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# Determining the roles of dendritic cells and ICAM-1 in the transpresentation of IL-15 to CD8 T cells

Spencer W. Stonier

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Kimberly S. Schluns, Ph.D., Supervisory Professor

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Stephen Ullrich, Ph.D

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Stephanie Watowich, Ph.D.

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Willem Overwijk, Ph.D.

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A

DISSERTATION

Presented to the Faculty of

The University of Texas

Health Science Center at Houston

And

The University of Texas

M. D. Anderson Cancer Center

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

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Houston, Texas

August 2011

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# Abstract

## Determining the roles of dendritic cells and ICAM-1 in the transpresentation of IL-15 to CD8 T cells

The maintenance and generation of memory CD8 T cells is dependent on the cytokine IL-15. IL-15 is delivered by a novel mechanism termed transpresentation: IL-15 is presented by a cell expressing IL-15R$\alpha$ to the CD8 T cell which responds via IL-2R$\beta$/$\gamma_c$. The identity of what cells transpresent IL-15 to support the survival and homeostatic proliferation of memory CD8 T cells is unknown. Using a transgenic mouse model that limits IL-15 transpresentation to DCs, I have demonstrated that DCs transpresent IL-15 to CD8 T cells. DCs transpresent IL-15 to CD8 T cells during the contraction of an immune response and also drive homeostatic proliferation of memory CD8 T cells. Additionally, I identified a role for ICAM-1 in promoting homeostatic proliferation. Wt memory CD8 T cells displayed impaired homeostatic proliferation in ICAM-1$^{-/-}$ hosts but not in models of acute IL-15-driven proliferation. In this way, the role of ICAM-1 in IL-15 transpresentation resembles the role for ICAM-1 in antigen-presentation: where antigen or IL-15 is limited, adhesion molecules are important for generating maximal responses. *In vitro* cultures between CD8 T cells and bone marrow-differentiated DCs (BMDC) activated with a TLR agonist established a model of proliferation and signaling in CD8 T cells that was dependent on IL-15 transpresentation and required ICAM-1 expression by BMDCs. Regarding the expression of IL-15, I demonstrated that in normal mice it is undetectable without stimulation but is elevated in lymphopenic mice, suggesting a role for T cells in regulating IL-15 expression. Overall, these studies have identified many novel aspects of the interaction between DCs and CD8 T cells that were previously unknown. The study of adhesion molecules in IL-15 transpresentation describes a novel role for these well-known adhesion molecules and it will be interesting for future studies to further characterize this relationship for other IL-15-dependent cell types.

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| DN | double negative |
| MHC | major histocompatibility complex |
| DC | dendritic cell |
| TLR | Toll-like receptor |
| $\gamma_c$ | common $\gamma$ chain, CD132 |
| IFN | interferon |
| SLEC | short-lived effector cell |
| MPEC | memory-precursor effector cell |
| Tcm | central memory |
| Tem | effector memory |
| OT-I | ovalbumin transgenic, [MHC class] I |
| OVA | ovalbumin |
| Tg | transgenic |
| VSV | vesicular stomatitis virus |
| SD | standard deviation |
| Wt | wild type |
| LN | lymph node |
| BM | bone marrow |
| IEL | intra-epithelial lymphocyte |
| CFSE | carboxyfluorescein succinimidyl ester |
| BrdU | bromodeoxyuridine |
| Ab | antibody |
| $\beta_2$m | $\beta_2$ microglobulin, crucial to MHC class I expression |
| pSTAT5 | phosphorylated STAT5 |

BMDC  bone marrow-differentiated DC

IP  intra-peritoneal

Poly I:C  poly inosinic:polycytidylic acid; TLR3 ligand

RAG  recombination activating gene

cGy  centiGray; measure of absorbed radiation

qtPCR  quantitative PCR

$\Delta C_T$  change in cross-threshold values between $\beta$-actin and IL-15 during qtPCR

$T_{reg}$  CD4+ regulatory T cell

LCMV  lymphocytic choriomeningitis virus

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**Introduction**


The immune system exists to protect host organisms against infectious organisms by eliminating these pathogens once they've been detected: a further goal is the establishment of a bulwark against the reoccurrence of a given pathogen.  The immune system uses a multi-pronged approach to control and eliminate infectious organisms and CD8 T cells are an important feature in this response as they are responsible for recognizing and killing cells that have become infected.  By killing infected cells, CD8 T cells remove cellular reservoirs that can produce and disseminate more virus.  What's more, after the immune response has resolved, these pathogen-specific CD8 T cells – now called memory CD8 T cells – are part of said bulwark as they can now more swiftly curtail the infection by virtue of having experienced the given pathogen before.  This abbreviated infection is important in that the overall immune system engagement is lessened, resulting in less immune pathology, and the pathogen itself is not allowed to disrupt the host as it otherwise would.  Maintaining this pool of pathogen-experienced memory CD8 T cells is therefore an important component to the future protection of an organism.  As various aspects of memory CD8 T cell biology are dependent on the cytokine IL-15, uncovering the mechanisms behind the unique delivery of this cytokine will be useful in developing methodologies to augment or enhance the CD8 T cell memory pool and could be translated into better host protection.

**Chapter 1: Background**

## 1.1    CD8 T cell development and immune responses

T cell development begins with T cell progenitors leaving the BM and migrating to the thymus. Upon arriving in the thymus, CD4 and CD8 double-negative (DN) T cell precursors undergo a series of developmental steps that are characterized by changes in CD44 and CD25 expression: CD44+ CD25- (DN1), CD44+ CD25+ (DN2), CD44- CD25+ (DN3), and CD44- CD25- (DN4) (1).  Accompanying these fluctuations in CD44 and CD25 expression is the rearrangement of T cell receptor (TCR) genes that ultimately yields the $\beta$-chain of the TCR(1). The genes encoding the TCR $\beta$-chain are sequentially rearranged, first by combining D-J genes, then V genes to the combined DJ genes.

The successful creation of the TCR $\beta$-chain enables a cell to exit the DN4 stage where the $\beta$-chain is then paired with a surrogate $\alpha$-chain, pre-T-cell receptor $\alpha$-chain (pT$\alpha$), and begins to express CD3 (2).  Developing T cells that have a functional TCR $\beta$-chain will gain expression of CD4 and CD8 and turn off $\beta$-chain gene rearrangement as well as undergo proliferation (2). Subsequent to proliferating, each developing T cell has the opportunity to arrange an $\alpha$-chain to pair with the pre-existing $\beta$-chain that will replace pT$\alpha$.  $\alpha$-chain rearrangements occur between V and J genes and these putative $\alpha$-chains pair with the $\beta$-chain to form an $\alpha\beta$-TCR (2).  The process of randomly rearranging V, D, and J genes results in a combinatorial diversity of antigens that T cells can recognize, which is crucial to protecting the host against a wide variety of pathogens.  At this stage in T cell development, developing T cells are found in the cortical region of the thymus where they come in contact with cortical epithelial cells. Cortical epithelial cells present antigens via major histocompatibility complex (MHC) class I and II for positive selection (3) of developing T cells with a functional TCR, i.e. capable of recognizing antigen.  The CD4 and CD8 co-receptors at this stage are important for allowing

cells bearing αβ-TCRs the ability to interact with MHC class I or II, and their preference for either will be in part determined by the nature of the antigens presented. MHC class I and II molecules possess different peptide binding abilities and this limits the variety of peptide antigens that either molecule can present. Positive selection occurs to allow a diverse set of TCRs to develop; however, it is also crucial to eliminate T cells that bear TCRs specific for self-antigens. T cells that recognize self-antigens presented by dendritic cells (DCs) and macrophages in the medulla of the thymus (4) will be deleted so that they do not mature further. This process of positive and negative selection ensures a pool of CD4+ and CD8+ single-positive T cells that recognize diverse antigens but will not react against host peptides.

CD8 T cells that survive thymic selection reside primarily in secondary lymphoid organs such as the lymph node where they continually interact with migrating DCs to scan for the antigen for which their T-cell receptor is specific. These naïve CD8 T cells are quiescent and require IL-7 and require interactions with MHC class I for survival (5,6). Immature DCs constantly sample antigen through phagocytosis and pinocytosis in peripheral tissues and migrate through secondary lymphoid tissues where they display the sampled antigens for the perusal of naïve CD8 T cells. The naïve T cells themselves are motile and will continue migrating through the lymphatic system, sampling the antigens that DCs in secondary lymphoid organs are presenting. In homeostatic conditions, these T cell:DC interactions will not yield an immune response. In an infection, peripheral DCs can detect pathogens through surface receptors called Toll-like receptors (TLRs) that recognize conserved bacterial or viral components, i.e. bacterial cell-wall components or double-stranded DNA in endosomes, resulting in their activation. TLRs are a tool the immune system has evolved to allow the detection of a wide variety of pathogenic species by simply recognizing conserved elements that different pathogens share in common. The result of this TLR recognition and signaling is the expression of pro-inflammatory cytokines that can recruit other cells to the site of infection,

3

as well as inducing the expression of costimulatory molecules. Activated DCs down-regulate their phagocytic functions and increase antigen processing as they migrate to the draining lymph node.

In the context of an infection where DCs have been activated, the nature of T cell:DC interactions in secondary lymphoid organs changes. Upon recognition of their cognate antigen, CD8 T cells form lasting contacts (up to ~48 hours) (7) with DCs where the T cell receives both TCR signals (signal 1) as well as CD28 costimulation (signal 2) via CD80/CD86 on the DCs (8,9). The prolonged interaction between CD8 T cells and DCs is important in efficiently activating the T cell as T cells that receive TCR stimulation without sufficient costimulation become anergic and are not functional (8,9). This is one way the immune system ensures T cells do not become active inappropriately – T cells seeing their specific antigen in the absence of "danger" signals (i.e. DCs not having responded via TLR) are essentially shut down. The level of inflammatory cytokines present at the time of the T cell receiving signal 1 and signal 2 have also been implicated in impacting the outcome of CD8 T cell differentiation, which will be discussed more in detail later.

TCR and CD28 signals serve to induce massive IL-2 production by activated CD8 T cells (9), which helps sustain the initial TCR-mediated proliferation that CD8 T cells undergo upon activation (10). Several notable phenotypic changes accompany activation: the up-regulation of IL-2R$\alpha$ (CD25) and the down-regulation of both IL-7R$\alpha$ (CD127) and CD62L (11). Up-regulation of IL-2R$\alpha$ increases the affinity with which CD8 T cells can bind IL-2 by pairing with IL-2R$\beta$ and the common gamma chain ($\gamma_c$) (12). Down-regulation of IL-7R$\alpha$ and CD62L disables the ability of the T cells to respond to IL-7 and enables T cells to leave lymphoid tissues and migrate to peripheral locations, respectively. Activated CD8 T cells then leave the secondary lymphoid organ and home to sites of infection where they begin killing cells bearing

their cognate antigen via the cytolytic granules perforin and granzyme B.  Additionally,

activated CD8 T cells express TNF-$\alpha$ and IFN-$\gamma$, which are involved in inducing apoptosis and

inflammation and increasing antigen presentation, respectively.  Antigen-specific naïve CD8 T

cells exist in very small numbers: typically, for a given antigen, there exists between 50 and

500 naïve CD8 T cells that can recognize that antigen (13).  In order to effectively combat the

infection, upon activation these antigen-specific CD8 T cells undergo massive proliferation,

which typically peaks one week after infection.  At the peak of the expansion phase, the

population of antigen-specific CD8 T cells can comprise 20-40% of the total CD8 T cell pool,

though this is not true for all CD8 T cell immune responses.


During the expansion phase, a subset of CD8 T cells will re-express IL-7R$\alpha$ while others begin

to express KLRG-1, a marker of cellular senescence (14)  The IL-7R$\alpha^+$KLRG-1$^-$ subset of CD8

T cells are known as memory-precursor effect cells (MPEC), and their polar counterparts,

those lacking IL-7R$\alpha$ but expressing KLRG-1, are known as short-lived effector cells (SLEC)

(15).  Following the zenith of expansion, the antigen-specific CD8 T cell population undergoes

a contraction period during which the vast majority of these cells will die via apoptosis.  TGF-$\beta$

production during the expansion phase of the immune response lowers Bcl-2 levels in

activated CD8 T cells, contributing to their death in the contraction (16).  Addition of

exogenous IL-2 or IL-15 at this time can counteract the contraction (17) by providing survival

signals.  By virtue of IL-7R$\alpha$ expression, MPECs are able to respond to both IL-7 and IL-15

during the contraction, whereas SLECs are only responsive to IL-15.  This contributes to their

differential survival during the contraction phase and their subsequent differentiation into

memory.  Both MPECs and SLECs will both seed the memory pool but the SLEC population

declines over time whereas MPECs persist and can self-renew.  Approximately one month

after infection and directly following the contraction phase, surviving antigen-specific CD8 T

cells are now considered memory CD8 T cells.  Importantly, the number of cells that survive

the contraction – i.e. the pool of memory CD8 T cells – is much higher than the number of initial naïve antigen-specific precursors.  This is one of the factors that contributes to the rapid response of memory CD8 T cells in the case of a secondary infection.

Additional heterogeneity exists in memory CD8 T cells as defined by CD62L expression.  As mentioned previously, activated CD8 T cells lose CD62L expression but a subpopulation of CD8 T cells will regain CD62L once they have entered the memory phase.  CD62L+ memory CD8 T cells, known as central memory (Tcm), ostensibly differ from their CD62L- counterparts (effector memory, Tem) by the difference in homing potential conferred by CD62L expression (18).  Functional studies, however, have determined that Tcm undergo homeostatic proliferation and reside in secondary lymphoid tissues whereas Tem less efficiently undergo homeostatic proliferation but are robust producers of cytokines upon reactivation (18,19). Because they are found predominantly in peripheral tissues, Tem are thought to play a role primarily in being the front line of defense during a secondary pathogen challenge.  Unlike naïve CD8 T cells, memory CD8 T cells no longer require costimulation in order to be functional (20) and these peripherally-dwelling Tem can begin killing infected cells immediately.  It is thought that Tem help control the pathogen at the site of infection and Tcm will proliferate in response to antigen-bearing cells in secondary lymphoid tissues and help to increase the number of CD8 T cells available to combat the infection.

CD8 T cell memory persists in the absence of pathogen so memory maintenance is not dependent on lingering antigen (21).  Interestingly, in chronic immune responses, a pool of memory CD8 T cells exists but has become anergic through repeated contact with the pathogen.  One of the mechanisms that the immune system uses to maintain its population of memory CD8 T cells is what is known as homeostatic proliferation (22,23), so-called because it occurs in the absence of pathogen and is proliferation that renews the pool of memory cells,

rather than expanding it.  In mice lacking IL-15, it was determined that adoptively transferred memory CD8 T cells did not undergo homeostatic proliferation and consequently memory CD8 T cells were lost over time (22,24).  IL-15, then, is required to drive the proliferative renewal of memory CD8 T cells.

## 1.2     Interleukin 15

Interleukin 15 is a pleiotropic cytokine that is crucial for specific cells on both the innate and adaptive arms of the immune system.  Initially described as a T cell growth factor, IL-15 was found to share many properties with IL-2 (25), not the least of which are the receptors through which both cytokines signal – IL-2R$\beta$/$\gamma_c$ (26,27). In addition to these shared receptor components, IL-2 and IL-15 each have their own private binding receptors, respectively known as IL-2R$\alpha$ and IL-15R$\alpha$ (28,29).  The collective IL-2 receptors combine to achieve their high affinity binding (kD ~ $10^{-11}$ M) (28) while IL-15R$\alpha$ by itself has very high affinity for IL-15 (1.4 x $10^{-11}$ M ) (29,30).  In contrast to IL-2, engaging all three IL-15 receptors (IL-15R$\alpha$/IL-2R$\beta$/$\gamma_c$) does not enhance the binding affinity of IL-15 to its receptors (29). These biochemical characteristics of the interaction between IL-15 and its receptors become important when considering some rather unique phenomena associated with IL-15.

Due to the shared usage of IL-2R$\beta$ and the common $\gamma$-chain, both IL-2 and IL-15 induce similar signals in CD8 T cells.  Jak3 associates with $\gamma_c$ and phosphorylates STAT5, which then dimerizes and translocates to the nucleus where it functions as a transcription factor.  Jak1 is associated with IL-2R$\beta$ and phosphorylates STAT3 (reviewed in (31)).  An important STAT5 target in CD8 T cells is the anti-apoptotic protein Bcl-2, which contributes to survival of CD8 T cells during an immune response (16).  Overall, because of the receptors these cytokines use for signaling, signaling induced by IL-2 and IL-15 is largely the same.

IL-2 is produced by activated T cells during immune responses and acts a potent stimulator of cytokine production and T cell proliferation in an autocrine and paracrine manner. Interestingly, screens of mRNA expression in crude sorted cell populations revealed that T cells did not contain IL-15 mRNA expression (27), which leads to two very important distinctions between IL-2 and IL-15.  Firstly, IL-15 cannot therefore function in an autocrine manner, and, secondly, IL-15 must be provided to T cells by a different cell.  These two crucial differences set the stage for the subsequent discoveries regarding the method of action for IL-15 and serve to distinguish IL-2 and IL-15 in spite of their similar effects.


Because $T_{reg}$s constitutively express IL-2R$\alpha$ and require IL-2 for their survival (32,33), IL-2-deficient mice develop severe inflammation due to the lack of regulatory T cells (34).  However, the phenotype of IL-15-deficient mice is much milder than perhaps would be anticipated.  Rather, the immune disruptions seen in IL-15$^{-/-}$ mice manifested in the disappearance of specific types of cells but otherwise exhibited no pathology (35).  IL-15 is not necessary for thymic T cell development but is necessary specifically for CD8 T cells with a memory phenotype, that is to say CD8 T cells expressing high levels of CD44 (35).  CD4 T cells are not affected by the absence of IL-15.  Though they will not be discussed at length here, IL-15 is also shown to be crucial for NK and NK T cell development and the development of CD8$\alpha\alpha$-expressing intraepithelial lymphocytes (reviewed in (36) ).  The generation of IL-15R$\alpha^{-/-}$ mice revealed that IL-15 and IL-15R$\alpha$ are intimately linked as the phenotype of mice lacking IL-15R$\alpha^{-/-}$ closely resembled that of IL-15$^{-/-}$ animals (37).  The lack of IL-15 or IL-15R$\alpha$ mainly affects the development and/or maintenance of the aforementioned cell populations, suggesting that IL-15 is acting during homeostatic conditions, unlike IL-2 which is most typically present when there is an ongoing immune response.

## 1.3 IL-15 Transpresentation

CD8 T cells express high levels of IL-15R$\alpha$ yet it was discovered that they did not need to express IL-15R$\alpha$ in order to respond to IL-15 (38,39). Indeed, it was found that IL-15R$\alpha^{-/-}$ CD8 T cells adoptively transferred into wild type (Wt) mice proliferated as well as Wt CD8 T cells, so long as the host they were transferred into was replete with IL-15R$\alpha$. This observation may have been explained by the effects of IL-15 being indirect, i.e. the proliferation response in the CD8 T cells could be a byproduct of host cells being stimulated by IL-15. IL-15, however, had initially been described as a T cell growth factor *in vitro* so the bystander explanation did not seem quite adequate (38). In what would be some groundbreaking and enlightening experiments, it was discovered that IL-15 could actually be delivered via IL-15R$\alpha$ to cells expressing only IL-2R$\beta$/$\gamma_c$(40). A study using human monocytes revealed that coating these cells with IL-15 prior to culturing with CTLL-2 cells resulted in bioactive IL-15 as evidenced by thymidine incorporation, indicating there had been proliferation in the cultures (40). To buttress these findings, endogenously-produced IL-15 by activated monocytes expressing IL-15R$\alpha$ was found to be sufficient to drive a response in an IL-15R$\alpha$-deficient mast cell line, PT-18, that expressed only IL-2R$\beta$/$\gamma$ (40). Because the only IL-15R$\alpha$ available in this system existed on the monocytes, these experiments demonstrated that the response did not depend on the mast cells expressing IL-15R$\alpha$, rather that the cells driving the response – the activated monocytes – were the ones that required IL-15R$\alpha$. These experiments were the first to demonstrate the presentation of IL-15 in trans and began to shed light on what had become a truly perplexing series of observations. Transpresentation of IL-15 is depicted in Figure 1A, where IL-15 is delivered via IL-15R$\alpha$ to a CD8 T cell expressing IL-2R$\beta$/$\gamma_c$.


These series of experiments made strides in clarifying the role that IL-15R$\alpha$ has in mediating IL-15 responses. The stymieing observation that IL-15R$\alpha$ was not required on CD8 T cells for

**Figure 1.  IL-15 transpresentation by BM-derived cells**
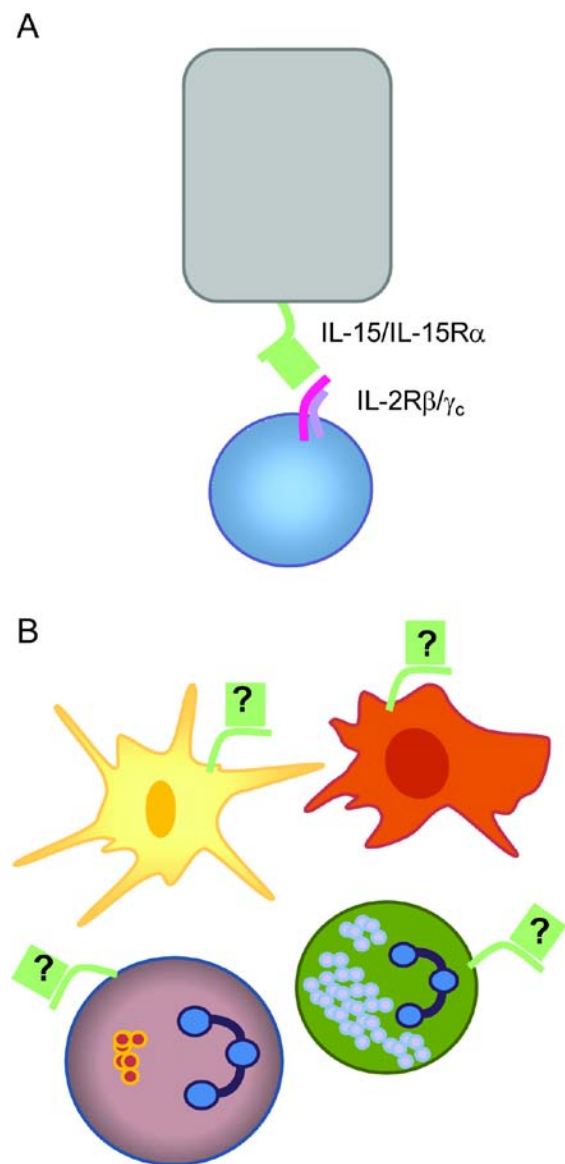
**Figure 1. IL-15 transpresentation by BM-derived cells**. A) Delivery of IL-15 in trans via IL-15R$\alpha$ by an unknown cell type. The CD8 T cell responds through IL-2R$\beta$/$\gamma_c$. B) Cartoon representation of various BM-derived cells and their potential to transpresent IL-15.

IL-15 driven responses now made sense – IL-15Rα was required to deliver IL-15, in a process that has since become known as IL-15 transpresentation. IL-15 transpresentation is an unprecedented cytokine delivery system that remains unique and is clearly distinct from the vast majority of cytokines that are simply secreted. The role of IL-15Rα as, essentially, a delivery mechanism also helps to explain how two virtually similar signals – IL-2 and IL-15 – can have discrete roles. Therefore, the delivery of these cytokines as well as the context in which they are found prise apart their roles in CD8 T cell biology.

Subsequent to the description of IL-15 transpresentation *in vitro*, several studies followed in short order that validated transpresentation *in vivo* but at the same time deepened the complexity already inherent in this system. In an adoptive transfer of IL-15Rα-sufficient and – deficient OT-I T cells, OVA-immunized mice generated and maintained normal levels of memory OT-I T cells (24,38); due to the host in each case being IL-15Rα sufficient, these studies demonstrated that transpresentation is indeed an *in vivo* phenomenon as well. Using various bone marrow chimeras, it was discovered that not only did certain cell types require IL-15 transpresentation, these cell types exhibited a strict preference for the nature of the cell providing the IL-15. For memory CD8 T cells, IL-15Rα$^{-/-}$ mice reconstituted with Wt BM after irradiation were sufficient to fully recapitulate the memory CD8 T cell population; IL-15Rα$^{-/-}$ BM, however, failed to restore this population when transferred into Wt irradiated mice (41). NK and NKT T cells required transpresented IL-15 from both radiation-sensitive and – insensitive sources whereas CD8αα+ intestinal intraepithelial lymphocytes (iIELs) required IL-15 transpresented by radiation-resistant cells only (39). IL-15 transpresentation, therefore, has very distinct roles in furnishing various immune cell niches in the variety of cells it affects as well as the nature of the specificity these cells exhibit for the IL-15 source. Additionally, the cells that transpresent IL-15 also must synthesize it themselves: IL-15 could then effect

transpresentation (42).  The heterogeneity of BM-derived cells and their potential to

transpresent IL-15 is depicted in Figure 1B.

**In chapter 2, I will address the issue of which specific cell type transpresents IL-15 to**

**memory CD8 T cells.  Our data demonstrate that DCs transpresent IL-15 to memory CD8**

**T cells mainly to drive homeostatic proliferation but DCs as well as other cells are**

**involved in providing IL-15 for the survival of CD8 T cells during the contraction of an**

**immune response.**

## 1.4     IL-15 Expression

The nature of the source of transpresented IL-15 for memory CD8 T cells was found to be

radiation-sensitive as well as BM-derived.  Given that IL-15 and IL-15R$\alpha$ must be coordinately

expressed, the cells that transpresent IL-15 must at the same time produce it. It can therefore

be understood that IL-15 protein expression by BM-derived cells is important for memory CD8

T cell homeostasis.  While the clarity of this observation is not in question, these findings only

give anecdotal and indirect data that BM-derived cells express IL-15.  As the BM gives rise to

several diverse lineages of hematopoietic cells, it is of great interest to learn what cells are

capable of producing IL-15 as they may be candidate transpresenting cells.  Much of the

current literature regarding IL-15 expression is confined to studies of RNA expression.

Moreover, many such studies analyzed IL-15 mRNA after stimulation with IFN-$\alpha$ or Toll-like

receptor agonists (43) and therefore were not analyzing normal, homeostatic conditions.  In

general, very little is known about steady-state IL-15 protein expression: the current body of

information regarding IL-15 mRNA expression is, unfortunately, largely irrelevant to the

homeostatic conditions under which memory CD8 T cells respond to IL-15.  More to the point,

direct studies of IL-15 protein expression are lacking and point to a significant gap in our understanding of IL-15 biology.

The lack of information available about IL-15 protein expression is likely a testament to many unsuccessful attempts that sought to determine its steady-state expression pattern. Several studies have determined that at various levels, the stability of IL-15 mRNA as well as its translation appear to be major factors in regulating IL-15 expression. Multiple isoforms of IL-15 mRNA exist, some of which are not transported to the cellular membrane even in the presence of IL-15R$\alpha$ and instead are found in the nucleus and cytoplasm (44). The presence of multiple AUG codons upstream of the 5' end inhibits IL-15 mRNA translation, while yet other posttranslational elements exist that further interfere with IL-15 production. The signal peptide for IL-15 inhibits its production – when the signal peptide for IL-2 was introduced into IL-15, IL-15 production massively increased (45). IL-15 production requires the presence of IL-15R$\alpha$, likely for its transport from the endoplasmic reticulum/Golgi apparatus to the cell surface after TLR stimulation (46). While this result was not determined under homeostatic conditions, it has already been demonstrated that IL-15 and IL-15R$\alpha$ must be co-expressed for various homeostatic processes so this remains a potential road block that must be passed before IL-15 can reach the cell surface. Though the precise mechanism is unclear, it was found that IL-15 constructs containing a carboxy-terminal FLAG epitope resulted in far more IL-15 production than those containing the native carboxy-terminal sequence, leading to the conclusion that the carboxy-terminus includes additional negative regulation elements (45). Overall, IL-15 is very highly regulated by multiple mechanisms before the mature protein is even synthesized. As IL-15 is a potent stimulant for CD8 T cells, these levels of regulation are likely

important for ensuring that IL-15 is transpresented only at appropriate times. Despite our understanding of what mechanisms regulate IL-15 express, very little is known about what constitutes "appropriate timing" for the delivery of IL-15 signals. The strict preference that various IL-15-dependent cell types have for the source of transpresented IL-15 (hematopoietic vs. parenchymal) is the best understanding we have of how IL-15 transpresentation is limited. Indeed, IL-15-responsive cells can be in IL-15+ environments but remain ignorant of the IL-15 if it's not presented by the proper cell type. Exactly how this preference is achieved is currently unknown.

**In chapter 4, I have endeavored to characterize the expression of IL-15 among diverse hematopoietic lineages. While IL-15 expression under normal, homeostatic conditions is so low that I was unable to detect it, I have demonstrated that IL-15 expression is increased in lymphopenic environments. Additionally, I show that IL-15 is expressed by several previously unheralded cell types.**

## 1.5 Adhesion molecules

Cells possess many ways of interacting with each other: indirectly, as in through the secretion of cytokines, or directly, via receptor:ligand interactions between two cells, to name two examples. One of the ways in which direct intercellular interactions are achieved is through the expression of adhesion molecules, which interact between the opposing cells and serve to tether the cells together. A major function of adhesion molecules is enabling cells to migrate between tissues via the bloodstream or lymphatic system. The expression of appropriate cognate

adhesion molecules allows migrating lymphocytes to bind to endothelial cells, which express ligands for the adhesion molecules on the lymphocytes. The interaction between endothelial cells and lymphocytes then enables the cell to migrate between endothelial cell junctions into tissues.

Adhesion molecules play critical roles in the migration of cells between tissues but are also critical in the induction of T cell immune responses. As mentioned previously, the recognition of antigen presented by MHC class I on dendritic cells causes the antigen-specific CD8 T cell to form lasting contacts with the dendritic cells. This long-lasting contact is mediated by the expression of adhesion molecules on the CD8 T cell and DC that interact to bind the cells tightly together. CD8 T cells express the adhesion molecule LFA-1, a heterodimer composed of CD18 ($\beta_2$) and CD11a ($\alpha_L$), which exists in two conformations. In naive CD8 T cells, LFA-1 is expressed at relatively low levels and found in a conformation that has low affinity for the ligand ICAM-1, found on DCs. Upon TCR stimulation, PLC-$\gamma$ and PKC-$\theta$ signaling can induce higher expression of LFA-1 and alter its conformation (47) to achieve the capability to bind ICAM-1 with high affinity. In addition to mediating lasting contacts between cells, adhesion molecules also have signaling properties. LFA-1 contributes additional Erk1/2 signals during TCR stimulation and provides signals to rearrange the cytoskeleton so as to direct the secretion of lytic granules for CTL function (48). For migrating CD8 T cells, the binding of chemokines such as CCL21 to the receptor CCR7, a G-protein coupled receptor, can induce the high affinity conformation of LFA-1 (49) through the GTPase RhoA (49).

CD8 T cells deficient in CD11a – one half of the LFA-1 heterodimer and thus lacking LFA-1 – display several deficiencies. It was discovered that CD11a$^{-/-}$ OT-I T cells were unable to respond to low amounts of peptide compared to Wt OT-I T cells (50), indicating that one function of LFA-1 is to enable greater T cell responsiveness when peptide (antigen) is limited. The converse argument therefore is that when antigen is in abundance, the role of LFA-1 may be marginal. The prolonged contact between DCs and CD8 T cells via LFA-1/ICAM-1 is likely the reason behind the efficacy of LFA-1 to foster TCR responses to limited antigen, enabling multiple rounds of stimulation in one interaction. Additionally, CD8 T cells lacking CD11a have reduced CTL function (51), likely related to the inability to polarize their cytolytic granules.

The large majority of literature involving the role of LFA-1/ICAM-1 in CD8 T cell biology is determined at the time of T cell activation but a few studies have investigated the impact that the lack of adhesion molecules can have in later phases of the CD8 T cell response. Several models have been used to address this question, sometimes yielding conflicting results. It was determined that although CD8 T cells express ICAM-1 as well as DCs, defects in CD8 T cell activation were due to the lack of ICAM-1 on DCs and not the CD8 T cells themselves (52). In most cases, when ICAM-1 was absent during the induction of an immune response, CD8 T cell activation was impaired at low doses of antigen, although differing results have been reported regarding IFN-$\gamma$ secretion, antigen-driven proliferation, and cytotoxicity (7,52). In all cases, however, ICAM-1$^{-/-}$ mice failed to generate normal levels of CD8 T cell memory, both in the frequency of memory cells as well as their quality (7,52). One study followed the expansion of antigen-specific CD8 T cells present in the draining lymph node after immunization

and showed that OT-I T cells in ICAM-1$^{-/-}$ mice expand the same as in Wt mice but did not survive as well, resulting in fewer memory OT-I T cells (7).  When these immune mice were rechallenged, the memory OT-I T cells produced less IFN-$\gamma$ and had decreased CTL function (7).  Similar results were obtained with the adoptive transfer of established OVA-specific memory CD8 T cells into ICAM-1$^{-/-}$ mice: in Wt mice, the memory cells were maintained, whereas in ICAM-1$^{-/-}$ hosts, the memory cells declined over time (53).  Though it was not linked with IL-15 transpresentation, this study also revealed that memory CD8 T cells transferred into ICAM-1$^{-/-}$ mice had higher expression of pro-apoptotic molecules and had higher Annexin-V staining (53).

**In chapter 3 I have addressed the role of ICAM-1 and LFA-1 in IL-15 transpresentation.  Based on the various models utilized, ICAM-1 is likely important for transpresentation mainly where IL-15 is limited but not where IL-15 is more readily available.  LFA-1, however, is highly important for the homeostatic proliferation of memory CD8 T cells, which may implicate that other adhesion molecule ligands for LFA-1 – in addition to ICAM-1 – play a role in IL-15 transpresentation.**

**Chapter 2 – Specific Aims**

**2.1     The role of DCs in the transpresentation of IL-15 to memory CD8 T cells**

The source of transpresented IL-15 for memory CD8 T cell generation and homeostasis is known to be BM-derived but has yet to be better characterized or definitively identified.  Given that IL-15 transpresented requires two cells to interact, **we hypothesize that DCs are the source of IL-15 for CD8 T cells**.  By the use of a transgenic mouse model generated in our lab that specifically allows DCs to transpresent IL-15, we will determine the role of DCs in IL-15 transpresentation to CD8 T cells.  IL-15 is involved in the survival of CD8 T cells during the contraction of an immune response as well as in the homeostatic proliferation of memory CD8 T cells so we will use a model of an acute viral infection to study the CD8 T cell immune response in the transgenic mice.

**2.2     The requirement for adhesion molecules in IL-15 transpresentation**

In the context of antigen presentation, adhesion molecules are important for maintaining contact between DCs and CD8 T cells for the efficient delivery of necessary signals for CD8 T cell responses.  IL-15 transpresentation can be likened to this mechanism, in the sense that instead of antigen being presented, it is the cytokine IL-15.  **We hypothesize, therefore, that ICAM-1 will be important for IL-15 transpresentation to memory CD8 T cells**.  We will utilize ICAM-1$^{-/-}$ mice to study IL-15-related phenomena, such as the generation of CD8 T cell memory and homeostatic proliferation.  Because ICAM-1 is also involved in cell trafficking, we will use *in vitro* cultures to address the role of ICAM-1 in IL-15 transpresentation as well.

## 2.3    Investigation of mechanisms controlling IL-15 expression among hematopoietic cells

 The observation that CD8 T cells require IL-15 transpresented by BM-derived cells invites a characterization of the expression patterns of IL-15 among these cell types.  As previously discussed, IL-15 expression is highly regulated and is a potent homeostatic cytokine.  In light of this, **we hypothesize that steady-state IL-15 expression is very low but is likely limited in its breadth of expression by hematopoietic cells**.  An antibody that has recently been demonstrated to be effective at detecting IL-15 will be used to determine both homeostatic and induced expression of IL-15 with the expectation that IL-15 protein expression will correlate with previously described mRNA expression.  Additionally, we will consider some mechanisms by which IL-15 expression is regulated.

**Chapter 3 – IL-15 transpresentation by dendritic cells**

## 3.1    Introduction

Immunological memory is an important feature of the body's ability to ward off infections that it

has previously encountered.  CD8 T cell memory is an integral component of said memory and

demystifying the requirements to generate and maintain memory CD8 T cells is therefore

critical to our understanding of how this protection arises and persists.  IL-15 is known to be

required for these phenomena but the identity of the cell(s) that provides IL-15 for these

processes remains unknown.  The discovery that IL-15 is transpresented by BM-derived cells

to memory CD8 T cells narrowed down the candidate cell types but as various diverse cell

lineages arise from the BM, a more targeted approach is necessary to more definitively

determine the IL-15 source.

## 3.2    Results.

### 3.2.1    "CD11c promoter drives IL-15R$\alpha$ expression preferentially by DCs

Since DCs are highly specialized for T cell interactions and stimulation as well as

express IL-15 and IL-15R$\alpha$ (43,54,55), we examined the contribution of IL-15 trans-

presentation by DCs on CD8 T cell development and the generation of memory CD8 T

cells. To target IL-15R$\alpha$ to DCs, a transgenic mouse model was generated in which IL-

15R$\alpha$ expression is driven by the murine CD11c promoter, a well-described promoter

with a high specificity to DCs (56).  We chose to control IL-15R$\alpha$ protein expression

rather than IL-15 to specifically address the role of IL-15 transpresentation.  To

eliminate IL-15R$\alpha$ expression from all other cells, the CD11c-IL-15R$\alpha$ Tg mice were

bred to the IL-15Rα$^{-/-}$ background. Once backcrossed, CD11c specific expression of cell surface IL-15Rα protein was assessed using immunofluorescence staining and flow cytometry.

Using this approach, two founders were identified that expressed IL-15Rα by CD11c+ cells: one founder line with IL-15Rα restricted to CD11c+ cells and a second founder line with expression spilling over onto CD11c-negative hematopoietic cells. Overall, the CD11c-driven expression of IL-15Rα closely correlated with the endogenous expression of CD11c, resulting in a model where DCs are the dominant cell type expressing IL-15Rα. Analysis of transgenic IL-15Rα expression showed that IL-15Rα was highly expressed by lineage (CD19, CD3, DX5)-negative, CD11b+ and CD11b-CD11c$^{hi}$ cells (CD8+ and conventional DCs respectively) and was moderate in lin$^-$ CD11c$^{lo}$ (putative DC precursors and pDC) in both Tg founder lines (Figure 2A). On CD11c-negative cells, which include monocytes (CD11b$^{lo}$SSC$^{lo}$), granulocytes (CD11b$^{lo}$SSC$^{hi}$), CD4, and CD8 T cells, Tg-1 had no expression of IL-15Rα while Tg 2 had levels similar to that of Wt cells (Figure 2B). As NK cells and IELs express CD11c, each of these cell types expressed IL-15Rα in both Tg founder lines at varying levels (Figure2B). Whereas previous studies have shown that IL-15Rα is expressed at a low level on CD11c+ cells (57), a comparison of IL-15Rα expression by DC subsets directly *ex vivo* had not been described before. Interestingly, this analysis demonstrates that IL-15Rα expression is relatively low and homogenous among DC subsets and other myeloid cells.

A similar technique was used to measure IL-15 on the cell surface using a recently available antibody. On lineage-negative myeloid cells, cell surface IL-15 was detected on CD11b$^+$ and CD8$^+$CD11c$^+$ cells (DCs) at similar levels in both Wt, Tg1, and Tg2

# Figure 2. Expression of IL-15 and IL-15Rα in CD11c-IL-15Rα Tg mice
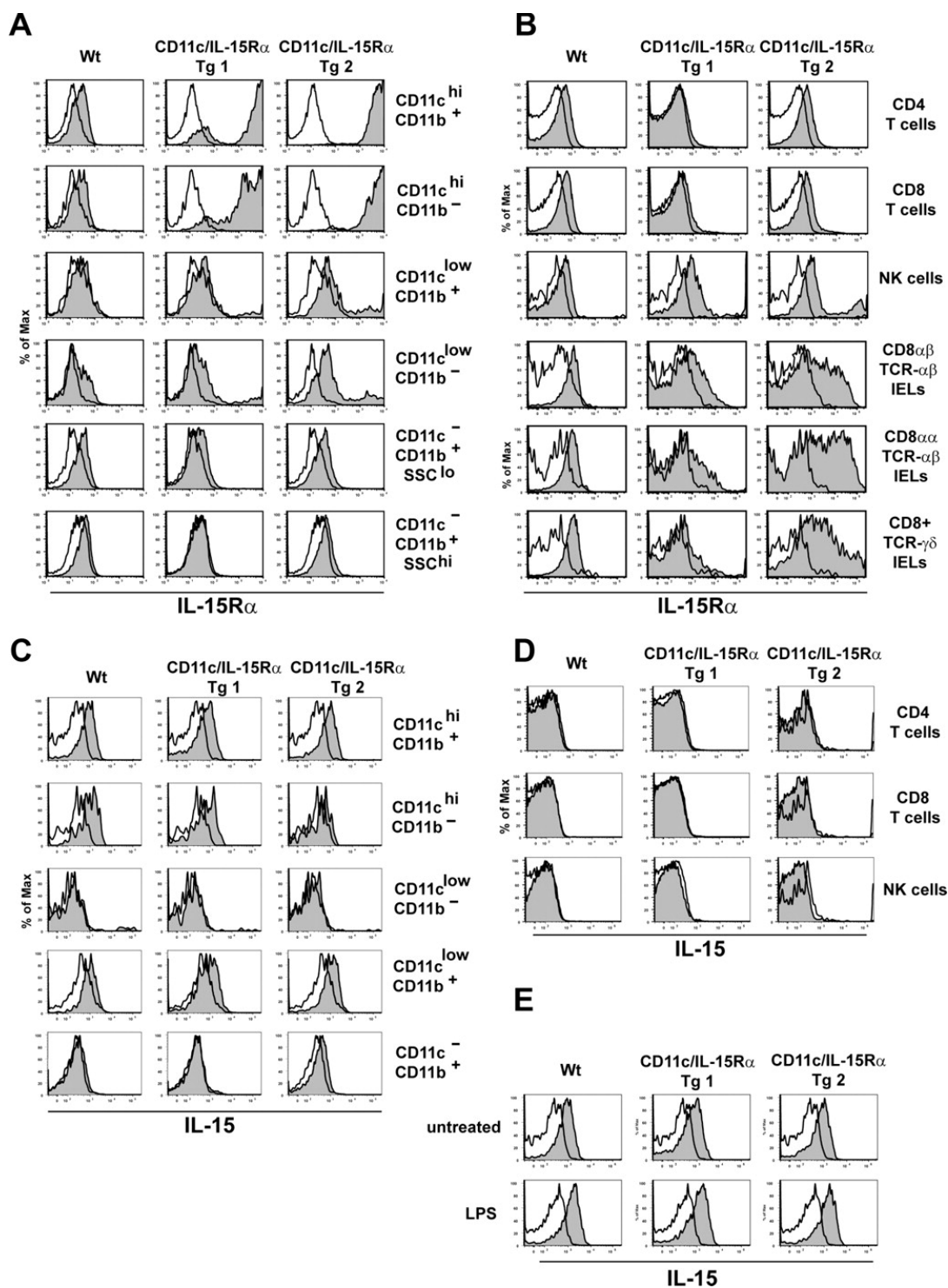
**Figure 2. Expression of IL-15 and IL-15Rα in CD11c-IL-15Rα Tg mice**

(A) Histograms show IL-15Rα expression on myeloid subsets in Wt, CD11c-IL-15Rα Tg 1 and Tg 2, and IL-15Rα$^{-/-}$ mice after gating on lineage (CD3, CD19, DX5) negative splenocytes. Filled histograms indicate IL-15Rα staining on the indicated populations, open histograms represent staining of the same population from IL-15Rα$^{-/-}$ mice. (B) Histograms show IL-15Rα staining on lymphoid populations from the spleen (top three panels) and IEL (bottom three panels). (C, D) 1x10$^{7}$ splenocytes from Wt, CD11c/IL-15Rα Tg 2, and IL-15$^{-/-}$ mice were cultured overnight in CM prior to staining. Filled histogram indicates IL-15 staining, the open histogram represents staining of cells from IL-15$^{-/-}$ mice. Histograms in panel C were first gated on lineage-negative cells as in panel A. (E) Splenocytes were treated as in C in the absence or presence of 1μg/ml LPS. **This research was originally published in *Blood*.** Stonier, S.W., Ma, L.J., Castillo, E.F., and Schluns, K.S. Dendritic cells drive memory CD8 T-cell homeostasis via IL-15 transpresentation. *Blood*. 2008; 112:4546-4554. **© the American Society of Hematology.**(58)

cells, although was not present on pDCs (Figure 2C). In WT and Tg 2 but not Tg 1

mice, IL-15 expression was observed at a lower level on CD11b+CD11c- cells

(monocytes and granulocytes) (Figure 2C) providing evidence that cells other than DCs

may transpresent IL-15.  Among lineage$^+$ cells (CD4 T cells, CD8 T cells, B cells, or NK

cells), IL-15 was not detected on the surface of cells isolated from WT or either CD11c-

IL-15R$\alpha$ Tg mice (Figure 2D).  In response to LPS stimulation, the amount of IL-15

upregulated was similar in DCs from both WT and Tg mice (Figure 2E) suggesting that,

in spite of elevated IL-15R$\alpha$ on DCs, IL-15 expression was not coordinately enhanced.

Altogether, these findings demonstrate that DCs and monocytes/granulocytes express

cell surface IL-15 in WT mice; however, DCs in the CD11c-IL-15R$\alpha$ Tg-1 mice are

likely the only cell transpresenting IL-15.

### 3.2.2   Naïve and memory-phenotype CD8 T cells are partially recovered in the presence of IL-15R$\alpha^+$ DCs

IL-15R$\alpha^{-/-}$ mice have reduced numbers of peripheral CD8 T cells with the deficiency in

memory-phenotype CD8 T cells affected more than the naïve CD8 T cells (37).

Decreased numbers of naïve CD8 T cells in the absence of IL-15R$\alpha$ are predominantly

due to the decreased survival of naïve CD8 T cells as only minor effects on the thymic

CD8 T cells are observed (59). To determine if CD11c-driven IL-15R$\alpha$ affects thymic T

cell development, thymocytes were analyzed from the CD11c-IL-15R$\alpha$ Tg mice and

compared to IL-15R$\alpha^{-/-}$ and Wt mice.  The proportions of the major thymocyte subsets

(CD4-CD8-, CD4+CD8+, CD4+, and CD8+ thymocytes) were similar in all groups of

mice (Figure 3A, and data not shown) indicating that transgenic expression of IL-15R$\alpha$

does not cause major disruptions in thymic T cell development.

**Figure 3. Phenotype of CD8 T cells as a function of CD11c-driven IL-15Rα expression.**

**Figure 3. Phenotype of CD8 T cells as a function of CD11c-driven IL-15R$\alpha$ expression.** Lymphocytes from thymus, spleen (SP), lymph node (LN), lung (LG), and bone marrow (BM) were isolated from the mice indicated (6-8 week old) and stained for cell surface markers to identify differences in T cell populations. (A) Representative flow cytometry plots from thymus and spleen. (B) The percentage of CD8 T cells along with the proportion of naïve (CD44$^{low}$) and memory-phenotype (CD44$^{hi}$) in various tissues. Results are averages with n=10 mice/group for Tg-1. For Tg-2, n=8 for spleen and n=2 for other tissues. § represents a significant difference between naïve T cells in Tg and Wt (P<0.05) while * indicates differences between memory phenotype in Tg and Wt (p<0.05). (C) The number of each IEL population recovered from small intestine of the indicated mice. The respective populations were identified after gating on CD45+ cells. Error bars represent SD from a representative experiment; values from either Tg mice are not significantly different than those from IL-15R$\alpha$-/- mice. (D) Total numbers of myeloid populations in the spleen of the indicated mice. Error bars represent SD, n=2, for one representative experiment. **This research was originally published in *Blood*.** Stonier, S.W., Ma, L.J., Castillo, E.F., and Schluns, K.S. Dendritic cells drive memory CD8 T-cell homeostasis via IL-15 transpresentation. *Blood*. 2008; 112:4546-4554. **© the American Society of Hematology.**(58)

Upon extending our analysis to peripheral tissues, differences in the CD8 T cells between the different groups of mice were apparent.  As previously reported, the percentage of CD8 T cells in the IL-15R$\alpha^{-/-}$ mice are decreased by approximately 50% compared to Wt mice (37), a trend observed not only in the spleen but also in the lymph node, lung, and bone marrow (Figure 3A, B).  In both CD11c-IL-15R$\alpha$ Tg lines, the percentages of splenic CD8 T cells were increased compared to those observed in the IL-15R$\alpha^{-/-}$ mice (~9% and 11% compared to only ~6%), but lower that in the Wt mice (~12%)(Figure 3A, B). This trend was consistently seen in the other tissues analyzed.  Since the total CD8 T cell population is comprised of both naïve and memory-phenotype CD8 T cells and IL-15 preferentially affects the memory CD8 T cells (54), the effects of the restricted IL-15R$\alpha$ expression on these two subpopulations was measured.  For the naïve CD8 T cells (CD44$^{low}$), the presence of IL-15R$\alpha$+DCs in Tg 1 increased the percentage of naïve CD8 T cells from ~50%, as observed in IL-15R$\alpha^{-/-}$ mice, to between 62-71% of Wt values, depending on the tissue (Figure 3B); however, this effect was more dramatic in the Tg2 line where the percentages of naïve T cells were almost similar to levels seen in Wt mice.  Among total CD8 T cells, percentages of memory-phenotype (CD44$^{hi}$) CD8 T cells in the CD11c-IL-15R$\alpha$ Tg mice approached parity with Wt levels in all tissues examined, ranging from 90-100% of Wt in both Tg lines(Figure 3B). Although the percentage of memory-CD8 T cells is almost recovered, a deficiency in the total levels of memory CD8 T cells still exists as a function of the deficit in naïve CD8 T cells in CD11c-IL-15R$\alpha$ Tg mice (Figure 3B). Altogether, these findings show that that the CD11c-IL-15R$\alpha$ transgenic environment partially restored the deficiencies in total CD8 T cells observed in the IL-15R$\alpha$ mice with the memory-phenotype CD8 T cells affected more than the naïve CD8 T cells.

Intestinal IELs, specifically TCR$\gamma\delta$ and CD8$\alpha\alpha$TCR$\alpha\beta$ cells, depend heavily on IL-15 for their normal development (37). To determine whether CD11c-driven expression of IL-15R$\alpha$+ affects IEL development, IELs were isolated from each group of mice and the respective populations were compared. Similar to IL-15R$\alpha^{-/-}$ mice, CD11c-IL-15R$\alpha$ Tg 1 and 2 mice were deficient in TCR$\gamma\delta$ and CD8$\alpha\alpha$TCR$\alpha\beta$ cells, whereas conventional CD8$\alpha\alpha$TCR$\alpha\beta$ cells were present in normal numbers (Figure 3C). While there are clearly IL-15R$\alpha$+ cells present in the intestines, the inability of these cells to recover the defects in IEL development is a sterling example of how cell specificity dictates responsiveness to transpresented IL-15.

Since IL-15R$\alpha$ expression by DCs could indirectly affect CD8 T cell development by altering the development and normal function of DCs, general characteristics of DCs were examined. Among lineage negative cells, no differences in the number or distribution of various DC subsets were observed in spleen (Figure 3D) among the four groups of mice. In addition, MHC class II expression by CD11c$^{hi}$DCs was not different (data not shown). Altogether, elevated IL-15R$\alpha$ expression did not alter the development or maturation of DCs.

### 3.2.3   IL-15R$\alpha$ expression by DCs contributes to the generation of virus-specific memory CD8 T cells

We next measured the generation and maintenance of antigen-specific memory CD8 T cells in response to a viral infection in our transgenic system. In separate experiments, Wt, CD11c-IL-15R$\alpha$ Tg-1, Tg-2, and IL-15R$\alpha^{-/-}$ mice were infected with 1x10$^5$ pfu VSV (vesicular stomatitis virus) and the levels of N-tetramer+ CD8 T cells in the peripheral

blood were measured at various times post-infection. During the expansion phase, no detectable differences in the percent of VSV-specific T cells were observed between the groups of mice (Figure 4A), which is consistent with previous studies showing IL-15R$\alpha$ is not required for normal Ag-specific CD8 T cell expansion (22,24). Following the contraction phase (30 days p.i.), the percentage of VSV-specific CD8 T cells present in the blood was increased in the CD11c-IL-15R$\alpha$Tg-1 mice, compared to IL-15R$\alpha^{-/-}$ mice, but was lower than that of Wt mice (Figure 4A), which was in contrast to Tg-2 mice, which had levels similar to Wt mice (Figure 4B). During the memory phase, both CD11c-IL-15R$\alpha$ Tg mice were able to maintain levels of VSV-specific CD8 T cells similar to Wt mice (Figure 4A,B). The discrepancy between the two Tg mice can be explained by the leaky expression of IL-15R$\alpha$ expression onto CD11c-negative cells, therefore suggesting that transpresentation by cells other than DCs participate in providing IL-15 to CD8 T cells during the contraction phase.

To determine if tissue-specific effects were evident, the level of VSV-specific CD8 T cells was measured in the spleen, lymph nodes, and lung. In all the tissues analyzed, the percent of VSV-specific memory CD8 T cells in CD11c-IL-15R$\alpha$ Tg-1 was greater than in the IL-15R$\alpha^{-/-}$ mice, but less than in the Wt mice (Figure 4C). When IL-15R$\alpha$ is present on both CD11c+ and CD11c- cells (Tg-2), the percentage of viral-specific CD8 T cells in the tissues was similar to that of Wt mice (Figure 4D). As expected, due to pre-existing defects in total CD8 T cells, total numbers of Ag-specific CD8 T cells were also deficient (Figure 4E and data not shown). Overall, the effect of transpresentation by DCs in different tissues was commensurate to the trends observed in the blood, indicating no tissue-specific effects.

CD27, KLRG-1, IL-7R$\alpha$, CD62L are cell surface markers that have been used to define subpopulations of effector and memory CD8 T cells(14,15,60). During the transition from effector to memory T cell, CD27+ KLRG-1-, IL-7R$\alpha^{hi}$ CD8 T cells have been identified as those having a greater potential to differentiate into memory T cells. In contrast, KLRG-1+CD27-IL-7R$\alpha$- cells are thought to represent short-lived effectors that rely temporarily on IL-15 for their survival (15). Therefore, to determine whether IL-15R$\alpha$+ DCs alter the differentiation of these subsets, expression of CD27, KLRG-1, and IL-7R$\alpha$ by antigen-specific CD8 T cells was analyzed at various times after infection in the CD11c-IL-15R$\alpha$ Tg-1 mice. We chose to use the combination of CD27 and KLRG-1 as CD27+KLRG-1- cells were more easily defined than IL-7R$\alpha$+ KLRG-1- cells. At the peak of the expansion (7 days p.i.), no differences in the proportion of VSV-specific CD8 T cells defined by KLRG-1 and CD27 were observed between the different groups (Figure 4F). Interestingly, from 19-60 days p.i., the VSV-specific CD8 T cells in the IL-15R$\alpha$ deficient mice were predominantly CD27-KLRG-1+ whereas both CD27+KLRG-1+ and CD27+KLRG-1- populations were abundant at similar levels in both the CD11c-IL-15R$\alpha$ and Wt mice (Figure 4F). Considering all memory CD8 T cells utilize IL-15, our findings suggest that IL-15R$\alpha$+ DCs preferentially drive the survival of short-lived CD27-KLRG-1+ memory T cells while other IL-15R$\alpha$+ cells may promote development of long-lived memory CD8 T cells.

Whereas KLRG-1, CD27 and IL-7R$\alpha$ identify T cell subsets during the transition from the effector phase to the memory phase, high CD62L expression on a subset of memory T cells is associated with the ability to migrate to lymphoid tissues and an enhanced self-renewal, i.e. central memory (18,61). To determine whether CD11c-directed expression of IL-15R$\alpha$ affects the generation of central and effector memory subsets, CD62L expression on VSV-specific memory CD8 T cells was examined.

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# Figure 5. Role of IL-15R$\alpha^+$ DCs in a viral immune response.

**Figure 5. Role of IL-15Rα⁺ DCs in a viral immune response.**

The kinetics of an anti-viral CD8 T cell response was compared among different groups of mice by measuring the percent of VSV-specific CD8 T cells present in the peripheral blood as determined by N-tetramer staining: (A) CD11c-IL-15Rα Tg-1, (B) CD11c-IL-15Rα Tg-2. (C,D) Dot plots show the percent of N-tetramer+ cells versus CD44 expression after gating on CD8⁺ cells present in SP, LN, and LG 25 weeks p.i. in C) Tg-1, and (D) Tg-2. (E) Graphs show total numbers of N-tet+ cells recovered from the various tissues in Wt, Tg 1 and IL-15Rα⁻ᐟ⁻ mice. Error bars represent SD, n=2. Numbers above the bars represent percent of Wt mice.  (F) KLRG-1 and CD27 expression on N-tet+ CD8 T cells in peripheral blood at indicated times after infection.  (G) Histograms show CD62L expression by N-tet+ CD8 T cells in various tissues 25 weeks p.i.. **This research was originally published in *Blood*.** Stonier, S.W., Ma, L.J., Castillo, E.F., and Schluns, K.S. Dendritic cells drive memory CD8 T-cell homeostasis via IL-15 transpresentation. *Blood*. 2008; 112:4546-4554. **© the American Society of Hematology.**(58)

Depending on the tissue site of isolation, VSV-specific memory CD8 T cells had a similar pattern of CD62L expression among all groups of mice (Figure 4G and data not shown). This suggests that IL-15Rα did not impact the proportion of memory CD8 T cell subsets, as defined by CD62L expression, that arise during an acute viral infection.

### 3.2.4   IL-15Rα⁺ DCs drive homeostatic proliferation of memory CD8 T cells

Whereas we demonstrated that IL-15Rα+ DCs alone were not completely sufficient in generating normal levels of memory CD8 T cells, it was unclear whether IL-15Rα+ DCs could still drive normal homeostatic proliferation. To examine the homeostatic proliferation of endogenous Ag-specific memory CD8 T cells, VSV-infected mice from each group (Wt, CD11c-IL-15Rα Tg 1, and IL-15Rα⁻ᐟ⁻) were given BrdU in their drinking water at 40 days p.i. for 4 weeks.  After this course of treatment, BrdU incorporation of the VSV-specific memory CD8 T cells in various tissues was measured. In all the tissues analyzed, VSV-specific CD8 T cells incorporated similar levels of BrdU in CD11c-IL-15Rα Tg 1 and Wt mice (Figure 5A).  In contrast, VSV-specific memory CD8 T cells had little BrdU incorporation in the IL-15Rα⁻ᐟ⁻ mice. Although the extent of BrdU incorporation was slightly different among tissues, the effect of the transgenic IL-15Rα+ by DCs drove almost normal levels of cell division. The similar BrdU incorporation in VSV-specific memory CD8 T cells between Wt and CD11c-IL-15Rα Tg 1 mice is evidence that IL-15Rα+ DCs are pivotal in mediating homeostatic proliferation of memory CD8 T cells.

The possibility exists that the ability of memory CD8 T cells to undergo homeostatic proliferation is affected earlier in the immune response by the transgenic expression of

**Figure 4. Homeostatic proliferation of memory CD8 T cells.**
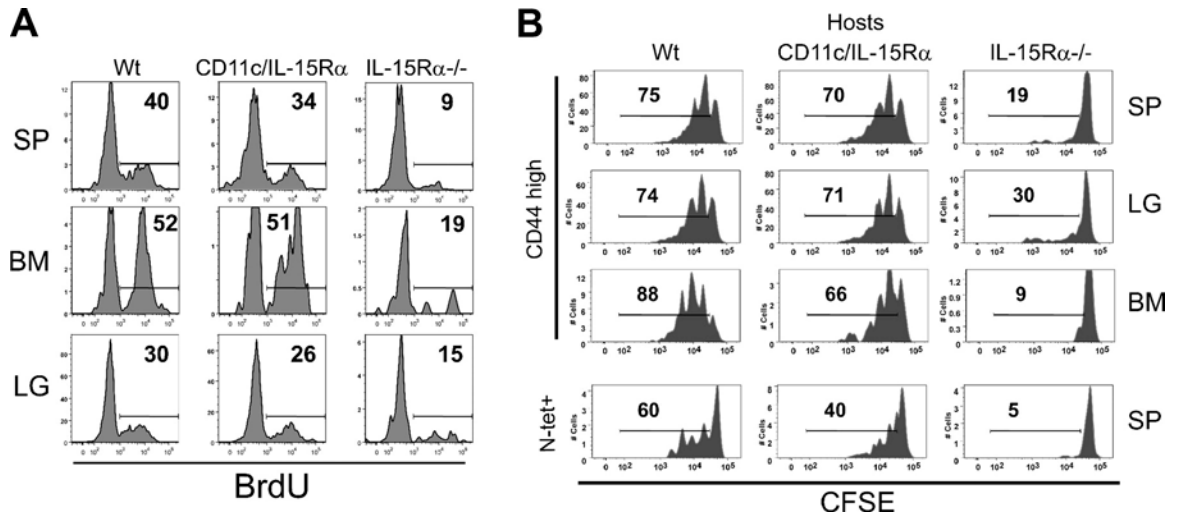
**Figure 4. Homeostatic proliferation of memory CD8 T cells.**

(A) Histograms show the percentage of VSV-N specific memory CD8 T cells that have incorporated BrdU. (B) Spleen cells from normal, CD45.1$^+$, VSV-infected mice were enriched for CD8 T cells, labeled with CFSE, and transferred to various CD45.2$^+$ hosts. Two months after transfer, lymphocytes from SP, LG, and BM were analyzed for CFSE intensity. Top panels show CFSE dilution in CD45.1$^+$ CD44$^{hi}$ CD8+ T cells (memory-phenotype) in various tissues. Bottom row depicts CFSE dilution in splenic N-tet+ cells. **This research was originally published in *Blood*.** Stonier, S.W., Ma, L.J., Castillo, E.F., and Schluns, K.S. Dendritic cells drive memory CD8 T-cell homeostasis via IL-15 transpresentation. *Blood*. 2008; 112:4546-4554. **© the American Society of Hematology.**(58)

IL-15Rα. We therefore examined the proliferation of VSV-specific memory CD8 T cells

generated in a normal environment. CD8 T cells were enriched from congenic Wt mice

(CD45.1+) previously infected with VSV, CFSE-labeled, and adoptively transferred into

CD11c-IL-15Rα Tg-1, Wt, and IL-15Rα$^{-/-}$ mice (all CD45.2+). Approximately two

months later, CFSE dilution of the donor CD8 T cells was measured. In both the Tg

and Wt mice, the memory-phenotype CD8 T cells divided 2-3 times in the spleen, lung,

and lymph node in a similar manner (Figure 5B). Similar results were observed with

CD11c-IL-15Rα Tg-2 line (data not shown). In contrast, the IL-15Rα$^{-/-}$ host was unable

to support division of the memory-phenotype CD8 T cells (Figure 5B). Among the

VSV-specific memory CD8 T cells and memory CD8 T cells in the BM, both the Wt and

Tg mice were able to induce cell division; however, cell division was greater in the Wt

than in the Tg hosts (Figure 5B). Overall, the exclusive expression of IL-15Rα to DCs

provides a sufficient signal for memory CD8 T cells to proliferate under homeostatic

conditions.


### 3.2.5   IL-15Rα+ DCs are not the only hematopoietic cells participating in

### homeostatic proliferation of memory CD8 T cells

The homeostatic proliferation of memory CD8 T cells was almost completely recovered

in mice that express IL-15Rα exclusively by CD11c+ cells (Figure 6B), indicating that

other cells types transpresent IL-15 to memory CD8 T cells. Since our previous

studies showed that IL-15Rα by parenchymal cells could play a minor role in

homeostatic proliferation (41), we wanted to determine if a host containing IL-15Rα+

DCs along with IL-15Rα+ parenchymal cells would be sufficient to completely restore

the homeostatic proliferation of memory CD8 T cells. Therefore, three groups of BM

chimeras were generated with each group containing different combinations of IL-15Rα

on either DCs and/or parenchymal cells: 1) Wt BM in Wt hosts (IL-15R$\alpha$ expressed by all cells), 2) CD11c-IL-15RTg-1 in Wt hosts (IL-15R$\alpha$ expressed by DCs and parenchymal cells), 3) IL-15R$\alpha^{-/-}$ BM in Wt hosts (IL-15R$\alpha$ expressed only by parenchymal cells).  After reconstitution, CD8 T cells (~10x10$^6$) from a cohort of VSV-infected Thy1.1+ mice (at least 40 days p.i.) were isolated, labeled with CFSE, and transferred into the various groups of chimeras.  Six weeks later, homeostatic proliferation of the donor memory CD8 T cells was assessed by measuring CFSE dilution. Memory CD8 T cells divided 1-3 times in the Wt chimeras (Wt into Wt) representing the normal level of homeostatic proliferation over this time-period (Figure 5).  In the chimeras containing IL-15R$\alpha$+ DCs along with IL-15R$\alpha$+ parenchymal cells (CD11c-IL-15R$\alpha$Tg-1 into wt), the memory CD8 T cells divided but not to same extent as in the Wt chimeras (Figure 6).  Little cell division of memory CD8 T cells was observed in the chimeras containing IL-15R$\alpha$ only in the parenchyma (IL-15R$\alpha^{-/-}$ into Wt) (Figure 5). These differences indicate that homeostatic proliferation of memory CD8 T cells is not completely restored in the presence of IL-15R$\alpha$+ DCs and parenchymal cells and suggest that IL-15R$\alpha$+ hematopoietic cells other than DCs participate in the response.

## 3.3    Discussion

Generating a model with DC-restricted IL-15R$\alpha$ expression has allowed us to demonstrate that DCs are not only important for presenting antigen for T cell stimulation but also for presenting IL-15 during later stages of CD8 T cell differentiation.  This function of DCs in memory CD8 T cell homeostasis had been speculated but never demonstrated. Our study has also demonstrated that IL-15 transpresentation by DCs has specific roles during CD8 T cell differentiation in that DCs were most effective at inducing homeostatic proliferation of established memory

**Figure 6. Contribution of CD11c-negative hematopoietic cells in memory CD8 T cell homeostasis.**
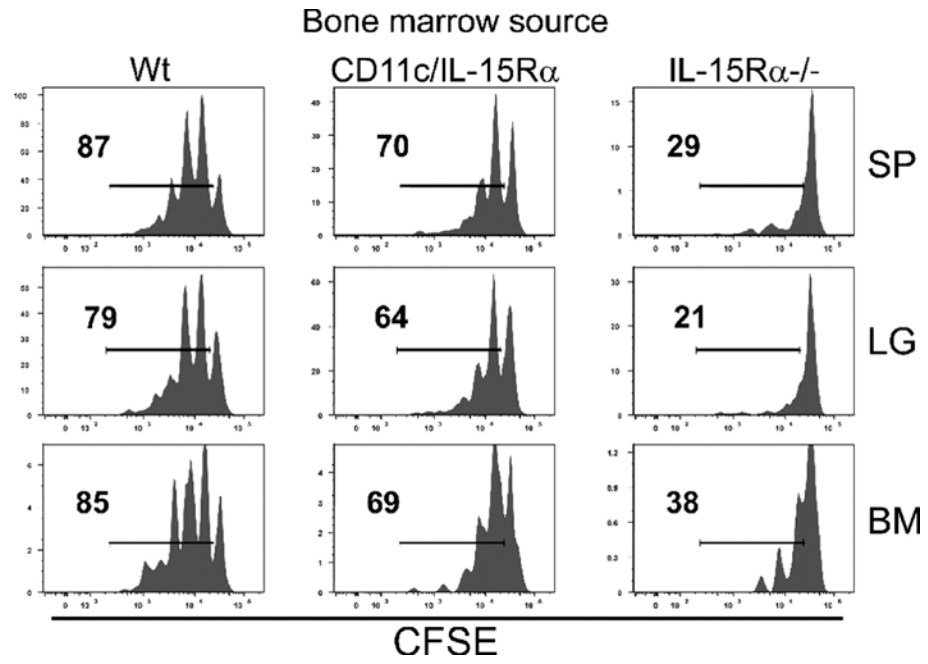
**Figure 6. Contribution of CD11c-negative hematopoietic cells in memory CD8 T cell homeostasis.** BM cells were isolated from IL-15Rα$^{-/-}$, CD11c-IL-15RαTg-1, and Wt mice (all CD45.2+) and transferred into lethally irradiated Wt hosts (CD45.1+). CD8 T cells were isolated from VSV-infected Thy1.1+ cells, labeled with CFSE, and transferred into the various BM chimeras after lymphocyte reconstitution (between 8-12 weeks after BM transfer). Six weeks after transfer, CFSE dilution of donor cells was measured. **This research was originally published in *Blood.*** Stonier, S.W., Ma, L.J., Castillo, E.F., and Schluns, K.S. Dendritic cells drive memory CD8 T-cell homeostasis via IL-15 transpresentation. *Blood*. 2008; 112:4546-4554. **© the American Society of Hematology.**(58)

CD8 T cells, while having less of a role in generating memory CD8 T cells and the homeostasis of naïve CD8 T cells. In contrast, transpresentation by DCs had no effect on IEL development.  In concurrent analysis of our model, we have also found that IL-15 transpresentation by DCs heavily influenced NK cell development while only minimally affecting NKT cell development (62). Altogether, our study has shown that cell specificity is pivotal in dictating IL-15 responsiveness by transpresentation and that DCs are an integral cell type transpresenting IL-15 to memory CD8 T cells.

The enhanced ability of DCs to transpresent IL-15 as differentiation progresses could be an indication that developing memory CD8 T cells become more accessible to DCs and preferentially receive IL-15 signals in a DC-enriched environment.  This is contradictory to studies touting BM as the predominant site for memory CD8 T homeostasis, as BM is a site with a very low frequency of DCs. The importance of BM in providing homeostatic signals is based on the presence of memory CD8 T cells undergoing a higher rate of cell division (63-65), which could be explained by preferential migration of actively dividing central memory CD8 T cells.  If BM is the predominant site for memory CD8 T homeostasis, we would predict an exaggerated defect in homeostatic proliferation of memory CD8 T cells in the BM of our Tg model, where only DCs transpresent IL-15.  On the contrary, homeostatic proliferation of memory CD8 T cells was restored rather efficiently in the BM of the CD11c-IL-15R$\alpha$ Tg mice suggesting that memory CD8 T cells did receive adequate IL-15 signals elsewhere and then preferentially migrated to the BM.  Moreover, memory CD8 T cells that are sequestered in the spleen and LN with FTY720 treatment undergo normal homeostatic proliferation despite this tissue restriction (66). This result, along with our findings, suggest that memory CD8 T cells efficiently receive homeostatic signals in many tissues besides the BM.

In addition to the specific effects on the homeostatic proliferation of established memory CD8 T cells, DCs appeared to have a distinct role in generating memory CD8 T cells. During the contraction phase, IL-15 begins to become important by mediating survival of Ag-specific CD8 T cells as these cells are not proliferating (67). Specifically, a subpopulation of short-lived effectors (SLECs) defined as KLRG-1$^+$, are predominantly affected by the lack of IL-15 (68). In our model, IL-15 transpresentation by DCs limited the contraction of the Ag-specific memory CD8 T cells by increasing the proportion of SLEC-like cells. Considering all memory CD8 T cells utilize IL-15, our findings yield two possible scenarios: SLECs may be more responsive or accessible to DCs providing IL-15.

The limited requirements for DCs transpresenting IL-15 to naïve and differentiating memory CD8 T cells highlights the idea that cells other than DCs are providing IL-15 to CD8 T cells during these earlier phases. For naïve CD8 T cells, this isn't surprising as previous studies using BM chimeras indicated that IL-15R$\alpha$ expression by parenchymal cells is important for naive CD8 T cells (41). Our current studies provide further evidence for this, as the ability to generate a normal frequency of naïve CD8 T cells could not be substituted by CD11c+ and/or CD11c- hematopoietic cells even when IL-15R$\alpha$ is expressed at high levels. For differentiating memory CD8 T cells, these cells do not appear to utilize IL-15 transpresented by parenchymal cells but rather receive IL-15 signals from both CD11c+ and CD11c- hematopoietic cells. In comparison of the two CD11c-IL-15R$\alpha$ Tg lines, the main difference observed was during the contraction phase. When IL-15R$\alpha$ expression was restricted to DCs, the contraction of the Ag-specific CD8 T cells was more exaggerated resulting in less memory CD8 T cells generated; however, when IL-15R$\alpha$ expression was present on DCs along with other

hematopoietic cells, the contraction and generation of memory CD8 T cells was normal. Therefore, our study provides the first evidence that some unknown CD11c-negative hematopoietic cells participate in the generation of memory CD8 T cells *in vivo*.

The incomplete abilities of DCs to transpresent IL-15 to CD8 T cells was not only present during the generation of memory CD8 T cells, but could also be observed to a small degree during the homeostasis of memory CD8 T cells. While parenchymal cells can have a minor contribution in homeostatic proliferation (41), the combination of IL-15R$\alpha$ on both DCs and parenchyma did not completely restore normal levels of homeostatic proliferation, again providing evidence that hematopoietic cells other than DCs transpresent IL-15 to memory CD8 T cells during the memory phase.

While this current study has provided evidence that other hematopoietic cells transpresent IL-15 to CD8 T cells, the clear effectiveness of the DCs suggests that DCs may be superior at IL-15 transpresentation than other cell types; however, this could have been due to the imprecise specificity of the transgenic promoter. Whereas high expression of CD11c is specific to DCs, low levels of CD11c are sometimes expressed on non-DCs, such as activated T cells, NK cells, and IELs (69). In our Tg models, even when some IL-15R$\alpha$ is expressed on these cells, it was not accompanied by IL-15 expression. As IL-15 is not transferred from other transpresenting cells even under inflammatory circumstances (46), this IL-15R$\alpha$ does not appear to be transpresenting IL-15. Furthermore, since previous studies have shown that IL-15R$\alpha$ expression by T cells is dispensable for generation and maintenance of memory CD8 T

cells and the IL-15 dependent development of IELs (39,41,70), the little IL-15R$\alpha$ expression that is expressed by T cells is likely irrelevant.

Another caveat of our model is the supraphysiological levels of IL-15R$\alpha$ present on DCs that could make the DCs more efficient. Indeed, recent reports demonstrate that the presence of IL-15R$\alpha$ can stabilize IL-15 protein and increase bioactivity (71); however, our studies do not support the idea that higher IL-15R$\alpha$ translates to more IL-15 transpresentation as IL-15R$\alpha^{hi}$DCs did not have higher levels of IL-15 than IL-15R$\alpha^{lo}$DCs. Altogether, our observations suggest that IL-15 expression appears to be endogenously controlled and is the limiting factor in transpresentation.

In summary, we have developed an *in vivo* model whereby DCs are likely the only cell able to transpresent IL-15. Using this model, DCs are shown to be effective in mediating the generation and maintenance of memory CD8 T cells via IL-15 transpresentation, thus identifying a novel function of DCs. Although efficient at transpresenting IL-15 to memory CD8 T cells, DCs were not the only hematopoietic cells acting on memory CD8 T cells. Lastly, the inability of IL-15 transpresented by DCs to recover all the IL-15 functions in CD8 T cells is a sterling example of how cell specificity dictates responsiveness to transpresented IL-15. In general, these findings will enable future studies to better focus on mechanisms regulating T cell homeostasis.

**This research was originally published in *Blood*.** Stonier, S.W., Ma, L.J., Castillo, E.F., and Schluns, K.S. Dendritic cells drive memory CD8 T-cell homeostasis via IL-15 transpresentation. *Blood*. 2008; 112:4546-4554. **© the American Society of Hematology."**"(58)

A study conducted by Mortier *et al* used another system to analyze cell-specific IL-15 transpresentation (72). In this model, Cre-lox technology was employed to remove the ability to transpresent, in contrast to our model which bestowed this ability. Their findings largely mirrored ours: when recombination and deletion in IL-15R$\alpha$-loxp mice was directed by CD11c-Cre mice, memory CD8 T cells declined over time (72). Homeostatic proliferation was not measured in these mice, only the frequency of OT-I T cells in the blood. Nonetheless, their findings corroborated ours in that DCs are important in transpresenting IL-15 for memory CD8 T cell maintenance. Additionally, LysM-Cre x IL-15R$\alpha$-loxp mice exhibited a partial recovery of antigen-specific, contracting CD8 T cells; in these mice the lack of transpresentation is due to the absence of IL-15R$\alpha$ on macrophages, although LysM promoter usage is not strictly limited to macrophages (72). Therefore, our study showed that DCs alone were not sufficient to fully protect contracting CD8 T cells, and the study by Mortier *et al* suggests that macrophages are the other side of the coin, so to speak.

**Chapter 4 – ICAM-1 and IL-15 transpresentation**

## 4.1 Introduction

The transpresentation of IL-15 requires close contact between cells. The nature of transpresentation being an intercellular interaction – at a basic level – then likely also involves the adhesion of transpresenter to responder. How long this interaction lasts, and whether or not several "transpresentation events" occur during one interaction, are large unknowns in the field. Additionally, the requirements for this interaction have never been addressed in regards to what molecules are required on the cell surface to manufacture or maintain the interaction.

Given that IL-15R$\alpha$ is not expressed in high amounts on the surface of DCs – or any other myeloid cell type for that matter – and the fact that IL-2R$\beta$/$\gamma_c$ are not highly expressed either, it stands to reason that a cellular interaction generated solely by IL-15 transpresentation is a poor basis for two cells to interact. Therefore, we propose that adhesion molecules – specifically, ICAM-1 and LFA-1 – are important for IL-15 transpresentation. In this scenario, cells would first likely interact via adhesion molecule receptor:ligand pairings, which would then enable IL-15 transpresentation. As memory CD8 T cells express high amounts of LFA-1 and require IL-15 for their maintenance, the high expression of LFA-1 may be a feature that gives them additional ability to interact with cells transpresenting IL-15. It has already been demonstrated that mice lacking ICAM-1 fail to support CD8 T cells during the contraction of an immune response and subsequently generate lower levels of memory (7). This pattern is reminiscent of IL-15R$\alpha^{-/-}$ mice, which may suggest a relationship between ICAM-1 and IL-15R$\alpha$. Therefore, we have set out to investigate the role of ICAM-1/LFA-1 in IL-15 transpresentation to memory CD8 T cells.

## 4.2. Results

### 4.2.1 BMDCs need ICAM-1 to transpresent IL-15 to memory CD8 T cells

Because ICAM-1 is involved in cell trafficking as well as antigen presentation, we first looked for evidence that ICAM-1 plays a role in IL-15 transpresentation in a closed system where migration would not be a factor. To address this, cultures of CD8 T cells and BM differentiated DCs were utilized as a model of IL-15 transpresentation in isolation, enabling the study of IL-15 transpresentation while avoiding any potential unforeseen effects due to altered migration. To investigate IL-15 responses *in vitro*, CD8 T cells were enriched from congenically-marked mice and cultured in the presence of BrdU with poly I:C-activated bone marrow-differentiated DCs (BMDCs) from Wt and ICAM-1$^{-/-}$ BM. After three days of culture, the frequency of CD44$^{hi}$ CD8 T cells was higher among CD8 T cells cultured with Wt DCs compared to ICAM-1$^{-/-}$ DCs (Figure 7A), showing that survival of memory CD8 T cells required ICAM-1 expression by DCs. More importantly, BrdU incorporation was apparent only among CD44$^{hi}$ CD8 T cells (~22% of CD44$^{hi}$) cultured with Wt DCs, demonstrating a response specific to memory phenotype CD8 T cells (Figure 7A,B). In contrast, BrdU incorporation was virtually absent in CD44$^{hi}$ CD8 T cells cultured with ICAM-1$^{-/-}$ DCs (Figure 7A,B). Memory CD8 T cells also failed to incorporate BrdU when cultured with Wt DCs in the presence of ICAM-1 and CD122 blocking Ab or DCs generated from IL-15R$\alpha^{-/-}$ BM (Figure 7C,D). These findings provide evidence that BrdU incorporation in this system is indeed mediated by IL-15 and not related to development differences in DCs generated from IL-15R$\alpha^{-/-}$ or ICAM-1$^{-/-}$ mice. Given that BrdU incorporation was confined to memory CD8 T cells and dependent on IL-15R$\alpha$ by DCs (Figure 7C,D), these results demonstrate the

**Figure 7.  BrdU incorporation in memory CD8 T cells requires IL-15Rα and ICAM-1 expression by BMDCs.**
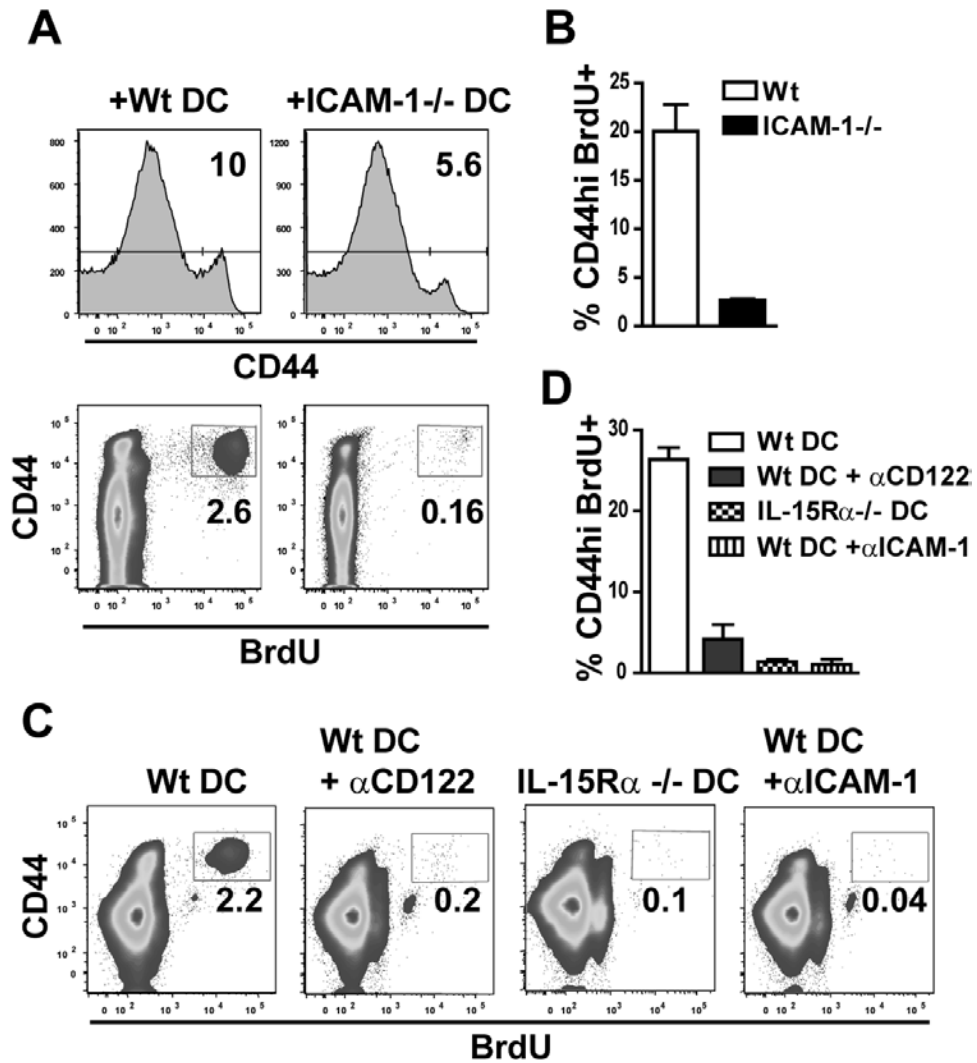
**Figure 7. BrdU incorporation in memory CD8 T cells requires IL-15R$\alpha$ and ICAM-1 expression by BMDCs.** A) Top row show shows percent of memory CD8 T cells (CD44$^{hi}$) on TCR$\beta$+ CD8$\alpha$+ CD45.1+ gated cells present after coculturing with Wt or ICAM-1$^{-/-}$ BMDCs. Bottom row shows percent of cells incorporating BrdU, gated as above. Plots are representative of 2 replicates per group. B) Histograms show the average BrdU incorporation in CD44$^{hi}$ CD8 T cells. Error bars represent SD of 2 replicates per group. C) Representative flow cytometric plots show BrdU incorporation in CD8 T cells cocultured with BMDC in the presence of the indicated Ab. D) Graph shows the average BrdU incorporation in CD44$^{hi}$ CD8 T cells. Error bars represent SD of 2 replicates per group. Data is representative of 3 independent experiments.

importance of ICAM-1 in a response mediated by IL-15 transpresentation. We also differentiated DCs using BM from $\beta_2m^{-/-}$ mice to determine if MHC class I expression was involved in this proliferation response. To our surprise, CD8 T cells cocultured with $\beta_2m^{-/-}$ BMDCs failed to proliferate (Figure 8), resembling cocultures with both IL-15R$\alpha^{-/-}$ and ICAM-1$^{-/-}$ BMDCs. MHC class I expression, therefore appears to be important in this coculture system, though exactly what role it plays is unclear.

Because of the lack of BrdU incorporation among memory phenotype CD8 T cells cultured with ICAM-1$^{-/-}$ BMDCs, we next asked if IL-15 signaling was compromised in CD8 T cells after culture with ICAM-1$^{-/-}$ DCs. To this end, serum-starved CD8 T cells were cocultured with poly I:C-activated BMDC for 1h and then analyzed for STAT5 phosphorylation, a major signaling event induced by IL-15 (73,74). CD8 T cells cultured in the absence of DCs show virtually no pSTAT5 expression whereas CD8 T cells cultured with rIL-15 showed a dramatic induction of pSTAT5 expression, in both the memory and naïve populations. When cultured with Wt DCs, the CD8 T cells showed a clear increase in pSTAT5 expression (Figure 9A,B) with the CD44$^{hi}$ CD8 T cells expressing higher levels of pSTAT5 than CD44$^{lo}$ CD8 T cells (39% compared to 15%, respectively), which again is consistent with memory CD8 T cells being more responsive to IL-15 signals (54). In striking contrast, the induction of pSTAT5 signaling was severely impaired in both memory and naïve CD8 T cells when cultured with BMDCs derived from IL-15R$\alpha^{-/-}$ or ICAM-1$^{-/-}$ BMDC (Figure 9A,B). These data demonstrate that the absence of ICAM-1 inhibits the transmission of IL-15-specific signals by impairing IL-15 transpresentation. Coupled with the lack of IL-15-driven BrdU incorporation in memory CD8 T cells in the absence of ICAM-1, these findings demonstrate a crucial and previously unappreciated role for ICAM-1 in IL-15 transpresentation responses.

**Figure 8. BMDCs need $\beta_2$m expression to drive proliferation of memory-phenotype CD8 T cells.**
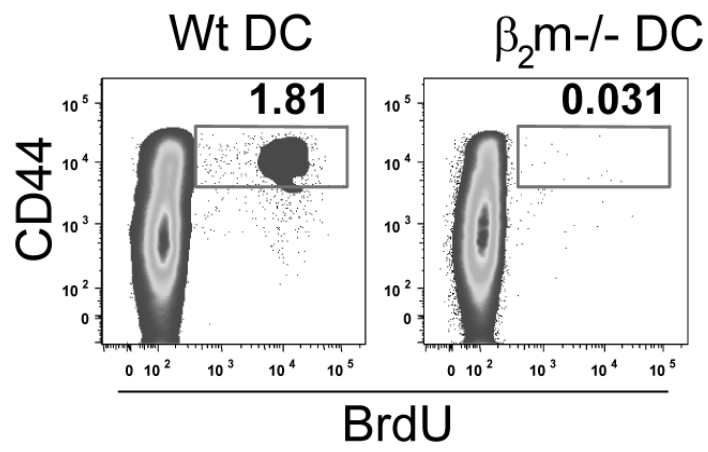
**Figure 8. BMDCs need $\beta_2$m expression to drive proliferation of memory-phenotype CD8 T cells.** A) BMDCs were activated at d7 of culture with 150 $\mu$g/ml poly I:C for 12-16 hours. CD8 T cells were enriched from Wt mice and cultured with BMDCs after poly I:C stimulation for 3 days in the presence of BrdU. Plots are gated on TCR$\beta$+ CD8$\alpha$+ CD45.1+ cells and measure the incorporation of BrdU on CD44 hi CD8 T cells. Plots are representatives of triplicate cultures and two independent experiments.

**Figure 9. STAT5 phosphorylation in CD8 T cells induced by BMDCs requires IL-15Rα and ICAM-1 expression by DCs.**
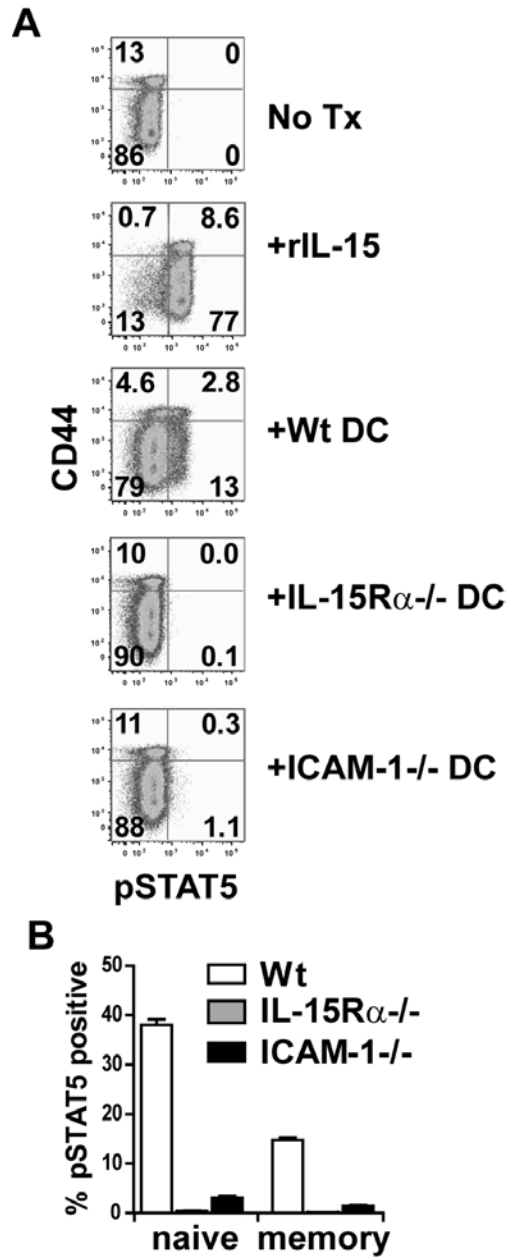
**Figure 9. STAT5 phosphorylation in CD8 T cells induced by BMDCs requires IL-15R$\alpha$ and ICAM-1 expression by DCs.** A) Dot plots show phophorylated STAT5 versus CD44 expression after gating on CD8$\alpha$+ TCR$\beta$+ CD45.1+ cells in the cultures indicated. B) Graph shows the average frequency of memory (CD44$^{hi}$) or naïve (CD44$^{lo}$) CD8 T cells expressing phospho-STAT5 after coculturing with the indicated BMDCs. In panels A and B, error bars represent SD of 3 replicates per group. Experiment shown is representative of three experiments performed.

### 4.2.2 ICAM-1 and LFA-1 are important for memory CD8 T cell homeostatic proliferation and maintenance

Having demonstrated that ICAM-1 is important for IL-15 transpresentation in a closed system, we wanted to examine IL-15 transpresentation responses *in vivo*. Homeostatic proliferation of memory CD8 T cells is a well-established readout of IL-15 transpresentation so we endeavored to analyze the role of ICAM-1 in this response. Since ICAM-1/LFA-1 interactions have multiple functions on T cells that can impact the differentiation and behavior of memory CD8 T cells, we chose to examine memory CD8 T cells generated in a normal environment. To this end, CD8 T cells were enriched from Wt mice infected at least 50 days earlier with VSV, CFSE-labeled, and transferred into either ICAM-1$^{-/-}$ or Wt recipients. Approximately 30 days post-transfer, homeostatic proliferation and the total number of the donor VSV-specific and memory phenotype CD8 T cells were analyzed. Among the donor memory phenotype CD8 T cells, CFSE dilution was less in ICAM-1$^{-/-}$ mice than in Wt mice in both spleen and BM (Figure10A). Unfortunately, the low number of donor VSV-specific memory CD8 T cells made it difficult to clearly assess differences in their level of proliferation (data not shown); however, the frequency and total number of VSV-specific CD8 T cells was decreased in ICAM-1$^{-/-}$ mice (Figure 10B), suggesting that homeostasis and/or survival of these CD8 T cells was impaired in the absence of ICAM-1. Alternatively, the homeostatic proliferation of antigen-specific OT-I memory CD8 T cells was examined. After memory OT-I T cells were generated *in vivo* following VSV-OVA infection, CD8 T cells were again CFSE-labeled and transferred into ICAM-1$^{-/-}$ and WT mice. Within the ICAM-1$^{-/-}$ mice, the memory OT-I T cells exhibited a consistent reduction in cell division compared to OT-I T cells in Wt mice in all tissues (Figure 10C, D). Reduced division in ICAM-1$^{-/-}$ hosts was observed in both central and effector memory subsets (data not shown). Similar to that observed with VSV-specific memory CD8 T cells, ICAM-1$^{-/-}$ hosts had fewer numbers of donor memory OT-I T cells in each tissue

**Figure 10. Homeostatic proliferation of memory CD8 T cells is impaired in ICAM-1$^{-/-}$ hosts.**
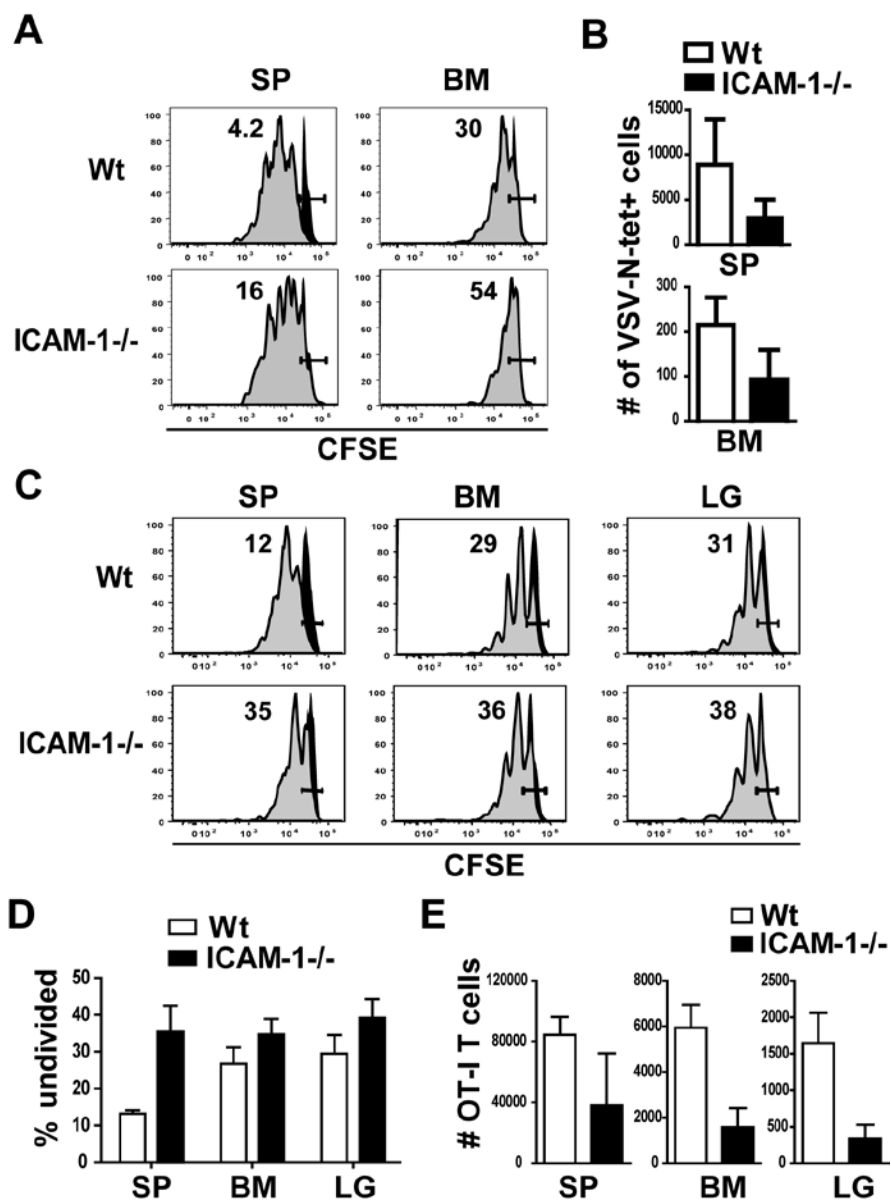
**Figure 10. Homeostatic proliferation of memory CD8 T cells is impaired in ICAM-1$^{-/-}$**

**hosts.** A) CD8 T cells were enriched from mice infected at least 60 day previous, CFSE-labeled and transferred into Wt and ICAM-1$^{-/-}$ mice for 29 days. Histograms show CFSE dilution of CD8+ CD45.1+ CD44$^{hi}$ CFSE+ cells in spleen (SP) and bone marrow (BM). The black underlay represents undivided naïve CD8 T cells. Gating represents the frequency of cells that have not divided. Plots are representative of 2 mice per group. B) Histograms showing the absolute numbers of N-tetramer specific CD8 T cells after adoptive transfer into Wt and ICAM-1-/- hosts. Error bars represent S.D. of 2 mice per group. C) CD45.1+ OT-I memory CD8 T cells were CFSE labeled and adoptively transferred into CD45.2+ mice for 21 days. Histograms show CFSE dilution of OT-I cells in SP, BM, and Lung (LG). Gate indicates the frequency of cells that have not divided. The black underlay marks undivided donor CD8 T cells. Plots are representative of 3-4 mice per group. D) Graphs represent the frequency of cells gated as above that failed to divide. Error bars represent S.D of 3 mice per group. E) Total numbers of OT-I+ memory CD8 T cells in the indicated tissues 21 days post-transfer. Error bars represent S.D. of 3 mice per group.

compared to WT hosts, implying survival of the memory CD8 T cells is also compromised (Figure 10E). To address whether decreased numbers in the BM and lung were due in part to defects in migration, donor cell numbers were also examined 24 hours after transfer before effects on homeostasis could occur and no defects in migration to lung or BM were observed (data not shown). By virtue of homeostatic proliferation being exclusively driven by IL-15, we conclude from these data that IL-15 transpresentation is indeed impaired in the absence of ICAM-1, which is in agreement with our *in vitro* data.

The absence of ICAM-1 did not completely inhibit the homeostatic proliferation of memory CD8 T cells as we had anticipated. However, since LFA-1 on CD8 T cells can interact with other ICAMs, we next wanted to determine if the absence of LFA-1 on the CD8 T cells would alter their ability to undergo homeostatic proliferation. We enriched CD8 T cells from unmanipulated CD11a-deficient mice, which we then labeled with CFSE and transferred into Wt mice. 2-3 weeks after transfer, we again looked in the spleen and BM to determine the extent of homeostatic proliferation the transferred cells had undergone. As we had anticipated, ~80% of memory-phenotype CD8 T cells lacking CD11a failed to undergo homeostatic proliferation, in contrast to Wt memory-phenotype CD8 T cells which had undergone several rounds of division (Figure 11). These data and the adoptive transfers into ICAM-1$^{-/-}$ mice raise the possibility that other adhesion molecules in addition to ICAM-1 are involved in IL-15 transpresentation; LFA-1 expression, however, is essential on CD8 T cells.

### 4.2.3 Endogenous immune responses in ICAM-1$^{-/-}$ mice are normal

IL-15R$\alpha^{-/-}$ mice develop lower levels of CD8 T cell memory than WT counterparts in response to VSV and indeed a similar phenomenon has been observed using adoptively transferred OT-I T cells into ICAM-1-deficient mice (7). We sought to recapitulate these findings by infecting

**Figure 11. Homeostatic proliferation of CD11a$^{-/-}$ memory CD8 T cells is impaired.**
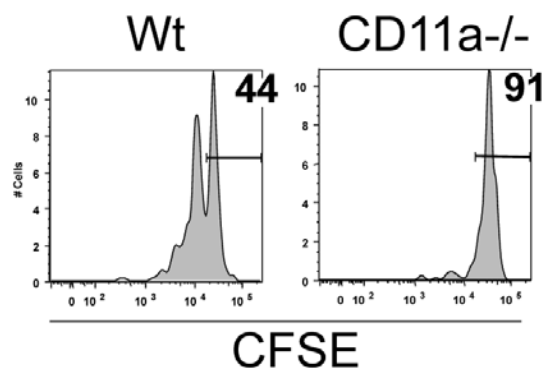
**Figure 11. Homeostatic proliferation of CD11a$^{-/-}$ memory CD8 T cells is impaired.** CD8 T cells were enriched from the spleens of unmanipulated Wt and CD11a$^{-/-}$ mice. Histograms are gated on CD45.2+ CD44hi CD8 T cells. Interval gate measures the frequency of cells that have failed to proliferate after an 18 day transfer period. Plots are representative of 3 mice per group.

ICAM-1$^{-/-}$ mice and analyzing the development of endogenous VSV-specific CD8 T cell memory but also extended our study to analyze the CD8 T cell memory maintenance. CD8 T cells express ICAM-1 and while the role of ICAM-1 on CD8 T cells is likely for adhesion, ICAM-1 indeed has a well-established role in antigen presentation which is presumably compromised in this model. Despite this caveat, we investigated whether VSV-specific CD8 T cells generated in ICAM-1$^{-/-}$ mice display defective memory CD8 T cell homeostasis. Surprisingly, we found that the expansion, contraction, and memory maintenance of endogenous CD8 T cells in ICAM-1$^{-/-}$ mice appeared normal in response to VSV infection (Figure 12A,B). At all time points measured, the level of antigen-specific CD8 T cells was not significantly different between Wt and ICAM-1$^{-/-}$ mice. In addition, BrdU incorporation in memory CD8 T cells in ICAM-1$^{-/-}$ mice over a two week period was not defective and actually was increased somewhat compared to normal mice, regardless of tissue localization (Figure 12B and data not shown). We next tested the ability of ICAM-1$^{-/-}$ memory CD8 T cells to produce IFN-$\gamma$ after stimulation with peptide. Previous studies indicated that IFN-$\gamma$ production by memory CD8 T cells generated in ICAM-1$^{-/-}$ mice is defective so we wanted to determine if this was the case in our model as well. Using a peptide encoding for the immunodominant epitope of the VSV N-protein, we show that in contrast to previous studies, ICAM-1$^{-/-}$ memory CD8 T cells produced IFN-$\gamma$ at equivalent or slightly better levels than Wt memory cells (Figure 12C,D). Taken together, endogenous antigen-specific memory CD8 T cells generated in ICAM-1$^{-/-}$ mice display no defects that we could determine and may even perform slightly better than their Wt equivalents.

### 4.2.4    IL-15 expression is elevated in ICAM-1$^{-/-}$ mice

As endogenously-generated ICAM-1$^{-/-}$ memory CD8 T cells are able to respond to IL-15-driven phenomena equivalent to their Wt counterparts, we wanted to determine if levels of IL-15 protein expression were different in these mice. We reasoned that if IL-15 expression was

**Figure 12. Endogenous immune responses to VSV in ICAM-1^{-/-} mice are normal.**
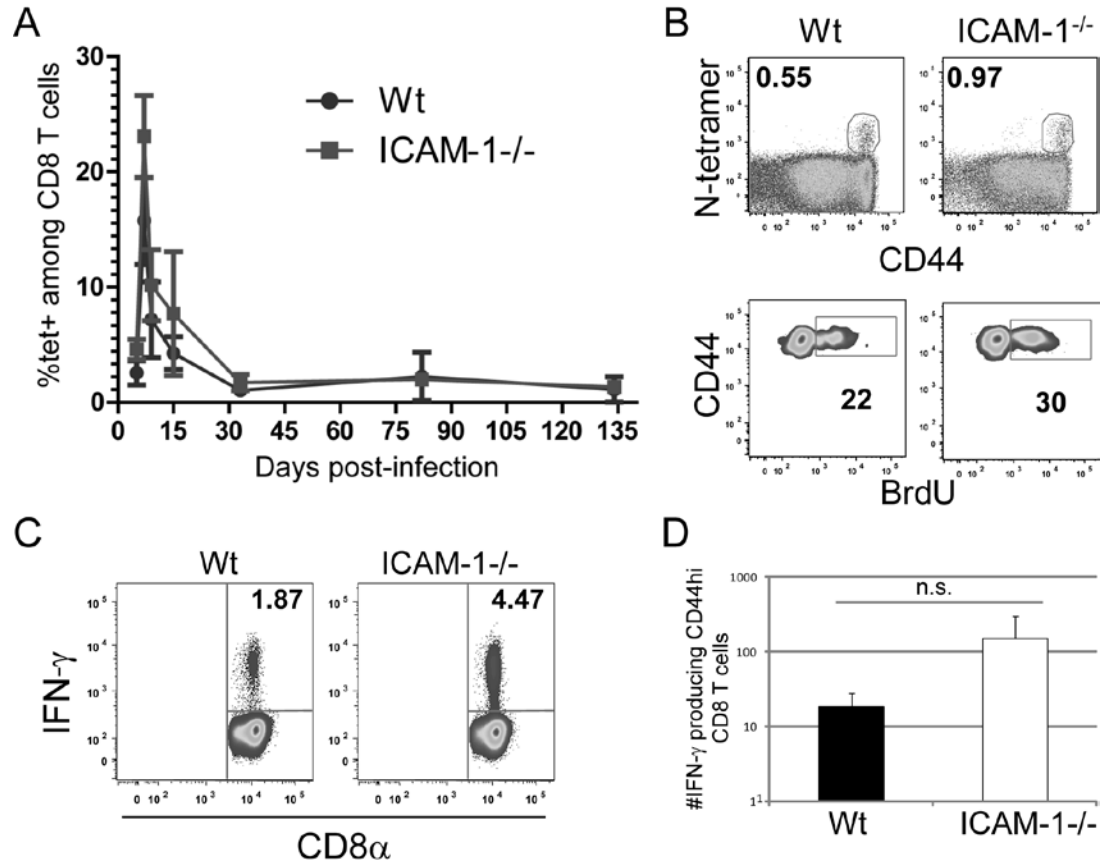
**Figure 12. Endogenous immune responses to VSV in ICAM-1$^{-/-}$ mice are normal.** A) Wt

and ICAM-1$^{-/-}$ mouse were infected with 1x10$^5$ pfu VSV. The frequency of antigen-specific

CD8 T cells were measured in peripheral blood using a tetramer reagent. Error bars represent

standard deviation of 4-5 mice per group. Plot is representative of 3 independent experiments.

B) Upper panel: the frequency of antigen-specific CD8 T cells in the spleen 4 months after

infection. Lower panel: mice were fed 0.8 mg/ml BrdU in their drinking water for a two-week

period, after which time mice were sacrificed and incorporation of BrdU assessed. Plots are

representative of 4-5 mice per group and 2-3 independent experiments. C) 3x10$^6$

Splenocytes from various VSV-immunized Wt and ICAM-1$^{-/-}$ were incubated for five hours with

a peptide encoding for the antigenic sequence of the VSV N protein in the presence of Golgi

stop. Plots are gated on CD44hi CD8 T cells and the frequency of cells producing IFN-$\gamma$ is

measured. Plots are representative of 3 mice per group. D) The enumeration of IFN-$\gamma$-

producing CD44hi CD8 T cells. Error bars are standard deviation of 3 mice per group.

increased in ICAM-1$^{-/-}$ mice, this could explain how less-efficient IL-15 transpresentation could still generate normal responses in memory CD8 T cells. We analyzed splenic DCs from Wt, IL-15$^{-/-}$, RAG-1$^{-/-}$ (positive control; **more on this in chapter 5**), and ICAM-1$^{-/-}$ mice for IL-15 expression directly *ex vivo*. As we had anticipated, IL-15 expression was indeed increased on DCs from ICAM-1$^{-/-}$ mice (Figure 13 A,D), albeit not to the same extent as in RAG-1$^{-/-}$ mice. These data raise the possibility that memory CD8 T cells generated in an ICAM-1$^{-/-}$ environment have more IL-15 available to them, which could explain why homeostatic proliferation and memory generation are not affected after virus infection in ICAM-1$^{-/-}$ mice.

### 4.2.5 ICAM-1 is not required for IL-15 transpresentation in acute models of homeostatic proliferation

Our initial findings linking ICAM-1 to transpresentation phenomena utilized models of CD8 T cell proliferation so we next wanted to determine if the main role for ICAM-1 in IL-15 transpresentation is to drive the proliferation of memory CD8 T cells. We used a model which was originally described as bystander proliferation, so-called because mice were treated with poly I:C, which induces IL-15 expression, and subsequently memory CD8 T cells proliferated. The proliferation required IL-15R$\alpha$ expressed by host cells and as such this model can be used as another model of transpresentation. We enriched CD8 T cells from congenically-marker Wt mice, CFSE-labeled the cells and transferred them into Wt and ICAM-1$^{-/-}$ mice. One day later, we treated the mice with 150 $\mu$g poly I:C and then analyzed the dilution of CFSE after 5 days. Previous studies used IL-15R$\alpha^{-/-}$ deficient hosts to analyze the role of IL-15R$\alpha$ in driving the proliferation (38); we however are using ICAM-1$^{-/-}$ hosts to determine if ICAM-1 expression affects the ability of memory CD8 T cells to respond to elevated levels of IL-15. We measured the frequency of cells in the spleen and bone marrow that had not divided, those that had divided between 2 and 5 times, as well as the cells that had divided so

**Figure 13. IL-15 expression on DCs is increased in ICAM-1$^{-/-}$ mice.**
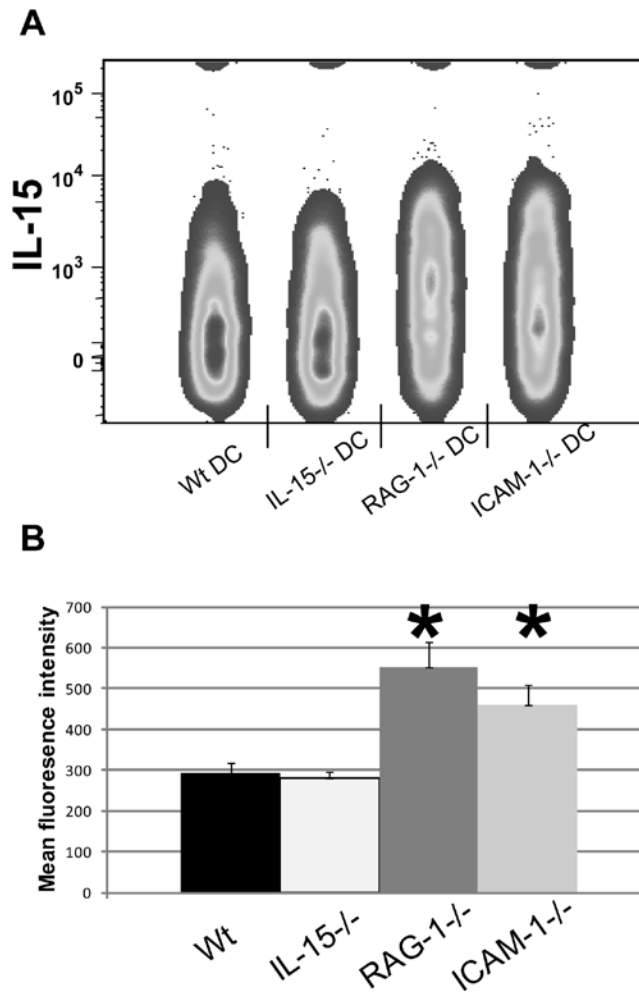
**Figure 13. IL-15 expression on DCs is increased in ICAM-1$^{-/-}$ mice.** A) Comparison of IL-15 expression among various mice. Flow cytometry plot showing the level of IL-15 staining (y-axis) on splenic DCs from Wt, IL-15$^{-/-}$, RAG-1$^{-/-}$, and ICAM-1$^{-/-}$. As indicated on the x-axis, each population shows representative staining of IL-15 from each mouse. Each population is representative of three mice per group and two independent experiments. B) Histogram shows the geometric mean of fluorescence intensity (MFI) on splenic DCs from the indicated mice. Error bars represent the standard deviation of three mice per group. The asterisk denotes an MFI that is statistically significant from IL-15$^{-/-}$ DCs ($p<.05$).

**Figure 14. Proliferation of memory CD8 T cells after up-regulating IL-15 expression by poly I:C is normal.**
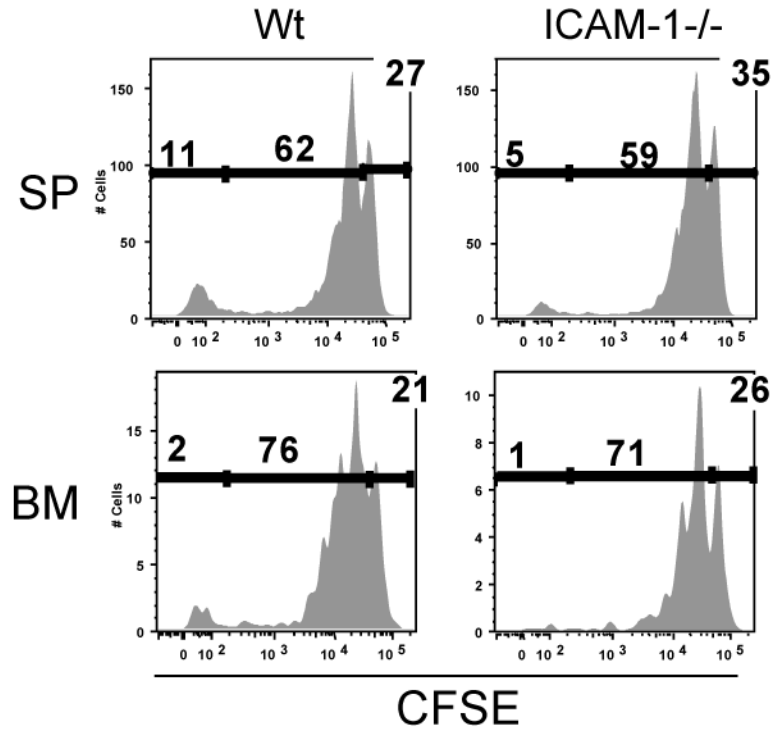
**Figure 14. Proliferation of memory CD8 T cells after up-regulating IL-15 expression by poly I:C is normal.**  A)  CD8 T cells were enriched from unmanipulated Wt mice, CFSE-labeled and transferred into Wt or ICAM-1$^{-/-}$ mice.  One day later, the recipient mice were injected with 150 $\mu$g poly I:C intraperitoneally to induce IL-15 expression.  Five days after poly I:C treatment, mice were sacrificed and proliferation assessed in the spleen and bone marrow.  Plots are gated on CD45.1+ CD44 hi CD8 T cells.  Interval gates measure the frequency of donor cells that have failed to divide, those that have divided 1-6 times, and those that have divided enough times to dilute their CFSE to undetectable levels.  Plots are representative of 3 mice per group from 2 independent experiments.

many times their CFSE was completely diluted.  In Wt and ICAM-1^{-/-}, there was no difference

in any of these demarcations of division (Figure 14), suggesting that ICAM-1 does not play a

role in poly I:C-induced IL-15 transpresentation.  Previously we had observed that ICAM-1^{-/-}

were deficient in their ability to induce the homeostatic proliferation of

transferred memory CD8 T cells; however in this model of more acute proliferation, ICAM-1^{-/-}

were equivalent to Wt mice in their ability to drive proliferation.


Another model of IL-15 driven proliferation involves the use of lymphopenic mice as hosts for

adoptively transferred CD8 T cells.  In a phenomenon referred to as lymphopenia-induced

proliferation (LIP), T cells transferred into lymphopenic hosts respond to the homeostatic

cytokines IL-15 and IL-7 that are more accessible when there are no other T cells with which

to compete (75).  This response involves the activation of naïve CD8 T cells by IL-7 and TCR

stimulation, followed by IL-15-induced proliferation of memory-phenotype CD8 T

cells(6,76,77).  As our interest is in IL-15 and not IL-7, our experiments were conducted in the

presence of a blocking antibody against IL-7R$\alpha$ so that the transferred cells cannot respond to

IL-7.  We crossed RAG-1^{-/-} mice with ICAM-1^{-/-} and IL-15R$\alpha$[-/-] mice to establish two

environments where IL-15 is more available but 1) cell adhesion is compromised or 2) IL-15

cannot be presented , respectively.


We generated memory CD8 T cells as before using VSV, enriched CD8 T cells from immune

mice, CFSE-labeled the cells and transferred into the various mice: RAG-1^{-/-}, RAG-1^{-/-} x ICAM-

1[-/-], and RAG-1^{-/-} x IL-15R$\alpha$[-/-].  Before transfer, anti-IL-7R$\alpha$ was added to the CD8 T cells, and

the recipient mice received 0.5 mg of anti-IL-7R$\alpha$ prior to transfer as well.  After 5 days we

assessed proliferation in the spleen and bone marrow on antigen-specific as well as memory-

phenotype CD8 T cells.  Proliferation of antigen-specific memory CD8 T cells in both tissues

was not very robust but the frequency of cells that did not divide was equivalent among RAG-

1$^{-/-}$ and RAG-1$^{-/-}$ x ICAM-1$^{-/-}$ mice (35%) (Figure 15A), indicating that LIP is not compromised in the absence of ICAM-1.  RAG-1$^{-/-}$ x IL-15R$\alpha^{-/-}$ recipients, on the other hand, failed to support normal amounts of proliferation (60% undivided, Figure 14A), in keeping with our expectation that the increased availability of IL-15 would be functionless without IL-15R$\alpha$ to deliver it. Proliferation among memory-phenotype CD8 T cells was far more robust, with the majority of the population undergoing at least two rounds of division (Figure 14 B,C).  Due to the extensive proliferation characteristic of this model, we were able to measure the frequency of memory-phenotype CD8 T cells within several generations.  Once again, both RAG-1$^{-/-}$ and RAG-1$^{-/-}$ x ICAM-1$^{-/-}$ mice drove equivalent amounts of proliferation as evidenced by the similar frequencies of memory-phenotype CD8 T cells within each generation (Figure 14B,C).  RAG-1$^{-/-}$ x IL-15R$\alpha^{-/-}$ mice again displayed defective proliferation, with the vast majority of memory cells remained in the parent generation (Figure 14 B,C).  Based on these two models of acute IL-15-driven proliferation as well as homeostatic proliferation, the role of ICAM-1 in IL-15 transpresentation to memory CD8 T cells seems to be important mainly in situations where IL-15 expression is low.  Where IL-15 is more highly expressed or more available, however, ICAM-1 does not appear to be important in IL-15 transpresentation.

## 4.3     Discussion

Previous studies of memory CD8 T cell homeostasis and maintenance have clearly established the role of IL-15 transpresentation in these processes but our understanding of how the interaction between transpresenter and responder occurred remained unclear. As such, we sought to explain how IL-15 transpresentation occurs by addressing the role that specific adhesion molecules have in enabling transpresentation to memory CD8 T cells. Our finding that ICAM-1/LFA-1 is important in enabling IL-15 transpresentation is the first study to link adhesion molecules with IL-15 transpresentation.  The involvement of adhesion molecules in IL-15 transpresentation spans a crucial gap in our understanding of this process and may

**Figure 15.  Lymphopenia-induced proliferation does not require ICAM-1 expression.**
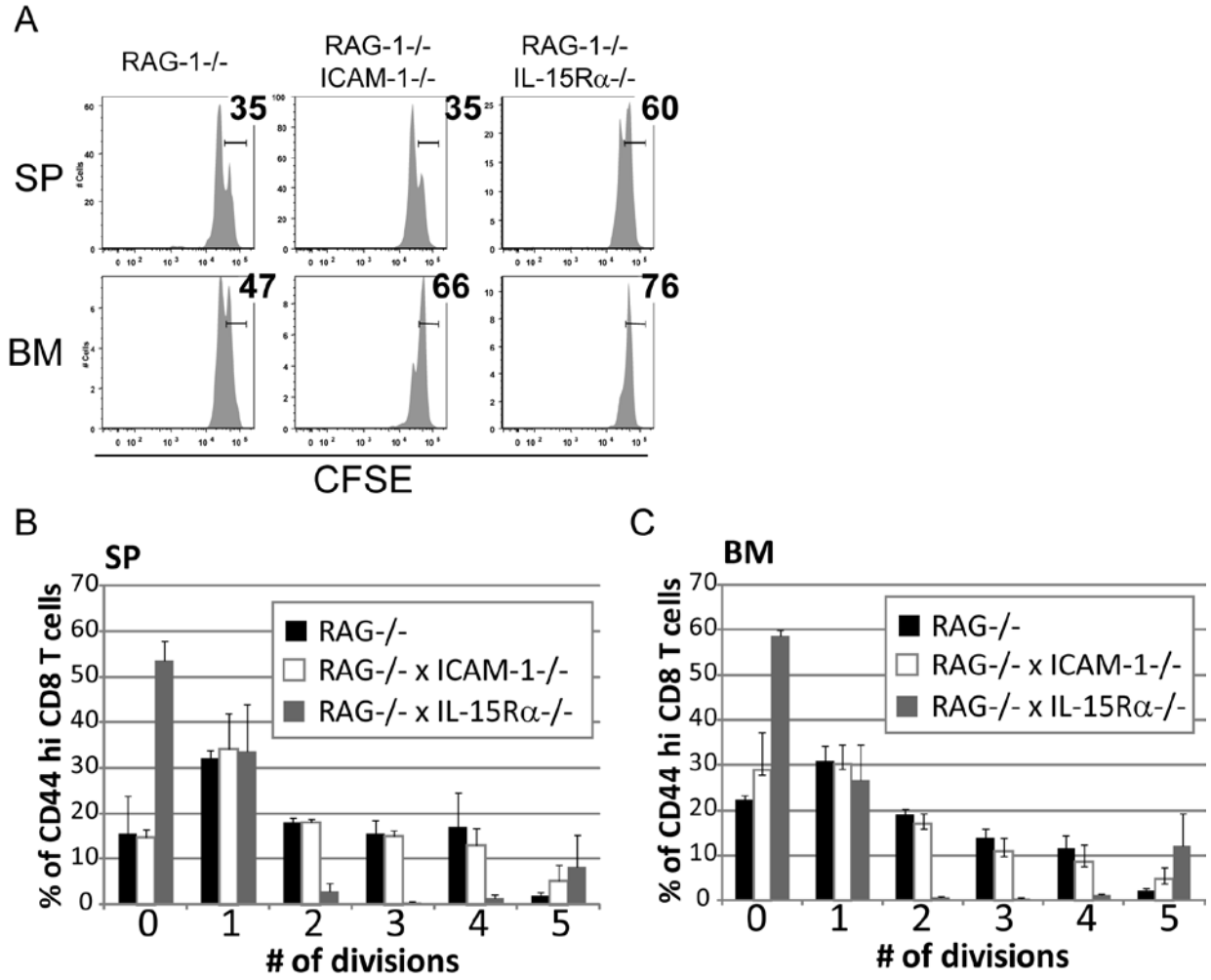
**Figure 15.  Lymphopenia-induced proliferation does not require ICAM-1 expression.**  A)

CD8 T cells were enriched from VSV-immunized mice, CFSE-labeled and transferred into

RAG-1$^{-/-}$, RAG-1$^{-/-}$ x ICAM-1$^{-/-}$, and RAG-1$^{-/-}$ x IL-15R$\alpha^{-/-}$ mice in the presence of CD127

blocking Ab.  Plots are gated on TCR$\beta$+ CD8$\alpha$+ CD44 hi N-tetramer-specific T cells from the

spleen and bone marrow 5 days post-transfer.  Interval gate measures the frequency of cells

that have not divided.  Plots are representative of 3 mice per group.  B, C)  Histograms

showing the frequency of memory-phenotype CD8 T cells that have divided the indicated

number  of times in the various host mice in the spleen (B) and BM (C).  Error bars are

standard deviation of three mice per group.  Data is representative of two independent

experiments.

serve as the basis for furthering our understanding of how the specificity observed in IL-15 transpresentation is achieved.  For example, the inability of a given cell to respond to or transpresent IL-15 may be related to its lack of appropriate adhesion factors and as such could be a mechanism that has developed to control IL-15 responses. The recent discoveries identifying the cells that transpresent IL-15 to CD8 T cells as well as NK/NKT cells and intestinal intraepithelial lymphocytes has now given us a platform upon which to begin elucidating factors which enable a particular cell type to transpresent IL-15 (62,72,78,79).  The present study therefore represents a potential new avenue for understanding the characteristics that enable cells to respond to transpresented IL-15 and could lead to the design of strategies to heighten the efficacy and specificity of therapies that seek to generate IL-15 responses.  Additionally, as ICAM-1 interacts with LFA-1, modulating the affinity of LFA-1 could be used to enhance IL-15 signals in *ex vivo* stimulation of T cells for the preparation of therapeutic adoptive T cell transfers.

The decreased level of homeostatic proliferation observed in adoptive transfers into ICAM-1$^{-/-}$ mice was not as striking as that which has been described in IL-15R$\alpha^{-/-}$ mice, where homeostatic proliferation is almost completely compromised (22,24).  This result suggests that ICAM-1 is not absolutely necessary for IL-15 transpresentation and may function mainly to enhance transpresentation.  Alternatively, cell adhesion may be integral for IL-15 transpresentation but mediated by multiple adhesion molecules simultaneously.  For example, in addition to acting as the ligand for ICAM-1, LFA-1 can also bind with ICAM-2 and ICAM-3.  Furthermore, while DCs are a major cell type transpresenting IL-15 to memory CD8 T cells (58,72), other cells types such as macrophages and BM stromal cells may utilize other adhesion molecules to facilitate IL-15 transpresentation.  As memory CD8 T cells in the lung are less dependent on IL-15 transpresented by hematopoietic cells (41) and DCs are rare in

the BM, our data showing the importance of ICAM-1 in the spleen could reflect a specific role for ICAM-1 between memory CD8 T cells and DCs.

Because ICAM-1/LFA-1 is involved in T cell activation (50,52) and utilized during the contraction (7,53), memory CD8 T cells generated in the absence of ICAM-1 may have an altered ability to undergo homeostasis that is unrelated to the role of ICAM-1 in IL-15 transpresentation. Indeed, it has been reported that the absence of ICAM-1 by APCs during priming promotes the differentiation of effector memory over central memory (52). In addition, Chang *et al* (80) showed that the asymmetric partitioning of IL-7R$\alpha$, an important fate determinant (14), is lost upon cell division in the absence of ICAM-1.

The elevated level of IL-15 expression in ICAM-1$^{-/-}$ mice is a truly intriguing finding due to the differential effects on memory CD8 T cells we observe when memory is generated in Wt mice versus when memory is generated in ICAM-1$^{-/-}$ mice. Memory CD8 T cells generated in Wt mice and transferred into ICAM-1$^{-/-}$ mice proliferate less and have reduced survival, whereas both phenomena are unaffected with endogenously-generated ICAM-1$^{-/-}$ memory. One potential explanation for this is that memory generated in the different hosts is programmed differently, such that requirements for IL-15 and transpresentation are altered by virtue of the environment in which the memory CD8 T cells are raised. Our data showing that memory CD8 T cells respond normally to elevated levels of IL-15 after *in vivo* poly I:C treatment (Figure 14) and in lymphopenia-induced proliferation (Figure 15) suggest though that memory CD8 T cells generated in ICAM-1$^{-/-}$ mice may be inherently different from those generated in Wt mice. Whether or not this difference is cell intrinsic or –extrinsic – indeed, even what the nature of this alteration could be – remains subject to speculation. Since our results do not mirror previous findings using different model systems, we suspect that compensatory mechanisms exist that may mask the functions of ICAM-1, which are dependent on the type of pathogen,

strength of stimulation and/or inflammatory milieu. Taken together, these issues confound the study of homeostasis of endogenous memory CD8 T cells generated in ICAM-1$^{-/-}$ mice as one cannot exclude the possibility that the absence of ICAM-1 alters the programming of memory CD8 T cells or generates a population that has adapted to an ICAM-1$^{-/-}$ environment.

Our data showing an absolute requirement for ICAM-1 in IL-15 transpresentation *in vitro* is in contrast to the partial requirement observed *in vivo* and could reflect a requirement that is specific for DCs. Alternatively, these cultures measure acute responses that are different from the much slower homeostatic proliferation occurring *in vivo* (81). Therefore, cumulative IL-15 signals, occurring inefficiently over time and by different cell types, may obscure the *in vivo* role of ICAM-1 in IL-15 transpresentation. Another interesting feature of this coculture system is the requirement for MHC class I on BMDCs. Whether the proliferation seen by memory-phenotype CD8 T cells is in response to auto antigens presented by the DCs remains to be determined. IL-15 can potentiate CD8 T cell responsiveness to weak TCR stimulation (82), which could be an explanation for why MHC class I is involved. This feature of IL-15 may be especially relevant when seeking to engender an immune response against self antigens. It will be interesting to determine if antigen-specific memory CD8 T cells proliferate in this system as well. If antigen-specific memory CD8 T cells fail to proliferate as well, that will demonstrate that this response is not simply the proliferation of autoreactive CD8 T cells. Antigen-specific memory CD8 T cells can proliferate robustly in MHC class I-deficient hosts in response to IL-15/IL-15R$\alpha$ complexes (83) so this will be a good litmus test to determine if ICAM-1 truly is essential for IL-15 transpresentation in our model.

Other factors such as various TNFR family members, i.e. CD27, 4-1BB among others, are reported to influence memory CD8 T cells as well (84-86). 4-1BB is a particularly interesting member of this family in that it has been linked specifically with the homeostatsis of memory

CD8 T cells. 4-1BB is mainly expressed on CD8 T cells that have recently been activated by TCR stimulation (87) but its expression can also be modulated by IL-15 stimulation (85,86). Two studies have linked signals derived from 4-1BB stimulation with the survival and/or proliferation of memory CD8 T cells. Although we did not test this, memory CD8 T cells generated in ICAM-1$^{-/-}$ mice may have altered expression or responsiveness through 4-1BB that could contribute to lack of effect we saw in that particular model. Aside from that possibility, through lasting contact mediated by ICAM-1/LFA-1, it is possible that simultaneous or sequential signals are delivered via these TNFR family members to the T cells and could help explain some inconsistencies that have been seen regarding the roles of these molecules (86). It will be interesting for future studies to explore the relationship between ICAM-1/LFA-1 and how these molecules might interact alongside IL-15.

## 5.1    Introduction

The functions of IL-15 serve to maintain homeostasis, which necessarily requires tight regulation of IL-15 so that the balance of homeostasis isn't disturbed.  IL-15 potently stimulates CD8 T cells (among others) when it is in excess, which has been determined using transgenic mouse models (88,89).  Consequently, the regulation of IL-15 expression is crucial to maintaining homeostasis.  As discussed in **section 1.4**, many layers of regulation exist to control IL-15 levels.  Likely as a consequence of the various levels of regulation, very little is known about steady-state IL-15 expression because it is presumably quite low – potentially undetectable.  Furthermore, even under stimulatory conditions, reports of IL-15 protein expression are sparse.  While IL-15 mRNA expression has long served as the benchmark for our understanding of IL-15 expression, there exists a substantial bridge to cross before IL-15 mRNA becomes a mature, bioactive protein.

Because of the importance of IL-15 in generating and maintaining memory CD8 T cells, an undertaking to study and characterize IL-15 protein expression – both steady-state and induced – is crucial as this will better enable the rational design of therapies or vaccines. Reconciling IL-15 mRNA expression data with protein expression will perhaps elucidate mechanisms behind the efficacy of certain therapeutic interventions over others and may uncover further unappreciated applications for these therapies.

## 5.2    Results

### 5.2.1    IL-15 Expression *in vitro*

Previous attempts in our lab to measure cell-surface IL-15 expression using a flow cytometry-based detection method had been unsuccessful using all of the available IL-15 antibodies (data not shown). However, two published reports demonstrating IL-15 staining (46,90) using an antibody that had recently become available prompted us to deploy this reagent in our assays as well. As shown in Figure 2E, after overnight activation of bulk splenocytes from Wt and IL-15$^{-/-}$ mice with LPS, we could successfully measure IL-15 protein expression on DCs (58). An intriguing aspect of this experiment was that even when splenocytes were not stimulated – just incubated in media – we could detect IL-15 expression on DCs in these cultures as well (Figure 2E) (58). Prompted by these data, we measured IL-15 protein expression on other spleen and bone marrow cell populations after overnight culture. Among spleen populations, we observed IL-15 staining on eosinophils in addition to DCs (Figure 16A), but not on neutrophils or pDCs (data not shown). In the BM, neutrophils, eosinophils and macrophages all expressed IL-15 after overnight culture (Figure 16B). Additionally, we cultured cells from the SP and BM with LPS and IFN-$\alpha$ to correlate IL-15 protein expression levels with previous studies that observed increases in IL-15 mRNA expression after culturing with these stimuli. Interestingly, the expression of IL-15 after culture did not change radically with LPS and IFN-$\alpha$ treatment: eosinophils in the spleen as well as BM macrophages displayed the greatest sensitivity to these stimuli whereas DCs and neutrophils were less affected (Figure 16A,B). We also incubated splenocytes from IL-15$^{-/-}$ and IL-15R$\alpha^{-/-}$ mice overnight with or without LPS to verify that the IL-15 staining was associated with IL-15R$\alpha$. Indeed, IL-15 expression was absent on DCs from both types of splenocytes (Figure 16C), indicating that the IL-15 we are detecting is at the cell surface and requires IL-15R$\alpha$ expression.

**Figure 16. Expression of IL-15 protein is detectable after overnight culture and with various stimuli.**
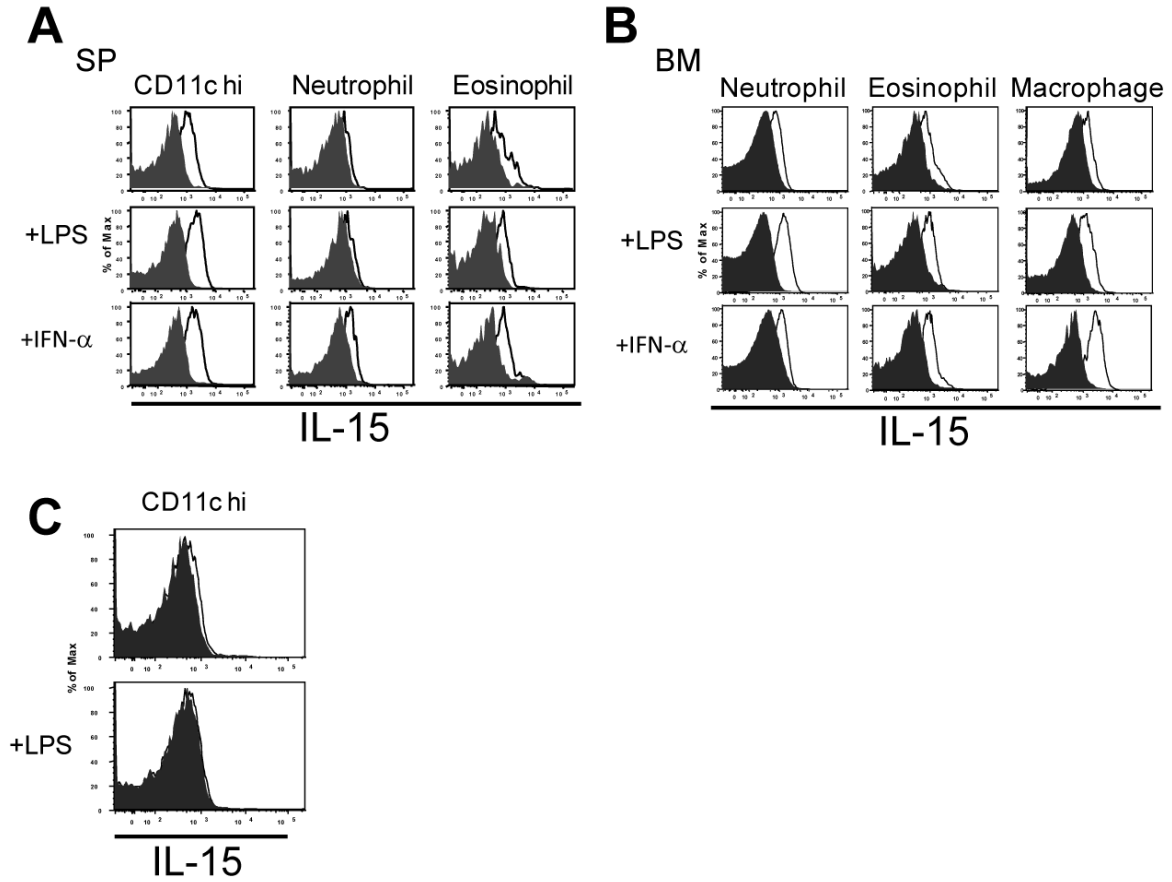
**Figure 16. Expression of IL-15 protein is detectable after overnight culture and with various stimuli.** A,B) $1 \times 10^7$ cells from the spleen (A) and BM (B) of Wt and IL-15-/- mice were incubated overnight in the absence or presence of 1 $\mu$g/ml LPS or 300 U/ml IFN-$\alpha$. Filled histograms represent background staining of IL-15 on IL-15$^{-/-}$ cells; open histograms are IL-15 staining on Wt cells. Plots are representative of 2-3 experiments. C) $1 \times 10^7$ splenocytes from IL-15$^{-/-}$ and IL-15R$\alpha^{-/-}$ were incubated overnight in the presence or absence of LPS as before. Filled histograms represent IL-15 staining on IL-15$^{-/-}$ cells and open histograms represent staining on IL-15R$\alpha^{-/-}$ cells.

### 5.2.2 Steady-state IL-15 expression

Given the success of this particular antibody to detect transpresented IL-15 *in vitro*, we then sought to measure IL-15 expression directly *ex vivo* on various hematopoietic cell types. From our studies as well as others, we know – albeit indirectly – that DCs make IL-15; however, other cells must make IL-15 as well because IL-15 expression by DCs alone is not sufficient to drive all aspects of IL-15 biology. Measuring IL-15 expression directly *ex vivo* is also more informative than after culture because memory maintenance occurs in the absence of overt stimulation, implying that IL-15 is expressed at some level under steady-state conditions. As the spleen and bone marrow are major sites of homeostatic proliferation, we identified several hematopoietic lineages that reside in these tissues and measured the expression of IL-15 on the cell surface. Curiously, in neither tissue we were able to detect IL-15 expression directly *ex vivo* on any of the various cell types identified (Figure 17A,B,C). Some of these populations, such as eosinophils, neutrophils and basophils, have never been linked with roles in IL-15 biology and the lack of IL-15 expression is therefore not surprising. More interesting is that monocytes and DCs – two cell types heavily linked with IL-15 expression – were negative as well (Figure 17A,B). While the former observation could be expected, the latter observation was more startling as we and others have shown that these cells do indeed make and transpresent IL-15, but these data suggest that either the expression is too low to detect by flow cytometry, or these cells do not make IL-15 under steady-state conditions. CD8 and CD4 T cells, B cells and NK cells also did not express IL-15 (Figure 17C).

While levels of IL-15 mRNA have been measured in monocytes and DCs (43), a direct comparison of IL-15 mRNA between different hematopoietic lineages has never been analyzed. Our first approach to measure IL-15 protein expression by flow cytometry could not

**Figure 17. Steady-state expression of IL-15 is undetectable in Wt mice.**
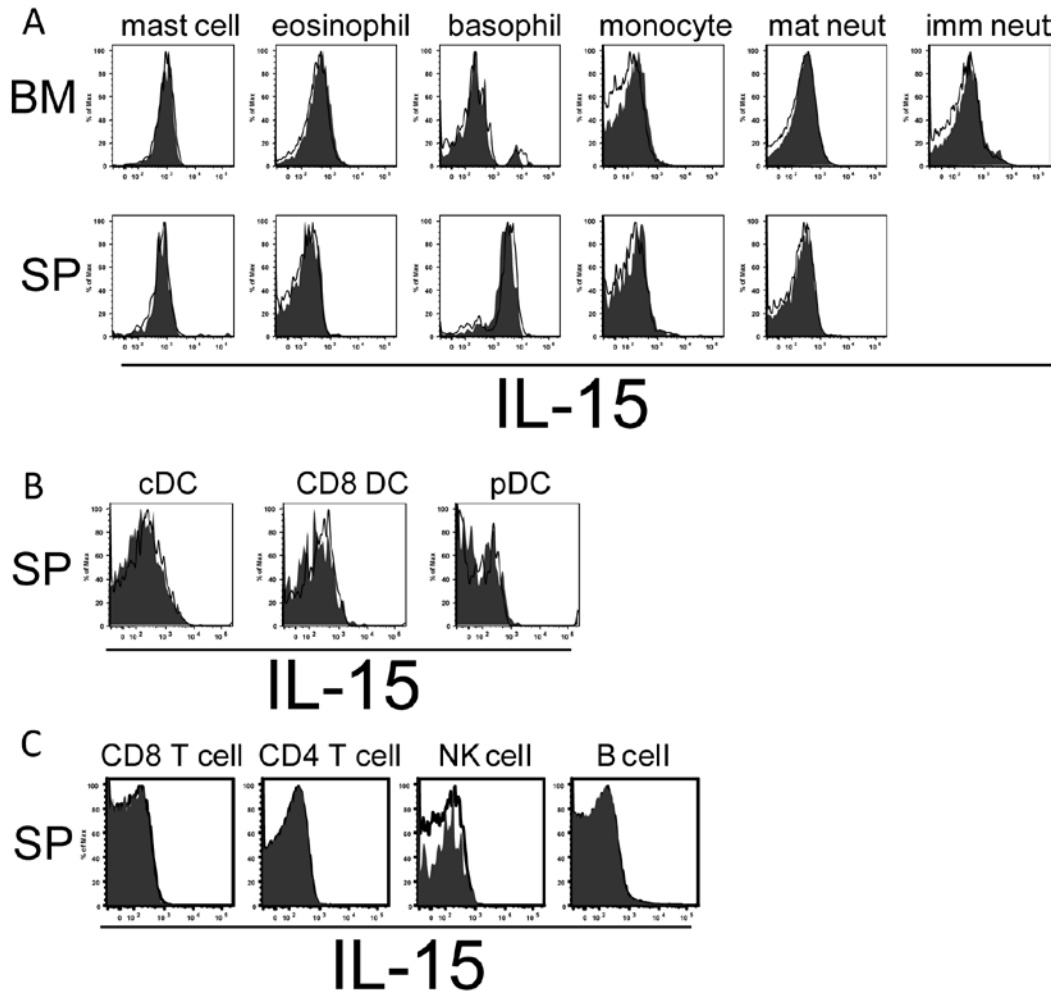
**Figure 17. Steady-state expression of IL-15 is undetectable in Wt mice.** A) IL-15 expression on granulocyte populations in the bone marrow and spleen directly *ex vivo*. B) IL-15 expression on splenic DC populations directly *ex* vivo. Data are representative of at least four independent experiments (A, B). C) IL-15 expression on lymphocytes. Filled histograms represent IL-15$^{-/-}$ staining, open histograms represent Wt staining. Data are representative of two independent experiments.

detect any cytokine: in lieu of this, we turned to qtPCR to measure mRNA levels. As

previously mentioned, mRNA levels are informative but certainly do not always mean the

mature protein is produced, regardless of expression levels. Nevertheless, we sorted cell

types from the spleen and bone marrow according to the gating strategy in Figure 18 and

isolated RNA. After cDNA synthesis, we performed qtPCR to measure the amount of IL-15

mRNA expression among these cell types. A cursory analysis of the mRNA expression

reveals that most of the cell types we sorted do contain some level of IL-15 mRNA, with the

exception of pDCs (Figure 19A,B). The truly perplexing observation is that basophils and

neutrophils in the spleen and BM and BM mast cells produce the highest levels of IL-15

mRNA, (Figure 19A,B) yet, as mentioned before, these cells have never been considered to

have a role in IL-15 transpresentation. Monocytes, cDCs, and CD8$\alpha$+ DCs, on the other hand,

express relatively low levels of IL-15 mRNA (Figure A,B). Again, these data are rather

shocking in that the cells that actually transpresent IL-15 express very little of its mRNA.

Collectively, however, these data tell us that IL-15 mRNA levels do not strictly correlate with

protein expression, or indeed transpresentation ability.


### 5.2.3   IL-15 expression is increased in RAG-1$^{-/-}$ mice

The lack of IL-15 expression on DCs under homeostatic conditions coupled with the apparent

IL-15 expression after culture led us to hypothesize that the DCs were receiving a signal in

culture that causes them to produce IL-15. We began to question the role of T cells in IL-15

expression *in vivo*. Some evidence exists that CD8 T cells may be "sinks" for IL-15 and,

consequently, in a CD8 T cell-replete mouse, the IL-15 that is produced may be used up by

CD8 T cells (91). Alternatively, CD8 T cells may provide a negative feedback signal to DCs to

stifle IL-15 expression so that it isn't expressed inappropriately. To test if T cells in general

influence IL-15 expression, we isolated cells from the spleen and BM from Wt and RAG-1$^{-/-}$

**Figure 18. Gating strategy and markers used to identify various myeloid cell populations for cell sorting.**
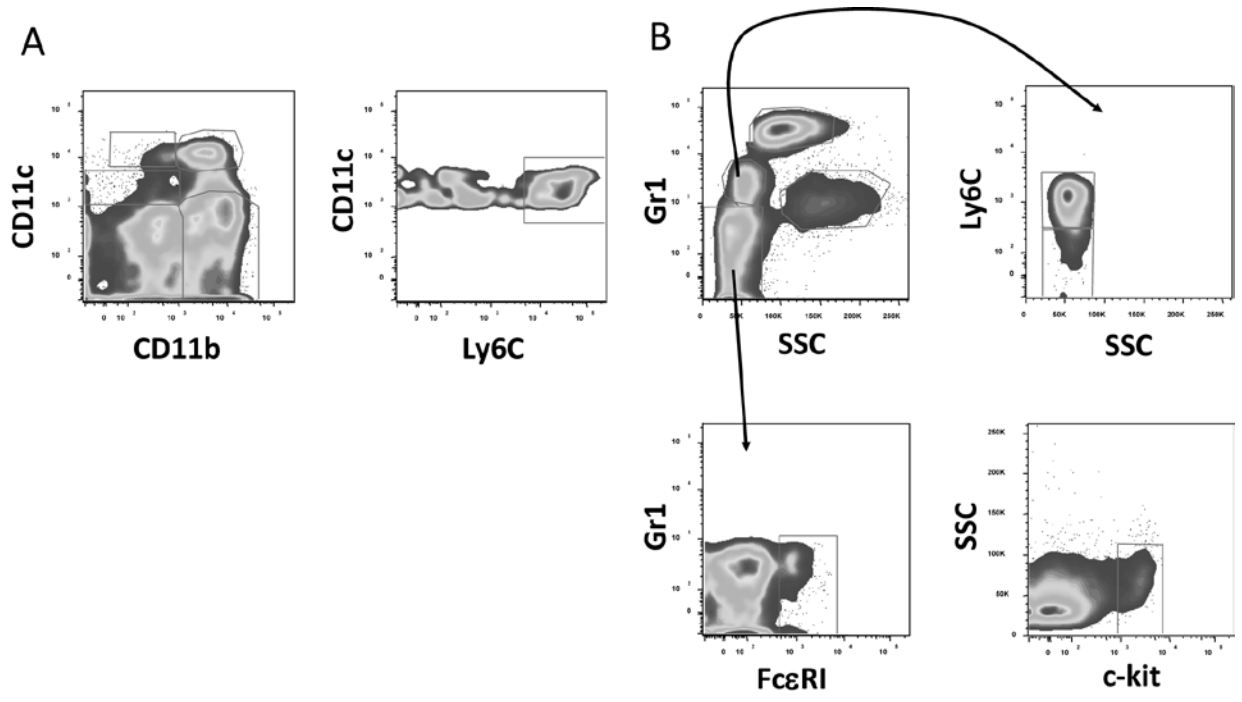
**Figure 18. Gating strategy and markers used to identify various myeloid cell populations for cell sorting.** All plots are gated on B220-, TCRβ- cells in the spleen. A) cDC: CD11c hi, CD11b+; CD8 DC: CD11c hi, CD11b-; pDC: CD11c int, CD11b-, Ly6C+. B) Mature neutrophil: CD11b+, Gr1 hi, SSC int; monocyte: CD11b+, Gr1 int, Ly6C+, SSC lo; immature neutrophil: CD11b+, Gr1 int, Ly6C-, SSC lo; eosinophil: CD11b+, Gr1 int, SSC hi; basophil: CD11b+, Gr1-, FcεRI+; mast cell: CD11b-, c-kit+.

**Figure 19. IL-15 mRNA is widely expressed among different BM-derived cell types.**
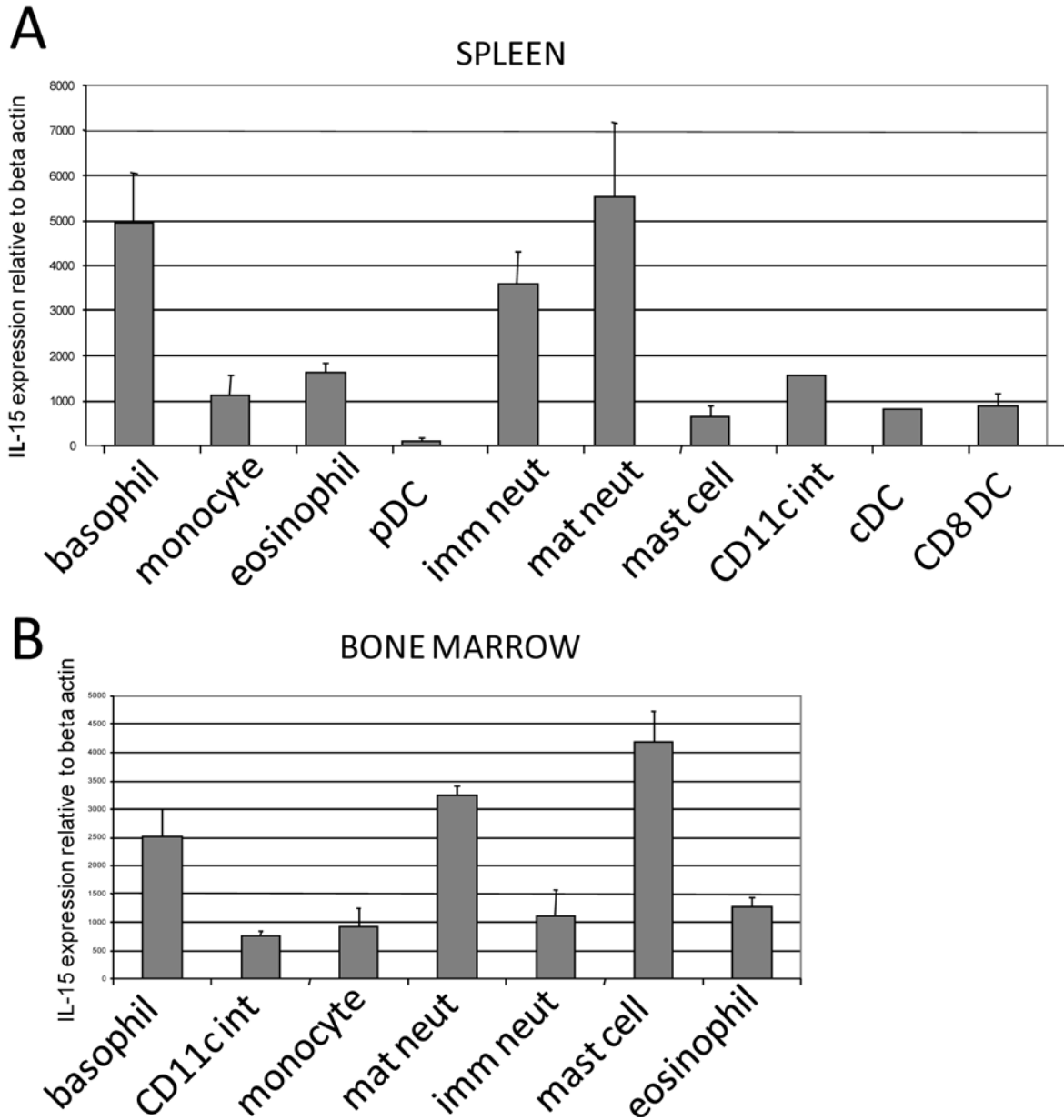
**Figure 19. IL-15 mRNA is widely expressed among different BM-derived cell types.** A,B) qtPCR was done to determine the amount of IL-15 message in various sorted myeloid cell populations from the spleen (A) and BM (B). Histograms represent IL-15 expression relative to β-actin as determined by calculating average $\Delta C_T$ values of triplicate assays for each cell population. Data are representative of two independent experiments (both sorting of cells and qtPCR). Error bars are derived from the standard deviation in $\Delta C_T$ values.

mice and stained them directly *ex vivo* for IL-15 expression. To our surprise, we saw that in RAG-1$^{-/-}$ mice, both cDCs and CD8$\alpha$+ DCs in the spleen express cell surface IL-15, evidenced both by the flow plots and by measuring the MFI of IL-15 expression (Figure 20A,B). Additionally, IL-15 was also detected on BM eosinophils, monocytes and neutrophils (Figure 20C). These data are the first to demonstrate IL-15 staining that did not involve exogenous stimulation, and also to demonstrate that IL-15 is expressed by more cells than would be anticipated. Having already observed that IL-15 protein expression is increased on DCs in RAG-1$^{-/-}$ mice, we measured IL-15 mRNA expression to determine if different levels of IL-15 transcript are the cause of the increase in protein expression. We sorted DCs and neutrophils from the spleen as before and used qtPCR to measure IL-15 transcripts. In both populations, IL-15 mRNA expression was not significantly different (Figure 20D), meaning the different amounts of IL-15 found on the cell surface was not related to differential transcription.

### 5.2.4  Total body irradiation results in increased IL-15 expression

We hypothesized that it was the lymphopenic state inherent to RAG-1$^{-/-}$ mice that resulted in increased IL-15 expression and next sought to analyze another model of lymphopenia that is commonly used: total body irradiation. Total body irradiation is typically used to ablate lymphocytes prior to BM transplantation to ward off potential host versus graft responses, as well as to provide a niche into which the new BM can expand. We used this approach to establish lymphopenia in Wt and IL-15$^{-/-}$ mice to determine if IL-15 protein expression is increased in this model as well. Mice were given a sub-lethal dose of total body irradiation (700 cGy) to ablate lymphocytes and were analyzed one day later for IL-15 protein expression. After irradiation, we saw increases in IL-15 expression on several hematopoietic cells, including BM monocytes, neutrophils, and eosinophils, as well as splenic DCs (Figure 21A,B). The effect was most pronounced in the BM, with BM monocytes showing the greatest increase in protein expression (Figure 21 A,B). These results indicate that after total body

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**Figure 20. IL-15 expression is increased in RAG-1$^{-/-}$ mice.**
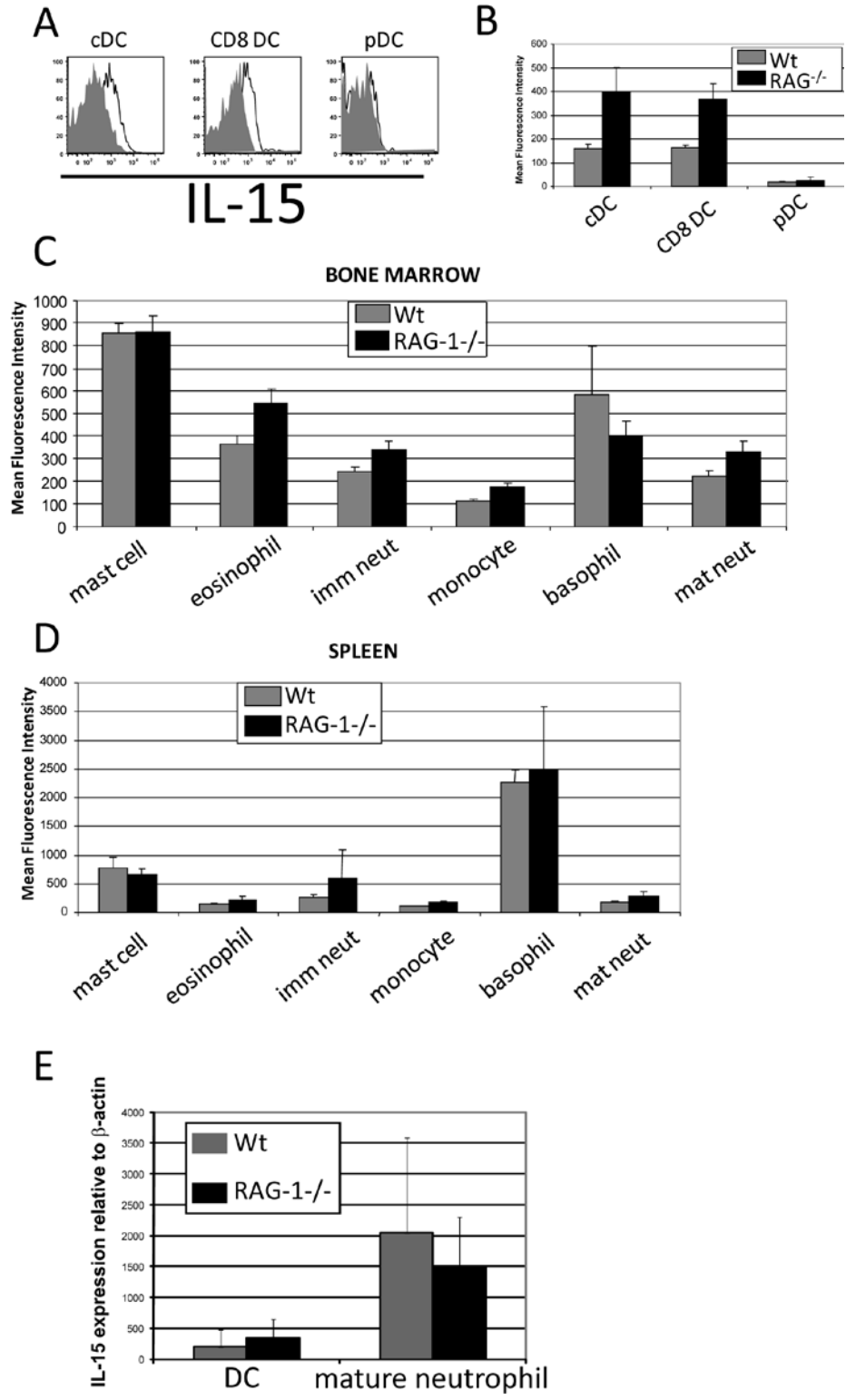
**Figure 20. IL-15 expression is increased in RAG-1$^{-/-}$ mice.** A) DC populations were isolated from the spleens of Wt and RAG-1$^{-/-}$ and stained for surface IL-15 expression. Filled histograms represent staining on Wt cells and open histograms represent staining on RAG-1$^{-/-}$ cells. B, C, and D) Bar graphs showing the MFI of IL-15 expression on Wt and RAG-1$^{-/-}$ cell populations from the indicated tissue. Cell populations in (B) are from the spleen. Results are representative of 3-4 experiments and 2-5 mice per group. E) Sorted splenic DC and neutrophil populations were analyzed by qtPCR for expression of IL-15 transcripts. Results are representative of two independent experiments.

**Figure 21. Sublethal irradiation induces IL-15 expression.**
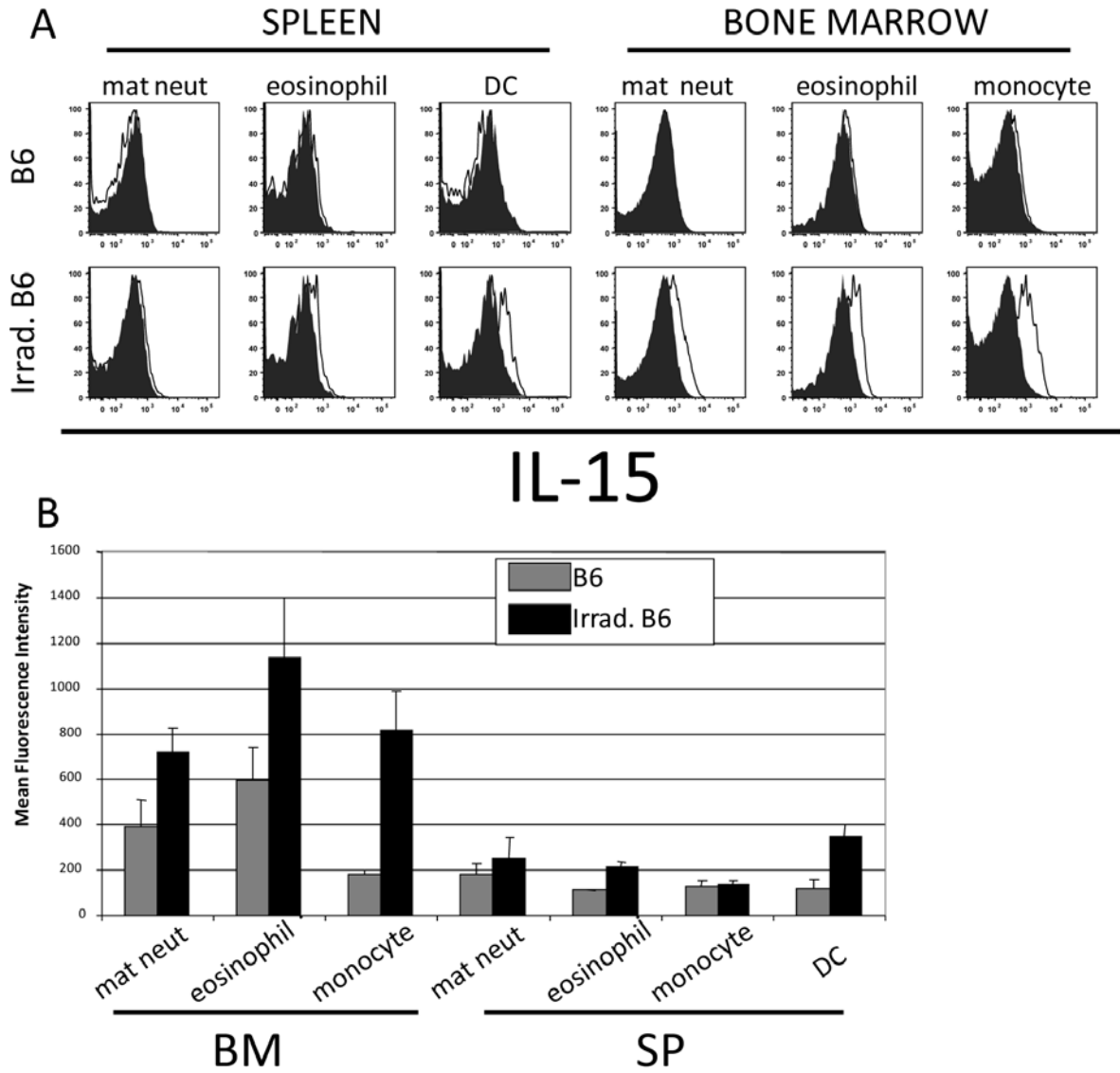
**Figure 21.  Sublethal irradiation induces IL-15 expression.**  A) Histograms show IL-15

staining on spleen and bone marrow cell populations one day after receiving a sublethal (700

cGy) dose of irradiation.  Filled histograms represent background staining on irradiated IL-15$^{-/-}$

cells and open histograms represent staining on irradiated Wt cells.  B) Bar graphs showing

the MFI of IL-15 staining on Wt or irradiated Wt cell populations from the spleen and bone

marrow.  Error bars represent standard deviation of 2-3 mice per group.

irradiation, accompanying the ablation of lymphocytes is an increase in expression of IL-15.

Whether or not the increase in IL-15 expression on cells such as neutrophils or eosinophils is

consequential remains to be determined, but certainly an increase in IL-15 on monocytes and

DCs would be important to consider for their ability to transpresent.

## 5.3    Discussion

We have now determined that IL-15 expression in Wt mice is too low to detect, or possibly is

simply not expressed.  We have, however, observed that in lymphopenic conditions, IL-15 is

increased, not only on DCs but other hematopoietic cell types as well.  While anecdotal

evidence has identified a role for IL-15 transpresentation by DCs, it had yet to be shown that

DCs produce cytokine homeostatically, in the absence of overt stimulation.

Our data here are the first to observe that IL-15 expression is increased in lymphopenic

environments.  Studies of adoptive transfers of CD8 T cells into RAG-1$^{-/-}$ mice have observed

that the subsequent proliferation undergone by the CD8 T cells is due in part to IL-15, but this

was explained as the increased availability of cytokine, not an increase in cytokine expression.

Here, however, we've shown that IL-15 expression is indeed increased in lymphopenic

environments, which leads to some very intriguing possibilities regarding IL-15 regulation.  The

increased expression of IL-15 by DCs may indicate that the presence of T cells - or CD8 T

cells in particular – down-regulates IL-15 expression.  The CD8 T cells may "use" all of the IL-

15 as well.  Both of these scenarios would explain why IL-15 is so low as to be undetectable in

normal mice.  However, another particularly piquant possibility is that the regulation of IL-15

expression by DCs is not necessarily related to CD8 T cells, but rather CD4 T cells.  It was

demonstrated that in the absence of CD4+ CD25+ T cells, memory CD8 T cells transferred

into RAG-1$^{-/-}$ mice proliferated more than when CD4+ CD25+ T cells were present (92).  The

population of CD4+ CD25+ T cells is known to contain regulatory T cells and it is likely that the

presence of $T_{reg}$ cells modulated the proliferation of memory CD8 T cells.  The exact

mechanism of this suppression was not fully explored, but we posit a situation wherein

regulatory T cells ($T_{reg}$s) modulate IL-15 expression on DCs, which in turn modulates the

degree of proliferation.  In an intact animal, $T_{reg}$s may regulate the constitutive expression of

IL-15 and in so doing, limit our ability to detect the cytokine.  Additionally, while $T_{reg}$s are

known to modulate the immune system by TGF-$\beta$, they also act in cell contact-dependent

mechanisms as well.  Indeed, our data showing ICAM-1$^{-/-}$ mice have increased IL-15

expression would agree with this theory.  Because the IL-15 expression is not as high in

ICAM-1$^{-/-}$ mice as it is in RAG-1$^{-/-}$ mice, if indeed $T_{reg}$s are involved in regulating IL-15 it is likely

through mechanisms additional to their contact-dependent suppression mechanisms.  While

we have yet to definitely determine a role for $T_{reg}$s in the regulation of memory CD8 T cell

homeostasis, it remains a highly intriguing possibility.


As we've now shown, cells such as eosinophils and neutrophils have the ability to express IL-

15.  These two examples of previously unheralded yet potential sources of IL-15 raise several

questions in the field of IL-15.  We've shown that various CD11b+ CD11c- spleen populations

express IL-15R$\alpha$ (Figure 2A)(58), and eosinophils and neutrophils fall into this staining pattern.

As mentioned previously, another study corroborated our earlier findings regarding DCs

transpresenting IL-15 to memory CD8 T cells using various Cre-lox mice(72).  One of the

promoters used to drive Cre recombinase expression was LysM, a promoter used by

macrophages as well as granulocytes (eosinophils, basophils, neutrophils) (93).  While this

group reportedly was unable to detect IL-15R$\alpha$ expression on neutrophils, apparently

eosinophils were not analyzed.  The working assumption then was that LysM-directed IL-

15R$\alpha$ deletion affected only macrophages but the possibility exists that eosinophils, and

potentially neutrophils, were affected in their ability to transpresent IL-15 as well.

Unfortunately, currently there is no way to specifically deplete eosinophils so it will be difficult

to prise apart the role they may have in IL-15 transpresentation from macrophages. We did attempt, however, to study homeostatic proliferation in neutropenic mice, which we established by antibody depletion as well as G-CSFR$^{-/-}$ BM chimeras (data not shown) but neither model satisfactorily depleted neutrophils. In these models, in any event, homeostatic proliferation was not affected.

Based on our detection of elevated IL-15 expression in RAG-1$^{-/-}$ mice, our interpretation of the finding that IL-15 is increased after irradiation is that the lack of T cells influences the level of IL-15 expression. An alternative explanation is an emerging concept that radiation can induce IFN-$\alpha/\beta$ expression, which has been demonstrated to be important for the control of tumor growth after radiotherapy (94). This certainly fits with our data that DCs upregulate IL-15 expression after stimulation with IFN-$\alpha$. It will be interesting to determine which of these factors – lymphopenia or IFN-$\alpha$ stimulation – is the true cause of the increase in IL-15 expression. Nevertheless, our observation that IL-15 is increased would certainly be of interest to the field of tumor biology because IL-15 has been shown to enhance the ability of the immune system to control tumor growth (91). As our approach used total body irradiation, the effects are likely too widespread to attribute to any one factor so a more directed delivery of radiation would be a superior approach.

## Chapter 6 – Future Directions and General Discussion

### 6.1    Summary

### 6.1.1   Results of chapter 3

In aim 1 I sought to determine the role that DCs play in transpresenting IL-15 to memory CD8

T cells, based on the hypothesis that DCs are the main cell transpresenting IL-15.  DCs are

mainly known as antigen-presenting cells and despite some evidence that they express IL-15

transcripts, little was known about whether or not they could indeed transpresent IL-15.

Therefore, my goal was to describe a new feature of DCs, this one related to their role as

"cytokine-presenters."  Using a transgenic model of IL-15R$\alpha$ expression, I took away the ability

of all cells in the mouse to transpresent IL-15, excepting DCs.  In this model I observed that

DCs can very efficiently recover the loss of the CD44 hi memory-phenotype pool of CD8 T

cells that is found in mice globally lacking IL-15R$\alpha$ (Figure 3B).  Transpresentation by DCs did

not recover IL-15-dependent CD8 T cell development in the intestine (Figure 3C), however,

demonstrating that the delivery of IL-15 is mediated by specific cell types – DCs could not

substitute for the natural IL-15 source.  In the contraction phase of an antiviral immune

response, IL-15 transpresentation by DCs enhanced the survival of contracting cells relative to

IL-15R$\alpha^{-/-}$ mice (Figure 4A,F), which subsequently enabled the generation of a higher level of

memory (Figure 4A,  comparing CD11c/IL-15R$\alpha$ Tg1 and IL-15R$\alpha^{-/-}$).  Specifically, DCs

supported the survival of cells that were KLRG-1+ and CD27-, cells that failed to survive in IL-

15R$\alpha^{-/-}$ mice.  Exclusive transpresentation by DCs did not fully recoup the survival afforded by

Wt mice (Figure 4), however, indicating that cells other than DCs transpresent IL-15 to CD8 T

cells for their survival during the contraction.  I found that the main role for DCs in IL-15

transpresentation to memory CD8 T cells was for driving homeostatic proliferation, a function

necessary for the long-term survival of established memory CD8 T cells.

## 6.1.2   Results of chapter 4

In aim 2, my goal was to determine if there was any relationship between IL-15 transpresentation and adhesion molecule expression, specifically ICAM-1.  I had hypothesized that ICAM-1 expression could be integral to IL-15 transpresentation, or could simply function to enhance IL-15 transpresentation.  I found that in an *in vitro* model of IL-15 transpresentation, ICAM-1 expression by BMDCs was essential to drive the proliferation of memory-phenotype CD8 T cells.  Somewhat contrastingly, I found that ICAM-1 was not required for *in vivo* homeostatic proliferation of Wt memory CD8 T cells.  In these adoptive transfers, I found that CD8 T cells – specifically memory CD8 T cells – did not undergo homeostatic proliferation to the same extent in ICAM-1-deficient mice that cells transferred into Wt mice did.  Essentially, fewer memory CD8 T cells divided in ICAM-1$^{-/-}$ hosts.  Accompanying this reduced proliferation was less survival as evidenced by fewer memory CD8 T cells recovered in ICAM-1$^{-/-}$ mice. Further *in vivo* experiments using similar adoptive transfer methodology but analyzing more acute proliferation responses revealed that ICAM-1 expression is not required at all in situations where IL-15 expression is elevated.  Specifically, the use of poly I:C to stimulate IL-15 expression showed no reduction in the proliferation of memory CD8 T cells.  Similarly, RAG-1$^{-/-}$ x ICAM-1$^{-/-}$ mice also drove normal levels of proliferation.  To summarize these findings, I have determined that ICAM-1 expression is important but not required for IL-15 transpresentation where normal, homeostatic levels of IL-15 are present.  Where IL-15 expression is increased, however, ICAM-1 is not involved in IL-15 transpresentation.  Never before had the role of ICAM-1in CD8 T cells been addressed using these types of assays and readouts.  Whereas most lines of inquiry focus on the role of ICAM-1 in infections, its role in homeostatic processes outside of homing had not been considered.

Using VSV as an infectious agent I found that there were no IL-15-related deficiencies in ICAM-1-deficient mice. The kinetics of the expansion and contraction of antigen-specific CD8 T cells were the same in both Wt and ICAM-1$^{-/-}$ mice (Figure 12A). The level of VSV-specific memory CD8 T cells in the spleen (Figure 12B) and BM (not shown) was actually slightly higher in ICAM-1$^{-/-}$, as was the amount of BrdU incorporation among memory cells over a two week assay. I also showed that cytokine production was not impaired in memory CD8 T cells generated in ICAM-1$^{-/-}$ mice. These findings regarding immune responses in ICAM-1$^{-/-}$ mice disagree with the current literature in that previous studies have shown the generation of CD8 T cell memory to be defective in the absence of ICAM-1.

### 6.1.3   Summary of chapter 5

Early findings that for memory CD8 T cells, IL-15 expression and transpresentation were required by BM-derived cells invited a relatively straightforward question – which cells derived from hematopoietic progenitors express IL-15? Throughout my graduate studies I attempted to address this question but found no success. Through the use of the transgenic mouse I've described in **chapter 3**, I indirectly showed that DCs must express IL-15. However, it also became apparent that cells other than DCs transpresent IL-15 as well, which necessitated the revivification of my efforts to describe IL-15 expression within the hematopoietic compartment. Despite many attempts, I was forced to conclude that IL-15 expression at steady-state conditions is too low to detect, potentially due to a lack of sensitivity in my assays or by active down-regulation. By qtPCR I demonstrated that IL-15 mRNA is rather widely expressed various BM-derived cells and that whereas DCs are important in IL-15 transpresentation, the level of transcript they contain is relatively low. The serendipitous observation that IL-15 is apparently spontaneously expressed after overnight culture enabled the detection of IL-15 expression on DCs among other BM-derived cells. Additionally, in RAG-1$^{-/-}$ mice, I observed

that DCs as well as eosinophils and neutrophils express IL-15. IL-15 expression by neutrophils and eosinophils is an unanticipated finding and had not been previously described or even suspected. I was not able to assess the functional consequence of IL-15 expression by these cells due to the lack of models that selectively lack neutrophils or eosinophils. Nevertheless, these cells may be involved in providing IL-15 during the contraction of an immune response as transpresentation by DCs alone did not recoup a Wt phenotype. Sublethal irradiation also induced an increase in IL-15 expression on monocytes, DCs and eosinophils; however, the exact mechanism behind this has yet to be fully described. Overall I determined that IL-15 is more widely expressed than previously thought and was able to build a comparative analysis of IL-15 expression in various environments.

## 6.2  Future Directions

### 6.2.1   IL-15 transpresentation and DCs

As the CD8 T cell immune responses progresses, it becomes increasingly more dependent on IL-15 transpresentation by DCs. While transpresented IL-15 isn't important for the initial expansion phase, CD8 T cells need IL-15 during the contraction for their survival. DCs provide some of the needed IL-15 but macrophages also apparently supply IL-15 at this time. In the memory phase, however, IL-15 transpresented limited to DCs is almost completely sufficient to drive memory CD8 T cell homeostatic proliferation. The reason behind the progressive dependence on IL-15 provided by DCs is unknown. Memory CD8 T cells are found to reside in B cell zones, marginal zones, T cell zones as well as the red pulp (95,96). Subsets of memory CD8 T cells also have specific microniches: Tcm reside in T cell zones and Tem reside in marginal zones and in the red pulp (96). The consequence of memory CD8 T cells residing in B cell zones is unknown, but the lack of memory CD8 T cells in T cell zones in favor of marginal zones is intriguing. The spleen is home to two major types of antigen-

presenting DCs which can be distinguished by the differential expression of CD8$\alpha$. CD8$\alpha$+

DCs reside primarily in T cell zones of the spleen and CD8$\alpha$- DCs are found in the marginal

zones and red pulp (97). While our study of DC-specific IL-15 transpresentation did not

distinguish between these two subsets of DCs, it remains possible that the workload of IL-15

transpresentation falls on one subset and not the other. The localization of memory CD8 T

cells in the red pulp and marginal zones and the localization of CD8$\alpha$- DCs in these areas

suggests that if indeed a differential workload exists, it likely falls on CD8$\alpha$- DCs. Previous

studies did not determine if these two cells do colocalize in these areas but this would be a

relatively straightforward relationship to establish if indeed it does exist. A strategy that could

be employed to determine if one DC subset is more important for transpresentation of IL-15

could involve the use of our CD11c/IL-15R$\alpha$ Tg mice. The use of CD8-Cre mice (98) that have

been crossed to IL-15R$\alpha$-loxp (72) mice would eliminate IL-15R$\alpha$ expression on CD8 T cells

but also on CD8$\alpha$+ DCs. These mice can be crossed to our CD11c/IL-15R$\alpha$ Tg mice (58) to

have CD8$\alpha$+ DCs that are unable to transpresent IL-15, which can be compared to mice in

which both DC subsets can transpresent IL-15. If the hypothesis that CD8$\alpha$- DCs are the main

transpresenters of IL-15, the lack of ability to transpresent IL-15 by CD8$\alpha$+ DCs should be of

no consequence. As it has already been determined that CD8 T cells do not require IL-15R$\alpha$

expression (38,39), the absence of IL-15R$\alpha$ on the triple-transgenic mice should not influence

the outcome. To normalize for this complication, memory OT-I T cells on the IL-15R$\alpha^{-/-}$

background could be CFSE-labeled and adoptively transferred into both mice.


## 6.2.2   Adhesion molecules in IL-15 transpresentation

As I demonstrated in **chapter 3**, ICAM-1 does play a role in IL-15 transpresentation under

homeostatic conditions: however, ICAM-1 expression is not strictly required to generate IL-15

responses. Because ICAM-1$^{-/-}$ mice are commercially available, we used these mice in our

studies first.  As mentioned previously, however, ICAM-1 pairs with LFA-1 on CD8 T cells, but LFA-1 can also pair with ICAM-2 and ICAM-3.  Both ICAM-2 and ICAM-3 are expressed on lymphocytes as well as ICAM-1 so it is possible that IL-15 transpresentation involves some combination of these molecules, or potentially all three.  The finding that memory CD8 T cells lacking CD11a largely failed to undergo homeostatic proliferation supports this hypothesis (Figure 11).  While ICAM-3$^{-/-}$ mice have not been generated, ICAM-2$^{-/-}$ and ICAM-1$^{-/-}$ ICAM-2$^{-/-}$ mice exist and could be utilized to study the role of ICAM-2, singly or in conjunction with ICAM-1, in IL-15 transpresentation.

The concept of IL-15 transpresentation resembling the synapse formed during antigen presentation invites similar studies to be performed involving the imaging of this interaction. However, as IL-15 transpresentation is still a poorly understand event, this undertaking is likely to be difficult.  One study that utilized conjugations between NK cells and poly I:C-activated DCs showed IL-15R$\alpha$ clustering at the synapse; however, IL-15R$\alpha$ was found to cluster on NK cells in this model and not on DCs as may be expected (99).  Another interesting aspect of this study revealed that blocking ICAM-1, -2, and -3 in addition to LFA-1 and LFA-3 to prevent synapse formation inhibited NK cell survival over a 9 day period (99).  This reduction in survival was related to IL-15R$\alpha$ expression on NK cells (99), but nonetheless suggests that adhesion molecules are indeed important in IL-15 responses.  Adapting this model of conjugate-formation to study CD8 T cell and DC interactions would be very useful in further establishing a relationship between adhesion molecules – whether it's one or all ICAM molecules – and IL-15 transpresentation.

The affinity of LFA-1 binding can be regulated by altering the conformation of the molecule, such that when closed, LFA-1 binds with low affinity.  Conversely, LFA-1 binds with high affinity when open.  As discussed in **chapter 1.5**, TCR signals as well as chemokine signals

can regulate this affinity. Additionally, a molecule associated with memory CD8 T cells, Ly6C, has also been shown to regulate the function of LFA-1(100,101). The ligand for Ly6C has not been determined, however it is expressed on B cells (102). As memory CD8 T cells primarily reside in B cell zones of the spleen (95,96), it is possible that they are receiving a signal that could modulate the affinity of LFA-1 via Ly6C stimulation. In so doing, memory CD8 T cells would be more likely to form stronger contacts with DCs, which could be essential for IL-15 transpresentation. While this remains pure speculation at this point, investigating the relationship of Ly6C and LFA-1 as modulators of a memory CD8 T cell's ability to receive transpresented IL-15 could be very exciting as it would demonstrate an unanticipated orchestration of events requiring crosstalk between multiple cell types.

### 6.2.3    Regulation of IL-15 expression

Our observation that IL-15 is increased in RAG-1$^{-/-}$ mice necessitates a thorough investigation to determine the cause for the altered expression. As discussed before, the reason behind the increase in IL-15 could be due to the absence of either CD8 T cells or CD4 T cells. We attempted some antibody depletion experiments in Wt mice to determine if the absence of either – or both – T cell subset gave us the same result. These experiments yielded inconsistent results but done with more care or slightly different methodology may be effective. So far as we've been able to determine, the IL-15 in RAG-1$^{-/-}$ is constitutively elevated. The time required after T cell depletion for any potential increase in IL-15 is therefore difficult to assess. We also administered a blocking antibody against TGF-$\beta$ as this is one way in which T$_{reg}$s are known to act but these experiments did not yield any increase in IL-15; however, as before, timing may be an issue as well. The finding that IL-15 is increased in ICAM-1$^{-/-}$ mice may suggest that ICAM-1 is involved in IL-15 regulation via cell-cell interactions, although again whether this is related to the ability of CD4 or CD8 T cells – or another cell altogether –

remains to be determined.  It is difficult to properly assess the influence T$_{reg}$s have on IL-15

expression directly because any mouse where T$_{reg}$s are lacking suffers from severe

inflammation.  To more specifically address the role TGF-$\beta$ has on the expression of IL-15 in

DCs or macrophages/granulocytes, TGF-$\beta$RII loxp mice (103) could be crossed to CD11c Cre

(104) and LysM Cre (93) mice to render DCs and macrophages/granulocytes insensitive to

TGF-$\beta$ signals.  If expression of IL-15 is detected in these mice it would strongly suggest that

TGF-$\beta$ production is actively suppressing IL-15 expression in normal mice, likely through the

function of T$_{reg}$s.


We used flow cytometry to measure IL-15 expression; however, this could also be approached

by the use of microscopy and immunofluorescence.  We focused on cell surface IL-15 as this

is where IL-15 is bioactive, but it would be very interesting to determine if IL-15 is maintained

in intracellular storage.  We show that IL-15 is not detectable on the cell surface under

homeostatic conditions, however we know that it is active.  It is possible that, given the right

conditions and right signals, DCs could shuttle IL-15 to the cell surface to deliver this signal.

Alternatively, DCs may constitutively display IL-15 on its surface and the interaction with a

CD8 or CD4 T cell could instruct the DC to internalize the IL-15.  The detection of IL-15

intracellularly could support either scenario – DCs hold the IL-15 in waiting or DCs internalize

IL-15.  This could be addressed by culturing DCs from RAG-1$^{-/-}$ mice, which we know to

express IL-15, with CD4 (CD25+ or -) or CD8 T cells and assaying for the presence or

absence of cell surface IL-15.  As clathrin-mediated endocytosis is a commonly used pathway

of membrane protein internalization, inhibitors that block this pathway, such as monensin or

dynasore, could determine if surface IL-15 is endocytosed via this mechanism.


**6.3    General Discussion**

The importance of IL-15 and CD8 T cell memory may be difficult to appreciate.  Why, after all, is immunological memory important when we can survive the infection in the first place?  In order to generate memory, one has to have undergone an immune response, so if that doesn't kill us, why bother with CD8 T cell memory?  Most models that are used to study CD8 T cell memory involve acute viral or bacterial infections – VSV, LCMV and *Listeria monocytogenes* are three commonly used examples of such pathogens.  When infected, mice generate immune responses that are fully sufficient to combat these pathogens and either by virtue of the inherent immunogenicity of the pathogen or by the use of recombinant proteins carried by the pathogen, antigen-specific memory CD8 T cells are conveniently generated.  Such banal "lather, rinse, repeat" approaches – while undeniably convenient and necessary to our understanding – do not engender our full appreciation for the power of immune memory.  What if we encounter a pathogen that kills us before the adaptive immune system can mount a response?  Or, despite the induction of an adaptive immune response, we still succumb?  In these cases, we have no protection.  Through the use of vaccinations, however, we can generate immune responses to various pathogens that would otherwise be lethal and in so doing give ourselves a measure of protection.  By successfully eliciting a CD8 T cell response after vaccination, a pool of memory CD8 T cells will arise that will enable swifter control of an otherwise lethal pathogen.  This memory response could function either to eliminate infected cells rapidly enough to halt the spread of pathogen, or could delay the progress of the infection so that other components of the immune system can be engaged.  To illustrate this point, infection of mice all the way up the evolutionary ladder to humans with certain strains of the Ebola virus results in a rate of lethality rate as high as 90% (105), despite the ability of mice to generate an adaptive immune response (106).  However, protection from a lethal infection in naïve mice can be achieved by adoptively transferring CD8 T cells from immunized mice prior to challenge (107).  This example highlights the power and necessity of CD8 T cell memory.  Of course, our knowledge of the power and utility of vaccination is only an application of a feature of the immune system that evolved long before humans began manipulating it for their

gain.  It is likely that the importance of CD8 T cell memory – in evolutionary terms – mainly functioned to lessen the severity of previously encountered pathogens and in so doing garnered better "fitness."  However, our understanding of how we can apply this knowledge with today's technology is where the real power of CD8 T cell memory can be appreciated.

The findings presented herein have addressed several outstanding questions within the field of CD8 T cell memory.  I have established a new relationship between DCs and CD8 T cells, that of IL-15 transpresentation.  Cytokine presentation – in contrast to antigen presentation – requires a reevaluation of the known interactions between CD8 T cells and DCs, and macrophages as well.  Antigen presentation has a rather well defined context: typically, DCs will carry antigen to secondary lymphoid organs where the antigen will be presented to T cells.  Cytokine presentation, on the other hand, happens in two distinct phases, both of which are poorly understood.  CD8 T cells receive IL-15 both during the contraction phase and memory phase from DCs and macrophages.  Now that this is established, we can begin looking at interactions between these cells as well as where they are located in tissues.  The micro niches wherein memory CD8 T cells have been demonstrated to reside gives us an indication as to with which cell – DC or macrophage – they are likely to interact.  This of course can be determined directly by immunofluorescence staining but it can be extended to determine if IL-15 signals are being transmitted at the time of cellular colocalization and interaction.  The presence of IL-15R$\alpha$ on one side of the synapse and a signaling molecule such as phosphorylated STAT5 on the other would indicate the transmission of IL-15 signals and would be another testament to the ability of DCs or macrophages to transpresent IL-15.  Various hematopoietic cells share the expression of a wide variety of cell surface markers which can make their identification by flow cytometry very difficult.  An approach such as this, using microscopy and immunofluorescence, may serve to disambiguate the identity of cytokine

presenting cells as tissue localization as well as context may better serve to identify

hematopoietic cells (108-111).

The relationship between IL-15 expression and irradiation is a particularly interesting finding as

it can have various clinical implications.  As mentioned before, IL-15 has been demonstrated

to be important for the control of tumor growth.  Perhaps instead of using irradiation as a

means to induce DNA damage and therefore cell death, it can be utilized more as an immune

modulator.  If indeed the irradiation is working via the induction of IFN-$\alpha$ expression to up-

regulate IL-15, this may be used as a more controlled and targeted way to utilize IFN-$\alpha$ as well

as IL-15.  A limited but focused increase in IFN-$\alpha$ expression would cut down on the side

effects associated with this treatment and perhaps have better efficacy.  Radiation delivered to

the tumor itself may result in DCs within the tumor microenvironment that – by virtue of their

increased IL-15 expression – can induce CD8 T cell responses to self-antigens that would

otherwise be left alone.  Our *in vitro* culture model showed that DCs need to express MHC

class I in addition to ICAM-1 and IL-15R$\alpha$ to elicit proliferation of memory-phenotype CD8 T

cells.  This may be a model of how such a scenario could work, albeit in our model IL-15 up-

regulation was achieved by poly I:C stimulation.

Overall I've demonstrated a new relationship between DCs and CD8 T cells in the context of

the cytokine IL-15.  Under steady state conditions, IL-15 transpresentation is enhanced by the

expression of ICAM-1 but where IL-15 expression is increased, ICAM-1 is not necessary for

IL-15 transpresenation (Figure 22).

**Figure 22**.  **The role of ICAM-1 in IL-15 transpresentation.**
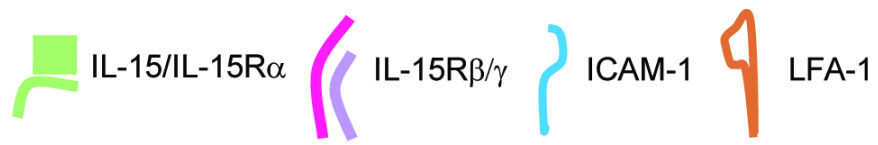


Normal homeostatic IL-15 expression
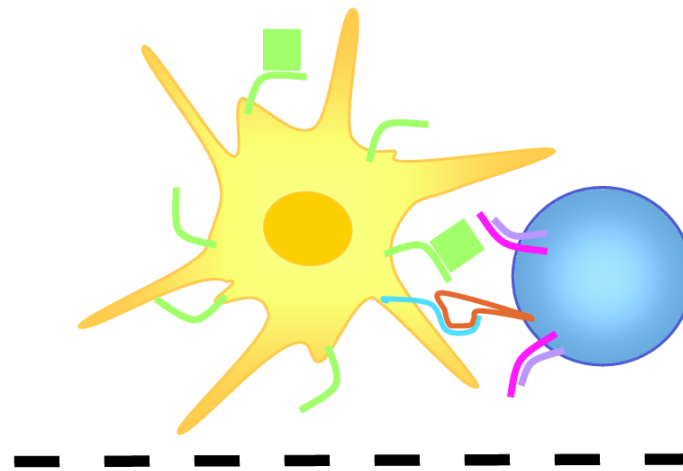
Heightened IL-15 expression
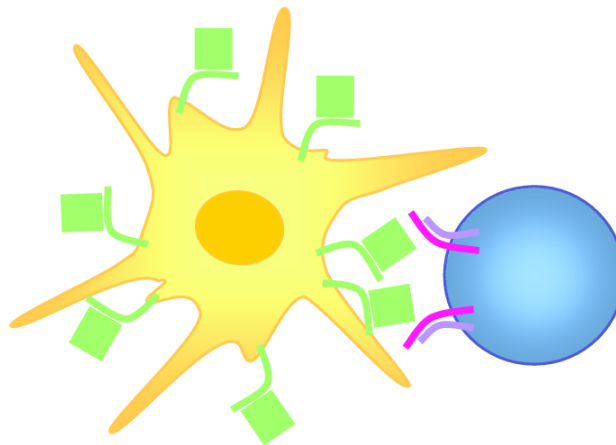
**Figure 22**.  **The role of ICAM-1 in IL-15 transpresentation.**  Model depicting the relationship between dendritic cells and CD8 T cells and the role of ICAM-1 in IL-15 transpresenation.

# Chapter 7 – Materials and Methods

## 7.1     Mice

C57Bl/6 (both CD45.2+ and Thy1.1+) and ICAM-1$^{-/-}$ mice were purchased from The Jackson

Laboratory (Bar Harbor, ME).  CD45.1+ C57Bl/6 mice were purchased from Charles River

(Wilmington, MA) through the National Cancer Institute (NCI) program.  IL-15$^{-/-}$ mice were

purchased from Taconic.  CD11a$^{-/-}$ mice were generously provided by Qing Ma (UT MD

Anderson Cancer Center, Houston, TX).  CD45.1+ OT-I transgenic RAG-1$^{-/-}$ and IL-15R$\alpha^{-/-}$

mice were bred in-house.  RAG-1$^{-/-}$ x ICAM-1$^{-/-}$ and RAG-1$^{-/-}$ x IL-15R$\alpha^{-/-}$ strains were

developed in house by crossing ICAM-1$^{-/-}$  and IL-15R$\alpha^{-/-}$ mice to the RAG-1$^{-/-}$ background.

Bone marrow chimeras were generated as previously described(41).  For irradiation

experiments, mice were irradiated with 750 cGy.  All mice were bred and maintained under

specific-pathogen free conditions at MD Anderson Cancer Center (Houston, TX).  All

experiments were performed with the approval of the Institutional Animal Care and Usage

Committee of MD Anderson Cancer Center.

## 7.2      Generation of CD11c/IL-15R$\alpha$ Tg mice

"The full length murine IL-15R$\alpha$ cDNA was cloned by PCR from cDNA generated from

C57Bl/6 spleen RNA using TA Topo cloning kit (Invitrogen). The IL-15R$\alpha$ cDNA was

then subcloned downstream of the CD11c promoter by ligating into the pCD11c-pDOI-

5 vector(56) (graciously provided by Thomas Brocker) at EcoRI site. The final 7.4 kb

CD11c-IL-15R$\alpha$ fragment was removed from the vector backbone by XhoI and NotI

digestion and used for microinjection into C57Bl/6 pronuclei for generation of

transgenic mice. Microinjection was performed by the Genetically-Engineered Mouse

Facility at the UT MD Anderson Cancer Center. Tg-positive mice were identified by PCR of tail DNA and bred to IL-15R$\alpha^{-/-}$ background." (58) **This research was originally published in *Blood*.** Stonier, S.W., Ma, L.J., Castillo, E.F., and Schluns, K.S. Dendritic cells drive memory CD8 T-cell homeostasis via IL-15 transpresentation. *Blood*. 2008; 112:4546-4554. **© the American Society of Hematology.**

## 7.3    Endogenous immune responses

For studies of a viral immune response, Wt, IL-15R$\alpha^{-/-}$, and ICAM-1$^{-/-}$ mice were infected intravenously with $1\times10^5$ pfu VSV-Indiana. Peripheral blood was collected via retro-orbital bleeds at the indicated times. Red blood cells were lysed with 155 mM NH$_4$Cl buffered with 130 mM Tris. Antigen-specific CD8 T cells were detected using an H-2K$^b$ tetramer (National Institutes of Health [NIH] tetramer facility) loaded with the VSV nucleoprotein epitope (RGYVYQGL) as previously described (41).

## 7.4    In vivo homeostatic proliferation assays

Proliferation of endogenous memory CD8 T cells was assessed by BrdU incorporation. VSV-infected mice were given BrdU in their drinking water (0.8 mg/ml) for 5-6 weeks. Cells were stained for surface markers as usual followed by staining using BrdU Flow Kit (BD Biosciences) according to manufacturer's instructions. For transfer studies of homeostatic proliferation, congenically-marked CD8 T cells were enriched from spleens of unmanipulated or immunized mice, where indicated. To establish antigen-specific CD8 T cell memory, C57BL/6 mice were infected i.v. with $1\times10^5$ pfu VSV and in some cases boosted at least 30 days post infection with VSV-New Jersey. Alternatively, memory OT-I T cells was generated as described(112). Spleens were harvested at least 30 days following most recent infection and CD8 T cells were enriched using a CD8 T cell negative enrichment kit (Invitrogen,

Carlsbad, CA).  Enriched CD8 T cells were labeled with 2 mM CFSE, and injected i.v. into

various recipients (between 5 and 10x10$^6$ cells/mouse).  The enrichments typically yielded a

purity of 80-85% with minor cellular contamination that included NK cells and DCs.


## 7.5    Acute proliferation assays *in vivo*

Where indicated, CD8 T cell memory was generated as previously described.  CD8 T cell

enrichments and CFSE labeling were performed as before.  For poly I:C-induced bystander

proliferation, CD8 T cells were transferred into Wt and ICAM-1$^{-/-}$ mice.  One day post-transfer,

recipient mice were given 150 $\mu$g poly I:C (InvivoGen, San Diego, CA) IP.  Five days later,

proliferation was assessed.  For lymphopenia-induced proliferation, prior to transfer RAG-1$^{-/-}$ ,

RAG-1$^{-/-}$ x ICAM-1$^{-/-}$ and RAG-1$^{-/-}$ x IL-15R$\alpha^{-/-}$ mice were injected with 0.5 mg blocking Ab

against IL-7R$\alpha$.  Additionally, IL-7R$\alpha$ was blocked on enriched CD8 T cells prior to transfer.

Mice were given an additional dose of anti- IL-7R$\alpha$ during the five day incubation, after which

tissues were harvested and proliferation analyzed.


## 7.6    Cocultures of CD8 T cells and BMDCs

BMDCs were generated with GM-CSF as previously described(113). Prior to culturing with

CD8 T cells, BMDCs were activated with 100 $\mu$g/ml poly I:C for 12-16 hours. For BrdU

incorporation studies, enriched CD8 T cells were cocultured with poly I:C-stimulated BMDCs

in complete media (CM [RPMI with 10%FCS, 10mM HEPES, 50$\mu$M 2-ME]) supplemented with

1 mM BrdU for three days.  BrdU incorporation was detected using the BrdU Flow Kit (BD

Biosciences) according to manufacturer's instructions. For phosphorylated STAT5 (pSTAT5)

studies, enriched splenic CD8 T cells (CD45.1+) were serum starved for 5h prior to coculturing

with DCs (3:1 DC:CD8 ratio).  Cells were cocultured for 1h then fixed immediately in 1x

Lyse/Fix Buffer (BD Biosciences), then frozen overnight in PBS and permeabilized with Perm

Buffer III (BD Biosciences).  pSTAT5 was detected using an anti-STAT5 (pY694) antibody (BD

Biosciences) according to the manufacturer's instructions.


## 7.7　　Flow cytometry analysis

IL-15R$\alpha$ and IL-15 were detected with goat anti–IL-15R$\alpha$-biotin (R&D Systems, Minneapolis,

MN) and rabbit anti–IL-15 biotin (Peprotech, Rocky Hill, NJ), respectively, followed by

streptavidin-APC (Jackson ImmunoResearch Laboratories, West Grove, PA). Background

staining was determined by staining analogous populations from either IL-15R$\alpha^{-/-}$ or IL-15$^{-/-}$

mice or with a biotinylated Ig control (Jackson ImmunoResearch Laboratories). Prior to

staining for IL-15 and IL-15R$\alpha$, cells were preincubated with Fc block (BD Biosciences, San

Jose, CA). The following monoclonal, fluorescently-labeled antibodies (mAbs) were purchased

from either BD Biosciences, eBioscience (San Diego, CA), or BioLegend (San Diego, CA):

CD44, CD62L, CD8$\alpha$, CD19, CD3, DX5, CD4, CD11b, CD11c, CD127, Thy1.1, KLRG-1,

CD27, CCR3, F4/80, Gr-1, Fc$\epsilon$RI, B220, Ly6C, TCR$\beta$, CD45.1 and MHC class II (I-Ab). LPS

stimulation for IL-15 expression was performed with bulk splenocytes in CM, in the presence

or absence of 1 $\mu$g/mL LPS (Sigma-Aldrich, St Louis,MO) for 24 hours.  IFN-$\alpha$ stimulation was

performed as with LPS but with 300 U/ml IFN-$\alpha$ (source).

Flow cytometry data were acquired using the Fortessa, LSRII, and FACSCalibur flow

cytometers (all BD Biosciences, San Jose, CA) and analyzed with FlowJo software (Treestar,

Ashland, OR). Lymphocyte percentages and total cell numbers were calculated and evaluated

using the Student $t$ test. Values of $p$ less than .05 were considered statistically significant.

## 7.8    qtPCR

RNA was isolated from sorted cell populations using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.  cDNA was synthesized using the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA).  iQ SYBR supermix (Bio-rad, Hercules, CA) was used to conduct quantitative PCR assays which were all done in triplicate.  Expression of IL-15 was determined by the following formula, applied to each reaction:

$$1.8^{(C_T \beta\text{-actin} - C_T \text{ IL-15})} \times 10^6$$

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**Vita**

Spencer William Stonier was born in Corning, NY on May 21, 1982 to Lynn and Sabine Stonier.  He graduated from Campbell-Savona High School as valedictorian in 2000, after which he attended the Rochester Institute of Technology (RIT) in Rochester, NY.  At RIT he pursued a Bachelor's Degree of Science in Biology, which he completed in 2004 with high honors.  He had two concentrations alongside his pursuit of the Biology degree, which were in German and Spanish.  In September of 2004 he moved to Texas where he worked as a research assistant in the lab of Dr. Kimberly Schluns.  He enrolled in the Graduate School of Biomedical Sciences at the University of Texas Health Science Center at Houston/MD Anderson Cancer Center in May 2006 where he has pursued a Ph.D. in the Immunology program under the mentorship of Dr. Kimberly Schluns.

# Publications

1. Stonier, S.W., Ma, L.J., Castillo, E.F., and Schluns, K.S.  "Dendritic cells drive memory CD8 T-cell homeostasis via IL-15 transpresentation."  Blood.  2008. 112:  4546-4554.

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