expression specifically in DCs resulted in a significant, but incomplete loss in sIL-15 complexes (Figure 11A). The CD8$\alpha^+$ DC subset has been shown to have high baseline and VSV-inducible expression of IL-15; furthermore these DCs have high baseline expression of CD40 relative to other DC subsets (58,59,115). The development of CD8$\alpha^+$ DC subset is dependent on the basic-leucine zipper transcription factor ATF-like protein 3 (BATF3) and mice deficient in BATF3 lack the CD8$\alpha^+$ and CD103$^+$ DC subsets (76). We obtained BATF3-deficient mice and investigated the ability for CD40-stimulation to induce sIL-15 complexes in the absence of these DC subsets. Interestingly, despite the moderate induction of sIL-15 complexes in CD40-stimulated Flt3L-DC cultures, lack of BATF3-dependent DC lineages completely eliminated the ability for αCD40 to induce sIL-15 complexes *in vivo* (Figure 11B). This indicates that BATF3-dependent DC lineages are the dominant source of CD40-induced sIL-15 complexes *in vivo*. Therefore, despite CD40 stimulation inducing sIL-15 complexes by both Type I IFN and non-IFN pathways, this *in vivo* induction absolutely requires the presence of BATF3-dependent DCs.

**Figure 11: BATF3-dependent DCs are the dominant source of sIL-15 complexes in response to CD40 stimulation.**

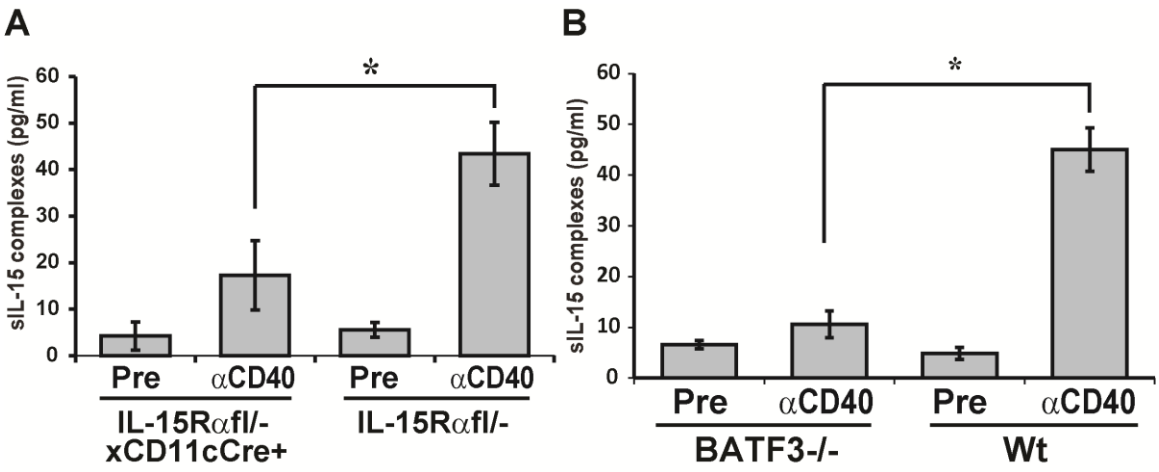



**Figure 11: BATF3-dependent DCs are the source of sIL-15 complexes in response to CD40 stimulation.** A) Serum levels of sIL-15 complexes in IL-15R$\alpha^{fl/-}$ and IL-15R$\alpha^{fl/-}$ x CD11cCre+ mice were isolated from peripheral blood before treatment (Pre) and 24 hours post CD40-stimulation (FGK4.5, 50µg / mouse i.p.). (n=2-5 mice/group), one representative experiment of two is shown. B) Serum levels of sIL-15 complexes in Wt and BATF3-/- mice were analyzed in pre-bleeds and 24 hours post CD40-stimulation (FGK4.5, 50µg / mouse i.p.). (n=5-7 mice/group), one representative experiment of three is shown. Levels of sIL-15 complexes in serum were measured using ELISA. All error bars represent SD. * indicates $p<0.05$.

**3.3 Discussion.**

Our demonstration that IFN-α increases sIL-15 complexes in BMDCs and requires IFNAR signaling identifies Type I IFNs as one direct signaling pathway that induces sIL-15 complexes. Since IFN-α is often elevated in Systemic lupus erythematosus and Psoriasis (124,125), these are conditions that likely exhibit elevated local and/or systemic sIL-15 complexes. Furthermore, we found that continual Type I IFN-signaling *in vivo* can sustain production of sIL-15 complexes, implicating the absence of a negative feedback loop at the level of sIL-15 complex generation. This is substantial as it suggests that diseases with deregulated Type I IFN signaling would likely exhibit persistent production of sIL-15 complexes that theoretically should be stimulating CD8 T cell and NK cell responses and contributing to disease activity. Therefore, IFN-mediated increases in sIL-15 complexes could be one mechanism responsible for increased CD8 T cell and NK cell activity during these types of diseases.

As shown in this study, VSV infection and CD40 stimulation induce a robust but transient generation of sIL-15 complexes. Type I IFNs are induced upon a VSV infection (73), and we determined that CD40 stimulation also induced IFNα protein expression *in vivo* (Figure 5F). Interestingly, the production of sIL-15 complexes after VSV infection was unaffected in the absence of Type I IFN signaling, while CD40-induced sIL-15 complexes were reduced but still generated to a significant level in IFNAR-/- mice. This shows that additional pathways contribute to the inducible production of sIL-15 complexes; and in the case of VSV, this pathway is independent of both Type I IFN and CD40 signaling. While these results are surprising, they are not unprecedented as *in vivo* injection of LPS has been shown to increase IL-15 transcription in IFNAR-/- mice (62). Interestingly, although increased transcription of IL-15 during a VSV infection requires Type I IFN signaling (58), the abundance of sIL-15 complexes in VSV-infected IFNAR-/- mice provides evidence that the cleavage of IL-15 complexes is not

necessarily linked to the transcription of IL-15 and IL-15Rα. Alternatively, increases in IL-15 transcription mediated by IFNAR independent pathways may be occurring earlier than previously investigated. In fact, IL-15 transcription increases in IFNAR-/- mice as early as 4 hrs after LPS treatment (126). These results indicate that increases in sIL-15 complexes are a much more global event than previously realized. With the broad number of signaling pathways that are induced by these stimuli, especially in the case of active VSV infection, our results suggest the presence of numerous possible redundant pathways to generate sIL-15 complexes. Altogether, these findings show Type I IFNs directly regulate IL-15 responses, but that additional Type I IFN-independent pathways exist, which are fully capable of inducing the generation of sIL-15 complexes.

CD40 stimulation was previously shown to induce DC-specific IL-15Rα expression and IL-15-dependent anti-tumor responses (66,69). We determined that CD40 stimulation also induces the generation of systemic sIL-15 complexes. Furthermore, similar to VSV infection, CD40 stimulation also did not absolutely require Type I IFN signaling for the induction of sIL-15 complexes. These results provide additional support for an alternative sIL-15 complex-inducing pathway, which may involve signals stimulated by TLRs, additional inflammatory cytokines, and/or cell death which is further analyzed in Chapter 4. As agonistic antibodies stimulating CD40 are promising cancer immunotherapy agents, future analyses should examine the potential role of CD40-induced sIL-15 complexes in the induced anti-tumor immune responses (70).

Whereas Type I IFNs increase the transcription of IL-15 and IL-15Rα and the cell surface expression of IL-15Rα/IL-15 complexes (58,61,62,64,111,112), our current study demonstrates that Type I IFNs also upregulate cleavage of cell surface IL-15Rα/IL-15. Evidence

that IFN-α directly regulates cleavage of IL-15 complexes include our demonstration that ADAM17 is increased in BMDCs by IFN-α and is required for IFN-α-mediated increases in sIL-15 complexes *in vitro*. Despite these *in vitro* results, though our novel inducible ADAM17 knockout model we surprisingly determined that ADAM17 activity was not necessary for the *in vivo* generation of sIL-15 complexes in response to TLR3 or CD40 stimulations. We did observe a slight but significant loss in the fold induction of sIL-15 complexes in response to Poly I:C, but not to CD40. Interestingly, this mirrors these pathways' relative dependence upon Type I IFN signaling for this induction. Nonetheless, these results clearly indicate that ADAM17 is not the sole protease capable of cleaving IL-15Rα *in vivo*.

The finding that ADAM17 is capable of cleaving IL-15 complexes is not unexpected, as previous studies demonstrated through *in vitro* cultures that ADAM17 induces cleavage of membrane-bound IL-15Rα (52,117); our stimulations of ADAM17 knockout BMDCs corroborate these previous results. However, we found that a compensatory and/or entirely separate cleavage pathway exists *in vivo*, which clearly does not require or involve ADAM17. Based on these results it is interesting to speculate a model in which Type I IFN signaling is capable of inducing ADAM17-dependent cleavage of cell-surface bound sIL-15 complexes, while *in vivo* an additional Type I IFN-independent pathway uses an additional unknown protease to induce cleavage. Our primary candidate for this compensatory protease is the closely related protease ADAM10 as previous studies have demonstrated that activation of ADAM10 by Ionomycin in the absence of ADAM17 is sufficient to induce cleavage of multiple ADAM17-dependent substrates (121). Although, it should be noted that we did not observe a compensatory ability for PMA to induce *ex vivo* cleavage of the ADAM17 ligand CD62L in ADAM17 deficient peripheral B cells (Figure 7A, B). Regardless, these results clearly point to an alternative or compensatory mechanism for this induction *in vivo* which should be further investigated in future studies.

Our findings in concert with those of Le Gall et. al. together have the potential to at least partially explain the lack of efficacy of ADAM17-targeted therapies in IL-15 dependent diseases in clinical trials (121). Additional studies will need to further examine what we know about ADAM17 and other additional potential proteases in the cleavage of cell surface bound IL-15Rα.

Unfortunately, the role of this burst in sIL-15 complexes early in an infection is unclear as there is no current *in vivo* model that allows one to segregate IL-15-dependent responses mediated by sIL-15 complexes from those mediated by transpresentation. Our data showing that increases in surface IL-15 and sIL-15 complexes are concurrent events suggest both mechanisms are feasible in eliciting IL-15-mediated responses. Nevertheless, it is not unreasonable to speculate that these sIL-15 complexes could enhance immune responses by stimulating NK cells and memory CD8 T cells generated from a prior encounter, as these are two populations very responsive to recombinant sIL-15 complexes (45). These endogenously produced sIL-15 complexes also have the ability to act immediately as they do not require a cell-cell interaction to mediate responses; this is in contrast to transpresentation, which requires immune cells to locate their cellular source for IL-15. Additionally, even though naive CD8 T cells are not as responsive to IL-15 as memory CD8 T cells, it is possible that early exposure to sIL-15 complexes could influence T cell priming. A recent study by Tamzalit F. et al (127) provides evidence that cleavage of IL-15Rα/IL-15 complexes from the surface is important for efficient translocation and internalization of IL-15 complexes to opposing cells during transpresentation. If this is the case, then the production of sIL-15 complexes we observe may be a biomarker of concurrently enhanced IL-15 transpresentation. But more importantly, this scenario doesn't discount those circulating sIL-15 complexes, which have agonistic activity and could be acting on IL-15-responsive cells. Recent studies from the laboratory of Dr. John Harty have demonstrated that viral-induced IL-15 has a significant by-stander impact on both the

trafficking and proliferation of memory CD8 T cells but not naïve CD8 T cells in an antigen independent manner (84,101). In Chapter 4 we will address the functional role of inflammatory lymphopenia-induced IL-15, as we have observed an analogous dominant role for lymphopenia-induced IL-15 on the proliferation of memory CD8 T cells (Figure 23).

Macrophages and DCs were determined to be the principal cellular sources of sIL-15 complexes in response to VSV infection and CD40 stimulation respectively. Taking into account the cell surface IL-15 expression data, where macrophages exhibited the highest cell surface IL-15 expression after VSV-infection (Figures 3A, B), it is not unexpected that we also find these cells are the primary producers of sIL-15 complexes under the same conditions (Figure 10). This may seem a bit trivial, as one would likely expect cell surface IL-15 expression to correlate with the production of sIL-15 complexes; however, our data provides definitive evidence, rather than mere correlation of the role of LysM+ cells in the generation of sIL-15 complexes during VSV-infections *in vivo*. In a previous report, VSV induced the highest up-regulation of IL-15 transcription in the CD8$\alpha$+ DC subset (58). While we also observed an induction in DC cell surface expression (Figure 3B), CD8$\alpha$+ DCs were not specifically analyzed. In the absence of DC-derived IL-15 we did observe slightly reduced sIL-15 complexes (Figure 10), which does not discount DCs a potential source of sIL-15 complexes; however, the monocyte/macrophage subsets were clearly the dominant IL-15 protein expressing cells following VSV infection.

In response to CD40 stimulation, loss of DC-specific IL-15 (Figure 11A) resulted in significantly reduced sIL-15 complexes. Additionally, loss of BATF3-dependent DCs (Figure 11B) resulted in a complete loss of CD40-induced sIL-15 complexes. Therefore BATF3-DCs are likely the source of CD40-induced sIL-15 complexes. Alternatively, it is possible that the CD40 stimulation induces BATF3-dependent DCs to produce an intermediate factor which then

prompts an additional cell type to generate sIL-15 complexes; however, the dominant cytokine induced by CD40-stimulation, IL-12, was unable to induce sIL-15 complexes from Flt3L-DCs (Figure 5D). In any sense, it is clear that BATF3-dependent DCs are required for the generation of sIL-15 complexes in response to CD40 stimulation.

In summary we have characterized the dynamic induction of inflammatory IL-15, comprising both transpresented IL-15 and sIL-15 complexes which are correspondingly induced in response to IFN-$\alpha$ stimulation, VSV infection, and CD40 stimulation. Although Type I IFN signaling directly induced the generation of sIL-15 complexes, its activity was surprisingly not required for this induction in response to VSV infection or CD40 stimulation pointing to the existence of additional pathways for the generation of sIL-15 complexes. During *in vitro* stimulations, the metalloprotease ADAM17 was determined to be responsible for and fully capable of generating sIL-15 complexes; although its activity was not required for the production of the majority of sIL-15 complexes in response to *in vivo* CD40 or TLR3 stimulations. These results indirectly acknowledge the presence of additional or compensatory proteases which are responsible for the observed induction of sIL-15 complexes *in vivo*. We have also determined that macrophages and DCs are bona fide cellular sources of transpresented IL-15 and sIL-15 complexes in response to VSV infection and CD40 stimulation, respectively. Overall, we have observed multiple levels of redundancy in the generation of sIL-15 complexes including multiple pathways for induction, cleavage by multiple proteases and the production from multiple hematopoietic cell types.

**Chapter 4:**

This chapter is partially based upon and reproduced from the following journal article: Anthony SM, Howard ME, Hailemichael Y, Overwijk WW, and Schluns KS. Soluble interleukin-15 complexes are generated *in vivo* by type I interferon dependent and independent pathways. PLoS One. 2015 Mar 10; 10(3):e0120274. As per PLoS policy, no permission is required to reprint.

## Chapter 4: Examine the regulation of IL-15 expression during lymphopenia.

### 4.1 Introduction

IL-15 responses by T cells are elevated after the depletion of lymphocytes by total body irradiation (TBI) or high dose chemotherapy regimens (CTX) (102,107,128). Additional support for lymphopenia-mediated upregulation in IL-15 expression comes from the fact that endogenously lymphodeficient RAG-/- mice also exhibit elevated surface expression of IL-15 on DCs (129), in addition to having enhanced IL-15 dependent responses (102,130). Lymphopenia induces the proliferation of adoptively transferred T cells in addition to promoting a loss of tolerance against self-antigens, leading to enhanced IL-15-dependent anti-tumor responses (81). Lymphodepletion is widely used as a conditioning regimen for adoptive T cell therapies of melanoma in mouse models and clinical human treatments, and this is partially due to its ability to enhance IL-15 expression (102,107). The mechanisms of lymphopenia enhanced IL-15 expression have traditionally been viewed as a passive process, whereby the availability of the constant low level production of IL-15 is enhanced simply due to the loss or lack of endogenous lymphocytes utilizing this IL-15; this theory is often referred to as the cytokine sink (102). An additional report demonstrated that the anti-tumor effect induced by total body TBI is not passive and instead requires an active component of inflammation (131). Further studies found that in addition to enhancing IL-15 dependent anti-tumor responses, these lymphodepletion regimens directly induce a transient but systemic induction of sIL-15 complexes in mice and humans (104). Based on these results, IL-15 is clearly a dominant lymphopenia induced cytokine; **however, the signals mediating this induction and the cell types responsible for the generation of IL-15 under lymphopenia are currently unknown.** Using multiple mouse models of lymphopenia, we investigated the factors and cell types required for the generation of lymphopenia-induced IL-15 and sIL-15 complexes.

## 4.2 Results.

### 4.2.1 Lymphopenia-induced IL-15 expression

Serum sIL-15 complexes levels are reported to increase after TBI and chemotherapy coinciding with increased T cell and NK cell responses to IL-15 (104) implicating the induction of sIL-15 complexes is important during immunotherapy. Therefore, our first goal was to investigate the regulation of IL-15 complexes during various types of lymphopenia.

Similar to previous reports, TBI increased the levels of sIL-15 complexes in serum isolated from peripheral blood 24 hours post treatment (Figure 12A). In contrast to previous reports (104), we did not observe a significant increase in serum sIL-15 after cyclophosphamide chemotherapy (CTX) treatment at 72 hours post treatment in multiple experiments (Figure 12A). This lack of up-regulation of sIL-15 complexes was not due to insufficient drug delivery as CTX treatment lead to abundant lymphopenia, similar to the levels observed with TBI and published CTX-depletion levels (data not shown) (104). Analysis of earlier time points also did not reveal effects of CTX on serum sIL-15 complex levels (Figure 12B). Differences in the housing conditions of our mice may contribute to our inability to reproduce the findings of earlier studies. Unfortunately, the lack of significant increases in sIL-15 complexes by CTX precludes us from further investigating mechanisms in this model.

Despite RAG-/- mice being endogenously lymphopenic, the levels of sIL-15 complexes were equivalent to those at baseline (Figure 12A). To determine if baseline levels of sIL-15 complexes in untreated Wt mice were above the detection of the ELISA, serum from IL-15R$\alpha$-/- mice were analyzed and found to contain no detectable levels of sIL-15 complexes (Figure 12A).

**Figure 12: Lymphopenic RAG-/- mice do not have elevated sIL-15 complexes.**

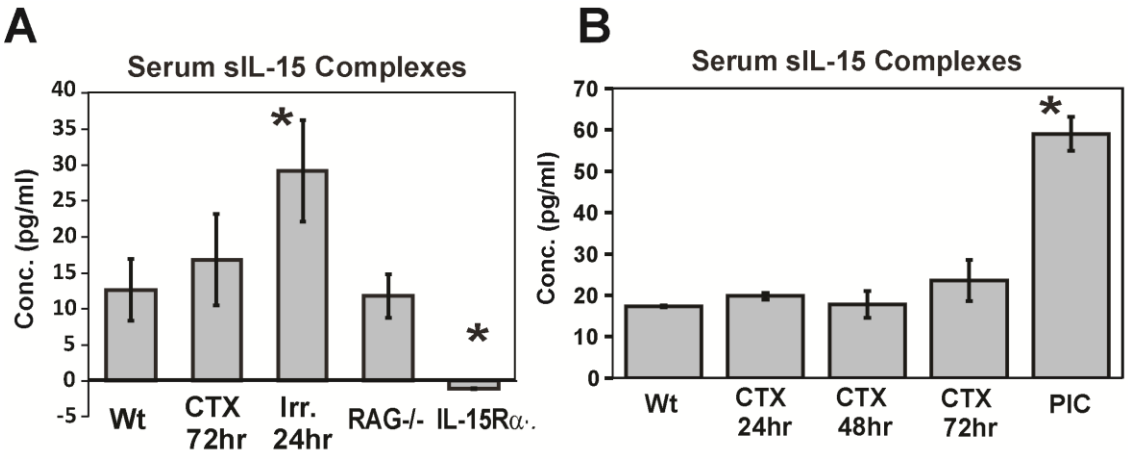



**Figure 12: Lymphopenic RAG-/- mice do not have elevated sIL-15 complexes.** A) Wt mice were subjected to TBI (800 RADS, 24 hrs) or CTX (200mg/kg, 72 hrs) or untreated endogenously lymphodeficient RAG-/-, or IL-15Rα-/- mice. Serum was isolated from peripheral blood at the indicated times post stimulation and sIL-15 complex levels were determined by ELISA. n=3-4 mice/group. Data is one representative of three experiments. B) Serum levels of sIL-15 complexes in Wt mice subjected to CTX (200mg/kg) at the indicated times post treatment. Serum from mice treated with Poly I:C (150µg i.p.) for 24 hrs was used as a positive control. n=2-3 mice/group, one representative of two experiments is shown. Error bars represent SD. * indicates $p<0.05$.

These results highlight the unappreciated finding that low levels of sIL-15 complexes are circulating during homeostatic conditions and therefore may be mediating IL-15 responses during the steady state. Overall, these results suggest that sIL-15 complexes are produced during the steady state and these levels are enhanced following TBI. In addition, the enhancement of sIL-15 complexes is not a feature common among all forms of lymphopenia, suggesting an additional component may be required for the generation of sIL-15 complexes.

We sought to further investigate the time-course and dose-response of sIL-15 complex production in response to TBI. Elevated sIL-15 complexes were noted by only 16 hrs post TBI (Figure 13A), reached peak levels at 24 hrs and returned to baseline 48 hrs post stimulation. Our observed time-course is similar to previously published results (104). Despite the induction of significant lymphopenia at only 800 RADS by splenocyte cell counts (data not shown), the levels of sIL-15 complexes were further elevated in response to higher doses of TBI (Figure 13B). Overall, we see that TBI quickly induces significant levels of sIL-15 complexes in a time and dose-dependent manner.

**Figure 13: TBI induces sIL-15 complexes in a time and dose-dependent manner**

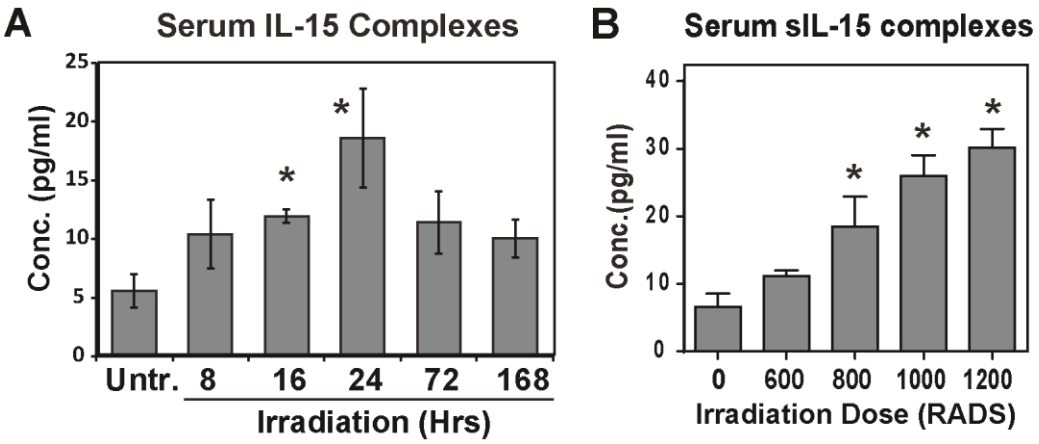



**Figure 13: TBI induces sIL-15 complexes in a time and dose-dependent manner** A) Wt mice were subjected to TBI (1000 RADS), serum was isolated from peripheral blood at the indicated times post stimulation and sIL-15 complex levels were determined by ELISA. n=2-4 mice/group. B) Serum was isolated 24 hours after treating Wt mice with varying levels of TBI. Levels of sIL-15 complexes in serum were measured using ELISA. n=3-4 mice/group. Induction of sIL-15 complexes after TBI has been observed in at least 10 experiments. Error bars represent SD. * indicates $p<0.05$. This figure is adapted and reproduced from the following journal article: Anthony SM, Howard ME, Hailemichael Y, Overwijk WW, and Schluns KS. Soluble interleukin-15 complexes are generated *in vivo* by type I interferon dependent and independent pathways. PLoS One. 2015 Mar 10; 10(3):e0120274. As per PLoS policy, no permission is required to reprint.

### 4.2.2 Inflammatory mediators of lymphopenia-induced IL-15

A role for inflammation and Type I IFN signaling in the support of TBI-mediated anti-tumor immunity has been previously established (131). This study determined that TBI-induced damage to the intestinal lining resulted in enhanced levels of circulating LPS from commensal bacteria and this circulating LPS alone was capable of facilitating enhanced anti-tumor responses; however, this induction in LPS was not evident until 6 days post TBI while we observed sIL-15 complexes as early as 16 hrs post TBI (Figure 13A). Therefore, we investigated the potential role of serum LPS as an inducer of sIL-15 complexes. Baseline LPS levels in untreated mice were equivalent to those observed in the aforementioned previous study (131). In contrast to previous reports, we did not observe any changes in serum LPS levels at any of the times during a week long time-course post TBI (Figure 14A) This is despite a significant induction of sIL-15 complexes which was observed in the same mice (Figure 13A). Our lack of LPS induction could be due to differences in the microbiota of our mice, but since LPS was not induced, this could not be a mechanism responsible for our observed induction of sIL-15 complexes.

TBI was previously shown to increase serum sIL-15 complexes and additional reports have shown Type I IFN mRNA increases after local irradiation (104,132), so we examined if Type I IFNs proteins were important for the induction of sIL-15 complexes after TBI. Systemic IFN-$\alpha$ protein levels were increased in mice given TBI (Figure 15A). To assess the importance of Type I IFN in the TBI-mediated induction of sIL-15 complexes, IFNAR-/- mice were analyzed. In the absence of IFNAR, sIL-15 complexes were not induced to a significant level after TBI (Figure 15B) suggesting Type I IFNs are a required signal inducing sIL-15 complexes in response to TBI.

**Figure 14: High dose TBI does not induce circulating LPS**

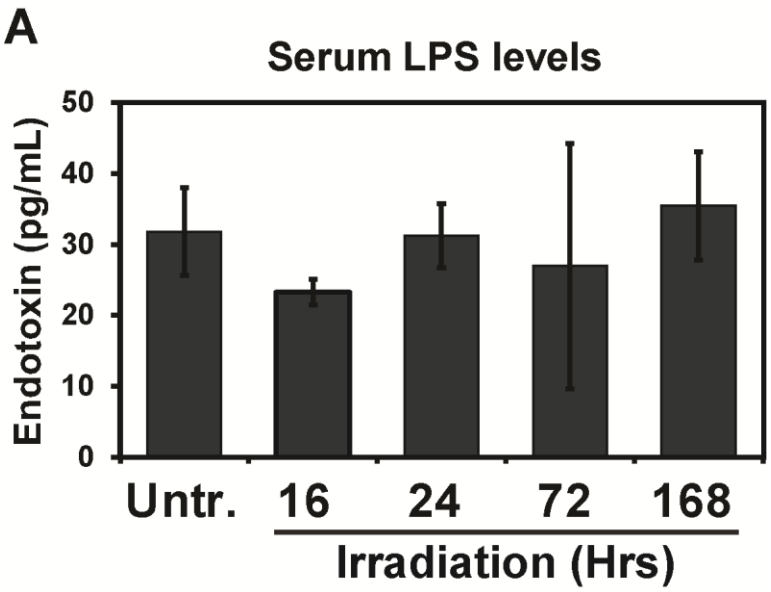



**Figure 14: High dose TBI does not induce circulating LPS**

A) Wt mice were subjected to TBI (1000 RADS), serum was isolated from peripheral blood at the indicated times post stimulation and levels of circulating serum LPS were measured using limulus amebocyte lysate assay. n=2-4 mice/group, one representative of two experiments is shown. Error bars represent SD.

**Figure 15: Type I IFN signaling is required for increasing sIL-15 complexes after TBI.**

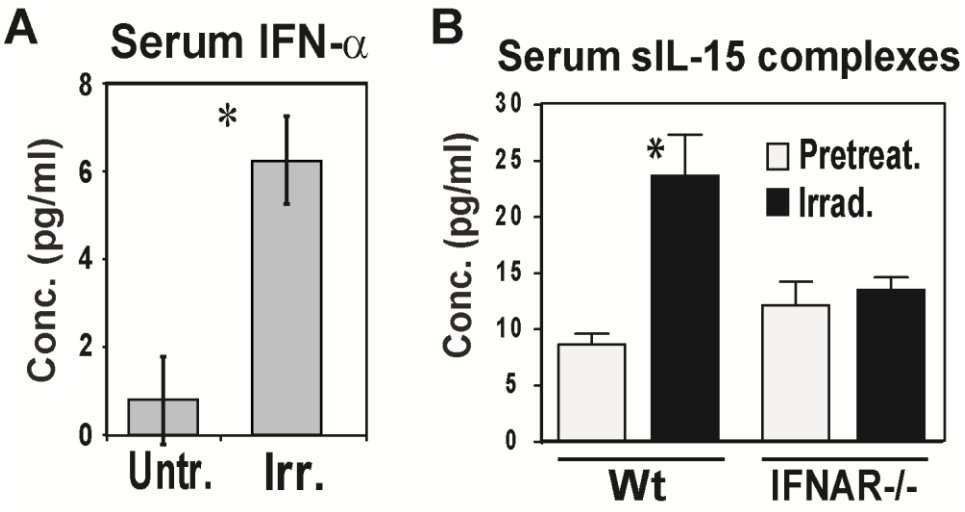



**Figure 15. Type I IFN signaling is required for increasing sIL-15 complexes after TBI.** (A) IFN-α levels were measured in serum isolated from Wt mice 24 hrs after treatment with 1000 RADS of TBI. One representative of two experiments is shown. (B) Serum was isolated from Wt and IFNAR-/- mice 24 hours after treatment with TBI (1000 RADS). Levels of sIL-15 complexes in serum were measured using ELISA. n=3-4 mice/group; one representative of 4 experiments. Error bars represent SD. * indicates $p<0.05$. This figure is adapted and reproduced from the following journal article: Anthony SM, Howard ME, Hailemichael Y, Overwijk WW, and Schluns KS. Soluble interleukin-15 complexes are generated *in vivo* by type I interferon dependent and independent pathways. PLoS One. 2015 Mar 10; 10(3):e0120274. As per PLoS policy, no permission is required to reprint.

An additional potential mechanism for lymphopenia-induced lymphocyte responses is due to depletion of the cellular cytokine sink (102). This theory postulates that as lymphopenia depletes the cell types dependent upon the homeostatic cytokines IL-7 and IL-15, removal of these cells passively leads to an enhanced bio-availability of IL-7 and IL-15. Several reports have directly found enhanced levels of circulating IL-15 in response to TBI or CTX lymphopenia-inducing regimens in mice and human patients (104,107); however, it is currently unknown what form of IL-15 is generated during lymphocyte depletion. Thus, we examined the ability for active complement-mediated depletion of lymphocytes to induce IL-15 complexes in the absence of gut breach. Mice were sufficiently depleted of lymphocytes using complement fixing antibodies anti-Thy1.2 mAb (300 µg 30H12 i.p.) alone or in concert with anti-NK1.1 (GK4.5, mAb). One day after Ab treatment, levels of serum sIL-15 complexes were significantly elevated in response to αThy1 treatment (Figure 16A) and further enhanced with the addition of αNk1.1. This result directly shows that depletion T cells in the absence of intestinal breach results in the generation of sIL-15 complexes *in vivo*.

Antibody-mediated depletion regimens require complement-mediated cell death of the targeted cells. Complement-mediated depletion is an active process which has been shown to result in systemic inflammation *in vivo* (133), therefore we examined the role of Type I IFN signaling in the αThy1-mediated induction of sIL-15 complexes. Interestingly, lack of Type I IFN signaling resulted in an approximate 50% reduction of induced sIL-15 complexes (Figure 16B) in response to αThy1 treatment. Hence, both TBI and complement-mediated depletion of T cells lead to a significant amount of cell death in a rapid manner; but their reliance on Type I IFN signaling to mediate these effects varied. Altogether these findings suggest that immunogenic cell death may be a signal leading to the induction of sIL-15 complexes.

**Figure 16: αThy1 mediated induction of sIL-15 complexes is partially dependent on Type I IFN signaling**

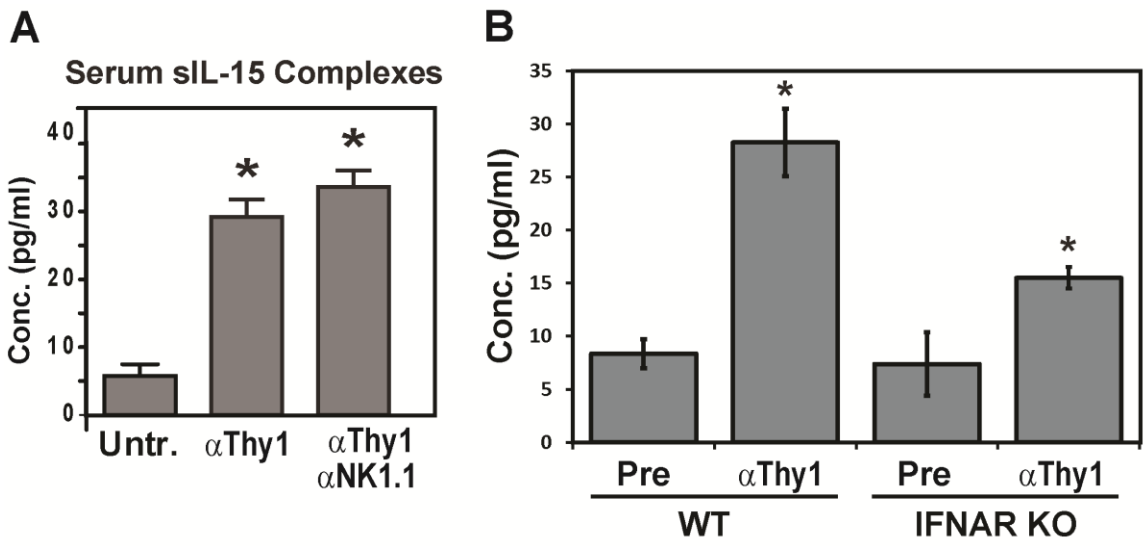



**Figure 16: αThy1 mediated induction of sIL-15 complexes is partially dependent on Type I IFN signaling** A) Wt mice were left untreated, subjected to αThy1.2 injection (300ug, i.p.) alone or in concert with αNK1.1 MAb. Serum was isolated from peripheral blood 24 hours post injection and IL-15 complex levels were determined by ELISA. n=2-3 mice/group, one representative of three experiments. (B) Serum was isolated from Wt and IFNAR-/- mice 24 hours after αThy1.2 injection (300ug, i.p.). Levels of sIL-15 complexes in serum were measured using ELISA. n=3-4 mice/group, one representative of three experiments is shown. Error bars represent SD. * indicates $p<0.05$.

### 4.2.3 STING pathway in the generation of sIL-15 complexes

As a field, we are beginning to appreciate the role which endogenous Damage-Associated Molecular Patterns (DAMPs) play in inducing innate and subsequent adaptive immune responses (4,10). The Stimulator of Interferon genes (STING) serves as an important recognition and adaptor of bacterial and viral DNAs leading to the induction of Type I IFNs (13,14). Activation of the STING pathway has also recently been demonstrated to be required in the absence of infection for the development of antitumor immune responses mediated by CD8 T cells (6,7). These responses were found to be completely dependent upon a tumor released DAMP (DNA) and Type I IFN signaling. As Type I IFN production is also a potent inducer of IL-15 (62) and sIL-15 complexes (Figure 1), we investigated the role of the STING pathway in the induction of sIL-15 complexes.

The STING ligand c-di-GMP significantly induced sIL-15 complexes from Flt3L BMDCs, more so than stimulation with IFN-$\alpha$ alone (Figure 17A). Injection of 25$\mu$g c-di-GMP by the i.v. route *in vivo* was superior to injections via the i.p. route in the ability to induce sIL-15 complexes in both circulation (Figure 17D) and in splenic homogenates (Figure 17B) therefore the i.v. route was used for all subsequent experiments. During a time-course analysis, c-di-GMP injection induced high levels of circulating sIL-15 complexes quickly, as all mice had significantly increased sIL-15 complexes in circulation and splenic homogenates within 12 hours of injection (Figures 17E, C), and these levels remained elevated 24 hours after injection. As the STING pathway is a known inducer of Type I IFNs, we examined the requirement of Type I IFN signaling in STING-induced sIL-15 complexes (13,14).

**Figure 17: Activation of STING induces sIL-15 complexes in a Type I IFN-dependent manner.**

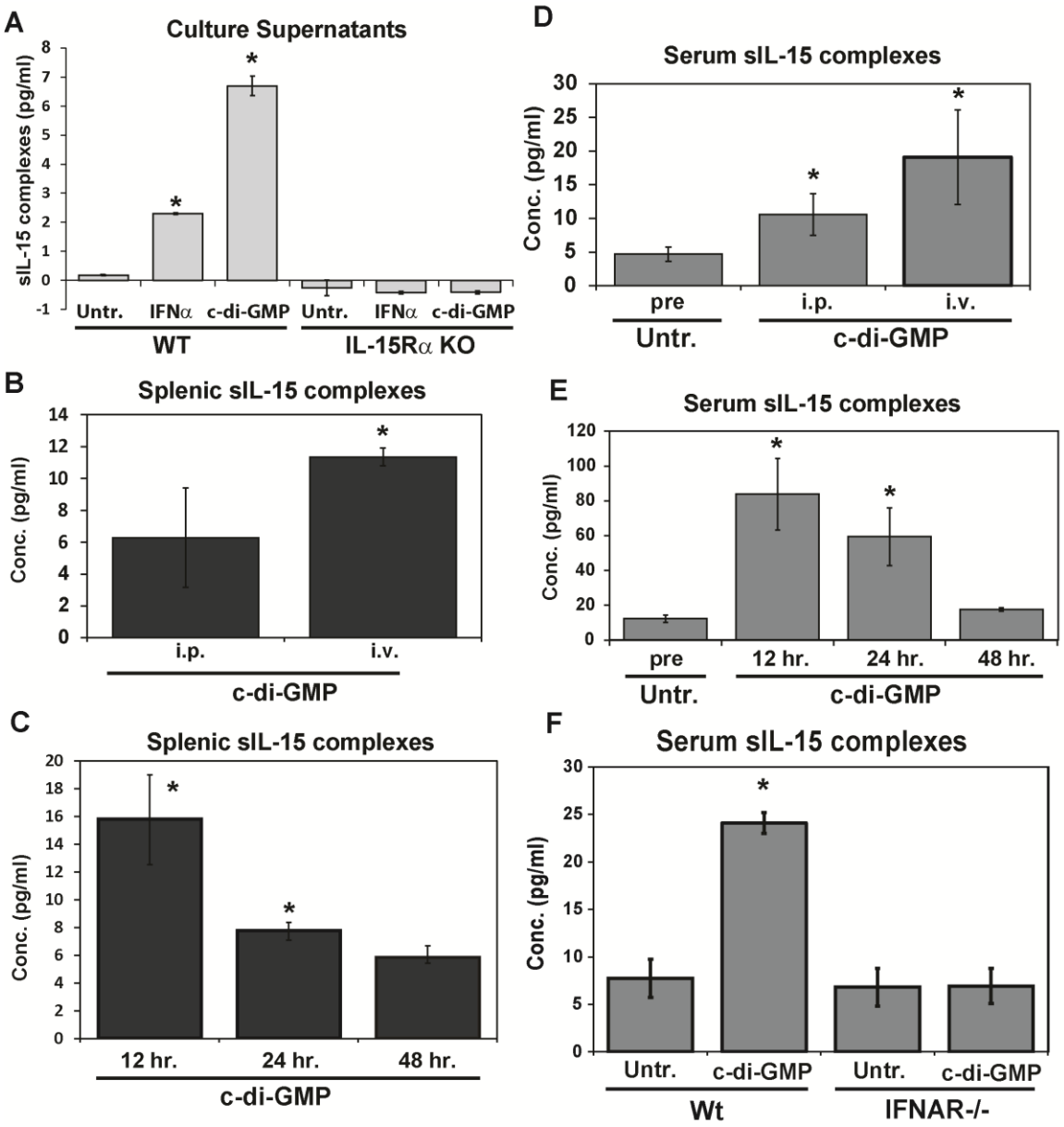


**Figure 17: Activation of STING induces sIL-15 complexes in a Type I IFN-dependent manner.** A) Flt3L BMDCs were generated from Wt or IL-15Rα-/- mice and left unstimulated, stimulated with IFN-α (300U/mL) or with the STING agonist c-di-GMP (25μg/mL) for 24 hours. Cell culture supernatants were isolated and measured for the levels of sIL-15 complexes by ELISA. n=2 wells/group, one representative of two experiments is shown. B) c-di-GMP (25μg) was injected *in vivo* via i.p. or i.v. routes and sIL-15 complexes were analyzed 24 hours post injection from peripheral blood (D) or from splenic supernatants (B). n=2-3 mice/group, one representative of two experiments is shown. C) c-di-GMP (25μg) was administered i.v. and the levels of sIL-15 complexes were analyzed from peripheral blood (E) or from splenic supernatants (C) at the indicated times post injection. n=2-3 mice/group, one representative experiment of two experiments is shown. F) c-di-GMP (25μg) was injected i.v. into Wt or IFNAR-/-mice, levels of sIL-15 complexes were analyzed 12 hours post injection. n=3 mice/group, one representative of two experiments is shown. Error bars represent SD. * indicates $p<0.05$.

In IFNAR-/- mice, the STING agonist was completely unable to induce sIL-15 complexes (Figure 17F), demonstrating that Type I IFNs are a required intermediate in STING-induced sIL-15 complexes. Collectively, this demonstrates that c-di-GMP is a potent inducer of sIL-15 complexes and requires Type I IFN signaling to mediate these effects.

As the STING pathway has been determined to play an important role in the induction of Type I IFN in response to PAMPs and DAMPs, we next investigated the requirement for STING signaling in TBI-induced sIL-15 complexes (6,7,13,14). We obtained commercially available STING-/- mice (Tmem173-/-) and examined the ability for TBI to induce sIL-15 complexes in the absence of STING signaling (134). Interestingly, lack of STING signaling resulted in an approximate 50% reduction in TBI-induced sIL-15 complexes (Figure 18). Hence, optimal TBI-induced sIL-15 complex generation requires Type I IFN signaling, and STING signaling. However, as Type I IFN signaling was completely required for this induction (Figure 15B), these results also suggest the presence of an additional Type I IFN-dependent but STING-independent, pathways involved in TBI-induced sIL-15 complexes.

**Figure 18: TBI-mediated induction of sIL-15 complexes is partially dependent on STING signaling**

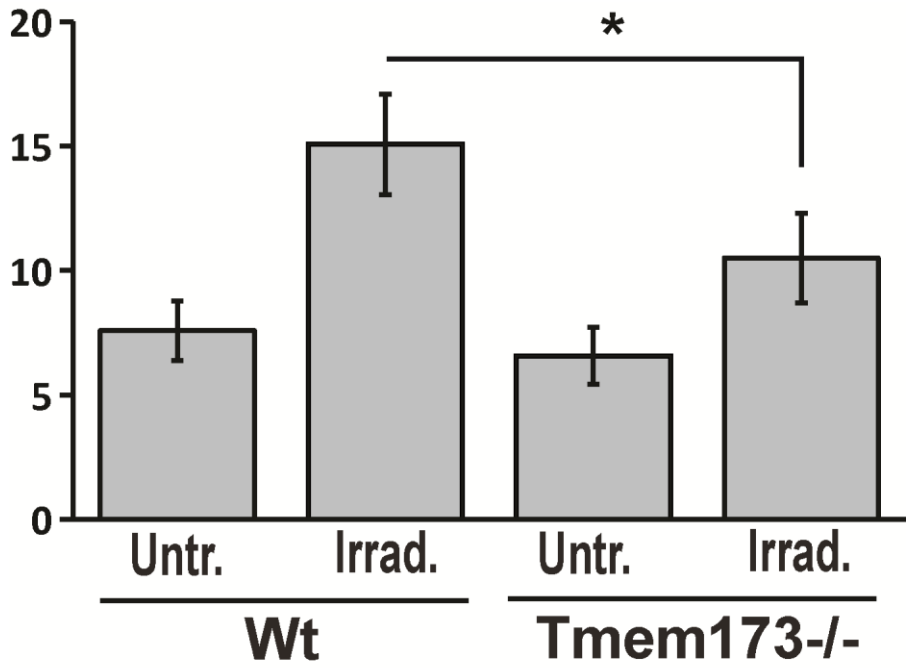



**Figure 18. TBI-mediated induction of sIL-15 complexes is partially dependent on STING signaling** (A) Serum was isolated from Wt and Tmem173-/- mice 24 hours after treatment with TBI (1000 RADS). Levels of sIL-15 complexes in serum were measured using ELISA. n=3-4 mice/group; data is one representative of 2 experiments. Error bars represent SD. * indicates $p<0.05$.

### 4.2.4 Cellular sources of TBI-induced IL-15

To this point we have established that sIL-15 complexes are produced during the steady state and these levels can be augmented by active inflammatory, but not in endogenously lymphopenic mice. Therefore, it would be beneficial to know the cell types producing these IL-15 complexes *in vivo* during these times of inflammatory lymphopenia. To examine cell specific IL-15 expression we utilized a transcriptional reporter mouse developed in the laboratory of Dr. Leo Lefrancois which expresses GFP under the control of the IL-15 promoter (58). Cells were isolated 24 hours after TBI (800 RADS) from spleen of Wt and transgenic mice. TBI-induced GFP expression in nearly all splenic cell types examined, including CD8$\alpha$+ DCs, (Figure 19A), CD11b+ conventional DCs (Figure 19B), monocytes (Figure 19C), and even in CD45- cells (Figure 19D). But an induction was not observed in Neutrophils (Figure 19E), which have been previously shown to express IL-15 mRNA at baseline and low levels of IL-15 protein upon *in vitro* stimulation (77). These results indicate that IL-15 transcriptional activity is induced by TBI in a nearly global fashion in both the hematopoietic and parenchymal compartments.

We further examined IL-15 expression by directly analyzing surface levels of transpresented IL-15 by myeloid cells in mice depleted of lymphocytes by TBI (800 RADS), chemotherapy (CTX, 200mg/kg, i.p.), or by gene disruption (RAG-/- mice). Lymphopenia induced by TBI or CTX was confirmed by measuring the total splenocyte counts (data not shown). Under all lymphopenic conditions examined cell surface IL-15 expression by myeloid cells was enhanced, relative to untreated Wt mice; however, differential cell-type expression was noted (Figure 20A, B). DCs (CD11b+CD11c+) from RAG-/- mice exhibited significantly elevated IL-15 cell surface expression, as previously shown (129), while IL-15 on monocytes and macrophages was increased but not elevated to a significant degree (Figure 20B).

**Figure 19: TBI-induces up-regulated IL-15 expression in DCs, Monocytes and Parenchymal cells in an IL-15 transcriptional reporter mouse.**

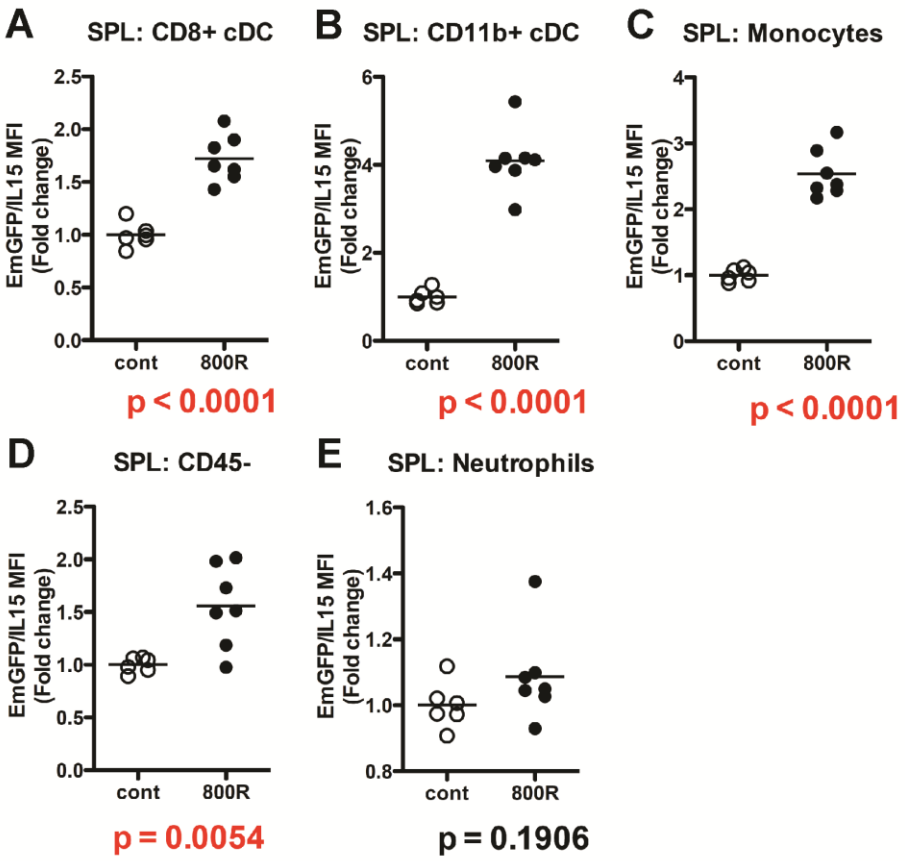


**Figure 19: TBI-induces up-regulated IL-15 expression in DCs, Monocytes and Parenchymal cells in an IL-15 transcriptional reporter mouse.** IL-15-GFP transcriptional reporter mice were left untreated (cont.) or subjected to 800 RADS of TBI (800R). Splenocytes were isolated 24 hrs after TBI and IL-15 transcriptional activity was examined in various populations by GFP intensity during immunofluorescence staining and flow cytometry. Due to differing levels of baseline GFP/IL-15 expression between different cell types, data is shown as the average fold difference in MFI of GFP expression in TBI-treated vs. untreated littermate control mice for each of the respective cell types analyzed. n=3-4 mice/group, data is representative of two experiments. Error bars represent SEM. The significance levels between control and treated mice are listed below each respective plot.

* Figure 19 was generated through an ongoing collaboration and performed by Dr. Sara Colpitts (UConn Health Science Center). These experiments were designed by Scott Anthony, Dr. Sara Colpitts and Dr. Kimberly Schluns

**Figure 20: Transpresentated IL-15 on DCs, macrophages and monocytes differs during various types of lymphopenia.**

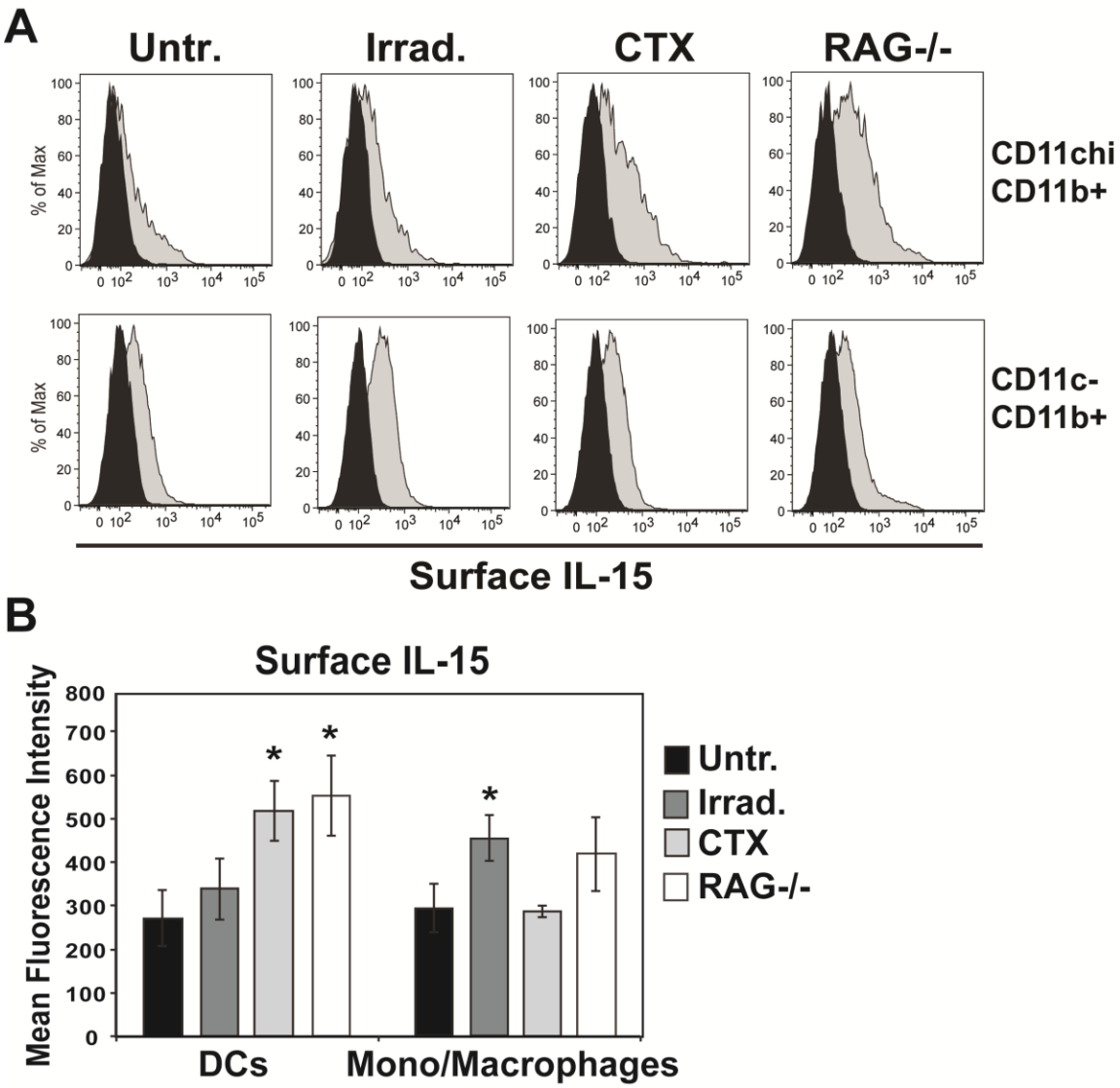


**Figure 20: Transpresentated IL-15 on DCs, macrophages and monocytes differs during various types of lymphopenia.** A) Wt mice were subjected to TBI (800 RADS, 24 hrs) or CTX (200mg/kg, 72 hrs) or untreated endogenously lymphodeficient RAG-/- mice. Splenocytes were harvested at the indicated times post stimulation and IL-15 cell surface staining was determined by immunofluorescence staining and flow cytometry. Histograms depict representative staining with control Ig (black histogram) and anti-IL-15 Ab (grey histograms) in Dendritic Cells (top) and monocytes/macrophages (bottom) as previously discussed (Figure 3). B) Graph shows average MFI of IL-15 expression in DCs and monocytes/macrophages from indicated mice. n=3 mice/group, one representative of three experiments is shown. Error bars represent SD. * indicates $p<0.05$.

In CTX-treated mice, but not in irradiated mice, DCs displayed increased cell-surface IL-15 expression (Figure 20A, B). In contrast, IL-15 expression by CD11b+ monocytes was increased after TBI and in RAG-/- mice, but not in response to CTX treatment (Figure 20A, B). This indicates that the diverse types of lymphopenia all enhance the expression of IL-15, which has been well characterized, as all of these conditions facilitate enhanced IL-15-dependent responses of adoptively transferred lymphocytes; however, we surprisingly discovered that each type of lymphopenia results in a different profile of cellular sources for these observed increases in IL-15 expression. Whereas cell surface IL-15 by DCs from RAG-/- mice was increased (Figure 20A, B), the levels of serum sIL-15 complexes was not increased in RAG-/- mice (Figure 12A) suggesting enhanced IL-15 responses in RAG-/- mice are likely mediated by increased IL-15 transpresentation. Nonetheless, the weak induction of sIL-15 complexes by CTX together with the differential increases in cell surface IL-15 suggest that the mechanisms regulating IL-15 after TBI are distinct from the mechanisms regulating sIL-15 complexes after chemotherapy. Overall, these results suggest that increased surface IL-15 is common feature of lymphopenia, whereas sIL-15 complexes are induced by signals specific to cell death.

We next examined the cellular requirements of TBI-induced sIL-15 complexes using our cell-specific IL-15R$\alpha$ deficient mice. IL-15R$\alpha^{fl/fl}$, IL-15R$\alpha^{fl/fl}$ x CD11cCre+/+ and IL-15R$\alpha^{fl/fl}$ x LysMCre+/+ mice were pre-bled for serum, subjected to TBI (1000 RADS) and the levels of soluble IL-15 complexes were examined 24 hours post-TBI. A significant induction of sIL-15 complexes was observed in response to TBI in all mice, although interestingly, this induction was substantially less in both the IL-15R$\alpha^{fl/fl}$ x CD11cCre+/+ and IL-15R$\alpha^{fl/fl}$ x LysMCre+/+ mice than in the IL-15R$\alpha^{fl/fl}$ mice (Figure 21).

**Figure 21: TBI-induced sIL-15 complexes are primarily derived from Macrophages and DCs**

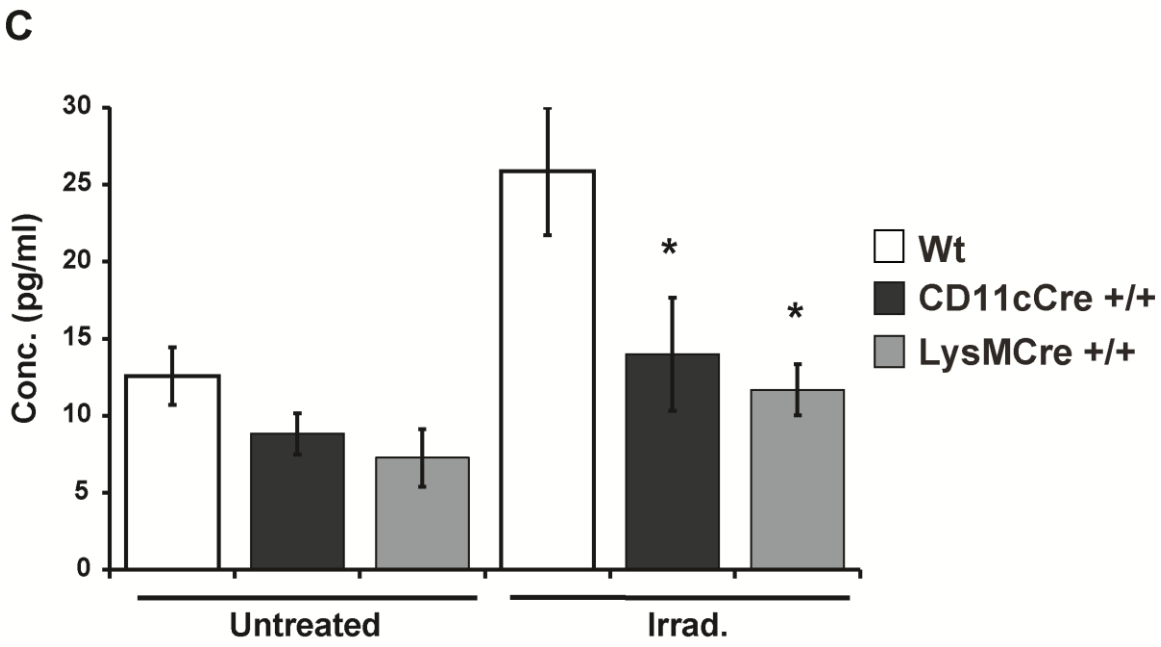



**Figure 21: TBI-induced sIL-15 complexes are primarily derived from Macrophages and DCs** Serum levels of sIL-15 complexes in Wt (White), IL-15R$\alpha^{fl/fl}$ x CD11cCre+/+ (Black) and IL-15R$\alpha^{fl/fl}$ x LysMCre+/+ (Grey) mice were isolated from peripheral blood before treatment (Untreated) and 24 hours post TBI (1000 RADS) (n=2-5 mice/group), one representative experiment of three is shown. Levels of sIL-15 complexes in serum were measured using ELISA. Error bars represent SD.* indicates $p<0.05$.

Although this does not discount the contribution of sIL-15 complexes from non-hematopoietic sources, these results indicate that dendritic cells, macrophages and monocytes together are major cellular sources of sIL-15 complexes in response to TBI.

As we determined that αThy1- induced sIL-15 complexes, we examined the cellular sources of αThy1-induced sIL-15 complexes using the same cell-specific IL-15Rα deficient mice. IL-15Rα$^{fl/fl}$, IL-15Rα$^{fl/fl}$ x CD11cCre+/+ and IL-15Rα$^{fl/fl}$ x LysMCre+/+ mice were pre-bled for serum, subjected to αThy1.2 (300µg i.p.) and the levels of soluble IL-15 complexes were examined 24 hours post-injection. A significant induction of sIL-15 complexes was observed in response to αThy1 in all mice (Figure 22). Furthermore, this induction was only slightly reduced in the absence of DC or monocyte/macrophage derived IL-15 compared to the Wt controls (Figure 22). These results indicate that dendritic cells, macrophages and monocytes contribute to the production, but are not dominant sources of sIL-15 complexes in response to αThy1 treatment.

**Figure 22: αThy1-induced sIL-15 complexes are primarily derived from non-hematopoietic sources**

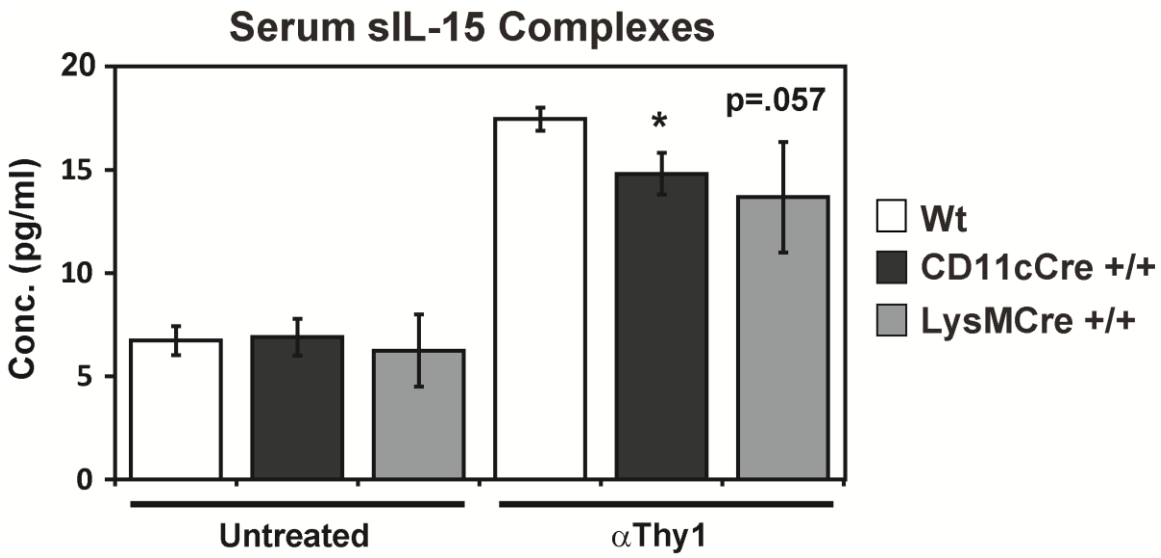



**Figure 22: αThy1-induced sIL-15 complexes are primarily derived from non-hematopoietic sources** Serum levels of sIL-15 complexes in Wt (White), IL-15Rα$^{fl/fl}$ x CD11cCre+/+ (Black) and IL-15Rα$^{fl/fl}$ x LysMCre+/+ (Grey) mice were isolated from peripheral blood before treatment (Untreated) and 24 hours post αThy1.2 injection (300ug, i.p.). n=3-5 mice/group. Levels of sIL-15 complexes in serum were measured using ELISA. * indicates $p<0.05$.

### 4.2.5 Lymphopenia-induced T cell proliferation is dependent on inflammatory IL-15.

During this project, we have examined and characterized the regulation of lymphopenia induced IL-15 expression at the levels of transcription (Figure 19), transpresentation (Figure 20) and sIL-15 complex generation (Figure 12). Although numerous previous studies have determined a critical role for IL-15 in T cell proliferation and survival, and during anti-tumor immune responses; the overall direct effects of inflammatory lymphopenia-induced IL-15 are not well established. We have not addressed the effects of lymphopenia-induced inflammatory IL-15 on responding lymphocytes because we lack a model to distinguish the effects of sIL-15 complexes from those due to transpresented IL-15; however, we can address the role of IL-15 on T cells during the window in which sIL-15 complexes are induced using αThy1-mediated lymphocyte depletion. As we previously determined that treatment with the T cell depleting αThy1.2 monoclonal antibody induces sIL-15 complexes we investigated the effects of αThy1.2-induced inflammatory IL-15 on T cell proliferation.

To perform this experiment, our first objective was to generate Thy1.1+ (CD90.1) memory CD8 T cells, which are resistant to αThy1.2-mediated depletion. To this end, naïve (CD44-) Thy1.1+ transgenic Pmel-1 CD8 T cells (specific for Gp100 peptide 25-33 in the context of H2-$D^b$) were adoptively transferred to naïve hosts and one day later were infected with recombinant VSV expressing Gp100 (135,136). Once these cells were allowed to differentiate through the effector phase and into the memory phase (>30 days post infection), CD8+ CD44hi Thy1.1+ memory cells were isolated, negatively enriched, CFSE-labeled and adoptively transferred to Wt or IL-15Rα-/- mice both on the Thy1.2 background. One day post transfer, all mice were injected with αThy1.2 (30H12, 300µg i.p.) to induce inflammatory IL-15 expression by the depletion of host T cells.

**Figure 23: Lymphopenia-induced T cell proliferation is dependent on inflammatory IL-15.**

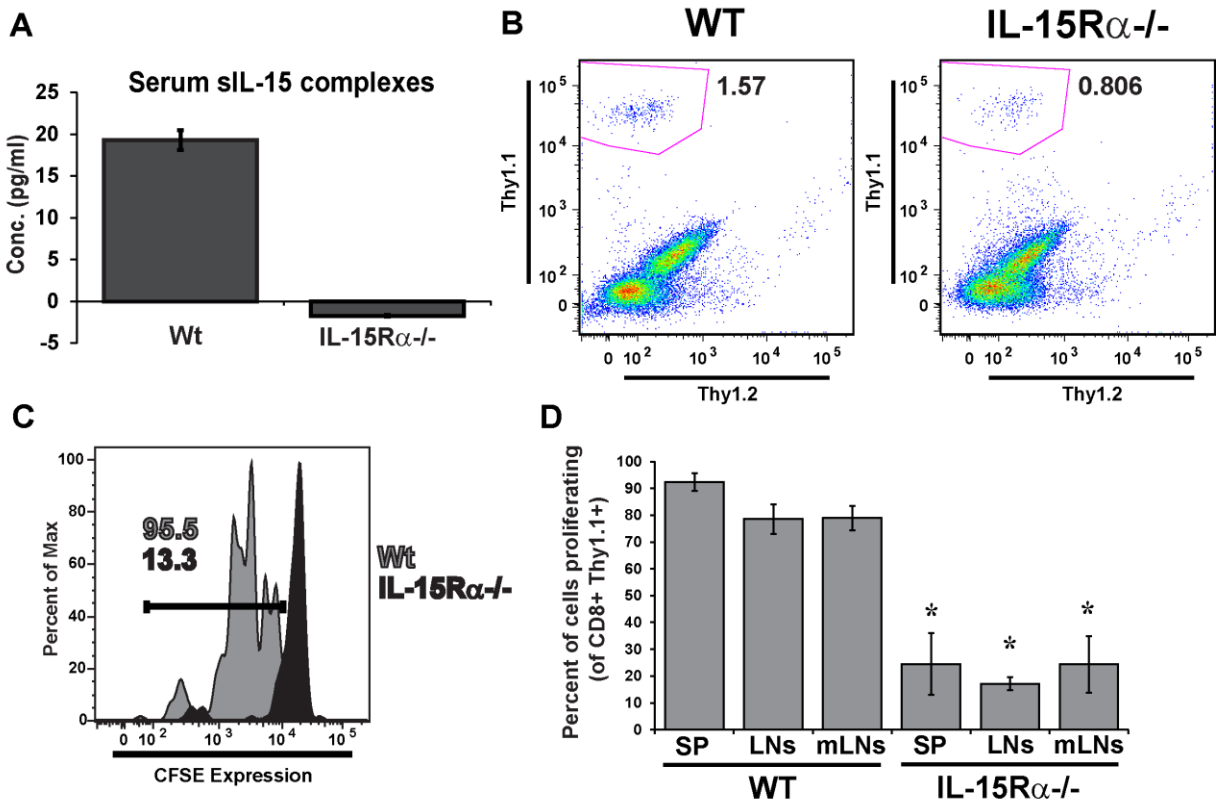


**Figure 23: Lymphopenia-induced T cell proliferation is dependent on inflammatory IL-15.** Memory Pmel-1 CD8 T cells (<30 days post infection) on Thy1.1 (CD90.1) background were negatively enriched from SPs, CFSE labeled and adoptively transferred to Wt or IL-15Rα hosts on Thy1.2 background (CD90.2) (150,000 cells/mouse). One day later, all mice were subjected to depletion of endogenous T cells via injection αThy1.2 (30H12, 300µg i.p.). A) 24 hours post injection serum was isolated from peripheral blood and IL-15 complex levels were determined by ELISA. B) Peripheral blood was isolated 4 days post stimulation and examined for presence of endogenous and adoptively transferred CD8 T cells, C) Mice were sacrificed 5 days post stimulation and lymphoid tissues (SP, LNs, mLNs) were analyzed for proliferation of memory Pmel-1 T cells by CFSE dilution. Histogram depicts CFSE dilution in memory Pmel-1 T cells in SP of a representative Wt (Grey) or IL-15Rα-/- (Black) mouse. D) The relative percentage of cells which had undergone at least one proliferation by CFSE dilution in response to αThy1.2 stimulation was calculated for all tissues analyzed. n=3 mice/group, one representative of two experiments is shown. Error bars represent SD. * indicates $p<0.05$.

This treatment does not deplete the Thy1.1+ memory CD8 T cells and will allow the T cells to respond to the IL-15 upregulated within the environment. At 24 hours post injection sIL-15 complexes were elevated in peripheral blood from Wt mice and absent in IL-15Rα-/- mice (Figure 23A). Five days post Ab treatment the majority (<75%) of Thy1.1+ cells had proliferated in Wt mice (Figure 23C). Despite the presence of all other inflammatory cytokines, this αThy1.2-induced T cell proliferation was fully dependent upon inflammatory IL-15, as few of the cells (<25%) underwent proliferation in the IL-15Rα-/- mice (Figure 23C). This dependence on inflammatory IL-15 for proliferation was also observed in SP, LNs and mesenteric LNs 5 days post Ab treatment with fewer than 25% of T cells having proliferated in all tissues examined in IL-15Rα-/- mice. On the contrary, nearly all of the T cells had undergone at least 1-2 proliferation cycles in the tissues of Wt mice (Figures 23 C, D). This directly confirms that lymphopenia-induced inflammatory IL-15 induces the proliferation of memory CD8 T cells.

**4.3 Discussion.**

The induction of lymphopenia is well known to enhance lymphocyte responses to homeostatic cytokines, such as IL-15 and IL-7 (102,128,137); however, the mechanism(s) responsible for these observed enhanced IL-15 responses has been unclear. Hence, the demonstration that sIL-15 complexes are induced by TBI (104) gives credence to the idea that elevated sIL-15 complexes are contributing to enhanced lymphocyte responses during lymphopenic conditions. Increasing lymphocyte responses during lymphopenia is very clinically relevant as lymphodepletion is widely used as an integral component of immunotherapy in the treatment of cancers. While studies have clearly demonstrated that IL-15 is critical for enhanced anti-tumor responses in mouse models of adoptive transfer (83), as mentioned before, there are currently no models to discern whether these IL-15-mediated responses are due to transpresented IL-15 or sIL-15 complexes. Therefore, the potential mechanisms mediating IL-15 responses during lymphopenic conditions should now include those elicited by transpresented IL-15, sIL-15 complexes, or the combination of both. A human clinical trial study observed a stepwise enhancement of IL-15 in response to increasing levels of lymphodepletion (107). Although these combined TBI and CTX lymphodepleting regimens did result in the highest anti-tumor efficacy with Adoptive T cell therapy (ACT), they also lead to the patients being immunocompromised for life. Therefore, understanding the mechanisms whereby these processes lead to enhanced IL-15 expression would be a first step towards developing potentially alternative means of enhancing anti-tumor responses.

Lymphopenia inducing regimens have largely been used interchangeably during experimental protocols, as all commonly used methods and models of lymphopenia support the survival of adoptively-transferred CD8 T cells. Our results show that at the level of transpresentation, IL-15 protein was increased under all lymphopenic conditions examined

(Figure 20B). Interestingly, the cell type's transpresenting IL-15 under these different conditions drastically differed. While TBI induced a significant increase of cell surface IL-15 expression in monocytes and macrophages, it had no significant effect on DCs (Figure 20B), while CTX conditioning induced high levels of cell surface IL-15 expression in DCs but resulted in no change in IL-15 expression in monocytes and macrophages. This indicates that while each of the forms of lymphopenia result in increased IL-15 transpresentation, the cell types responsible for these effects varied between treatments. As these regimens are utilized to support the survival and proliferation of adoptively transferred lymphocytes, these observed differences would likely exhibit significant effects on adoptively transferred lymphocytes. A previous study determined that the loss of DC specific IL-15 expression had dominant effects on Tcm cells while loss of macrophage specific IL-15 expression resulted in a specific loss of Tem cells (23). While this study took place under homeostatic conditions, the results can be extrapolated to predict that enhanced DC-specific IL-15 expression would likely have a more significant effect on Tcm cells and vice versa. Therefore, if the goal of lymphodepletion is to the facilitate the survival of adoptively transferred lymphocytes, in regard to the augmented IL-15 expression, it is likely that CTX treatment would better support Tcm cells while TBI-treatment would better support Tem cells. These results may partially explain the enhanced efficacy with the combination of CTX and TBI (107), at least at the level of IL-15 expression, as the combination would be predicted to induce significant expression of IL-15 from both DCs and macrophages. The direct analysis of mice treated with the combination of CTX and TBI was out of the scope of our current study and was not investigated; however future studies could determine if this combination therapy does indeed enhance the production of IL-15 from both DCs and macrophages.

Increased levels of sIL-15 complexes were found in TBI- and Thy1-treated mice, but not in CTX-treated or RAG-/- mice. We found that sIL-15 complexes were generated at 24 hours in

a dose-dependent manner, as increased doses of TBI resulted in significantly enhanced production of sIL-15 complexes. Although a previous study indicated that CTX-induced sIL-15 complexes, we did not observe this effect across all investigated time points, despite the induction of lymphopenia (104). The reason for this lack of induction is currently unknown, but may be due to differential composition of microbiota. The fact that we observed an induction of sIL-15 complexes in response to other previously published stimuli (Figure 2F, Figure 12A) indicates that this is not due to the inability of our mice to generate sIL-15 complexes. Despite the observed differences in the sources of lymphopenia-induced IL-15 by transpresentation, we observed a consistent TBI-mediated induction of IL-15 expression in monocytes throughout all models examined including the IL-15 transcriptional reporter model (Figure 19C) and cell-surface IL-15 expression (Figure 20B). In addition, the levels of TBI-induced sIL-15 complexes were significantly reduced in IL-15R$\alpha^{fl/fl}$ x LysMCre+/+ mice, showing that monocytes and macrophages express IL-15 not only at the transcriptional level, but also transpresent IL-15 protein and are a dominant source of sIL-15 complexes in response to TBI. The role of DCs as a source of TBI-induced IL-15 was a bit more complicated as the analysis of DCs showed a lack of TBI-induced up-regulation in transpresented IL-15, although they showed high expression in the transcriptional reporter model and TBI-induced sIL-15 complexes were significantly hampered in the absence of DC-specific IL-15R$\alpha$ expression. In addition to hematopoietic sources, we also observed increased IL-15 expression in the non-hematopoietic cells (CD45-) of TBI-treated transcriptional reporter mice. Regardless, these results suggest that increased transpresentation of IL-15 is an element conserved across all types of lymphopenia, while the induction of sIL-15 complexes requires an element of inflammatory cell death. Altogether, these data suggest that TBI-induced IL-15 expression arises in DCs, macrophages and non-hematopoietic cells.

We further investigated the role of several inflammatory inducers and pathways in the TBI and Thy1-mediated induction of sIL-15 complexes. In contrast to a previous report, circulating levels of LPS were not augmented in our mice in response to TBI (Figure 14) (131). Despite the lack of LPS induction, we still observed an increase in sIL-15 complexes following TBI (Figures 12, 13). We are not suggesting that this mechanism is not capable of inducing IL-15 dependent responses as LPS was also previously shown to directly enhance IL-15 expression and sIL-15 complexes *in vivo* (43). Since the observed increases in circulating LPS occurred 6 days post TBI, and we detected elevated sIL-15 complexes by only 16 hrs post TBI (Figure 13A) gut breach and LPS induction is not likely responsible for this early induction in sIL-15 complexes (131).

Both TBI and αThy1 treatments required Type I IFN signaling, to a different degree, for the observed induction of sIL-15 complexes. TBI absolutely required Type I IFN signaling, while αThy1 injection was able to induce sIL-15 complexes in IFNAR-/- mice corresponding to 50% of those observed in Wt mice. This suggests alternative mechanisms in the induction of sIL-15 complexes by these two lymphodepletion regimens. Despite their significant differences in activity, this partial reliance on Type I IFN signaling of αThy1-complement mediated induction of sIL-15 complexes was similar to what we observed during CD40-induced agonistic Ab stimulation (Figure 5E). Likewise, the cellular sources of sIL-15 complexes differed, as TBI-induced sIL-15 complexes were highly dependent on both DC and monocyte/macrophages, while loss of IL-15 expression in either DC or macrophage lineages had a small reduction in sIL-15 complex generation in response to αThy1 treatment. Therefore, DCs and macrophages are responsible for the majority of sIL-15 complexes produced in response to TBI, but are not in response to αThy1. While IL-15 expression is well characterized in the hematopoietic system,

our results provide evidence that parenchymal cells maybe an important source of sIL-15 complexes in response to lymphodepetion regimens.

The STING pathway has been recently shown to be a critical pathway for the induction for inflammation in response to cell death and subsequently for antitumor immune responses (6,7). Stimulation with the STING agonist c-di-GMP-induced sIL-15 complexes *in vivo* and directly in Flt3L-DCs. The ability for STING activation to induce sIL-15 complexes was entirely dependent upon Type I IFN signaling. As TBI-induced anti-tumor responses are also dependent upon Type I IFN signaling, we investigated the role of STING signaling in TBI-induced sIL-15 complexes. Interestingly, loss of STING signaling resulted in a significant, but not complete loss in the generation of sIL-15 complexes. These results clearly indicate that the STING pathway is involved in the generation of sIL-15 complexes in response to TBI, but also that an additional Type I IFN dependent STING-independent, pathway exists. Further experiments should be performed to examine the role of inflammatory IL-15 in the previously observed TBI-induced antitumor responses. Our results indicate that activation of the STING pathway strongly induces sIL-15 complexes; as STING agonists are currently being pursued as adjuvants in antitumor immune responses, the role of inflammatory IL-15 during these stimulations should be also further investigated.

We determined that inflammatory lymphopenia-induced the strong proliferation of memory CD8 T cells (Figure 23). This proliferation was almost completely dependent on IL-15 signaling, as this proliferation was absent in cells transferred to IL-15R$\alpha$-/-mice despite the presence of all other inflammatory factors. This discovery mirrors the results discovered in the lab of Dr. John Harty where dominant roles for inflammatory IL-15 were discovered to mediate their effects selectively on memory CD8 T cells (84,101). Those studies along with our current analysis shows a dominant role for inflammatory IL-15 on responding memory CD8 T cells. I

speculate that memory CD8 T cells will be more sensitive to inflammatory IL-15 because these cells have higher baseline expression of CD122, which has been shown to confer an increased responsiveness to IL-15 (138). In this situation memory CD8 T cells and NK cells would be the dominant targets for inflammatory IL-15. The selective role of inflammatory IL-15 on memory CD8 T cells is not well established, partially because the majority of antigen specific responses are investigated at the effector to primary memory states while the contribution of factors mediating secondary responses through memory cells are being actively discovered. Clearly inflammatory IL-15 has effects on memory CD8 T cells, although additional future work is still needed to determine the specific roles of sIL-15 complexes vs. transpresented IL-15 during inflammation.

In summary, we have found that several pathways inducing active depletion of lymphocytes, including TBI and αThy1 induce a transient burst in circulating sIL-15 complexes, while many additional lymphopenic conditions, including CTX treatment and naturally lymphopenic RAG-/- mice do not exhibit soluble IL-15 complexes above baseline levels. Although Type I IFN signaling-induced sIL-15 complexes, the requirement for this signaling pathway greatly varied between stimulations as Type I IFN signaling was required for TBI-induced sIL-15 complexes, but was only partially required for sIL-15 complex generation in responses to αThy1-treatment. In addition, we identified a direct role for lymphopenia-induced inflammatory IL-15 on the proliferation of CD8 memory T cells. Overall, this demonstrates that inflammation, specifically Type I IFN signaling, is a major, but not absolutely required factor for lymphopenia to induce sIL-15 complexes. These findings along with previous studies showing TLR stimulation and lymphodepletion induce sIL-15 complexes lead us to conclude that increases in sIL-15 complexes is an event associated with inflammation and immune activation. Moreover, the wide-ranging nature of these stimuli suggests that induction of sIL-15 complexes during immune

stimulation is a frequent event. These observations provide an unrealized mechanism for enhanced IL-15 responses observed in response to Type I IFNs and other inflammatory settings.

# Chapter 5: General Discussion and Future Directions

## 5.1 Summary

### 5.1.1 Global Induction of sIL-15 complexes

In chapter 3, I described my efforts to identify the mechanisms responsible for the generation of sIL-15 complexes, based on the hypothesis that Type I IFN signaling and ADAM17 were required for the generation of sIL-15 complexes. Past studies have primarily investigated the role for IL-15 during homeostasis, which is attributed to the dominant roles of IL-15 on homeostatic proliferation, yet a diverse assortment of infectious pathogens and TLR agonists were known to enhance IL-15 expression. My overall goal was to determine the pathways and cell types required for the generation of inflammatory IL-15 expression. Type I IFN signaling was also a known inducer of IL-15 expression, and it was previously shown that metalloprotease activity were required for inducible cleavage of IL-15Rα. However, the respective roles for Type I IFN signaling and ADAM17 activity in the generation of sIL-15 complexes were unknown. Through direct stimulation by IFN, I discovered that this pathway is directly capable of regulating the ADAM17-mediated cleavage of sIL-15 complexes. Additional analyses led to the surprising discovery that Type I IFN signaling was not required for the *in vivo* generation of sIL-15 complexes in response to several diverse stimuli. This indicates that additional unknown, IFN-independent pathways are capable of inducing sIL-15 complexes. Using an inducible model for conditional deletion of ADAM17 I further determined that redundancy likewise exists in the ability to cleave IL-15 complexes as mice exhibiting functional deficiency of ADAM17 were not completely impaired in the ability to generate sIL-15 complexes. Active infection with VSV enhanced IL-15 transpresentation by monocytes, macrophages and DCs. Utilizing mice with a conditional deletion of IL-15Rα, I removed the ability for either DCs or

macrophages to generate IL-15. In this model, I observed that VSV infection induces sIL-15 complexes from macrophages, while CD40 stimulation induces the generation of DC-derived sIL-15 complexes. This indicates that different stimuli induce the generation of sIL-15 complexes from varying cellular sources. Overall, these results indicate that sIL-15 complexes are a common feature of immune stimulations and arise from multiple different cell types.

### 5.1.2 Regulation of IL-15 during lymphopenia

All known forms of lymphopenia enhance the persistence and survival of adoptively transferred lymphocytes. This brings about the question, what is the mechanism for this observed effect? The majority of past studies investigating this question have revolved around the ability for adoptive transferred T cells to enhance anti-tumor responses. These anti-tumor effects are correlated with enhanced levels of sIL-15 complexes; yet, the pathways and cell types responsible for these effects were unknown. I discovered that although all examined lymphopenic models resulted in enhanced transpresentation of IL-15, only the models with an aspect of inflammation induced sIL-15 complexes, with the exception of CTX treatment, which may induce only minimal inflammatory signals. Throughout my studies in chapter 3, I observed that IFN signaling was a dominant mechanism capable of directly generating sIL-15 complexes. I similarly found that the optimal production of sIL-15 complexes in response to TBI required the activation of both the IFN and STING pathways. I also discovered that complement-mediated T cell depletion induces sIL-15 complexes in a manner partially dependent on IFN signaling. Overall, these results indicate a dominant role for inflammatory cell death in the generation of TBI-induced sIL-15 complexes. Using an IL-15 reporter system, we determined that IL-15 transcription was broadly induced in nearly all cells examined, including both hematopoietic and non-

hematopoietic sources. Further analysis with our conditional IL-15Rα-/- mice led to the discovery that TBI-induced sIL-15 complexes are greatly reduced in the absence of either macrophage- or DC-derived IL-15, indicating that both cell types are major cellular sources of sIL-15 complexes. To date our study is first to fully characterize the cell types and pathways responsible for the full complement of lymphopenia-induced IL-15 expression. In addition, utilizing an adoptive transfer model and Thy1-induced lymphodepletion, I provided evidence for a role in lymphopenia-induced inflammatory IL-15 on the proliferation of CD8 memory T cells. This finding is novel as it is the first account directly demonstrating the role of lymphodepletion-induced inflammatory IL-15 on memory CD8 T cells. Our findings are in agreement with several recent studies indicating an *in vivo* role for endogenous inflammatory IL-15 on memory CD8 T cells (84,101).

## 5.2 Future Directions

### 5.2.1 Mechanisms generating soluble Interleukin-15 complexes

During our studies, we determined that ADAM17 activity is induced by IFN; however, Type I IFN signaling was not absolutely required for the generation of sIL-15 complexes *in vivo*. The discovery that ADAM17 activity is not required for the generation of the majority of sIL-15 complexes *in vivo* was quite surprising as our *in vitro* data clearly corroborates with a previous study (52) in showing that the inhibition of ADAM17 activity blocked the cleavage of membrane bound IL-15Rα. Although we determined a lack of ADAM17-inducible activity, this was in peripheral B cells and we did not examine the expression of ADAM17 in the IL-15 producing subsets themselves. Therefore, our inducible knockout mouse has the potential to still express ADAM17 activity in these cell populations. Further analysis should be conducted on these cells, by inducing deletion *in vivo* and specifically isolating DCs,

monocytes and macrophages and directly examining their relative protein expression of ADAM17. Alternatively, the iRhom2-/- mouse has specific defects in ADAM17 activity in the hematopoietic system, while the activity of ADAM10 is unaltered (51). As CD40 stimulation was found to rely on DCs and generated sIL-15 complexes in a manner completely independent of ADAM17, the CD40 agonist would be a prime candidate to be investigated in iRhom2-/- mice. Future studies should re-examine the cleavage of IL-15 complexes, potentially with a screen for highly specific available protease inhibitors; however, caution should be used in interpreting the results as there was a clear disconnect between what we observed *in vitro* and *in vivo*. As ADAM10 has been shown to compensate for the cleavage of ADAM17-dependent substrates (121), future experiments should examine if sIL-15 complexes can be induced during the blockade of both ADAM17 and ADAM10. Despite these circumstances, it clearly looks as if there is a compensatory mechanism occurring *in vivo* regarding the generation of sIL-15 complexes.

We originally generated the inducible ADAM17$^{fl/fl}$ x ER$^{T2}$-Cre mice in the hopes that it would be a new model which retains transpresented IL-15 while having a lack of inducible sIL-15 complexes, as that the prior evidence suggested ADAM17 was the sole protease cleaving IL-15 complexes. Although this model showed that ADAM17 was not essential for cleaving sIL-15 complexes, it has allowed us to clearly establish that there are multiple levels of redundancy in the ability to generate sIL-15 complexes, including at the levels of inflammatory mediators, upstream pathways and the cleavage machinery. These studies have brought to light that the regulation of sIL-15 complexes is much more complicated than previously believed, as we did not find any one signal or pathway that was an absolutely required rate limiting step. Therefore, I believe that future approaches should focus directly on altering the ability for IL-15R$\alpha$ to be cleaved. Although the proteases capable of cleaving the IL-15R$\alpha$ are still unknown, these cell-surface proteases have a relatively conserved

mechanism of cleaving substrates at regions adjacent to the cell-membrane. Future studies should look to create a mouse where all of the IL-15Rα is not capable of being cleaved. In fact, a recent study involves a construct of IL-15Rα/IL-15 which is not cleaved during *in vitro* stimulations (127). We have noticed substantial differences between experiments performed *in vitro* and *in vivo* pertaining the requirements for sIL-15 complexes. Therefore, whether this information can be translated to a model in which IL-15Rα is fully retained *in vivo* is unknown but should be attempted to determine the definitive *in vivo* roles for endogenously-generated sIL-15 complexes.

We determined that CD40 stimulation directly induced sIL-15 complexes in Flt3L-DCs, additionally the induction of sIL-15 complexes *in vivo* in response to CD40 required BATF3-dependent DCs. It is possible that CD40 stimulation indirectly induces sIL-15 complexes *in vivo* through an intermediate factor and the obvious choice would be IL-12; yet, IL-12 alone was unable to induce sIL-15 complexes *in vitro*. Therefore this sIL-15 complex inducing pathway is likely not dependent on IL-12 signaling. Despite our current results, future studies should conclusively determine the potential role of IL-12 signaling in the induction of sIL-15 complexes through the use of IL-12Rβ2-/- mice. As CD40 signaling requires non-canonical NFκB signaling for the stimulation of DCs, it is likely that this additional pathway is dependent upon non-canonical NFκB signaling for the induction of sIL-15 complexes (71). Future experiments should also analyze the role of non-canonical NFκB signaling in the CD40-induced generation of sIL-15 complexes in mice and cells lacking the upstream NF-kB-inducing kinase, which is required for non-canonical NFκB signaling.

### **5.2.2** IL-15 expression during lymphopenia

TBI induces inflammatory cell death while also inducing lymphopenia. Previous studies determined that TBI-induces increases in IL-15 transpresentation by DCs and sIL-15 complexes *in vivo*. (104). We have discovered a dominant role for Type I IFN which is partially dependent on activation of the STING pathway for this induction. Although STING was only partially required, the *in vivo* significance for this sIL-15 complex inducing pathway is unclear. Clearly the activation of the STING pathway is capable and required for the optimal generation of sIL-15 complexes in response to TBI. As this pathway is crucial for productive CD8 T cell responses against inflammatory tumors, the role in which inflammatory IL-15 plays in this response needs to be further studied. Woo et. al. determined increased IL-15 mRNA expression with anti-tumor responses, but the direct role that IL-15 plays in these responses is unknown (7). The knowledge that the immune system is capable of inducing natural anti-tumor responses and Type I IFNs are critical suggests that the role of IL-15 in natural anti-tumor responses is in need of further investigation. In fact, a study determined that regardless of tumor stage, the relative local expression of IL-15 alone was a significant prognostic indicator of long term survival in colon cancer patients, (92). It is possible that this induction of IL-15 in the tumor microenvironment may just be a side effect of the local Type I IFN produced in the tumor microenvironment, but regardless this local IL-15 would be fully capable of enhancing multiple attributes of anti-tumor CD8 T cell responses.

Although we determined a role for lymphopenia-induced inflammatory IL-15 on the proliferation of memory CD8 T cells, further investigation into the roles of inflammatory IL-15 on other lymphocyte subsets is needed. In addition, future experiments should directly compare the responses induced by inflammatory and non-inflammatory lymphopenia;

explicitly in their relative abilities to induce the proliferation and trafficking of memory CD8 T cells. In addition, as NK cells are very response to IL-15 and express high levels of CD122, the roles of inflammatory IL-15 on NK cells should likewise be further investigated.

### 5.3 General Discussion

With this project we have addressed several long-standing questions regarding the regulation of IL-15 under inflammatory conditions and lymphopenia. Overall, we have characterized the mechanisms generating sIL-15 complexes, including the role of inflammatory mediators and required cell types for their induction. The function of sIL-15 complexes has been scrutinized since their initial discovery since despite being produced in response to TLR stimulation, it was determined that these endogenously-produced sIL-15 complexes had no effect on NK cells in a co-culture setting and that IL-15-mediated activation still required a cell-cell contact mechanism (43). Since small amounts of recombinant sIL-15 complexes induce the activation and proliferation of IL-15 responsive cells, it seems logical that these endogenously-generated sIL-15 complexes would play a role during active immune responses. In fact, since the start of this project, many companies have focused on enhancing anti-tumor responses with IL-15 and sIL-15 complexes. Our data shed light into the required factors for the generation of the endogenous forms of these complexes. The finding that continual IFN signaling promotes the persistent production of sIL-15 complexes has implications in the fields of autoimmunity and natural antitumor responses. There is much data implicating that the positive outcome of the current immunotherapies for cancer treatment correlates with the presence of an ongoing intra-tumoral Type I IFN response. Therefore, I predict that if DCs or macrophages are in the tumor microenvironment, they will be responsible for the local production of IL-15 and sIL-15 complexes, which could be at least partially responsible for the observed

antitumor responses. Further investigation should more directly investigate the role of IL-15 in antitumor immune responses.

Our finding that sIL-15 complexes are produced at detectible levels in the absence of stimulation is something that past studies have also observed but failed to properly acknowledge the significance of these findings. A recent study directly compared the levels of sIL-15 complexes in the serum to the levels of IL-15 complexes found in the entire spleen in response to mucosal stimulation with a TLR agonist (101). The authors determined that the peak in serum sIL-15 complexes was equivalent to that observed in the entire spleen, denoting this peak in sIL-15 complexes is significant. In addition, the levels of sIL-15 complexes we have observed at homeostasis are 40-60% of those observed during the steady-state from all of the splenocytes combined from this study. These collective results suggest a potential paradigm shift; that in addition to playing significant roles during inflammation, sIL-15 complexes may play a role in IL-15-dependent responses occurring during homeostatic conditions. More studies will obviously need to be conducted to directly understand the relative contributions of transpresented IL-15 vs. sIL-15 complexes during inflammation and homeostasis.

We determined there is redundancy in the ability to generate sIL-15 complexes *in vivo*, as inhibition of ADAM17-activity had little effect on the ability for Poly I:C and CD40 stimulations to induce sIL-15 complexes. Regardless if a single or multiple additional proteases are responsible for this effect, this suggests that directly targeting ADAM17 activity is not a viable approach to modulate the expression of sIL-15 complexes *in vivo*. In addition, our findings in concert with those of Le Gall et. al. (121) together have the potential to at least partially explain the lack of efficacy of ADAM17-targeted therapies in IL-15 dependent diseases in clinical trials, as it is likely that this compensatory mechanism was

able to take over during these conditions in the blockade of ADAM17. Additional studies will need to further examine what we know about ADAM17 and other additional potential proteases in the cleavage of cell surface bound IL-15Rα, specifically through the use of *in vivo* animal models.

A substantial but transient induction of sIL-15 complexes was observed in response to all sIL-15 complex-inducing stimuli, albeit with slightly offsetting time-courses. This similar pattern was observed during stimulations with c-di-GMP, αCD40, αThy1, TBI and VSV infection. These results point to a conserved mechanism of not just the induction of sIL-15 complexes but also in the resolution phase of the generation of sIL-15 complexes. As we determined that continual Type I IFN signaling induces the sustained generation of sIL-15 complexes, this regulation is likely to be observed at the levels preceding the generation of IL-15 complexes.

It is quite interesting to note that although Type I IFN signaling directly induces sIL-15 complexes and many of the additional pathways inducing sIL-15 complexes also induce Type I IFNs; however, the requirement for Type I IFN signaling for this induction varies from absolutely necessary in the case of Poly I:C and TBI, to partially required in response to αThy1 and αCD40 stimulations to completely unnecessary in the case of VSV. These differences can be partially attributed to the cell types induced, although the dominant cell types generating sIL-15 complex were similar in the case of TBI and VSV despite a complete divergence in the requirement for Type I IFN signaling to mediate these effects. Therefore, despite Type I IFN signaling being a strong inducer of sIL-15 complexes, the overall generation of sIL-15 complexes is likely dictated by the presence of additional IL-15 inducing pathways.

At the very least, these results indicate that at least 3 distinct pathways are fully capable of inducing sIL-15 complexes; the first of which is Type I IFN signaling, which we have shown has the ability to directly mediate the generation of sIL-15 complexes. The second is a CD40-dependent pathway and DCs were required for the CD40-mediated generation of sIL-15 complexes. The third unknown pathway is induced by VSV-infection in the absence of Type I IFN and CD40 signaling pathways.

Overall, I've demonstrated a diverse assortment of stimuli utilize a variety of pathways and cell types to induce the generation of sIL-15 complexes. Moreover, I have established that inflammatory lymphopenia induces sIL-15 complexes which enhance the proliferation of memory CD8 T cells. These results have the potential for global impacts on memory CD8 T cell functions during viral infections, autoimmunity and during natural and induced anti-tumor responses. These findings lead me to conclude that the increase in sIL-15 complexes is a common event likely contributing to memory CD8 T cell responses during conditions involving inflammation and immune activation.

## Chapter 6: Materials and Methods

### 6.1 Mice.

C57Bl/6 mice were purchased from NCI/Charles River. RAG1-/- mice, IL-15R$\alpha^{fl/fl}$ (23), CD11cCre (123), LysM Cre (122) and Tmem173-/- mice (134) were purchased from Jackson Laboratories (Bar Harbor, MN). ADAM17 $^{fl/fl}$ (119), Rosa26-Cre-ER$^{T2}$ mice (120) were purchased separately from Jackson Laboratories (Bar Harbor, MN) and bred to generate ADAM17 $^{fl/fl}$ X ER-Cre Tg mice. IL-15R$\alpha$-/- mice (37) were originally generated and obtained by Dr. Averil Ma through Leo Lefrancois and backcrossed to the C57Bl/6 line. BATF3-/- mice were generated by Dr. Ken Murphy (76) and provided by Dr. Tomasz Zal. Thy1.1+ Pmel-1 TCR Transgenic (specific for Gp100 peptide 25-33 in the context of H2-D$^b$) mice were provided by Dr. Willem Overwijk (135). IFNAR1-/- mice were provided by Dr. Paul W. Dempsey (Department Of Microbiology and Molecular Genetics, University of California, Los Angeles and Dr. Tadatsugu Taniguchi, Department of Immunology, Tokyo University, Japan) to W. Overwijk and crossed to the C57Bl/6 background (12). IL-15 transcriptional reporter mice were generated by Dr. Leo Lefrancois (Department of Immunology, University of Connecticut, Farmington, CT) (58); experiments utilizing these mice were performed at the University of Connecticut Health Science Center. All other mice described were maintained under specific pathogen-free conditions at the institutional animal facility. The animal facility is fully accredited by the Association of Assessment and Accreditation of Laboratory Animal Care International. All animal procedures were conducted on mice between 6-20 weeks of age, in accordance with the animal care and use protocols (100409934 and 070807332) approved by the Institutional Animal Care and Use Committee at the UT MD Anderson Cancer Center. Retro-orbital blood was obtained from mice anaesthetized by inhalation with a mixture of 2% isoflurane/98% oxygen. For all manipulations, animals were monitored and efforts were made to minimize

suffering. At the designated times, the animals were sacrificed according to institutional guidelines and blood and tissues were collected for analyses.

### 6.2 *In vivo* injections

Mice were injected with pORF-IFNα5 (2 μg) (InvivoGen, San Diego, CA) to induce *in vivo* IFN-α production or empty vector plasmid pORF (InvivoGen) in 2mL saline via hydrodynamic injection as previously described (64). For VSV infection, mice were infected i.v with 1 x $10^6$ PFU (VSV, Indiana strain or VSV-Gp100 where indicated). For Poly I:C stimulation, mice were administered Poly I:C i.p. (150 μg, Sigma, St. Louis, MO). For stimulation of the STING pathway, mice were administered c-di-GMP at the indicated doses (Invivogen, San Diego, CA). For chemotherapy treatments, mice were administered cyclophosphamide dissolved in saline i.p. (200mg/kg). For TBI, mice were exposed to a cesium irradiation source at the indicated doses. CD40 stimulation was carried out by injecting i.p. anti-CD40 monoclonal Ab at the indicated doses (clone FGK 4.5, BioXcell, Upper Heyford, UK). Complement-mediated depletion of T cells and NK cells was performed by injecting i.p. anti-CD90.1 (300ug, clone 30H12, BioXcell, Upper Heyford, UK) alone or in concert with anti-NK1.1 mAb (300ug). Depletion was confirmed with flow cytometry of circulating peripheral blood cells (Data not shown). For serum isolations, peripheral blood was collected from sacrificed mice via cardiac puncture and on some occasions from the retro-orbital cavity prior to treatment from the same mice. Blood was allowed to clot and centrifuged to separate serum.

### 6.3 Tamoxifen treatment and analysis of ADAM17 activity

To conditionally knockdown ADAM17, ADAM17 $^{fl/fl}$ X ER-Cre Tg and ADAM17 $^{fl/fl}$ mice were treated with Tamoxifen (Sigma, St. Louis, MO)) (2mg/mouse i.p.) dissolved in Corn Oil every 2 days for a total of 5-6 treatments. To examine loss of ADAM17 functional activity, an *ex*

*vivo* stimulation protocol was designed based on the observations from a published report in which PMA-induced shedding of CD62L required ADAM17 activity (121). Therefore, after tamoxifen treatment, mice were screened for loss of ADAM17 inducible activity by PMA stimulation of peripheral blood lymphocytes before proceeding to *in vivo* experiments. Peripheral blood was isolated from all animals, red blood cells were lysed and peripheral lymphocytes were left unstimulated or stimulated with 25ng PMA (25ng/mL, Sigma, St. Louis, MO) in RPMI for 1 hr. The cell surface expression and MFI of L-selectin (CD62L) was examined on circulating B cells (CD19+ B220+) by flow cytometry. Following the last tamoxifen treatment, mice were rested for 1-2 weeks before further experimentation.

**6.4 Analysis of Cytokine and LPS Expression**

ELISAs specific for murine soluble IL-15R$\alpha$/IL-15 complexes (eBioscience, San Diego, CA) and murine IFN-$\alpha$ (detects all 14 IFN-$\alpha$ subtypes, PBL Biomedical Laboratories) were performed according to manufacturer's recommendations. Limulus amebocyte lysate (LAL) chromogenic Endotoxin quantitation assay (ThermoScientific, Rockford, IL) was performed according to manufacturer's recommendations. Cell surface IL-15 was detected in splenic myeloid cells isolated directly *ex vivo* as previously described (54). Briefly, cell surface IL-15 was detected with polyclonal rabbit anti-IL-15-biotin (Peprotech, Rocky Hill, NJ) followed by streptavidin-APC (Jackson ImmunoResearch). Background staining was determined by staining analogous populations with a biotinylated Ig control (Jackson ImmunoResearch). The following monoclonal (m) Abs were purchased from BD Biosciences (San Jose, CA), eBiosciences, or BioLegend: CD19, CD3, DX5, CD11b, CD11c, B220, CD19, CD90.1, CD90.2, CD8, NK1.1, and CD44. Expression of CD19, CD3 and DX5 was used to define lineage+ cells. Expression of CD19 and B220 was used to define B cells. Expression of CD8$\alpha$, CD90.1 and high CD44 expression was used to define memory Pmel-1 T cells. Flow cytometric data were

acquired with a LSRII (BD Biosciences) or LSR Fortessa (BD Biosciences) and analyzed with Flowjo software version 9.7.6 (Flowjo LLC, Ashland).

### 6.5 Bone marrow derived dendritic cells (BMDCs)

BMDMs were generated from bone marrow cells cultured for 4 days in DMEM supplemented with 20% FBS and 30% M-CSF (139). Flt3L-DCs were generated using Flt3L supplemented cultures as previously described (140). BMDCs were generated using GM-CSF stimulation as previously described (55). Briefly, BM cells were flushed from femurs of indicated mice, dissociated, and treated with Tris ammonium chloride to lyse red blood cells. BM cells were then cultured in RPMI Complete Medium (CM) at a concentration of $1x10^6$ cells/mL. CM RPMI contains 2.5, mM HEPES, $5.5x10^{-5}$ M 2-mercaptoethanol, 100U/ml penicillin, 100 μg/ml streptomycin, 5mM glutamine and 10% fetal calf serum supplemented with 10 ng/ml GM-CSF (R&D Systems, Minneapolis, MN) at 37° C with 5% $CO_2$. BM cells were passaged 1:2, 3-4 days later with fresh CM containing GM-CSF (10ng/mL). After 6 days, BMDCs were given fresh media for stimulations. For Flt3L-DCs, isolated BM cells were cultured in CM with 200ng/mL FLT-3L (R&D Systems, Minneapolis, MN) at 37° C with 5% $CO_2$ for 9 days. BMDCs and Flt3L-DCs were seeded at 1-2.5x10^6 cells/mL and stimulated with Poly I:C (50μg/mL, Sigma, St. Louis, MO) or IFNα (300U/mL, PBL Laboratories, Piscataway,NJ), anti-CD40 monoclonal Ab (30μg/mL, clone FGK 4.5, BioXcell, Upper Heyford, UK) IL-12 (50ng/mL, Peprotech, Rocky Hill, NJ) or with the STING agonist c-di-GMP (25μg /mL, Invivogen, San Diego, CA) for 24 hours. Supernatants were collected and analyzed for sIL-15 complexes using ELISA. For analysis of ADAM17 expression, CD11c+CD11b+ BMDCs were stained with ADAM17 Ab (purified polyclonal Rabbit IgG, eBioscience) or control Rabbit IgG (JacksonImmunoResearch), followed by Donkey anti-Rabbit APC (JacksonImmunoResearch).

For Tat-Cre *in vitro* recombination, BM cells were collected from the indicated mice, washed twice and cultured in serum-free media with TAT-Cre for 1 hour at 37° C with 5% $CO_2$ (10 μg per $1X10^6$ cells, Protein and Proteomics Core Facility, The Children's Hospital of Philadelphia). Transduced BM cells were then washed twice with serum free media followed by culturing in CM containing GM-CSF for 6 days to generate BMDC. After 6 days, BMDC were stimulated as described and culture supernatants were collected for analysis of sIL-15 complexes.

### 6.6 Analysis of Lymphopenia-induced proliferation on memory CD8 T cells

$4x10^5$ Naïve (CD44-) Thy1.1+ (CD90.1) Pmel-1 TCR transgenic CD8 T cells were adoptively transferred to naïve hosts and one day later were infected with recombinant VSV expressing Gp100 (135,136). CD8+ CD44hi Thy1.1+ memory cells (35-60 days post infection) were negatively enriched (Invitrogen, Carlsbad, CA), labeled with 2mM CFSE as previously described and adoptively transferred by i.v. injection to Wt or IL-15Rα-/- mice both on the Thy1.2 (CD90.2) background. Four and five days after αThy1.2 injection peripheral blood cells and various tissues respectively were analyzed for the presence of Pmel-1 T cells and CFSE dilution.

### 6.7 Statistical Analysis

Statistical differences were determined by a two-tailed Students *t* test. * indicates $p<0.05$. Analyses were performed using GraphPad Prism, version 6 (GraphPad Software, San Diego, CA) or Microsoft Excel 2010 (Redmond, WA)

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## Vita

Scott Matthew Anthony was born in Palo Alto, CA on January 5, 1984 to James and Debra Anthony. He graduated from Governor Thomas Johnson High School in 2002, after which he attended The Pennsylvania State University to study General Biology, which he completed in 2007. Following his undergraduate career, he joined the laboratory of Dr. Sina Bavari as a research fellow where he pursued research into the immune response to infections. His primary work concentrated on vaccines and therapeutics, including his research project into the mechanisms and cell types required for protective immunity to post-exposure infections to emerging viral pathogens. While in the laboratory of Dr. Bavari, he obtained his Master's Degree in Biotechnology from Johns Hopkins University. He enrolled in the University of Texas Health Science Center's Graduate School of Biomedical Sciences at Houston/MD Anderson Cancer Center in August 2010 where he has pursued a Ph.D. in the Immunology program under the mentorship of Dr. Kimberly Schluns.

**Publications:**


1. Singh S, Yang G, Schluns KS, **Anthony SM**, Sastry KJ. Sublingual Vaccination Induces Mucosal and Systemic Adaptive Immunity for Protection against Lung Tumor Challenge. *PLoS One.* 2014 Mar 5;9(3):e90001. PMID:24599269.
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5. Li J, Huang X, Dorta-Estremera S, Di Domizio J, **Anthony, SM**, Popkin D, Brohawn P, Yao Y, Schluns KS, Lanier LL, and Cao W. Neutrophils regulate humoral autoimmunity by restricting interferon gamma production from natural killer cells. *Cell Rep.* 2015 Aug 18;(7):1120-32. PMID:26257170.