THE DEVELOPMENT AND FUNCTION OF SELF-SPECIFIC CD8 T CELLS

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B.Sc., University of British Columbia, 2001

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

The Faculty of Graduate Studies

Microbiology

THE UNIVERSITY OF BRITISH COLUMBIA

July 2006

Abstract

A distinguishing feature of conventional T cells is that they are tolerant to self-peptides that are presented by self-major histocompatibility complex (MHC) molecules even though these cells are positively selected by low affinity interactions between their TCRs and self-peptide/MHC ligands in the thymus. Over the last few years several subsets of unconventional T cells have begun to emerge which often rely on strong interactions between their TCRs and self-antigens for both their development and function. Furthermore, these unconventional T cells often express receptors that are not commonly associated with T cells but rather are associated with cells of the innate immune system. The work in this thesis is focused on the discovery and characterization of a novel subset of CD8 T cells which demonstrate a high affinity for self-antigens and are thus self-specific. These self-specific CD8 T cells can be found in normal mice as well as in self-antigen expressing TCR transgenic mice. Self-specific CD8 T cells are selected by high-affinity interactions either in the thymus or extrathymically and possess a memory-phenotype. As a consequence of this memory-phenotype, which is associated with high expression of CD122 (IL-2Rβ), these cells can proliferate in an antigen-independent manner in response to stimulation with interleukin-2 or -15. Upon activation, the cells express several natural killer cell receptors including NKG2D, CD94, 2B4, and CD16. These NK receptors can act independently or in concert with the self-specific TCR leading to target cell killing and cytokine production. Self-specific CD8 T cells require self-antigen interactions not only for their development but also for the maintenance of their memory-phenotype and cytokine responsiveness. These cells become activated in response to infection or inflammation and this activation results in the innate production of cytokines such as IFNγ which are protective during infection. Thus, self-specific CD8 T cells represent a novel CD8 T cell subset that utilize their TCRs, cytokine receptors as well as NK receptors to provide early protection against infection and the elimination of either stressed or transformed cells.

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List of Abbreviations

α-GalCer	alpha-galactosyl ceramide
ADCC	antibody-dependent cell-mediated cytotoxicity
AICD	activation-induced cell death
Ag	antigen
APC	antigen-presenting cell
$CD8^{hi}$	female H-2^b H-Y TCR transgenic CD8 T cell
$CD8^{int}$	male H-$2^{b/d}$ H-Y TCR transgenic CD8 T cell
$CD8^{lo}$	male H-2^b H-Y TCR transgenic CD8 T cell
CFSE	carboxyfluorescein diacetate succinimidyl ester
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DN	$CD4^-CD8^-$ double negative
DP	$CD4^+CD8^+$ double positive
DLN	draining lymph node
GM-CSF	granulocyte macrophage colony stimulating factor
HSC	hematopoetic stem cell
ICOS	inducible co-stimulator
IEL	intra-epithelial lymphocyte
Ig	immunoglobulin
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
JNK/SAPK	jun-N-terminal kinase/stress-activated protein kinase
KIR	killer Ig-like receptor
LAK	lymphokine-activated killer
LAT	linker of activated T cells
LCMV	lymphocytic choriomeningitis virus
LLO	listeriolysin-O
LM	Listeria monocytogenes
LPS	lipopolysaccharide

MAPK	mitogen activated protein kinase
MHC	major histocompatibility complex
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor κB
NK	natural killer
NKT	natural killer T cell
NOD	nonobese diabetic
OM	oncostatin M
PI3K	phosphatidylinositol 3-kinase
PMA	phorbol 12-myristate 13-acetate
RAG	recombinase activating gene
T-bet	T-box expressed in T cells
TCR	T cell receptor
TLR	Toll-like receptor
TRAF	TNFR receptor-associated factor
TNF	tumor necrosis factor
TNFR	TNF receptor
TNFR2, p75	tumor necrosis factor receptor-2
WT	wild-type
ZAP-70	zeta-associated protein of 70 kDa

Acknowledgements

I would like to thank Hung-Sia Teh for giving me the opportunity to study immunology and providing me with a strong foundation for my future research. I thank Soo Jeet Teh for teaching me the basic principals in immunological research. I would also like to thank members of my thesis supervisory committee including Michael Gold, Ninan Abraham, and Fumio Takei. In addition I thank Mark Horwitz and Shelley Small for their support. Thank you to NSERC and MSFHR for providing me with funding for my research.

I have been blessed to be surrounded by great people and great scientists. I have learned a lot about science from other members of the Teh lab including John Priatel, Edward Kim, Darryl Oble, Mike Chow, and Xiaoxi Chen and I thank them all immensely for sharing their knowledge. In addition I thank all of the people in our department including (but not limited to) SLT, BR, HK, MR, and LO.

To my parents and brother thank you for providing me with everything that I needed to be the person I am today. I could not have accomplished this without your guidance and support. To my beautiful children, Khayali and Kaysan, thank you for making the long days worthwhile. The biggest thank you goes out to my beautiful wife Neilin whose love and support has guided me through all of the hard times. If it was not for you I would never have survived the disappointments of science nor had anyone to share in its triumphs. I dedicate this thesis to you.

Co-Authorship Statement

For the co-authored chapter 3, I contributed all of the experimental design, 80% of the research, all of the data analysis, and wrote the manuscript. For the co-authored chapter 4, I contributed all of the experimental design, 90% of the research, all of the data analysis, and wrote the manuscript. For the co-authored chapter 5, I contributed all of the experimental design, 90% of the research, all of the data analysis, and wrote the manuscript.

Chapter 1 Introduction

1.1 Preface

The foundation for this thesis is the observation that unusual T cell subsets exist which display characteristics of adaptive as well as innate immune cells. Even though the majority of T cells undergo a stringent selection process in order to eliminate any self-reactive cells the immune system still retains some self-reactive cells to perform functions distinct from those performed by the majority of conventional T cells. From this foundation it is reasonable to believe that the CD8 T cell population that is normally thought to be specific for foreign antigens may in fact contain some cells that display self-specificity. This thesis consists of work which clearly demonstrates the existence of CD8 T cells expressing self-reactive T cell receptors whose selection and maintenance depend on self-antigen interactions. Much of the following work describes the development and function of these self-specific CD8 T cells including their expression of natural killer cell receptors and their responses to infection.

1.2 Receptors of the Innate and Adaptive Immune system

1.2.1 Biology of NK cells

Natural killer (NK) cells are an integral part of the innate immune system. Activation of NK cells leads to the rapid production of cytokines as well as the destruction of infected or transformed cells. NK cells also have the ability to alter the adaptive immune response by either promoting the maturation of dendritic cells (DCs) that can prime T cells or by destroying immature DCs before they can present antigen to T cells (1, 2). The effector pathways used by NK cells are very similar to those used by T cells. NK cells, like cytotoxic CD8 T cells, use perforin and granzymes to lyse cells (3). Both cell types are also capable of producing IFNγ that has multiple effects on the immune response. Unlike T cells, NK cells can immediately kill target cells and produce cytokines without the need for prior restimulation, thus leading to their classification as cells of the innate immune system.

NK cell precursors are derived from hematopoetic stem cells (HSCs). The development of NK cells in adults is thought to occur mainly in the bone marrow where stromal cells provide cell-cell contact mediated signals as well as cytokine mediated signals important for NK cell development (reviewed in (3)). Most of what is known about NK cell development has been learned through the in vitro generation of NK cells from HSCs or NK precursors. Figure 1.1 provides a good overview about what is known about NK cell development from these mainly in vitro studies. The stromal cells that drive NK cell development can actually be replaced in these in vitro cultures by cytokines such as stem-cell factor (SCF), fetal liver kinase 2 ligand (FLK2), and IL-7 suggesting that these cytokines are required for NK cell development and are normally provided by stromal cells (4,

5). These cytokines drive the development of NK precursor cells whose further maturation can be driven by IL-2 or IL-15 (6, 7). NK precursor cells become immature NK cells and finally mature NK cells, a process characterized by the sequential induction of NK receptors. The acquisition of NK receptors is not random and is thought to be required to prevent the erroneous activation of maturing NK cells in response to normal host cells (reviewed in (8)).

NK cells use several germ-line encoded receptors to recognize infected, stressed or transformed cells. NK receptors can transduce inhibitory, activating, or costimulatory signals and NK cell activation is regulated through the integration of the signals from several NK receptors (9). The finding that NK cells were tightly regulated by inhibitory receptors for self-MHC class I molecules was a breakthrough in the field and resulted in the "missing-self hypothesis" which stated that NK cells could recognize and kill target cells that had lost expression of MHC class I, an event that is known to occur both during infection and transformation (10). Recently, the missing-self hypothesis has been modified to account for the fact that NK cells cannot kill cells that have lost MHC class I without the engagement of activating receptors (9). Thus NK cells do not recognize erythrocytes which lack MHC class I molecules because they do not express any ligands for activating NK receptors. In order for a target cell to be killed by an NK cell the signals from activating receptors have to outweigh signals from the inhibitory receptors. The two ways for NK activation to occur are through either decreasing inhibitory receptor engagement or increasing activating

receptor engagement either of which can happen during infection or transformation.

1.2.2 Activating and inhibitory receptors of NK cells

Inhibitory NK receptors have been extensively characterized in both mice and humans. Table 1.1 shows a few common human and mouse inhibitory receptors and their MHC class I ligands. These receptors can be organized into two structural categories: killer Ig-like type I integral membrane receptors (KIRs), encoded in the leukocyte receptor complex, and lectin-like type II integral membrane receptors, encoded in the NK gene complex (11-14). The later category includes Ly49 homodimers in mice and CD94-NKG2A heterodimers in both mice and humans (9, 15). Despite their structural differences, the inhibitory NK receptors share many functional characteristics. Similar to T cell receptors (TCRs), these inhibitory receptors bind to major histocompatibility (MHC) class I molecules (9). The KIR and Ly49 family members interact with particular allelic variants of classical MHC class I molecules. In contrast, the ligand for the CD94-NKG2A receptor is the non-classical MHC class I molecule HLA-E, or its murine ortholog Qa-1^b, complexed to a nonapeptide, Qdm, derived from the leader sequence of particular MHC class I heavy chains (16). Interaction of inhibitory receptors with MHC class I molecules prevents activation of NK effector function by eliciting a strong inhibitory signal mediated by an immunoreceptor tyrosine-based inhibitory (ITIM) motif in the cytoplasmic domains of these receptors.

When these inhibitory receptors are engaged, the ITIM is tyrosine phosphorylated and SHP-1 phosphatase is recruited and activated, which presumably dephosphorylates signaling molecules involved in the activation cascade (17-20).

Until recently, the molecular structures involved in NK triggering have long been a matter of debate. Thus, the identification of a panel of recognition structures expressed on NK cells directly involved in NK triggering, is a major breakthrough in the field. Table 1.2 provides a summary of common human and mouse activating NK receptors along with their ligands and the signaling pathways which they use. Individual NK cells express multiple different activating NK receptors of both Ig and lectin-like structural families that lack cytoplasmic ITIMs (21, 22). These activating receptors include the natural cytotoxicity receptors, the activating NK receptors, which include stimulatory KIRs in humans and certain Ly49 receptors, i.e. Ly49D (23) in the mouse, CD94-NKG2C heterodimers in both species (24), and as described later, NKG2D and 2B4 receptors. Most of these receptors contain charged amino acids in their transmembrane domains, permitting interaction with distinct signaling chains containing immunoreceptor tyrosine-based activation motifs (ITAMs). NK cells express multiple ITAM signaling chains, including DAP12, CD3ζ, and FcεRIγ (25). Ly-49D and Ly-49H associate with DAP12 (22), NKR-P1 associates with FcεRIγ (26), and CD16 associates with CD3ζ (27, 28) and FcεRIγ (29). When activating receptors are cross-linked, the ITAMs in the associated signaling chain become tyrosine phosphorylated. These ITAMs then recruit downstream

signaling kinases such as Syk and ZAP-70, triggering activation of cytotoxic, proliferative, and/or secretory responses (30, 31).

One recently characterized activation molecule expressed on both human and mouse NK cells is the lectin-like NKG2D molecule (32). NKG2D has recently been shown to exist in two alternatively spliced isoforms, NKG2D-S and NKG2D-L (33). Both isoforms can pair with the signaling molecule, DAP10 (33), which contains a YxxM motif thought to recruit phosphatidylinositol 3-kinase (PI3-K) (34). Only NKG2D-S pairs with DAP12 (33). This differential recruitment of DAP10 versus DAP12 provides NKG2D with both directly stimulatory and co-stimulatory functions, respectively (33, 35). Human NKG2D recognizes the stress-inducible MIC family (MICA and MICB) (32). In addition, it interacts with the ULBP family of molecules, which also bind the cytomegalovirus-encoded UL16 molecule (36). The ULBPs stimulate cytokine and chemokine production from NK cells, and expression of ULBPs in NK cell-resistant target cells confers susceptibility to NK cell cytotoxicity (37). Mouse NKG2D binds the minor histocompatibility molecule H60, as well as members of the retinoic acid-inducible (Rae-1) family of molecules (38, 39). These NKG2D ligands are not expressed by most normal cells but are up-regulated on numerous tumor cells upon infection or in response to DNA damage. Expression of an NKG2D ligand by target cells triggers NK cell cytotoxicity, IFNγ secretion, as well as nitric oxide release and TNF-α and β transcription by macrophages (37, 40). Studies have demonstrated that NKG2D serves as a co-stimulatory molecule for TCR-activated $CD8^+$ T cells due to the lack of DAP12 expression; however, it functions

as a primary activation receptor on NK cells which do express DAP12 (33-35, 41).

One of the first activating receptors described on NK cells was FcγRIIIα or CD16. CD16 is a low affinity Fc receptor (FcR) that binds to IgG and is involved in antibody dependent cell-mediated cytotoxicity (ADCC) in which an antibody coated target cell is destroyed by NK cells (42). Stimulation of CD16 on NK cells also results in the production of cytokines such as IFNγ, TNFα and GM-CSF (43). FcγRIIIα associates mainly with the ITAM-containing homo or heterodimers of CD3ζ and FcεRIγ (FcRγ) in humans (28) or solely with FcRγ homodimers in mice (44). The binding of IgG to CD16 results in the phosphorylation of the ITAMs in the signaling chains leading to the recruitment of kinases such as ZAP-70 and Syk (45). These kinases initiate a signaling cascade resulting in the lysis of antibody-coated target cells and cytokine production.

2B4 (CD244) is an NK receptor that is expressed by all NK cells in both humans and mice (9). Both 2B4 and its ligand CD48 are members of the CD2-family of Ig-related proteins (46). 2B4 contains a binding-site within its intracellular domain that upon phosphorylation can bind to the protein-tyrosine phosphatases SHP-1 and SHP-2 or to the adaptor molecule SAP (SH2D1A) (47). The function of 2B4 is quite controversial with reports suggesting that the receptor is either activating, co-stimulatory or inhibitory. The strongest evidence for an inhibitory role for 2B4 comes from studies with 2B4-deficient mice (48, 49). The NK cells from these animals display enhanced responses against CD48-positive targets. The evidence for 2B4 being involved in activation comes from

studies showing that cross-linking of 2B4 on IL-2-activated NK cells leads to stimulation of lytic activity (50-52), IFNγ secretion (52), and granule exocytosis (53). 2B4 is constitutively associated with the linker for activation of T cells (LAT) (54) and antibody-mediated engagement of 2B4 resulted in tyrosine phosphorylation not only of 2B4 but also of the associated LAT molecules. Furthermore, tyrosine phosphorylation of LAT leads to the recruitment of signaling molecules that include PLCγ and Grb2 (54). A recent report showed that NK cells express another adaptor protein related to SAP called EAT-2 that can associate with 2B4. Unlike SAP, EAT-2 transduces an inhibitory signal (55). Thus the seemingly opposite functions of 2B4 are probably regulated by the relative amounts of the adaptor proteins expressed by the cell.

The regulation of NK cell activation is complex and multifaceted. For NK cells, several receptors are likely involved in triggering and it is the sum of the signals which dictates whether or not an NK cell will respond. NK cells, unlike T cells are not restricted to one particular ligand and can potentially respond to several different target cells expressing different combinations of ligands for activating and inhibitory NK receptors.

1.2.3 Activating and inhibitory receptors of T cells

T cells are an integral part of the adaptive immune system. In contrast to NK cells, which provide early protective immune responses, T cells are more important in providing protection against microbial infections during the later

stages of the immune response. T cell activation is a complex process that involves activation through the TCR and co-stimulatory receptors (56-58). The αβTCR on T cells consists of the peptide/MHC-binding TCR α and β chains along with the CD3 signaling complex consisting of one CD3γ, one CD3δ, two CD3ε, and two CD3ζ chains (reviewed in (59)). The CD3 chains all contain one ITAM motif in their cytoplasmic tail with the CD3ζ chains containing 3 ITAMs each. When the TCR binds to peptide/MHC, the ITAMs in the CD3 chains become phosphorylated by src-family kinases. Lck, a member of the src-family kinases, is thought to be the major contributor to ITAM phosphorylation in T cells (60). The phospho-tyrosine residues within the ITAMs on CD3ζ then recruit the kinases ZAP-70 and Syk (61). These kinases then act on several downstream targets including LAT eventually leading to the activation of the MAP kinase pathway and ERK activation (reviewed in (62)). Ultimately the signals emanating from the TCR lead to the activation of several transcription factors including c-jun, c-fos, and NF-AT, which result in the transcription of genes involved in cell division and differentiation (reviewed in (63)).

Full activation of naïve T cells requires simultaneous engagement of the TCR and co-stimulatory molecules, resulting in triggering of T cell effector function, survival and proliferation (64). The best-characterized T cell co-stimulatory molecule is CD28, which recognizes B7.1 and B7.2 (65, 66). The expression of B7.1 and B7.2 is restricted mainly to the professional antigen-presenting cells (APCs) that are required for naïve T cell activation. CD28 recruits and activates various tyrosine-phosphorylated proteins, including the

kinases, Lck and Itk, and the phosphoinositol lipase PLCγ1 (57). CD28 signals through a YxxM motif present in its cytoplasmic domain, which is a consensus binding site for the p85 subunit of PI3-K (67-69). CD28-engagement leads to the induction of survival molecules as well as to the efficient production of interleukin-2 in antigen-activated T cells (70).

Another member of the CD28-family of co-stimulatory molecules expressed by T cells is ICOS (71). The ligand for ICOS, ICOS-L, is also expressed primarily by professional APCs (72, 73). ICOS also contains the PI3-K binding motif but unlike CD28, ICOS is induced on activated T cells and plays a role in T cell survival but has little influence on IL-2 production (71, 74). ICOS does however play a role in the production of IL-10 by CD4 T cells (71) and ICOS-L expression by tumor cells augments tumor-specific CD8 T cell responses (75).

T cells can also express co-stimulatory molecules that are not a part of the CD28-family of molecules. These other co-stimulatory molecules include members of the tumor necrosis factor receptor (TNFR) family. Members of this family include OX40, 4-1BB, CD27, and TNFR2. The expression of TNFR family members on T cells can be either constitutive, as is the case for CD27 and TNFR2, or inducible, like for 4-1BB and OX40 (76, 77). These receptors all share the ability of activating TNF receptor associated factors (TRAFs) and all of the receptors can activate NFκB (76, 78). The ligand for CD27, CD70, is expressed by activated APCs (79). The ligands for OX40 and 4-1BB, OX40L and 4-1BBL respectively, are also induced on activated APCs (80, 81). TNFR2 binds to TNFα

which is produced by activated T cells themselves and acts in an autocrine manner (77). CD27 and TNFR2 are involved early during T cell activation and OX40 and 4-1BB seem to be involved more in the survival of activated T cells (82-85). Nevertheless all of the TNFR family members play important roles in full T cell activation.

T cells also express inhibitory receptors such as CTLA-4 and PD-1 (reviewed in (86)). Both of these receptors are induced upon T cell activation and both are capable of dampening the T cell response. CTLA-4 binds to B7.1 and B7.2, the same ligands that bind CD28, but it does so with a much higher affinity than CD28 (87, 88). Thus one mechanism by which CTLA-4 may inhibit T cell activation is through competition with CD28 for binding to the B7 molecules (89). CTLA-4 has also been shown to recruit the phosphatase SHP-2 which leads to the dephosphorylation of proteins in the T cell activation pathway (90). The importance of CTLA-4 is clear when one looks at mice lacking this receptor. CTLA-4$^{-/-}$ mice develop severe immunopathology where all of their T cells display an activated phenotype and the mice die within a few weeks of birth (91, 92). PD-1-deficiency also leads to autoimmunity although it seems to be less severe than that seen with CTLA-4 (93).

PD-1 contains an ITIM motif that can potentially signal the recruitment of phosphatases that can dampen TCR signaling (94). The ligands for PD-1 are PD-L1 (B7-H1) and PD-L2 (B7-DC) (95-98). These ligands are expressed in both lymphoid and non-lymphoid tissue suggesting that PD-1 is involved in regulating T cell responses in secondary lymphoid organs and in the periphery (95, 96). PD-

1 is expressed only on activated T cells and is predominant on chronically stimulated T cells where it plays a role in their inability to respond to further stimulation (94, 99). In fact blocking of PD-1/PD-L1 interactions can rescue these exhausted T cells and allow them to respond normally to TCR stimulation (99). PD-1 engagement can cause cell-cycle arrest in T cells which can be overcome by the addition of exogenous IL-2 (100). Interestingly, PD-L1 and PD-L2 can also costimulate T cell responses suggesting that perhaps there are other receptors capable of binding to these ligands (97, 98). Regardless, it is clear that PD-1 plays an important role in dampening T cell activation and more importantly its function is not redundant with CTLA-4.

Although T cell activation is generally dictated by the recognition of peptide/MHC by the TCR it is clear that full T cell activation is regulated by other receptors which can either modulate TCR signaling or act independently of the TCR through the induction of survival molecules. Unlike for NK cells where no single activating receptor dictates a particular NK cells' specificity, the TCR is the sole determinant of a T cells' specificity.

1.2.4 NK receptor expression by memory-phenotype CD8 T cells

Even though NK cells and T cells perform quite distinct functions they appear to be related. Both cell types are derived from a common progenitor cell (101). NK cells are more like CD8 T cells in the sense that they both use perforin and granzymes to kill target cells and both cell types are efficient producers of

IFNγ (9). NK cells can immediately exert their function in the absence of prior activation differentiating them from CD8 T cells which need prior antigen-activation before they can produce cytokines or kill target cells. Another commonality between NK cells and T cells is the observation that both cell types use similar signaling molecules and signaling pathways for their activation. As discussed in the previous sections, both NK cells and T cells have activating, costimulatory, and inhibitory receptors and both cell types use the same proteins to transmit the signals from these receptors. It is possible then, that if T cells were to express NK receptors, that these receptors would in fact be functional. In fact, there is potential for NK receptors to either amplify or abrogate TCR signals depending on whether the NK receptor involved is inhibitory or activating. This possibility would require the expression of either inhibitory and/or activating NK receptors by T cells.

Interestingly, various T cell subsets, B cells and myeloid cells do in fact express inhibitory as well as activating NK receptors (102). NKG2D is expressed by all human $CD8^+$ T cells and γδT cells (32). In mice, NKG2D is expressed by activated, but not resting $CD8^+$ T cells, macrophages, and subsets of $TCR\alpha\beta^+$ and $NK1.1^-$ T cells (39). In contrast to NK cells, cross-linking of NKG2D on activated $CD8^+$ T cells failed to mediate redirected lysis of FcR-expressing target cells suggesting that NKG2D does not directly stimulate $CD8^+$ T cells (103). Furthermore, the activated $CD8^+$ T cells did not respond to NKG2D cross-linking by producing IFNγ or by fluxing Ca^{+2} (103). However, NKG2D cross-linking augmented the proliferative response of $CD8^+$ T cells to limiting doses of anti-

TCR mAb (41, 103). Thus, NKG2D can only provide a costimulatory signal for the activation of NKG2D$^+$ CD8$^+$ cells due to their lack of expression of DAP12. In this regard, the transgenic expression of DAP12 into CD8 T cells is sufficient to convert NKG2D into a directly activating receptor confirming that the function of NKG2D on CD8 T cells is governed by the adaptor molecules which the T cell expresses (104).

In addition to NK cells, 2B4 is expressed on monocytes, basophils, and subsets of $\gamma\delta$T cells and CD8$^+$ T cells (46, 52, 105). The biological function of 2B4 on CD8$^+$ T cells remains largely unclear. 2B4 is preferentially expressed on CD8$^+$ T cells with an activated/memory phenotype (106) and by T cells involved in non-MHC restricted cytotoxicity (106, 107). A recent study suggests that 2B4 on activated/memory CD8$^+$ T cells serves as a ligand for CD48, and by its ability to interact with CD48 provides costimulatory function for neighboring T cells (106). 2B4 engagement on CD8 T cells can also enhance the killing of CD48-positive target cells (108). Thus it seems that when 2B4 is expressed on memory-phenotype CD8 T cells it does in fact play a role in both their activation and their effector functions.

In both mice and humans, the expression and function of inhibitory NK receptors on memory-phenotype CD8 T cells has been described extensively (reviewed in (109, 110)). Expression of a Ly49A transgene has been shown to effect both activation and effector functions of CD8 T cells (111-113). Strikingly, the expression of a Ly49A transgene in CTLA-4-deficient mice can prevent the T cell lymphoproliferative disease in these animals demonstrating that the signals

mediated by these distinct receptors are similar (114). The expression of NK receptors has also been shown to be important for the survival and development of memory-phenotype CD8 T cells (115). It has been suggested that CD8 T cells can acquire the expression of inhibitory NK receptors only upon recognition of cognate antigen as CD8 T cells that are chronically activated such as those in healthy carriers of Epstein Barr virus or cytomegalovirus often exhibit a memory phenotype and express NK receptors (116). The fact that NK receptor expression is not detected on CD8 T cells in the thymus but is induced in the periphery is consistent with this idea (117). Although memory-phenotype CD8 T cells have been shown to express NK receptors their physiological roles during normal immune responses remain largely unclear. Thus it remains possible that memory-phenotype CD8 T cells may express NK receptors in order to dampen signaling by their TCRs which may be specific for self-antigens. Consistent with this observation is the fact that T cells specific for non-mutated self-antigens expressed by melanomas often express inhibitory NK receptors, even in healthy donors (118).

1.2.5 The role of interleukin-2 and 15 in NK and T cell homeostasis

CD122 is a cytokine receptor shared by both interleukin (IL)-2 and IL-15. A high level of CD122 expression is characteristic of both memory-phenotype CD8 T cells and NK cells and thus it is not surprising that IL-2 and IL-15 play pivotal roles in the activation and homeostasis of both NK cells and T cells. The

receptors for these cytokines share two subunits, CD132 or the common gamma chain (γc) and CD122 (IL-2Rβ) (119, 120). CD122 expressed with CD132 makes up the low affinity receptor for both IL-2 and IL-15. The high affinity receptor for IL-2 consists of an added chain CD25 (IL-2Rα) that is capable of binding to IL-2 with a much higher affinity than CD132 and CD122 alone (121). The same is true for IL-15 where a third receptor component IL-15Rα increases the affinity of the IL-15R for IL-15 (122). CD122 is the signaling chain for both the IL-2 receptor as well as the IL-15 receptor and its expression along with CD132 is sufficient to confer both NK cells and T cells with the ability to respond to IL-2 or IL-15 (reviewed in (123)). IL-2 is produced by activated T cells where it binds to the high affinity IL-2 receptor in an autocrine manner (reviewed in (121)). Signaling through IL-2 is involved in the expansion phase of T cell activation but it is also crucial for programming T cells to undergo contraction upon further stimulation, a process termed activation-induced cell death (AICD) (124). Provision of high doses of IL-2 to NK or T cells is capable of inducing the activation and proliferation of the cells and these lymphokine activated killer (LAK) cells exhibit a high degree of killer activity towards several self- and non-self target cells that are normally resistant to NK cells (125, 126). IL-15 is produced by multiple cell types (122) and plays a major role in the development and/or homeostasis of NK cells, CD1d-restricted NKT cells, which will be discussed later, and memory-phenotype CD8 T cells all of which express high levels of CD122 (127, 128). IL-15 and IL-15Rα-deficient mice have a drastic reduction in the number of NK, NKT, and memory-phenotype CD8 T cells due partly to the inability of the cells to

divide in vivo. One oddity of IL-15 signaling is the fact that IL-15Rα seems to be involved mainly in presenting IL-15 to CD122 and CD132 (129, 130). In fact this presentation of IL-15 by IL-15Rα can occur in *trans* where the IL-15Rα-expressing cell provides IL-15 to a neighboring cell that expresses only CD122 and CD132 (131). Thus in IL-15Rα-deficient CD8 T cells undergo normal expansion in an IL-15Rα-sufficient host. IL-15, like IL-2, induces the activation and proliferation of CD8 T cells but unlike IL-2, induces the survival rather than contraction of the responding cells (132). Thus although IL-2 and IL15 share similar signaling components the outcome of IL-2 and IL-15 stimulation varies significantly. However, it is important to remember that both IL-2 and IL-15 are capable of activating both NK cells and memory-phenotype CD8 T cells without the need for TCR or NK receptor stimulation and this cytokine-dependent activation likely plays an important role during the majority of immune responses.

1.3 T cell development

1. 3.1 Positive and negative selection of the naïve T cell repertoire

T cell development is a complicated process involving the seeding of the thymus with bone marrow derived progenitor cells followed by selection of T lineage committed thymocytes that have successfully rearranged their T cell receptor (TCR). It all begins when progenitor cells from the bone marrow make their way

into the thymus. These $CD4^-CD8^-$ double negative (DN) progenitors then go through a stepwise process characterized by the acquisition and loss of the cell surface molecules CD44 and CD25 (133). The cells progress from the DN1 ($CD44^+CD25^-$) stage to the DN2 ($CD44^+CD25^+$) stage and then the DN3 ($CD44^-CD25^+$) stage which are all accompanied by the stepwise movement of the cells from the cortico-medullary junction of the thymus towards the cortex and subcapsular regions. At the DN3 stage is where the thymocytes initiate the rearrangement of their TCRβ genes which upon successful pairing with the surrogate light chain, pTα, allows the cells to progress to the DN4 stage ($CD44^-CD25^-$). At this point the cells undergo extensive proliferation accompanied by the expression of both the CD4 and CD8 coreceptors. These $CD4^+$ $CD8^+$ double positive (DP) thymocytes then undergo rearrangement of their TCRα loci in order to prepare them for the next stages of development (134). Thymocytes expressing rearranged TCRs undergo another round of selection that ensures that their newly rearranged receptor has some reactivity to self-peptide in the context of self-MHC. The selection of weakly self-reactive thymocytes, or positive selection, leads to their survival and maturation into CD4 or CD8 single positive (SP) T cells. If a TCR cannot bind to self-peptide/MHC the thymocyte expressing that TCR will not receive any survival signals and thus will die by neglect. When developing thymocytes react too strongly with self-peptide/MHC they are eliminated by negative selection in order to ensure that they cannot harm the host. Thus there are several checkpoints to ensure that mature T cells can recognize foreign peptides in the context of self-MHC without being overtly

autoreactive. Figure 1.1 provides an overview of T cell development from the seeding of progenitor cells in the thymus through to their positive or negative selection.

Positive selection of T cells is crucial for the function of mature T cells. During the positive selection process $CD4^{+}CD8+$ double positive (DP) thymocytes expressing a rearranged TCR are screened for reactivity with self-peptide/MHC complexes expressed by thymic cortical epithelial cells (135). If the TCR has weak to moderate affinity for a self-peptide/MHC complex, the thymocyte will receive a signal through the TCR that induces survival and differentiation. Weak stimulation of the TCR on DP thymocytes results in the activation of NFAT and ERK which are thought to be critical for efficient positive selection (136, 137). The self-peptide/MHC complexes that mediate positive selection react weakly with the TCR and these weak signals are insufficient to activate mature T cells (134). In addition to mediating survival, positive selection also commits DP thymocytes to either the helper (CD4) lineage or killer (CD8) lineage depending on whether the TCR binds to either MHC class II or MHC class I molecules respectively (138). Thus positive selection is not only important for the survival of T cells but it also ensures that the T cell adopts the appropriate functional phenotype.

Positive selection ensures that T cells can recognize self-peptide/MHC with a low affinity. Negative selection, on the other hand, eliminates T cells in the thymus that react too strongly with self-peptide/MHC. Negative selection is thought to occur in the thymic medulla where thymic medullary epithelial cells

scans positively selected thymocytes for strong reactivity with self-peptide/MHC complexes (139). Strong TCR signaling in these thymocytes results in the phosphorylation and activation of ERK, P38, and JNK which leads to cell death. It is important that thymic medullary epithelial cells express a wide array of self-peptides derived from organ specific proteins in order to prevent autoimmunity as a consequence of inefficient negative selection. Perhaps the most important mediator of this self-peptide presentation is a recently described gene, *aire*. Mice and humans with a disruption in *aire* develop autoimmune disease characterized by multiorgan cell infiltrates (140). The cause of disease in *aire*-deficient mice is not only the inability of medullary epithelial cells to express organ-specific peptides but also in their inability to efficiently present these peptides to developing T cells (141). Thus it is clear that negative selection against a variety of self-peptides, including those restricted to specific organs, is crucial for maintaining tolerance to self. Emphasizing the importance of purging autoreactive T cells is the fact that even if negative selection fails in the thymus, peripheral (extrathymic) mechanisms exist that can induce T cell tolerance to self –antigens (142).

1.3.2 Extrathymic T cell development

The thymus has evolved as an organ dedicated to T cell development. It contains all of the supporting cells and has the appropriate architecture and microenvironment for the development and selection of T cells. The thymus is not

the only location of T cell development, however, as certain secondary lymphoid organs have been shown to support T cell development. The mesenteric lymph nodes are a particularly effective site of extrathymic T cell development although their contribution to T cell lymphopoesis is minimal in the presence of an intact thymus (143). The problem with extrathymic T cell development seems to be due to the inability of the LNs to attract T cell progenitors. Overexpression of the cytokine oncostatin M (OM) can overcome this problem and converts the LNs into potent sites of T cell development (144, 145). Extrathymic T cell development involves the positive and negative selection of DP T cells but the major difference between conventional thymic and unconventional extrathymic positive selection is the nature of the selecting cell (146). In the thymus, cortical epithelial cells are the main cell type mediating positive selection whereas in the LNs, positive selection is mediated by hematopoetic cells (147). This difference in the positively selecting cell type imparts functional differences in the selected T cells, including those that express the same TCR.

Extrathymically selected T cells are functionally distinct from intrathymically selected T cells. Extrathymic T cells express lower levels of TCR and have a natural memory phenotype characterized by expression of high levels of CD44 and CD122 (147, 148). These cells also undergo excessive homeostatic proliferation in response to lymphodepletion which results in the availability of excess cytokines such as IL-15. Another unusual characteristic of extrathymic T cells that may stem from their memory phenotype is their ability to respond rapidly to infection. In fact extrathymic T cell numbers peak at around day 4 post-

infection whereas conventional T cells peak at day 8 (149). Even though these cells proliferate rapidly they also undergo a greater degree of apoptosis when compared to thymus-derived T cells. Extrathymic T cells are able to rapidly produce cytokines such as IFNγ upon stimulation without the need for the cells to proliferate (149). In addition, extrathymic T cells are not as efficient as conventional T cells when it comes to eliminating pathogens or generating memory (149). Many, but not all, CD8 T cells restricted to the nonclassical MHC class Ib molecules can be selected by hematopoetic cells (150). Extrathymic T cells provide early protection against pathogens before classical CD8 T cells are activated to eliminate the infection. Consistent with this hypothesis is the fact that the MHC class Ib-restricted CD8 T cell response peaks 2-3 days before the classical MHC class Ia-restricted CD8 T cell response during infection with Listeria monocytogenes (151). At least some extrathymic T cells appear to be dependent on selection by self-antigens for their development (152).OM transgenic mice normally develop autoimmunity but fail to do so if the mice are either deficient for both CD4 and CD8 T cells or if the T cells express a transgenic TCR specific for a foreign antigen suggesting that TCR-specificity plays a role in disease (153). The observation that the in vivo delivery of IL-2 to athymic Balb/c *nu/nu* mice led to the development of an autoimmune disease suggests that these cells are self-reactive and can contribute to autoimmunity (154). It is unknown what proportion of memory-phenotype CD8 T cells in normal mice may actually develop extrathymically. This is an important question, however, since extrathymic CD8 T cells are clearly different from thymic CD8 T

cells in terms of their high affinity for self-antigens. Moreover it is important to determine if extrathymically derived T cells play a significant, non-redundant, role during immune responses.

1.4 Positive selection of unconventional T cells

As one can imagine there is a range of affinities that a particular TCR can display for self-peptide/MHC complexes that can lead to positive selection without causing negative selection and thus T cells inherently vary in the extent of their "self-reactivity". The majority of conventional naïve T cells have a low to intermediate affinity for self-peptide/MHC but several unconventional T cells, such as natural killer T (NKT) cells and CD8$\alpha\alpha$ IELs, display a high affinity for self-peptide/MHC making them self-reactive. Importantly, these agonist selected T cells behave differently than conventional T cells and usually perform regulatory functions. Figure 1.2 provides a schematic representation of TCR affinity and the selection of conventional as well as unconventional T cells.

1.4.1 NKT cell development and function

NKT cells in mice express an $\alpha\beta$TCR that recognizes lipid antigens in the context of the nonclassical MHC class Ib molecule CD1d. In mice these T cells have an invariant Vα14-Jα18 rearrangement which restricts them to CD1d (155). Due to

their CD1d restriction, NKT cells can be identified by staining with CD1d tetramers bound to the NKT cell ligand α-galactosyl ceramide (αGalCer) (156). These cells also express several NK receptors including NK1.1 (NKRP1) and are either $CD4^-CD8^-$ or $CD4^+$ (156). NKT cells are unlike conventional naïve T cells in that they exhibit an activated or memory phenotype even in germfree animals and respond very rapidly to infection without the need to divide and differentiate (157). αGalCer is a very potent agonist for NKT cells but it is not naturally found in any mouse or human pathogen. Under more physiological conditions, NKT cells can recognize other lipids derived from bacteria in the context of CD1d (158, 159). These cells can also be activated in response to self-lipids/CD1d in conjunction with inflammatory cytokines like IL-12 differentiating them from conventional T cells that solely recognize foreign antigens (160). Upon activation NKT cells are thought to play a major role in regulating the immune response through the early production of IFNγ or IL-4, which can promote either TH1 or TH2 responses, respectively (161). NKT cells are not only capable of cytokine production but they can also kill target cells that express the appropriate ligands (162). NKT cells can induce the maturation of DCs (163) and cause NK cells to proliferate and produce cytokines (164). These cells have been implicated in everything from protection against bacterial infections, to promoting anti-tumor immunity and preventing diabetes (reviewed in (165)). Thus NKT cells seem to have a special multifaceted role during immune responses.

The special role of NKT cells seems to stem from their unconventional selection in the thymus. Like conventional T cells, NKT cells are thought to be

derived from DP thymocytes that have undergone random gene-rearrangements (166, 167). They are also thought to undergo positive selection by self-lipids/CD1d. What makes the selection of NKT cells different from conventional T cells is the cell type that mediates their positive selection. Unlike conventional T cells that are selected by thymic cortical epithelial cells, NKT cells require DP thymocytes for their selection (156). In addition, the ligands that mediate the positive selection of NKT cells are actually agonist ligands and provide a positive selection signal that is at the threshold of negative selection (168). NKT cells also differ from conventional T cells in their requirements for specific genes that have relatively little effect on conventional T cells. For example, NKT cell development requires NF-κB1 expression (169) and also relies on the expression of the T-box family transcription factor T-bet (T-box expressed in T cells) (170). Thus it is clear that NKT cells are a distinct lineage from conventional T cells and that their functions are somehow tied to their agonist selection in the thymus.

1.4.2 TCRαβ$^+$ CD8αα Intestinal intra-epithelial lymphocytes (iIELs)

TCRαβ$^+$ CD8αα iIELs are another unconventional T cell type that plays a huge role in regulating the homeostasis of the immune system. These cells make up a very large part of both the human and mouse immune system. They reside in the lining of the gut lumen where they play a large role in maintaining gut integrity as they are the first line of defense against ingested particles (reviewed in (171)). Similar to NKT cells, TCRαβ$^+$ CD8αα iIELs have a natural memory phenotype

(172) and express functional NK receptors (173). TCR$\alpha\beta^+$ CD8$\alpha\alpha$ iIELs, however, are more heterogeneous in terms of their MHC reactivity when compared to NKT cells. These cells can be restricted to classical MHC class Ia, non-classical MHC class Ib, or to MHC class II molecules although it seems that the majority of the cells are in fact restricted to non-classical MHC class Ib (174). Even though these cells are selected by a wide range of MHC they are all thought to be selected by high affinity interactions between self-peptide/MHC. TCR$\alpha\beta^+$ CD8$\alpha\alpha$ iIELs often express overtly autoreactive TCRs that are deleted from the conventional T cell repertoire (175). In fact, using TCR transgenic models of TCR$\alpha\beta^+$ CD8$\alpha\alpha$ iIELs development, it is clear that these cells are in fact selected by high affinity agonist self-peptides (176, 177).

The function of TCR$\alpha\beta^+$ CD8$\alpha\alpha$ IELs is regulatory in nature as the cells have been shown to be crucial for preventing uncontrolled immune responses in the gut. In double-transgenic mice that express a MHC class Ia-restricted TCR specific for an LCMV peptide (GP33-41) as well as the cognate peptide under control of the MHC class I promoter there are large numbers of TCR$\alpha\beta^+$ CD8$\alpha\alpha$ IELs that can be activated in response to LCMV infection (177). Instead of these self-reactive IELs causing disease, the virus-activated cells produced TGFβ, an important immunoregulatory cytokine that negatively regulates immune responses. In another TCR transgenic system it was clearly demonstrated that TCR$\alpha\beta^+$ CD8$\alpha\alpha$ IELs expressing a self-reactive TCR could actively prevent the development of colitis by suppressing pathogenic CD4 T cells (178). This suppression was dependent on IL-10 production by the IELs and perhaps most

strikingly was completely dependent on the expression of cognate self-antigen in the protected mice. Thus it is clear that agonist selection of TCR$\alpha\beta^+$ CD8$\alpha\alpha$ IELs is crucial for their special regulatory function and that the presence of self-antigen plays a role in not only the selection of these cells but is also important for their function.

1.4.3 Agonist selection of unconventional CD8 T cells in male H-Y TCR transgenic mice

H-Y TCR transgenic mice have been widely used as an animal model system for the determination of mechanisms of positive and negative selection of T cells. In these mice, CD8 T cells that express the H-Y TCR are positively selected in the thymus of B6 (H-2^b) female mice (179). In B6 male mice, the H-Y TCR is negatively selected, leading to the massive deletion of double positive (DP) thymocytes (180). Interestingly, there is a population of H-Y TCR^+ $CD8^+$ T cells, which express a low level of CD8 (referred to as $CD8^{lo}$) (181), that are resistant to deletion in male mice (181). The self-specific $CD8^{lo}$ cells can develop via an extrathymic pathway (152) and express high levels of CD44 and CD122 that are characteristic of memory T cells. However, they differ from conventional memory T cells in that they are more refractory to activation by antigen as compared to naïve T cells (182, 183). Another unusual feature of these self-specific $CD8^{lo}$ T cells is that they can develop extrathymically and can proliferate in response to cytokines such as IL-2 and IL-15 in an antigen-independent manner (184).

However, the $CD8^{lo}$ cells are similar to conventional memory T cells with regard to the ability to rapidly produce IFNγ upon TCR stimulation (185) and with respect to the killing of susceptible target cells without the need for additional reactivation with antigen (186, 187).

The work done in the H-Y TCR transgenic mice is complemented by studies in non-TCR transgenic mice, which suggest that a similar population of CD8 T cells exists in normal mice (152, 183, 184). These unconventional CD8 T cells exhibit a memory-phenotype and appear to be selected by cognate self-antigen (152). Interestingly, they respond to cytokines produced during infection (184). In addition, these cells maintain the ability to rapidly produce cytokines and kill target cells without the need for prior stimulation (187). These characteristics are very similar to those of the other unconventional T cells described in the previous sections. These results suggest that perhaps the normal immune system contains a population of self-antigen specific CD8 T cells that display characteristics of both innate and adaptive cells.

1.5 Thesis objectives

The objectives of this thesis are:

1) Demonstrate self-reactivity in the memory-phenotype CD8 T cell population in normal mice.
2) Look for the expression of NK receptors on self-reactive CD8 T cells and determine if these NK receptors can alter TCR signaling.

3) Determine the relationship between self-specific CD8 T cells in H-Y male TCR transgenic mice and memory-phenotype CD8 T cells in normal mice.
4) Use the self-specific H-Y male CD8 T cells to learn more about the development, maintenance, and activation of self-specific CD8 T cells.
5) Assess the role of self-specific CD8 T cells in vivo during infection.

Chapter 2 starts by examining the characteristics of self-reactive memory-phenotype CD8 T cells from normal mice. Chapter 3 examines the development and function of self-specific CD8 T cells in male H-Y TCR transgenic mice and demonstrates that these cells are nearly identical to the self-reactive memory-phenotype CD8 T cells described in chapter 2. Chapter 4 describes the expression and function of an activating NK receptor, CD16, on self-specific CD8 T cells from non-transgenic B6 as well as from male H-Y TCR transgenic mice. This chapter also shows that NK receptors on self-specific CD8 T cells can work either independently or together with the TCR to induce killing and cytokine production. Chapter 5 is a series of in vivo experiments that demonstrates the ability of self-specific CD8 T cells to provide innate protection from infections. This chapter also demonstrates that the self-reactive TCR on self-specific CD8 T cells plays a role in the maintenance and function of this cell type in vivo.

1.6 Tables

Table 1 | **Inhibitory NK receptors for MHC**

Species	Receptor	Ligands
Mouse	Ly49	H-2K, H-2D
Mouse	CD94/NKG2A	Qa-1^b
Human	KIR2DL	HLA-C
Human	KIR3DL	HLA-Bw4, HLA-A
Human	CD94/NKG2A (CD159a)	HLA-E
Human	CD85j, CD85d	HLA class I

Inhibitory receptors contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic domains that recruit the intracellular phosphatases SHP-1 and/or SHP-2. The human KIR and mouse Ly49 receptors are encoded by several genes that demonstrate extensive allelic polymorphism. They recognize polymorphic determinants on their major histocompatibility complex (MHC) class I ligands. CD94/NKG2A (CD159a) receptors bind to ligands with limited polymorphism and CD85j reacts with a region in the α3-domain of human human leukocyte antigen (HLA) class I that is highly conserved in class I molecules. KIR, killer cell immunoglobulin-like receptors; NK, natural killer.

Table 1.1 Inhibitory NK receptors for MHC. Table from Cerwenka, A., and L.L. Lanier. 2001. Natural killer cells, viruses, and cancer. *Nat Rev Immunol* 1:41-49. (188).

Table 2 | **Activating NK cell receptor complexes and their ligands**

Receptor	Species	Signalling adaptor	Signalling Pathway	Ligand*
CD16	Mouse, human	FcεRIγ or CD3ζ	ZAP70/Syk	IgG
NKp30	Human	FcεRIγ or CD3ζ	ZAP70/Syk	?
NKp46	Mouse, human	FcεRIγ or CD3ζ	ZAP70/Syk	Influenza haemagglutinin, others?
NKR-P1C	Mouse	FcεRIγ or CD3ζ	ZAP70/Syk	?
KIR2DS	Human	DAP12	ZAP70/Syk	HLA-C, others?
CD94/NKG2C	Mouse, human	DAP12	ZAP70/Syk	HLA-E (Qa-1)
Ly49D	Mouse	DAP12	ZAP70/Syk	H-2D[d]
Ly49H	Mouse	DAP12	ZAP70/Syk	MCMV-induced?
NKp44	Human	DAP12	ZAP70/Syk	Influenza haemagglutinin, others?
NKG2D	Mouse, human	DAP10	PI3K	MIC, ULBP (RAE-1, H60)
CD244	Mouse, human	SAP	?	CD48

*Molecules in parentheses are the ligands for the mouse natural killer (NK) receptors. HLA, human leukocyte antigen; KIR, killer cell immunoglobulin-like receptors; MCMV, mouse cytomegalovirus; MIC, MHC-class-I-chain-related molecules; PI3K, phosphatidylinositol 3-kinase; RAE-1, retinoic acid early inducible 1; ULBP, UL16-binding protein.

Table 1.2 Activating NK cell receptor complexes and their ligands. Table from Cerwenka, A., and L.L. Lanier. 2001. Natural killer cells, viruses, and cancer. *Nat Rev Immunol* 1:41-49. (188).

1.7 Figures

Figure 1.1 Overall scheme of T-cell development in the thymus. Committed lymphoid progenitors arise in the bone marrow and migrate to the thymus. Early committed T cells lack expression of T-cell receptor (TCR), CD4 and CD8, and are termed double-negative (DN; no CD4 or CD8) thymocytes. DN thymocytes can be further subdivided into four stages of differentiation (DN1, CD44+CD25−; DN2,CD44+CD25+; DN3, CD44−CD25+; and DN4, CD44−CD25−) (189). As cells progress through the DN2 to DN4 stages, they express the pre-TCR, which is composed of the non-rearranging pre-Tα chain and a rearranged TCR β-chain (190). Successful pre-TCR expression leads to substantial cell proliferation during the DN4 to double positive (DP) transition and replacement of the pre-TCR α-chain with a newly rearranged TCR α-chain, which yields a complete αβ TCR. The αβ-TCR+CD4+CD8+ (DP) thymocytes then interact with cortical epithelial cells that express a high density of MHC class I and class II molecules associated with self-peptides. The fate of the DP thymocytes depends on signaling that is mediated by interaction of the TCR with these self-peptide–MHC ligands (191, 192). Too little signaling results in delayed apoptosis (death by neglect). Too much signaling can promote acute apoptosis (negative selection); this is most common in the medulla on encounter with strongly activating self-ligands on hematopoietic cells, particularly dendritic cells (193). The appropriate, intermediate level of TCR signaling initiates effective maturation (positive selection). Thymocytes that express TCRs that bind self peptide–MHC-class-I

complexes become CD8+ T cells, whereas those that express TCRs that bind self-peptide–MHC-class-II ligands become CD4+ T cells; these cells are then ready for export from the medulla to peripheral lymphoid sites. SP, single positive. Figure from Germain, R.N. 2002. T-cell development and the CD4-CD8 lineage decision. *Nat Rev Immunol* 2:309-322. (194).

Figure 1.2 Central-tolerance mechanisms. The affinity of the T-cell receptor (TCR) for self-peptide–MHC ligands is the crucial parameter that drives developmental outcome in the thymus. Progenitors that have no affinity or very low affinity die by neglect. This is thought to be the fate of most thymocytes. If the TCR has a low affinity for self-peptide–MHC, then the progenitor survives and differentiates, a process that is known as positive selection. If the progenitor has a high affinity for self-peptide–MHC, then several outcomes are possible. First, the progenitor can be selected against, a process that is known as negative selection. The main mechanism of negative selection is clonal deletion, but receptor editing and anergy have also been described. Second, there seem to be mechanisms that select for high-affinity self-reactive cells and result in differentiation into a 'regulatory'-cell phenotype. It is not known what determines whether a T cell is tolerized by negative selection or is selected to become a regulatory T cell (195). IEL, intestinal epithelial lymphocyte; NKT cell, natural killer T cell; T_{Reg} cell, $CD4^+CD25^+$ regulatory T cell. Figure from Hogquist, K.A., T.A. Baldwin, S.C. Jameson. 2005. Central tolerance: learning self-control in the thymus. *Nat Rev Immunol* 5:772-782. (196).

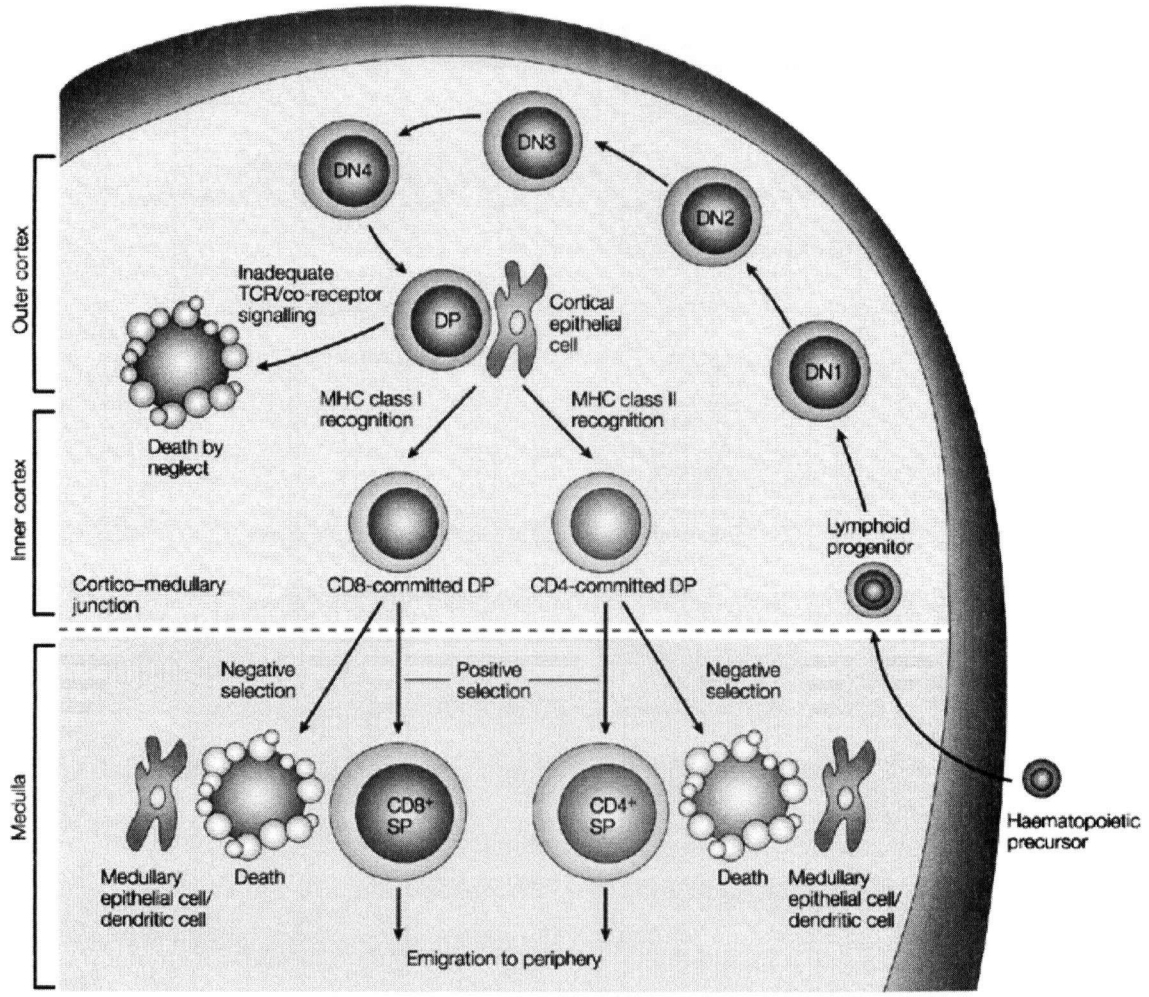

Figure 1.1 Overall scheme of T-cell development in the thymus. Figure from Germain, R.N. 2002. T-cell development and the CD4-CD8 lineage decision. *Nat Rev Immunol* 2:309-322. (194).

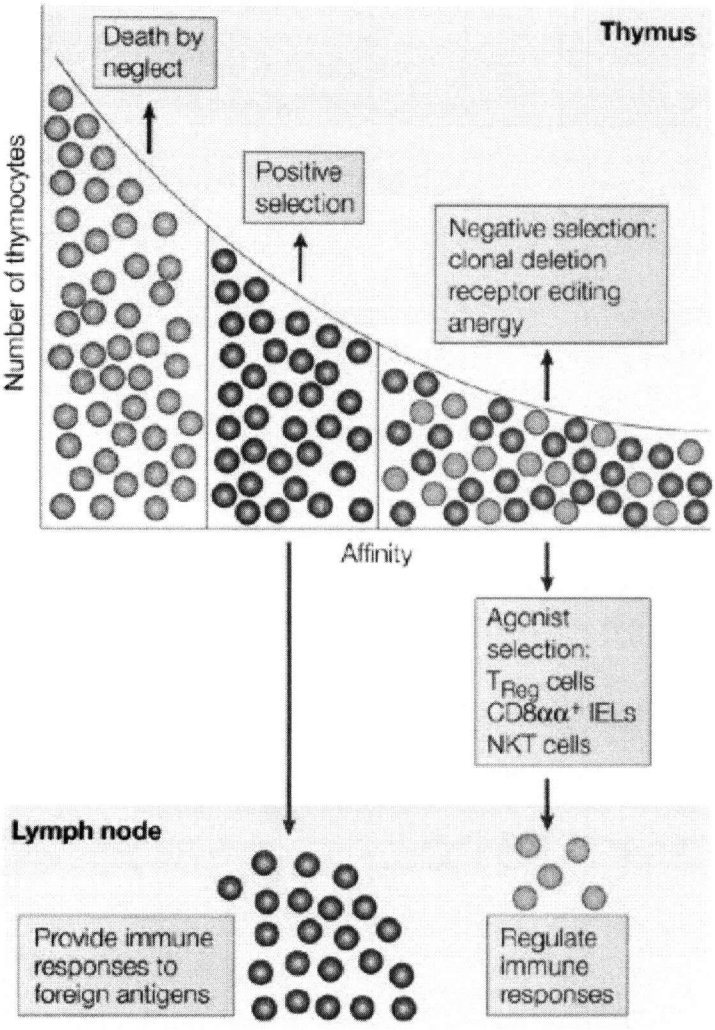

Figure 1.2 Central-tolerance mechanisms. Figure from Hogquist, K.A., T.A. Baldwin, S.C. Jameson. 2005. Central tolerance: learning self-control in the thymus. *Nat Rev Immunol* 5:772-782. (196).

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Chapter 2 IL-2 Activated $CD8^+CD44^{high}$ Cells Express Both Adaptive and Innate Immune System Receptors and Demonstrate Specificity for Syngeneic Tumor Cells[1]

2.1 Abstract

$CD8^+$ T cells depend on the $\alpha\beta$ TCR for antigen recognition and function. However, antigen-activated $CD8^+$ T cells can also express receptors of the innate immune system. In this study, we examined the expression of NK receptors on a population of $CD8^+$ T cells expressing high levels of CD44 ($CD8^+CD44^{hi}$ cells) from normal mice. These cells are distinct from conventional memory $CD8^+$ T cells and they proliferate and become activated in response to Interleukin 2 (IL-2) via a CD48/CD2-dependent mechanism. Prior to activation they express low or undetectable levels of NK receptors but upon activation with IL-2 they expressed significant levels of activating NK receptors including 2B4 and NKG2D. Interesting, the IL-2 activated cells demonstrate a preference in the killing of syngeneic tumor cells. This killing of syngeneic tumor cells was greatly enhanced by the expression of the NKG2D ligand, Rae-1, on the target cell. In contrast to conventional $CD8^+$ T cells, IL-2-activated $CD8^+CD44^{hi}$ cells express DAP12, an adaptor molecule that is normally expressed in activated NK cells. These observations indicate that activated $CD8^+CD44^{hi}$ cells express receptors of both the adaptive and innate immune system and may play a unique role in the surveillance of host cells, which have been altered by infection or transformation.

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[1] A version of this chapter has been published as:

Dhanji, S., and H.-S. Teh. 2003. IL-2-Activated CD8+CD44high Cells Express Both Adaptive and Innate Immune System Receptors and Demonstrate Specificity for Syngeneic Tumor Cells. *J Immunol 171:344*

2.2 Introduction

The cells of the adaptive immune system utilize functionally rearranged receptors to recognize foreign antigens. By contrast, cells of the innate immune system primarily use germ line encoded receptors to defend against infected or transformed cells. Interestingly, cells of the adaptive immune system can express some of these germ line encoded receptors (1). Furthermore, recent studies have indicated that in addition to providing immediate immune effector mechanisms to contain the spread of infections, the innate immune system also serves to prime the adaptive immune system to defend against infections that cannot be contained by the innate immune system (1).

Natural killer (NK) cells are an integral part of the innate immune system, producing cytokines to activate other cells of the immune system as well as directly recognizing and killing infected or transformed cells (2). NK cells use a combination of activating and inhibitory receptors in order to perform these functions (3-5). These activating and inhibitory receptors set a threshold for the activation of NK cells, with inhibitory signals predominating in the absence of infection (6). When a host cell is transformed or infected, the balance shifts towards activation, allowing NK cells to eliminate these hazardous host cells (6).

$\alpha\beta$ T cell receptor $(TCR)^+$ $CD8^+$ T cells play a crucial role in the adaptive immune system. The primary function of $CD8^+$ T cells is the lysis of virally infected target cells, via recognition of viral peptides that are presented by MHC class I molecules. Naive $CD8^+$ T cells require two distinct signals for activation:

signal one is provided by engagement of the TCR with its cognate ligand and signal two is provided by interaction of costimulatory receptors with their respective ligands on the antigen presenting cells (7, 8).

Recent studies showed that normal mice possess a subset of $CD8^+$ $\alpha\beta TCR^+$ T cells that express very high levels of CD44 (herein referred to as $CD8^+CD44^{hi}$ cells). These $CD8^+CD44^{hi}$ cells possess other markers of activated/memory cells such as the high expression of CD122 (IL-2 receptor β) and Ly6C and they can proliferate in response to IL-2 and IL-15 independently of TCR stimulation (9-11). The development of these $CD8^+$ T cells appears to be driven by the interaction of the $\alpha\beta$TCR with self-antigen and they can develop in the absence of a functional thymus (9).

$CD8^+CD44^{hi}$ cells have also been shown to express receptors characteristic of NK cells (12). Both activating and inhibitory NK receptors can be found on these cells. Receptors such as 2B4, which can be either activating or inhibitory (13), and whose expression on T cells can be induced by activation with various cytokines (14), have been found on $CD8^+CD44^{hi}$ cells (14). Inhibitory receptors such as the killer cell immunoglobulin-like receptor (KIR) in humans and the lectin-like Ly49 family in mice, have also been implicated in both the development and function of memory phenotype CD8 T cells (reviewed in (15)). The observation that NK receptor expression by CD8 T cells is restricted to cells with an activated/memory phenotype and the fact that T cells do not express NK receptors in the thymus (16), strongly suggests that only cells that have encountered cognate antigen are capable of their expression.

In this report, we characterized the phenotype and function of $CD8^+CD44^{hi}$ cells from normal mice. We showed that these cells only require IL-2 for proliferation and the acquisition of cytolytic activity. Interestingly, these IL-2-activated cells express both DAP10 and DAP12 adaptor molecules. DAP12 is normally expressed by activated NK, but not conventional $CD8^+$ T cells (17). The activated cells demonstrate preferential killing of syngeneic tumor cells. Expression of the NKG2D ligand, Rae-1δ, on the tumor cells led to greatly enhanced killing of the target cells.

2.3 Materials and Methods

Mice

Breeders for C57BL/6 (B6), Balb/c, and DBA/2 were obtained from the Jackson Laboratories (Bar Harbor, ME). These mice were bred at the Animal Unit in our department. Mice 8 to 12 weeks of age were used for the experiments described.

Abs and flow cytometry

The following mAbs were used: anti-CD4 (GK1.5), anti-CD8α (53-6.7), anti-CD8β (53.58), anti-CD3ε (2C11), anti-CD44 (PGP1), anti-TCRβ (H57.597), anti-γδ TCR, anti-CD43 (1B11), anti-CD94 (18D3), anti-NK-1.1 (PK136), anti-CD122 (TM-β1), anti-Ly6C (AL-21), anti-CD244.2 (2B4), and anti-NKG2D (18)]. Biotinylated mAbs were detected using streptavidin-PE. All Abs were obtained

from BD PharMingen (San Diego, California) except anti-NKG2D (18), which was a kind gift from Dr. Wayne M. Yokoyama (Howard Hughes Medical Institute, Washington University, St. Louis). Staining for NKG2D ligands was performed using the mNKG2D-Ig fusion protein (19), which was a kind gift from Dr. Lewis L. Lanier (University of California, San Francisco) followed by staining with a FITC-labeled anti-human Ig mAb. Cell staining and flow cytometry were performed according to standard procedures. The CellQuest software program (Becton Dickinson, Mountain View, CA) was used for data acquisition and analysis. For three-color analysis, a total of 20,000 live events were collected and analyzed.

Cell lines

Cell lines used were the RMA lymphoma (H-2^{b+}, Rae-1δ^{-}), RMA-Rae-1δ transfectant (H-2^{b+}, Rae-1δ^{+}), TAP-deficient RMAS (H-2^{b-}, Rae-1δ^{-}), RMAS-Rae-1δ transfectant (H-2^{b-}, Rae-1δ^{+}), A20 lymphoma (H-2^{d}) and P815 mastocytoma (H-2^{d}). The cell lines were cultured in IMEM (Life Technologies, Burlington, Canada), supplemented with 10% (v/v) FBS (Life Technologies), $5x10^{5}$ μM 2-ME and antibiotics (I-medium). The RMA-Rae1δ and RMAS-Rae1δ transfectants (20) were kind gifts from Dr. Lewis L. Lanier (University of California, San Francisco); these cell lines were passaged in I-medium and G418 (800μg/ml) to maintain high levels of Rae1δ expression.

Ex vivo staining

Single cell suspensions from the lymph nodes (LN) of mice were treated with anti-CD4 mAb and then depleted of $CD4^+Ig^+$ cells using Dynabeads M-450 Sheep anti-mouse IgG (Dynal Biotech, Lake Success, New York), according to manufacturers instructions. The cells were >95% $CD8^+$ and were then stained with the appropriate mAbs and analyzed by FACS.

$CD8^+$ T Cell purification and sorting

Single cell suspensions from the lymph nodes (LN) of mice were treated with biotinylated anti-CD8β mAb followed by positive selection using the MiniMACS system (Miltenyi Biotech, Auburn, California), according to the manufacturers specifications. The resulting cells were >95% pure $CD8\alpha\beta^+TCR\beta^+$ T cells. $CD8^+$ T cells purified by this method contained ~10% $CD44^{hi}$ cells. For purification of $CD8^+CD44^{hi}$ and $CD8^+CD44^{lo}$ T cells, MiniMACS purified $CD8^+$ T cells were stained with anti-CD8α-FITC and anti-CD44-PE and sorted on a Becton Dickinson FACS Vantage SE Turbo sort cell sorter. Cell sorting was performed by Andrew Johnson (University of British Columbia) and the sorted $CD8^+CD44^{hi}$ or $CD8^+CD44^{lo}$ cells were >98% pure. For some assays the following purification method was used to provide a source of $CD8^+CD44^{lo}$ cells: lymph node cells were incubated with mAbs specific for CD4, mouse Ig and CD44 followed by depletion with anti-mouse Ig-coated DYNA beads (Dynal). This method yielded a population of >98% $CD8^+CD44^-$ cells.

Natural Killer (NK) cell purification and activation

Natural killer cells were isolated by treating spleen cells with anti-CD4, anti-CD8, and anti-CD3 mAbs followed by treatment with sheep anti-mouse Ig in order to deplete $CD3^+CD4^+CD8^+Ig^+$ cells. The cells were about 60% $CD3^-$ $DX5^+NK1.1^+CD122^+$ and were then cultured in I-medium supplemented with IL-2 (200U/ml) for 5 days. On day 5 the cells were >99% $CD3^-DX5^+NK1.1^+2B4^+$, $CD122^+$.

CFSE labeling

Purified $CD8^+$ T cells ($1x10^7$/ml) were labeled with 1 μM CFSE (Molecular Probes, Eugene, OR) in PBS for 8 min at room temperature. After stopping the reaction with the addition of an equal volume of FCS, cells were washed four times with complete media prior to use.

Proliferation assays

For IL-2 proliferation, $1x10^5$ purified $CD8^+$ T cells of the indicated CD44 phenotype were cultured in I-medium + IL-2 (200U/ml) in 96-well U-bottom plates for 5 days. Blocking mAbs were added to the indicated cultures at 10 μg/ml. The cells were pulsed with 1μci of [^{3}H]thymidine for the final 6 hours to assess proliferation. For anti-CD3ε (2C11)-induced proliferation, $1x10^4$ purified $CD8^+$ T cells of the indicated CD44 phenotype were cultured in 96-well flat bottom plates coated with 2C11 (10μg/ml) and IL-2 (20U/ml) with or without the indicated

blocking mAbs (10μg/ml) for 4 days and pulsed with 1μCi of [^{3}H]thymidine for the final 6 hours to assess proliferation. For CFSE proliferation assays, the same conditions as above were used except the cells were CFSE-labeled and analyzed on day 4 by FACS.

CTL Assays

Target cells (RMA, RMAS, RMA-Rae1δ, RMAS-Rae1δ, A20, or P815) were labeled with ^{51}Cr (100μCi) for 1 hour at 37°C and then washed. 1x10^4 labeled targets were added to 96-well U-bottom plates containing activated CD8 or NK cells at the indicated ratios in a final volume of 200μl. After a 5-hour incubation, the supernatants were collected and counted. Spontaneous release varied from 8-15% of the maximum. All assays were performed in triplicate. Percent specific lysis was calculated as 100% x [cpm (experimental well) – cpm (spontaneous release)]/[(cpm (maximum release) – cpm (spontaneous release)].

RT-PCR

NK cells and CD8$^+$CD44^{hi} T cells were activated with IL-2 (200U/ml) and CD8$^+$CD44^{lo} cells were activated with anti-CD3 and IL-2 (20U/ml) for 5 days. Cells were then harvested, and total RNA was prepared according to the manufacturer's recommendations using the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNAs were generated from total RNA using the Protoscript cDNA synthesis kit according to manufacturers recommendations (NEB, Beverly, MA).

Left and right primer sequences, respectively, were as follows: DAP10: 5'-CAGGCTACCTCCTGTTCCTG-3' and 5'-GCCAGGCATGTTGATGTAGA-3'; DAP12: 5'-CTGGTGTACTGGCTGGGATT-3' and 5'-CTGGTCTCTGACCCTGAAGC-3'; Glyceraldehyde-3-phosphate dehydrogenase (GPADH): 5'-TGC(A/C)TCCTGCACCACCAACT-3' and 5'-(C/T)GCCTGCTTCACCACCTT-3'. The PCR products were subjected to electrophoresis on a 2% Agarose gel and visualized by ethidium bromide.

2.4 Results

Phenotypic characterization of $CD8^+CD44^{hi}$ cells from normal mice.

$CD8^+$ T cells from normal mice express varying levels of CD44. We first determined the cell surface phenotype of $CD8^+$ T cells from B6 mice, which expressed either high ($CD44^{hi}$) or low ($CD44^{lo}$) levels of CD44. The $CD8^+CD44^{hi}$ T cells comprised about 10 to 20% of total $CD8^+$ T cells in B6 mice with older mice possessing an increased proportion of these cells (n=40; the mice vary between 6 and 12 weeks of age). Figure 1 shows the relative expression of various cell surface markers by the $CD8^+CD44^{hi}$ and $CD8^+CD44^{lo}$ cells from the same B6 mouse. These data indicate that the $CD44^{hi}$ cells expressed elevated levels of CD44, Ly6C, CD122 and 1B11, a CD43 isoform characteristic of activated $CD8^+$ T cells (21). This activated/memory cell surface phenotype suggests that there is prior recognition of cognate antigen by the $CD44^{hi}$ cells. Interestingly, $CD44^{hi}$ cells expressed lower levels of the $\alpha\beta$TCR than the $CD44^{lo}$

cells. The lower expression of the αβ TCR is characteristic of NKT cells (22) as well as the self-specific $CD8^+$ T cells that can develop via an extrathymic pathway (11). However, unlike NKT cells, immediately ex vivo $CD8^+CD44^{hi}$ cells do not express NK1.1 or significant levels of other NK receptors such as CD94, 2B4 or NKG2D (Fig. 2.1).

$CD8^+CD44^{hi}$ T cells proliferate in response to IL-2 stimulation.

The expression of CD122 (IL-2Rβ) on $CD8^+CD44^{hi}$ T cells suggests that they might be capable of proliferating in response to cytokines such as IL-2 or IL-15. To test for this possibility we purified $CD8^+CD44^{hi}$ and $CD8^+CD44^{lo}$ T cells from B6 mice by cell sorting and labeled these cells with the fluorescent dye, CFSE and then cultured them with IL-2. The CFSE data in Fig. 2.2A indicate that only the $CD8^+CD44^{hi}$ proliferated in response to IL-2. Proliferation is relatively rapid, as some of the cells have undergone more than 6 rounds of cell division by 72 hr. By contrast, the vast majority of IL-2-activated $CD8^+CD44^{lo}$ cells did not divide during the 48 to 96 hr observation period. Measurement of cell proliferation by following the incorporation of ^{3}H-thymidine during the last 6hr of a 96 hr culture period also indicate that only the $CD8^+CD44^{hi}$ cells are capable of IL-2-induced proliferation (Fig. 2.2B). These results are consistent with a previous report showing that only $CD8^+$ T cells expressing high levels of CD44, but not antigen-specific memory $CD8^+$ T cells, can proliferate in response to IL-2 or IL-15 (23).

IL-2 activated $CD8^+CD44^h$ cells expressed NK receptors

Several studies have shown that activated $CD8^+$ T cells can express various types of NK receptors (24). However, these studies do not distinguish between the expression of NK receptors by either activated $CD8^+CD44^{hi}$ cells or $CD8^+CD44^{lo}$ cells. To determine whether $CD8^+CD44^{hi}$ cells express NK receptors after IL-2 activation, we examined the expression of NK receptors on IL-2-activated $CD8^+$ cells. Since only $CD8^+CD44^{hi}$ but not $CD8^+CD44^{lo}$ cells can be activated by IL-2 (Fig. 2.2) these results were equated with IL-2-activated $CD8^+CD44^{hi}$ T cells. Fig. 2.3 shows that IL-2 activated $CD8^+CD44^{hi}$ cells expressed high levels of 2B4 and CD94 but relatively low levels of DX5 and NK1.1. The activated cells also uniformly expressed a low level of NKG2D. This pattern of NK receptor expression is distinct from IL-2-activated NK cells, which express high levels of these NK receptors (data not shown). This pattern is also distinct from antigen-activated conventional $CD8^+CD44^{lo}$ T cells, which with the exception of NKG2D, do not express the other NK receptors (25). Previous studies have shown that both the S and L isoforms of 2B4 are induced upon IL-2 activation of $CD8^+$ T cells (14). Here we showed that IL-2-activated $CD8^+CD44^{hi}$ cells expressed high levels of 2B4. By contrast, anti-TCR activated $CD8^+CD44^{lo}$ cells did not express 2B4 (data not shown).

CD48 and CD2 are crucial for IL-2-induced proliferation of $CD8^+CD44^{hi}$ cells

2B4 is a member of the CD2 subset of Ig superfamily molecules and is the high affinity ligand for CD48 (26, 27). CD48 is also a member of the CD2 subset and is expressed by lymphocytes, monocytes and endothelial cells (28). Members of

the CD2 subset have been observed to interact with themselves or with other family members (28). CD48 has also been shown to function as a costimulatory molecule for T cells (27, 29). Engagement of 2B4 on NK cell surfaces with CD48 can trigger cell-mediated cytotoxicity, interferon-γ secretion, phosphoinositol turnover and NK-cell invasiveness (28). During the course of our experiments we noticed that IL-2-induced proliferation of $CD8^+CD44^{hi}$ cells was dependent on cell-cell contact (data not shown). However, since highly purified $CD8^+CD44^{hi}$ cells proliferated in response to IL-2 it is unlikely that contact with other lymph node cell types is required for IL-2-induced proliferation. As $CD8^+CD44^{hi}$ cells express high levels of CD48 and CD2 (Fig. 2.4A) and these cells expressed high levels of 2B4 upon activation (Fig. 2.3), we determined whether CD48, 2B4 or CD2 are important in mediating IL-2-induced proliferation. To test the role of these molecules in IL-2-induced proliferation we cultured purified $CD8^+$ T cells in IL-2 in the presence or absence of anti-CD48, anti-CD2, or anti-2B4 mAbs. Proliferation was determined either by measuring CFSE fluorescence (Fig. 2.4B) or by the incorporation of radiolabeled thymidine (Fig. 2.4C). The data indicate that anti-CD48 and anti-CD2 inhibited IL-2-induced proliferation to the same extent whereas the anti-2B4 mAb did not have any effect on the proliferative response. This observation indicates that CD48 and CD2, but not 2B4, are important in mediating IL-2-induced proliferation. We also determined the anti-proliferative effect of the anti-CD48 mAb on anti-CD3 + IL-2-induced proliferation, as assessed by CFSE dilution (Fig. 2.4B) or the incorporation of radiolabeled thymidine (Fig. 2.4C). Under these conditions, both the $CD8^+CD44^{hi}$ as well as

the $CD8^+CD44^{lo}$ populations will be activated. However, since the $CD8^+CD44^{lo}$ cells comprise ~90% of the starting population these cells constitute the vast majority of the responding cells. By contrast to IL-2-induced proliferation, the anti-CD3 induced proliferation was not inhibited by the anti-CD48 mAb demonstrating that anti-CD3-induced activation of $CD8^+CD44^{lo}$ cells is resistant to inhibition by anti-CD48 mAb. These data support the hypothesis that IL-2-induced activation of $CD8^+CD44^{hi}$ cells and anti-CD3-induced activation of $CD8^+CD44^{lo}$ cells occur via CD48-dependent and CD48-independent mechanisms, respectively.

Activated $CD8^+CD44^{hi}$ cells preferentially kill syngeneic tumors.

Previous studies suggested that the development of $CD8^+CD44^{hi}$ cells is dependent on interaction with self-antigens in peripheral lymphoid organs (9, 30, 31). Therefore, it was of interest to determine whether IL-2-activated $CD8^+CD44^{hi}$ cells demonstrate self-specificity as would be suggested by the killing of syngeneic tumor cells. RMA tumor cells are syngeneic to B6 mice and are lethal when injected into these mice (32). We first compared the ability of activated $CD8^+CD44^{hi}$ and $CD8^+CD44^{lo}$ cells from a B6 mouse to kill RMA target cells. Since only $CD8^+CD44^{hi}$ cells can be activated by IL-2 whereas both the $CD8^+CD44^{hi}$ as well as $CD8^+CD44^{lo}$ cells can be activated by anti-CD3 + IL-2, we used anti-CD3 + IL-2 as a common means of activating FACS purified $CD8^+CD44^{hi}$ and $CD8^+CD44^{lo}$ cells. The purpose of this experiment was to determine whether activated $CD8^+CD44^{hi}$ and $CD8^+CD44^{lo}$ cells differ in their ability to kill syngeneic tumor targets. We found that only anti-CD3-activated

$CD8^+CD44^{hi}$ cells demonstrate significant killing of RMA target cells (Fig. 2.5A) even though the $CD8^+CD44^{lo}$ cells were activated to the same extent as assessed by the expression of CD25 and CD69 (data not shown). These results indicate that these two activated $CD8^+$ subsets do indeed differ in their ability to kill syngeneic tumor target cells.

We then determined whether this preferential killing of syngeneic tumors applied to IL-2-activated $CD8^+CD44^{hi}$ cells from other mouse strains. Balb/c and DBA/2 mice are of the $H\text{-}2^d$ haplotype but differ in many non-MHC class I genetic loci. We determined the ability of IL-2-activated $CD8^+$ cells from Balb/c and DBA/2 mice to kill A20 and P815 tumor targets, which are derived from Balb/c and DBA/2 mice, respectively. Since IL-2 only activates $CD8^+CD44^{hi}$ cells we concluded that results derived from activation of purified $CD8^+$ T cells to be indicative of IL-2-activated $CD8^+CD44^{hi}$ cells. We found that IL-2-activated $CD8^+CD44^{hi}$ cells from Balb/c mice preferentially kill A20 targets (Fig. 2.5B, left panel) whereas P815 target cells were killed to a greater extent by IL-2-activated $CD8^+CD44^{hi}$ cells from DBA/2 mice (Fig. 2.5C, left panel). These data demonstrate that there is a preference for the killing of syngeneic tumors by IL-2-activated $CD8^+CD44^{hi}$ cells from Balb/c and DBA/2 mice.

We also examined the sensitivity of these tumor targets to killing by anti-CD3 + IL-2-activated $CD8^+CD44^{lo}$ T cells. To minimize the contribution of $CD8^+CD44^{hi}$ cells in these studies we depleted the $CD8^+$ cells of $CD44^+$ cells prior to culture (see Materials and Methods). We found that anti-CD3-activated $CD8^+CD44^{lo}$ T cells from either Balb/c (Fig. 2.5B, right panel) or DBA/2 (Fig.

2.5C, right panel) did not kill either target, even though these cells were highly activated. This observation likely reflects differences in the TCR repertoire of the $CD8^+CD44^{hi}$ and $CD8^+CD44^{lo}$ cells. Our data also supports the hypothesis that the $CD8^+CD44^{hi}$ population expresses TCRs with a strong bias towards self-antigens. By contrast, the TCRs of the conventional $CD8^+CD44^{lo}$ population are expected to be purged of anti-self ($H\text{-}2^d$) reactivity and therefore are unable to kill syngeneic ($H\text{-}2^d$) tumor targets. These data also implied that the $CD8^+CD44^{hi}$ and $CD8^+CD44^{lo}$ cells have differential requirements for self-antigens for their selection and development.

IL-2-activated $CD8^+CD44^{hi}$ cells express DAP12

The expression of NKG2D on IL-2 activated NK and $CD8^+CD44^{hi}$ cells as well as anti-CD3 + IL-2-activated $CD8^+CD44^{lo}$ cells were determined by staining with an anti-NKG2D mAb (18). Consistent with previous reports, we found that IL-2-activated NK cells expressed high levels of NKG2D whereas anti-CD3 + IL-2-activated $CD44^{lo}$ cells expressed lower levels (Fig. 2.6). IL-2-activated $CD44^{hi}$ cells also expressed lower levels of NKG2D (Fig. 2.6). Recent studies showed that activated NK cells express two alternative splice variants of NKG2D that associate differentially with DAP10 and DAP12 (17, 33). In NK cells, association of NKG2D with DAP10 provides a costimulatory signal whereas association of NKG2D with DAP12 confers a direct stimulatory signal (17, 33). Furthermore, activated $CD8^+$ cells from DAP10-deficient mice lack NKG2D expression suggesting that only DAP10 but not DAP12 is expressed by conventional $CD8^+$ T

cells (33). We determined the expression of DAP10 and DAP12 in activated NK cells, IL-2-activated $CD8^+CD44^{hi}$, and anti-CD3 + IL-2-activated $CD8^+CD44^{lo}$ cells from B6 mice. Consistent with previous reports, activated NK cells expressed both DAP10 and DAP12 whereas anti-CD3-activated $CD8^+CD44^{lo}$ cells expressed only DAP10. Interestingly, IL-2-activated $CD8^+CD44^{hi}$ cells expressed both DAP10 and DAP12. Thus, $CD8^+CD44^{hi}$ cells are more like NK cells with regard to DAP12 expression.

Expression of Rae-1δ enhances the lysis of syngeneic tumors by IL-2-activated $CD8^+CD44^{hi}$ cells.

We next determined whether NK receptors participate in this propensity to kill syngeneic tumor cells by IL-2-activated $CD8^+CD44^{hi}$ cells. Recent studies have shown that the activating NKG2D receptor plays a crucial role in the killing of syngeneic tumor cells (20, 32). The ligands for NKG2D, Rae-1 and H60, are expressed on infected or transformed cells (34). However, RMA tumor cells from B6 mice do not express Rae-1 (34). Rae-1δ is normally expressed in B6 mice (20) and we used RMA-Rae1δ transfectants (20) to determine whether NKG2D participates in the killing of syngeneic tumor cells. We tested the ability of IL-2-activated $CD8^+CD44^{hi}$ and anti-CD3 + IL-2-activated $CD8^+CD44^{lo}$ cells from B6 mice to kill RMA and RMA-Rae1δ target cells. We also used the peptide-transporter (TAP)-deficient cell lines, RMAS and RMAS-Rae-1δ, as sources of MHC class I-deficient target cells that do or do not express Rae-1δ. The expression of Rae-1δ on transfectant cell lines was confirmed by staining with a

murine NKG2D-Ig fusion protein (19)(data not shown). As an additional control for the specificity of killing we determined the cytolytic activity of IL-2-activated NK cells against these same target cells. NK cells were enriched by depleting B6 spleen cells of $CD4^+$, $CD8^+$, Ig^+ and $CD3^+$ cells by negative selection and the negatively selected cells were activated with IL-2. These activated cells were of the $CD4^-CD8^-CD3^-NKG2D^+DX5^+NK1.1^+2B4^+CD94^+$ cell surface phenotype (data not shown), consistent with the conclusion that this purification and activation scheme led to a pure population of effector NK cells.

The data in Fig. 2.7 indicate that the activated NK cells killed RMAS target to a greater extent than RMA cells. This is consistent with the conclusion that engagement of MHC class I molecules by inhibitory NK receptors likely contribute to the poorer killing of RMA target cells. More interestingly, and consistent with the observations of others (20, 25, 32), expression of Rae-1δ on either RMA or RMAS cells greatly increased their susceptibility to NK killing (Fig. 2.7). By contrast, IL-2-activated $CD8^+CD44^{hi}$ cells were relatively inefficient in killing either RMA or RMAS target cells (Fig. 2.7). This observation indicates that the TAP mutation has differential effects on target cell susceptibility to killing by NK or IL-2-activated $CD8^+CD44^{hi}$ cells. Interestingly, the presence of Rae1δ on either RMA or RMAS target cells also led to greatly enhanced killing by IL-2-activated $CD8^+CD44^{hi}$ cells. This observation suggests that the interaction of NKG2D with its ligand, Rae-1δ, greatly enhanced killing of syngeneic tumor cells by IL-2-acitvated $CD8^+CD44^{hi}$ cells.

By contrast, anti-CD3 + IL-2-activated $CD8^+CD44^{lo}$ cells are very inefficient killers of RMA and RMAS targets and Rae-1δ transfectants of these cell lines (Fig. 2.7). These data indicate that IL-2-activated $CD8^+CD44^{hi}$ cells possess the ability to kill syngeneic tumor targets and this killing is greatly enhanced by the expression of Rae-1δ.

2.5 Discussion

In this report we described a population of $CD8^+CD44^{hi}$ cells in normal mice that possess properties distinct from conventional memory T cells. These cells comprise between 10 and 20% of total CD8 T cells of normal mice. They proliferated in response to stimulation by exogenous IL-2 and differentiated into potent killer cells that demonstrate specificity for syngeneic tumors. These cells also upregulate several NK receptors including NKG2D upon activation with IL-2. Interestingly, IL-2-activated $CD8^+CD44^{hi}$ cells express DAP12, an adaptor molecule that is normally found in activated NK cells (35). Furthermore, tumor targets expressing a NKG2D ligand are exquisitely sensitive to killing by IL-2-activated $CD8^+CD44^{hi}$ cells.

Other studies also demonstrate that the $CD8^+CD44^{hi}$ cells that are present in normal mice are distinct from conventional memory T cells in many aspects. In contrast to MHC class I-restricted $CD8^+$ T cells, the development of $CD8^+CD44^{hi}$ cells can be thymus-independent (9, 11). The frequency of $CD8^+CD44^{hi}$ cells increases with age even when the mice are maintained under germ-free

conditions suggesting that the development of these cells is not foreign antigen-driven (36, 37). Memory $CD8^+$ T cells and $CD8^+CD44^{hi}$ cells also differ in their activation threshold. Whereas memory $CD8^+$ cells possess a lower activation threshold compared to naïve T cells, $CD8^+CD44^{hi}$ cells were shown to require a higher activation threshold relative to naïve $CD8^+$ T cells (11). Memory $CD8^+$ T cell also expressed lower levels of CD44 and CD122 compared to the $CD8^+CD44^{hi}$ (38) cells and the growth of $CD8^+CD44^{hi}$ cells, but not memory CD8 T cells, can be supported by IL-2 or IL-15 (11). These differences between $CD8^+CD44^{hi}$ cells and memory $CD8^+$ T cells strongly suggest that the $CD8^+CD44^{hi}$ cells are of a lineage that is distinct from conventional $CD8^+$ T cells. TCR transgenic mice provide a defined system for determining the developmental requirement of $CD8^+CD44^{hi}$ cells. In TCR transgenic mice these cells express only the transgenic TCR and can be easily tracked. It was found that the development of $CD8^+CD44^{hi}$ cells in TCR transgenic mice is independent of a thymus but is dependent on interaction with self-antigen in extrathymic tissues (9). Our observation that IL-2-activated $CD8^+CD44^{hi}$ cells demonstrate a preference in the killing of syngeneic tumor cells is also consistent with the notion that the development of these cells is dependent on selection by self-antigens. It was shown that in vivo delivery of large amounts of IL-2 to athymic nude mice results in an autoimmune disease (39). This observation is consistent with the hypothesis that the autoimmune disease is mediated by IL-2 activation of self-specific extrathymic $CD8^+CD44^{hi}$ cells. Large numbers of extrathymic T cells developed in oncostatin M transgenic mice (31). Interestingly, the presence of

cognate antigen is required for these $CD8^+$ T cells to acquire a memory phenotype (31). In normal mice, the self-ligands that are required for the development of $CD8^+CD44^{hi}$ cells remain to be determined. We found that small numbers of $CD8^+CD44^{hi}$ cells are also present in β2-microglobulin and TAP-deficient mice (our unpublished observations) suggesting that these cells may be restricted to non-classical MHC molecules. The report by Urdahl, et al. demonstrating that $CD8^+$ T cells with a memory phenotype can be positively selected on MHC class I^b molecules by hematopoietic cells is consistent with this notion (40). Several reports have also suggested that $CD8^+$ T cells that are specific for self-antigens, such as melanocyte differentiation antigens, can be isolated from healthy donors (41, 42). Interestingly, it was found that $CD8^+$ T cells that are capable of initiating tumor regression could also induce autoimmune reactions. This finding supports the hypothesis that self-specific $CD8^+CD44^{hi}$ cells may also contribute to autoimmune diseases (43).

The preferential killing of syngeneic tumor target cells by IL-2-activated $CD8^+CD44^{hi}$ but not anti-CD3-activated $CD8^+CD44^{lo}$ cells may reflect differences in the TCR repertoire of these two cell populations. However, analysis of TCR Vβ usage using the BD Pharmingen mouse Vβ TCR screening panel (a collection of mAbs to 17 Vβ's) by these two populations before and after activation reveals no significant differences in TCR Vβ usage by these cells (data not shown). This result indicates that there is no preferential usage of Vβ gene segments by these two populations. More importantly, they suggest that the TCR repertoire of $CD8^+CD44^{hi}$ cells is likely to be very heterogeneous and more sophisticated

analysis are required to determine whether there is a bias towards self-antigens in this heterogeneous TCR repertoire.

Immediately ex vivo $CD8^+CD44^{hi}$ cells do not express 2B4 (Fig. 2.1), but express high levels of 2B4 upon activation with IL-2 (Fig. 2.3). 2B4 has been shown to be expressed primarily on NK cells and a small subset of memory phenotype $CD8^+$ T cells (44-46), some of which can mediate non-MHC-restricted cytotoxicity (44, 45). The expression of 2B4 by $CD8^+$ T cells has been shown to correlate with the acquisition of effector functions (46) and was involved in proliferation (14). Since 2B4 is a high affinity receptor for CD48 it may serve as a receptor for CD48 and participate in IL-2-induced proliferation. However, we found that IL-2-induced proliferation was only inhibited by the anti-CD48 but not the anti-2B4 mAb (Fig. 2.4). A trivial explanation for the lack of inhibition by the anti-2B4 mAb is that this mAb does not block CD48/2B4 interaction. Arguing against this explanation is the observation that either the anti-CD48 or the same anti-2B4 mAb suppressed antigen-induced proliferation of $CD8^+$ T cells to the same extent; anti-CD2 mAb has no inhibitory effect in this system (14). Furthermore, there was no additive effect of the anti-CD48 and anti-2B4 mAbs in suppressing antigen-induced proliferation of $CD8^+$ T cells in this system (14). These observations suggest that the anti-2B4 mAb acts by inhibiting CD48/2B4 interaction. Therefore, a more likely explanation of our data for the lack of inhibitory effect of the anti-2B4 mAb is the late induction of 2B4 in IL-2-activated cells. By contrast, CD2 is expressed at a high level in ex vivo $CD8^+CD44^{hi}$ cells and anti-CD2 mAb inhibited IL-2-induced proliferation to the same extent as the

anti-CD48 mAb. This observation is consistent with the hypothesis that CD48 and/or CD2 are important in mediating IL-2-induced proliferation. CD48 is a glycosylphosphatidylinositol (GPI)-anchored molecule and it can aggregate lipid rafts when it is engaged (29). Thus, the anti-CD48 mAb could potentially exert its inhibitory effects by preventing the aggregation of lipid rafts. Mouse CD2 has also been shown to constitutively associate with lipid rafts (47) and so the anti-CD2 mAb may also exert its effect by preventing the aggregation of lipid rafts. Alternatively, the anti-CD48 mAb could serve as a ligand for the CD2 receptor. CD2 is implicated as an important co-stimulatory molecule in lymphocyte activation and proliferation (48). Furthermore, proline residues in the CD2 cytoplasmic domain have been shown to activate kinase activity such as PI3-kinase and the Tec-family tyrosine kinase, ITK (49, 50). In this alternative model, the anti-CD48 or the anti-CD2 mAb inhibits IL-2-induced proliferation by interfering with the CD2 signaling pathway. It is noted that the anti-CD48 mAb has no effect on anti-CD3-driven proliferation of $CD8^+$ T cells (Fig. 2.4). This observation provides independent support that $CD8^+CD44^{hi}$ cells and conventional $CD8^+$ T cells are dependent on distinct signaling pathways for growth.

Our data clearly shows the activating NK receptor, NKG2D, plays an important role in the lysis of syngeneic tumor targets by IL-2 activated $CD8^+CD44^{hi}$ cells (Fig. 2.7). The expression of the ligands for NKG2D, Rae-1 and H60 on tumor cells, leads to their rejection in vivo by both NK cells and $CD8^+$ T cells (20, 32). Interestingly, the expression of the ligands for NKG2D on tumor

cells in vivo, results in protection from subsequent challenge from parental (ligand-negative) tumors, suggesting a role for NKG2D in the activation of tumor-specific $CD8^+$ T cells (32). NKG2D has been shown to be exclusively costimulatory in $CD8^+$ T cells and directly stimulating in NK cells (17, 33). These differences have been attributed to the differential recruitment of adaptor molecules in NK cells versus $CD8^+$ T cells. Stimulation of NKG2D on NK cells primarily results in recruitment of DAP12 while stimulation in CD8 T cells, leads to recruitment of DAP10 (17, 33). Interestingly, we found that in contrast to anti-CD3-activated $CD8^+CD44^{lo}$ cells, which express only DAP10, IL-2-activated $CD8^+CD44^{hi}$ cells express both DAP10 and DAP12. Thus, the IL-2-activated $CD8^+CD44^{hi}$ cells are more NK-like in this regard. In this study we have shown that IL-2-activated $CD8^+CD44^{hi}$ cells from B6 mice can kill Rae-1^- RMA target cells, albeit with low efficiency. This observation suggests that the killing of syngeneic tumor target cells may be mediated in part by the $\alpha\beta$ TCR. The expression of Rae-1δ on RMA cells resulted in greatly enhanced lysis by IL-2-activated $CD8^+CD44^{hi}$ cells suggesting that the NKG2D receptor plays an important role in the killing of syngeneic tumor cells by these cells. Interestingly, deficiency in the TAP peptide transporter did not enhance killing of syngeneic tumor target cells by IL-2-activated $CD8^+CD44^{hi}$ cells. This observation is consistent with the notion that these activated cells either lack the inhibitory receptors that are expressed by NK cells and/or have a distinct combination of activating/inhibitory receptors from the activated NK cells. Alternatively, the ligands that are recognized by the $\alpha\beta$ TCR on IL-2-activated $CD8^+CD44^{hi}$ cells

may be independent of the TAP peptide transporter. The existence of $CD8^+CD44^{hi}$ cells in TAP-deficient mice (our unpublished observations) is consistent with this notion. Collectively, our data support the hypothesis that IL-2-activated $CD8^+CD44^{hi}$ cells express $\alpha\beta$ TCRs that are specific for syngeneic tumor target cells. In the absence of Rae-1 expression, the tumor targets are killed at relatively low efficiency. When the tumor targets express Rae-1, then there is synergy between the $\alpha\beta$ TCR and the NKG2D in the killing of syngeneic tumor target cells. Such a synergy between the $\alpha\beta$ TCR and activating NK receptors would render these cells particularly adept in the surveillance of host cells, which have been altered through infection or transformation.

2.6 Acknowledgments

We thank Soo-Jeet Teh for excellent technical assistance. We are grateful to Dr. Lewis Lanier (UCSF) for providing us with cell lines (RMA, RMA-Rae1δ, RMAS-Rae-1δ) and the NKG2D-Ig fusion protein and Dr. Wayne Yokoyama (HHMI, Washington University, St. Louis) for providing us with the anti-NKG2D mAb.

2.7 Figures

Figure 2.1 Cell surface phenotype of $CD8^+CD44^{hi}$ and $CD8^+CD44^{lo}$ T cells ex vivo. Lymph node cells from B6 mice were depleted of $CD4^+$ and Ig^+ cells and stained with antibodies against various cell surface markers. The filled histograms represent expression of the indicated cell surface molecule by gated $CD8^+CD44^{hi}$ cells. The unfilled histograms represent expression of the indicated cell surface molecule by gated $CD8^+CD44^{lo}$ cells from the same mouse.

Figure 2.2 Only $CD8^+CD44^{hi}$ cells proliferate in response to IL-2. (A) FACS sorted $CD8^+$ $CD44^{hi}$ and $CD44^{lo}$ T cells from B6 mice were labeled with CFSE and cultured with IL-2-supplemented media. Cell division, as assessed by CFSE dilution, was determined at 48, 72, and 96 hours by FACS analysis. (B) Sorted $CD8^+$ $CD44^{hi}$ and $CD44^{lo}$ T cells were cultured with IL-2 for 4 days and pulsed with ^{3}H-thymidine for the last 6 hours of culture. Error bars represent standard deviations of triplicate cultures.

Figure 2.3 Activated $CD8^+CD44^{hi}$ cells express several NK receptors. $CD8^+$ T cells were purified from B6 lymph nodes as described in Materials and Methods. The purified $CD8^+$ T cells were cultured with IL-2-supplemented media for 5 days and then stained with antibodies against various cell surface markers that are characteristic of NK cells. The filled histograms represent expression levels of the

indicated NK receptor by IL-2 activated $CD8^+CD44^{hi}$ cells and the unfilled histograms represent unstained controls.

Figure 2.4 CD48/CD2 interactions are required for IL-2-induced proliferation of $CD8^+CD44^{hi}$ cells. A) The expression of CD48 and CD2 on B6 $CD8^+CD44^{hi}$ cells ex vivo (filled histograms) vs. unstained controls (unfilled histograms). B) Purified $CD8^+$ T cells from B6 mice were labeled with CFSE and cultured in either IL-2-supplemented media (left panel) or plate-bound anti-CD3ε antibody (2C11) plus 20U/ml IL-2 (right panel) in the presence of 10μg/ml anti-CD48 mAb (solid line), anti-CD2 (dashed line), anti-2B4 (dotted line) or without any antibody (filled histogram) for 4 days and analyzed by FACS. C) Purified $CD8^+$ T cells from B6 mice were cultured in either IL-2-supplemented media (left panel) or plate-bound anti-CD3ε antibody (2C11) plus 20U/ml IL-2 (right panel) in the presence or absence of 10μg/ml anti-CD48 mAb for 4 days and then pulsed with ^3H-thymidine for the final 6 hours of culture. The error bars represent the standard deviation of triplicate cultures.

Figure 2.5 Activated $CD8^+CD44^{hi}$ cells preferential killed syngeneic tumor target cells. A) FACS-purified $CD8^+CD44^{hi}$ and $CD8^+CD44^{lo}$ cells from a B6 mouse were activated with anti-CD3 (2C11) and IL-2 (20U/ml) for 3 days and then used as effectors in a ^{51}Cr-release assay against RMA target cells, which are syngeneic to B6 mice. Error bars represent the standard deviation for triplicate cultures. B) (Left panel) Purified $CD8^+$ T cells from Balb/c mice were cultured in

IL-2-supplemented media for 5 days in order to activate $CD8^+CD44^{hi}$ cells. The cytolytic activities of the activated cells against A20 (syngeneic to Balb/c) or P815 (syngeneic to DBA/2) targets were then determined. (Right panel) Lymph node cells from Balb/c mice were depleted of Ig^+, $CD4^+$ and $CD44^+$ cells. Cells purified in this manner were ~98% $CD8^+CD44^{lo}$. They were cultured with plate-bound 2C11 in IL-2-supplemented media for 5 days and the cytolytic activities of the activated cells against A20 or P815 targets were then determined.) C) Same as (B) except that purified $CD8^+$ or $CD8^+CD44^{lo}$ lymph node cells from DBA/2 mice were used as the responding population. Error bars represent the standard deviation for triplicate cultures.

Figure 2.6 Activated $CD8^+CD44^{hi}$ cells express DAP12. NK cells were enriched by depleting B6 spleen cells of $CD4^+$, $CD8^+$, Ig^+ and $CD3^+$ cells by negative selection and the negatively selected cells were activated with IL-2-supplemented media. Purified $CD8^+$ cells from B6 mice were activated with IL-2-supplemented media and provided a source of IL-2-activated $CD8^+CD44^{hi}$ cells. $CD8^+CD44^{lo}$ cells were purified as described in Fig. 2.5 and activated with anti-CD3 + IL-2. mRNA was extracted from activated cells and RT-PCR for DAP10, DAP12 and GAPDH were performed. The data indicate that activated NK and $CD8^+CD44^{hi}$ cells express both DAP10 and DAP12 whereas activated $CD8^+CD44^{lo}$ cells only express DAP10.

Figure 2.7 NKG2D/Rae1 interaction enhances killing of syngeneic tumor targets by IL-2 activated $CD8^+CD44^{hi}$ cells. NK, $CD8^+CD44^{hi}$ and $CD8^+CD44^{lo}$ cells were purified and activated as described in Fig. 2.6. The ability of IL-2 activated NK cells (upper panel), $CD8^+CD44^{hi}$ cells (middle panel) or 2C11 + IL-2 activated $CD8^+CD44^{lo}$ cells to kill syngeneic target cells expressing a ligand for NKG2D was determined. The cells were >99% $CD3^-NK1.1^+DX5^+$ $2B4^+$, $CD94^+NKG2D^+$ for the purified NK cells and >98% $\alpha\beta TCR^+CD8^+$ for both groups of $CD8^+$ T cells. The cells were used as effectors in a ^{51}Cr-release assay against RMA, RMAS, RMA-Rae1δ, and RMAS-Rae1δ tumor cells. Error bars represent the standard deviation for triplicate cultures.

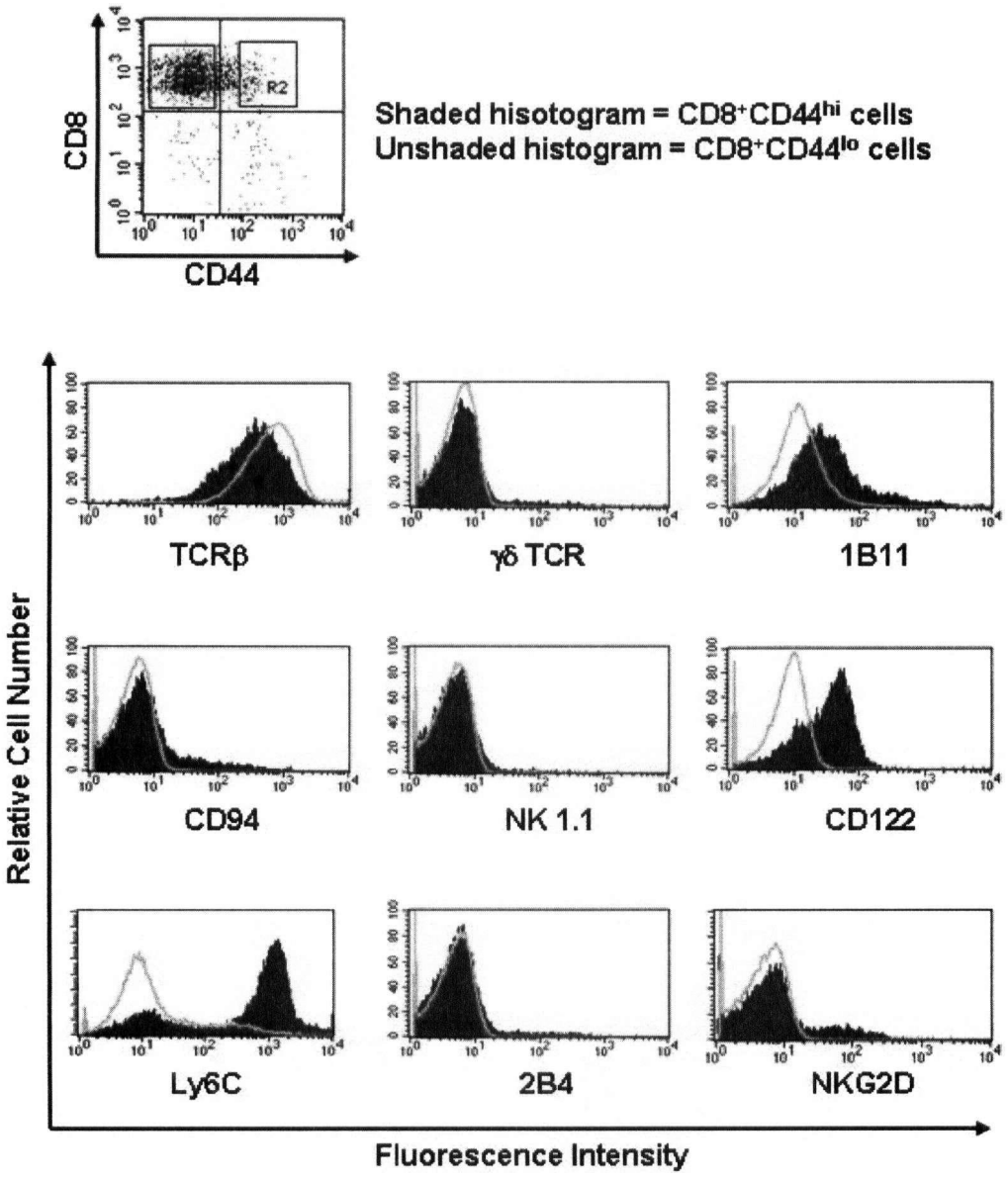


Figure 2.1 Cell surface phenotype of $CD8^{+}CD44^{hi}$ and $CD8^{+}CD44^{lo}$ T cells ex vivo.

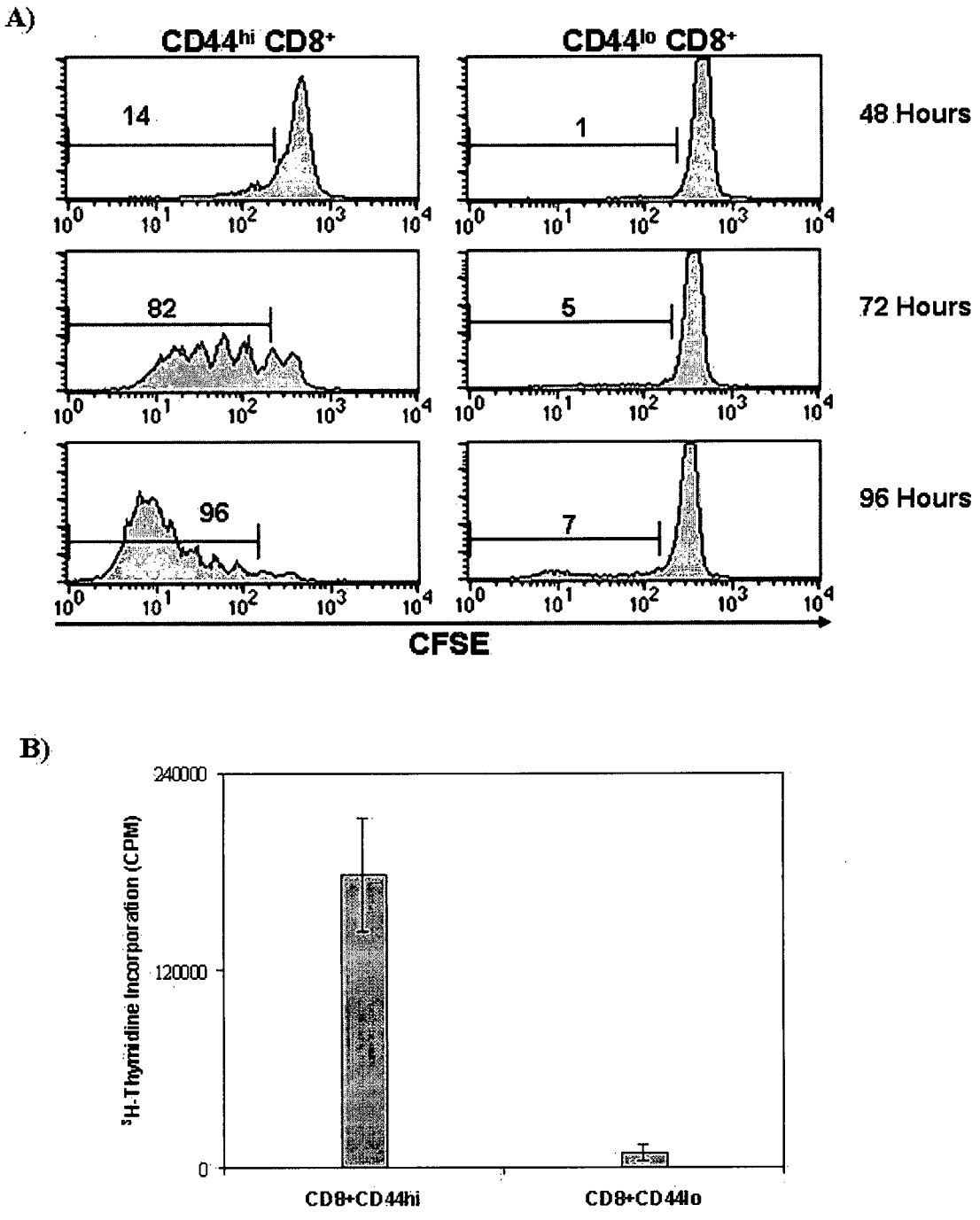


Figure 2.2 Only $CD8^{+}CD44^{hi}$ cells proliferate in response to IL-2.

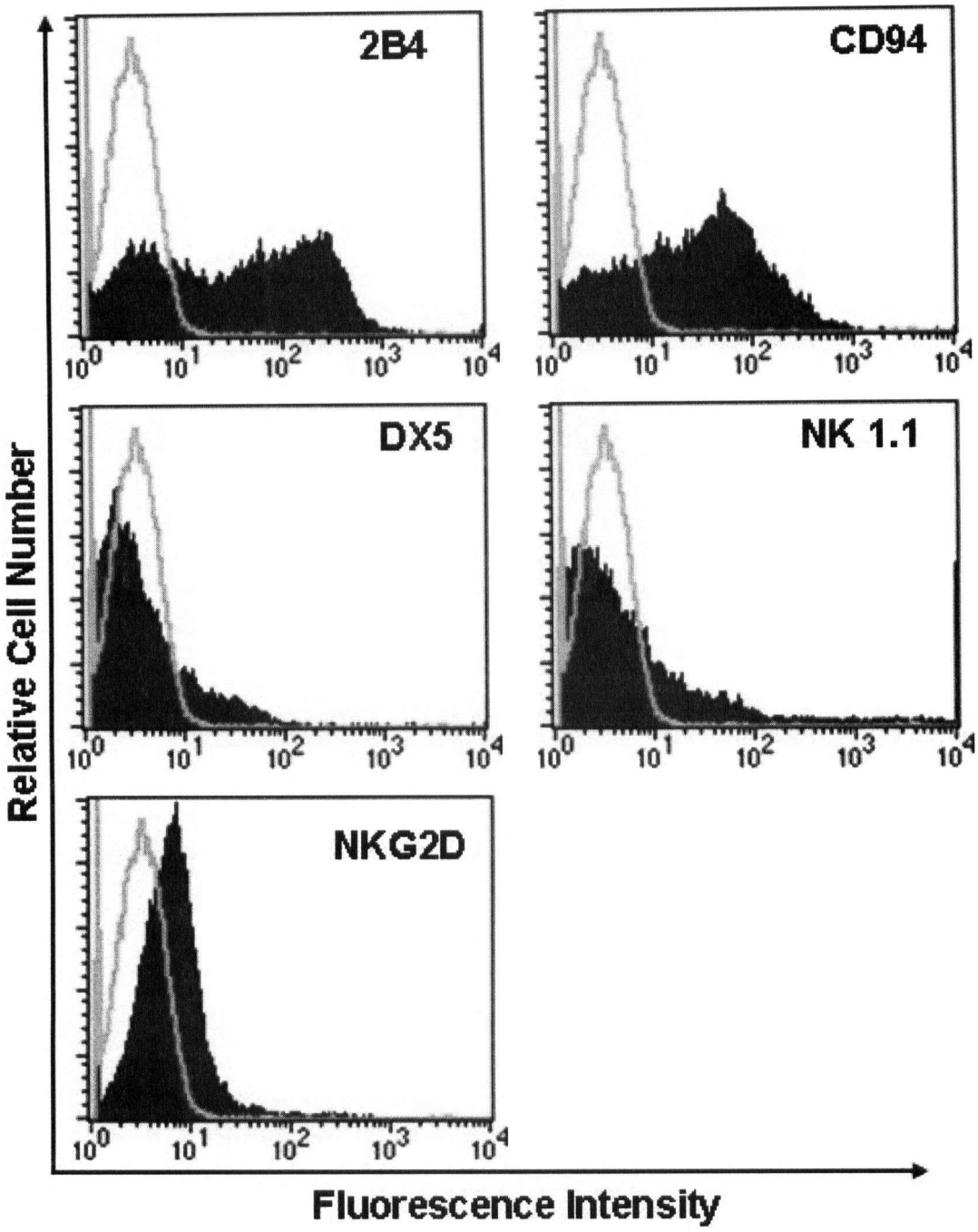


Figure 2.3 Activated $CD8^+CD44^{hi}$ cells express several NK receptors.

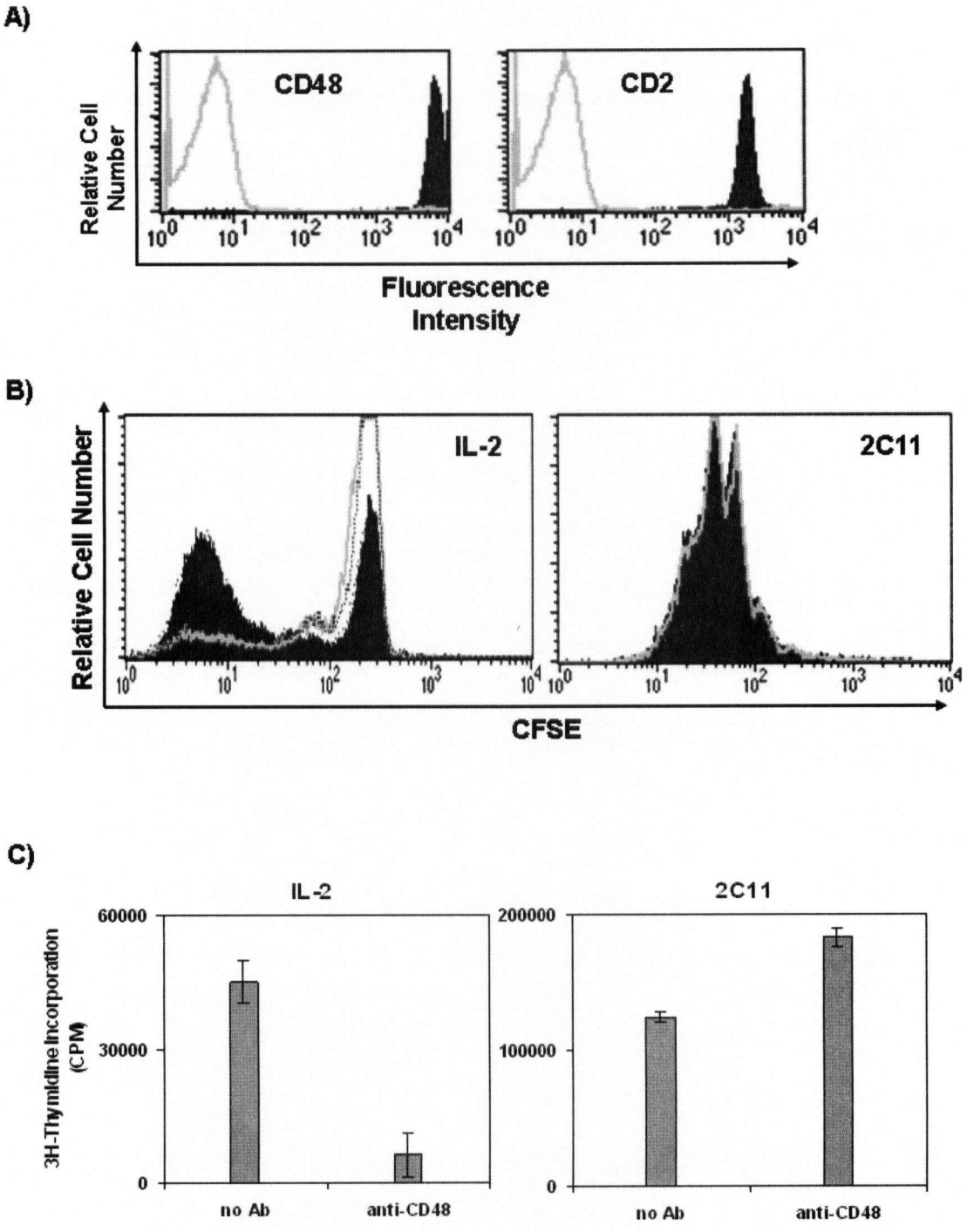
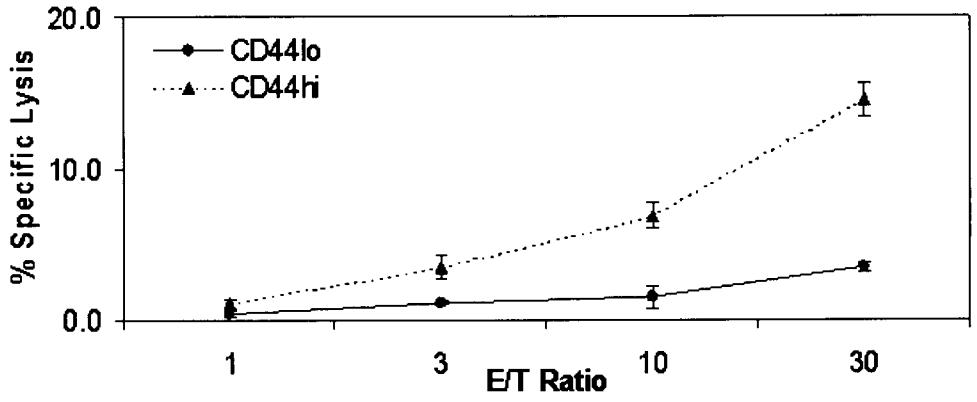


Figure 2.4 CD48/CD2 interactions are required for IL-2-induced proliferation of $CD8^{+}CD44^{hi}$ cells.

A)



B)

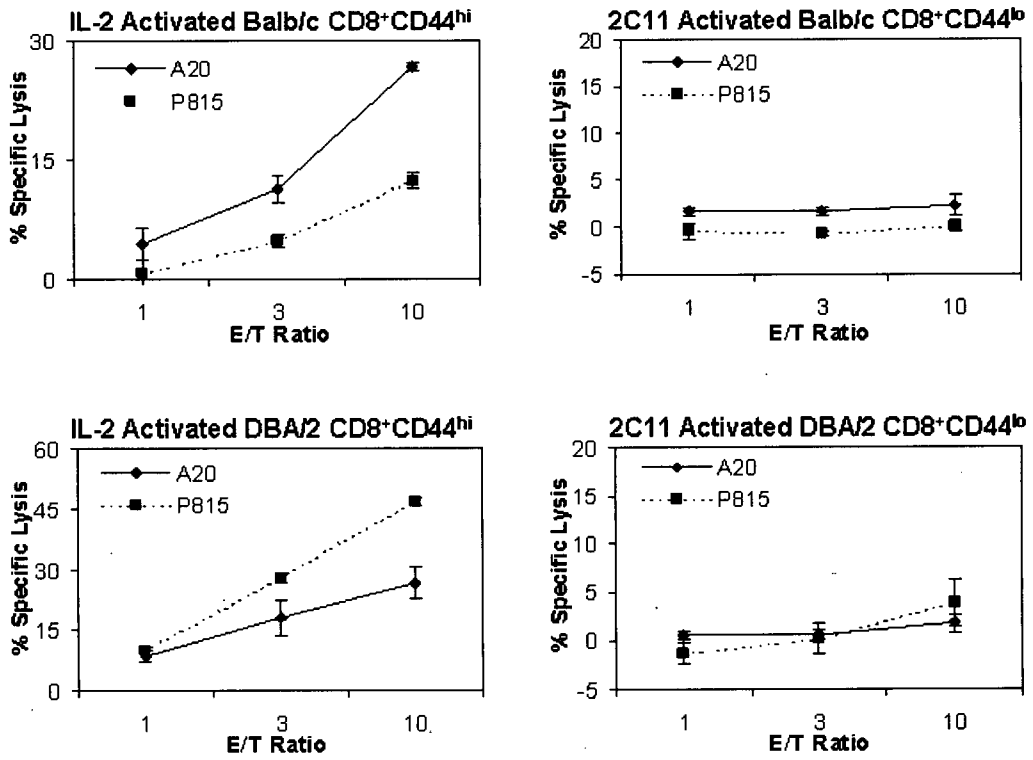
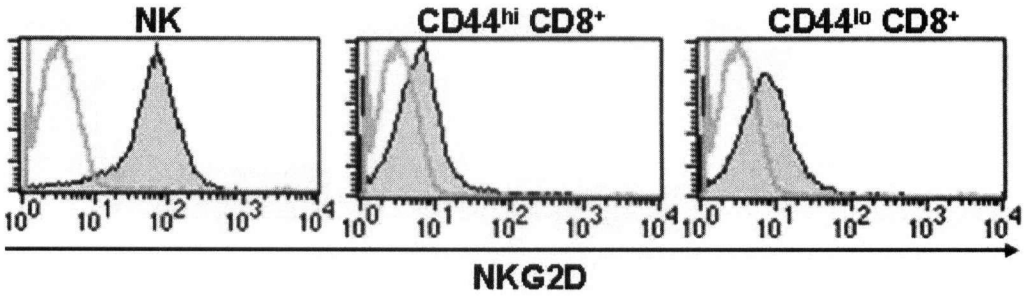


Figure 2.5 Activated $CD8^{+}CD44^{hi}$ cells preferential killed syngeneic tumor target cells.

A)



B)

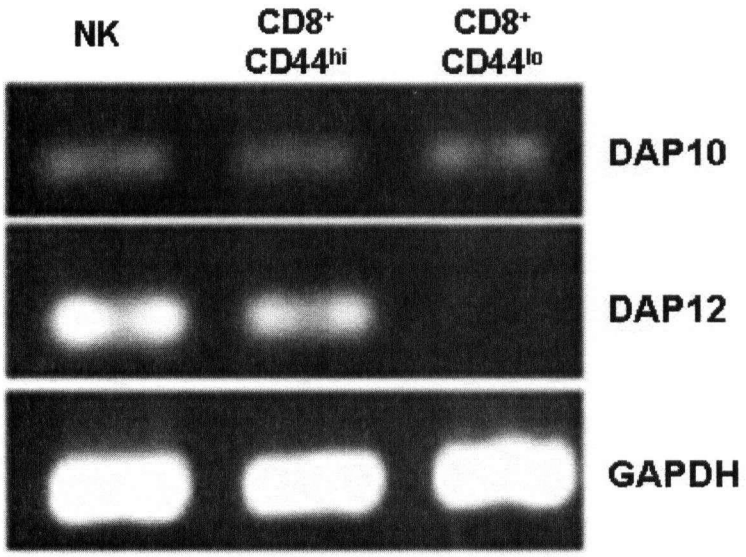


Figure 2.6 Activated $CD8^{+}CD44^{hi}$ cells express DAP12.

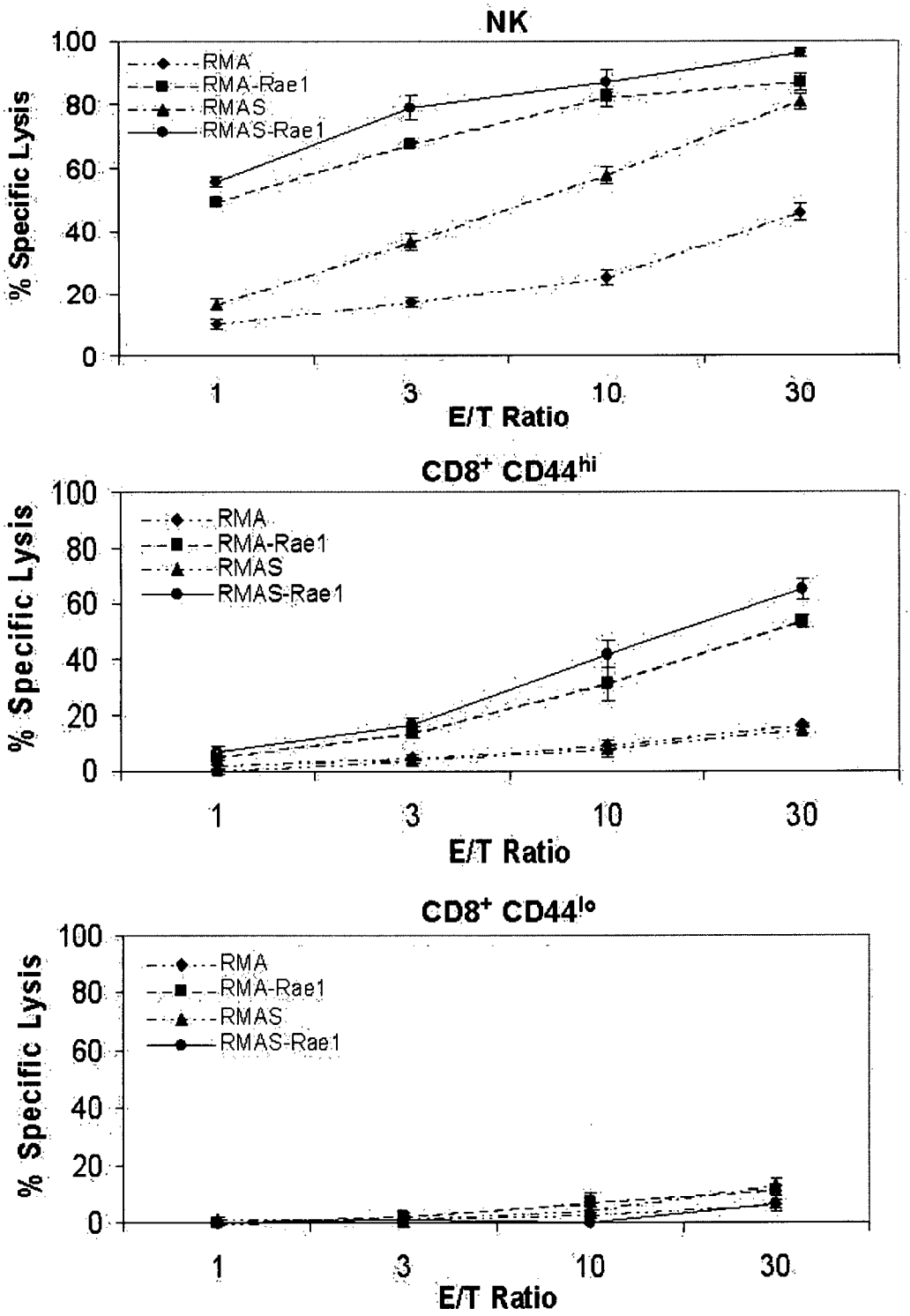


Figure 2.7 NKG2D/Rae1 interaction enhances killing of syngeneic tumor targets by IL-2 activated $CD8^{+}CD44^{hi}$ cells.

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Chapter 3 Self-reactive memory-phenotype CD8 T cells exhibit both MHC-restricted and non-MHC restricted cytotoxicity: A role for the T cell receptor and natural killer cell receptors[1]

3.1 Abstract

We have recently shown that IL-2-activated $CD8^+CD44^{hi}$ cells from normal mice express both adaptive and innate immune system receptors and specifically kill syngeneic tumor cells, particularly those that express NKG2D ligands. Here we show that $CD8^+$ T cells from antigen-expressing H-Y TCR transgenic mice also exhibit characteristics of both T cells and NK cells. Interaction with cognate self-antigen was required for the optimal expansion of these cells in peripheral lymphoid tissues. Although these cells possess a higher activation threshold relative to naïve T cells, they can be activated by cytokine alone in vitro. They also undergo bystander proliferation in response to a bacterial infection in vivo. Interestingly, upon activation, the cells express the NKG2D receptor as well as the DAP12 adaptor protein. We provide evidence that NKG2D can act additively with the TCR in the killing of target cells and it can also function as a directly activating receptor in non-MHC restricted killing of target cells. These properties of $CD8^+$ T cells from H-Y TCR transgenic mice are remarkably similar to $CD8^+CD44^{hi}$ cells that are found in normal mice. The H-Y TCR transgenic mice provide a well-defined system for characterizing the developmental biology and function of these cells.

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[1] A version of this chapter has been published as:

Dhanji, S., S. J. Teh, D. Oble, J. J. Priatel, and H. S. Teh. 2004. Self-reactive memory-phenotype CD8 T cells exhibit both MHC-restricted and non-MHC-restricted cytotoxicity: a role for the T-cell receptor and natural killer cell receptors. *Blood 104:2116*

3.2 Introduction

In this study we used mice that express a transgenic TCR that is specific for the male (H-Y) antigen presented by H-2D^b (1) as a model system for studying the developmental requirements and function of a population of self-specific CD8$^+$ T cells. H-Y TCR transgenic mice have been widely used as an animal model system for the determination of mechanisms of positive and negative selection of T cells. In these mice, CD8 T cells that express the H-Y TCR are positively selected in the thymus of female H-2^b mice (2). In male H-2^b mice, the H-Y TCR is negatively selected, leading to the massive deletion of double positive (DP) thymocytes (3). Interestingly, there is a population of CD8$^+$ T cells, which express a low level of CD8 (referred to as CD8^{lo}), that are resistant to deletion in male mice (4). The CD8^{lo} cells can develop via an extra-thymic pathway (5) and express high levels of CD44 and IL-2Rβ (CD122) that are characteristic of memory T cells. However, they differ from conventional memory T cells in that they are more refractory to activation by antigen as compared to naïve T cells (6, 7). Another unusual feature of these CD8^{lo} T cells is that they proliferate in response to cytokines such as IL-2 and IL-15 in an antigen-independent manner (8). However, the CD8^{lo} cells are similar to conventional memory T cells with regard to the ability to rapidly produce IFN-γ upon TCR stimulation (9) and in respect to the killing of susceptible target cells without the need for additional reactivation with antigen (10, 11).

$CD8^+$ T cells that develop via an extra-thymic pathway and possessing similar functional characteristics are also found in normal (non-TCR transgenic) mice (7). More importantly, cells of this phenotype in normal mice have also been shown to be important in responses towards viral and bacterial infections, suggesting that they may have an important role in the immune response (12, 13). We have recently showed that $CD8^+CD44^{hi}$ T cells from normal mice possess characteristics of both T cells and NK cells (14). These cells are activated in response to IL-2 alone and show self-reactivity in that they preferentially kill syngeneic tumor cells. Furthermore, $CD8^+CD44^{hi}$ T cells express NK receptors upon activation and engagement of these NK receptors enhances the ability of the cells to lyse syngeneic tumor cells. In this report we show that $CD8^+$ T cells from antigen-expressing H-Y TCR transgenic mice also exhibit characteristics of both T cells and NK cells and are remarkably similar to $CD8^+CD44^{hi}$ cells from normal mice with regard to their cell surface and functional phenotype. Thus, the H-Y TCR transgenic mice provide a well-defined system for characterizing the developmental biology and function of this interesting cell type.

3.3 Materials and Methods

Mice

Breeders for C57BL/6 (B6), B6-Tap-1$^{-/-}$, and DBA/2 were obtained from the Jackson Laboratories (Bar Harbor, ME). BDF_1 mice were F_1 mice from the mating of C57BL/6 mice with DBA/2 mice. The H-Y TCR transgenic (tg) mice were bred to the B6 background. Mice 8 to 12 weeks of age were used for the experiments described.

Abs and flow cytometry

The following mAbs were used: CD4 (GK1.5), CD8α (53-6.7), CD8β (53.58), CD3ε (2C11), CD44 (PGP1), H-Y TCRβ (F23.1), H-Y TCRα (T3.70), CD94 (18D3), NK-1.1 (PK136), CD122 (TM-β1), Ly6C (AL-21), CD244.2 (2B4), CD16/32, IL-7Rα (A7R34), NKG2D (A10 and CX5), and anti-NKG2D (15). Biotinylated mAbs were detected using streptavidin-PE. CD8α, H-Y TCRα, NKG2D (A10 and CX5), IL-7Rα, and CD94 were obtained from eBioscience (SanDiego, CA) All other abs were obtained from BD PharMingen (San Diego, CA) except anti-NKG2D (15), which was a kind gift from Dr. Wayne M. Yokoyama (Howard Hughes Medical Institute, Washington University, St. Louis). Cell staining and flow cytometry were performed according to standard procedures. The CellQuest software program (Becton Dickinson, Mountain View, CA) was used for data acquisition and analysis.

Cell lines

Cell lines used were the RMA lymphoma (H-2^{b+}, Rae-$1\delta^-$), RMA-Rae-1δ transfectant (H-2^{b+}, Rae-$1\delta^+$) and P815 mastocytoma. The cell lines were cultured in IMEM (Life Technologies, Burlington, Canada), supplemented with 10% (v/v) FBS (Life Technologies), $5x10^5$ μM 2-ME and antibiotics (I-medium). The RMA-Rae-1δ transfectant (16) was a kind gift from Dr. Lewis L. Lanier (University of California, San Francisco).

$CD8^+$ T cell purification and sorting

Single cell suspensions from the lymph nodes (LN) of mice were treated with biotinylated anti-CD8β mAb followed by positive selection using the MiniMACS system (Miltenyi Biotech, Auburn, CA), according to the manufacturers specifications. The resulting cells were >95% pure $CD8\alpha\beta^+TCR\beta^+$ T cells. For purification of H-Y TCRα $(T3.70)^+$ $CD8^+$ T cells single cell suspensions from the lymph nodes (LN) of H-Y male and female mice were treated with anti-CD4 mAb and then depleted of $CD4^+Ig^+$ cells using Dynabeads M-450 Sheep anti-mouse IgG (Dynal Biotech, Lake Success, NY), according to manufacturers instructions. After depletion the cells were stained with anti-CD8β-FITC and T3.70-PE and then sorted on a Becton Dickinson FACS Vantage SE Turbo sort cell sorter. Cell sorting was performed by Andrew Johnson (University of British Columbia) and the resulting cells were >95% pure.

Natural Killer (NK) cell purification and activation

NK cells were purified and activated as previously described (14).

CFSE Labeling

Purified $CD8^+$ T cells ($1x10^7$/ml) were labeled with 1 μM CFSE (Molecular Probes, Eugene, OR) in PBS for 8 min at room temperature. After stopping the reaction with the addition of an equal volume of FCS, cells were washed four times with complete media prior to use.

Adoptive transfer

Purified CFSE-labeled CD8 T cells ($1\text{-}2x10^6$) from $H\text{-}2^{b/b}$ male and female and $H\text{-}2^{b/d}$ male H-Y mice were injected into male or female B6 mice that had received a sub-lethal dose of irradiation (650 cGy) 24 h earlier. On day 7 spleens of the injected mice were depleted of $CD4^+Ig^+$ cells as above and stained with the indicated mAbs and analyzed by FACs. For Listeria infections, purified H-Y CD8 T cells ($3x10^6$) were labeled with CFSE and transferred into B6 male or female mice. 24h later the mice were challenged with *Listeria monocytogenes* (10,000 CFU). 5d after infection the mice were sacrificed and the spleens of the animals were analyzed. For the analysis of IFNγ production, $5x10^6$ splenocytes from infected and uninfected mice (day 5) were cultured in 1ml of I medium containing GolgiStop (BD PharMingen (San Diego, CA)) in the presence or absence of H-Y

peptide (1μM). After 6h incubation the cells were fixed, permeabilized and stained with anti-IFNγ, anti-CD8, and anti-H-Y TCR.

Proliferation assays

Purified H-Y CD8 T cells (10^4) were cultured with irradiated B6-Tap-$1^{-/-}$ splenocytes ($5x10^5$) and the indicated concentration of H-Y peptide (KCSRNRQYL)(17) in the presence or absence of IL-2 (20U/ml). The cells were pulsed with 1μCi of ^{3}H-thymidine for the final 6 hours of a 72 h culture. For CFSE-based proliferation assays, $1X10^5$ CFSE-labeled CD8 T cells were cultured in 96 well round-bottom plates in the presence of IL-2 (200U/ml), IL-15 (100ng/ml) or Tap-$1^{-/-}$ splenocytes (10^6), H-Y peptide (1μM) and IL-2 (20U/ml). CFSE-dilution (cell division) was assessed by FACs at the indicated times.

CTL Assays

CTL assays were performed as previously described (14). The CTL activity of activated CD8 T cells against RMA, RMA-Rae-1δ target cells was assessed at a ratio of 10 effector T cells to 1 target cell in a 4 h ^{51}Cr-release assay. For redirected lysis experiments, day 4 activated CD8 T cells were preincubated with the indicated mAb (10μg/ml) for 15 min. The CTL activity of Ab-coated $CD8^+$ T cells against FcR+ P815 target cells was then determined in a 4-5 h ^{51}Cr-release assay. Spontaneous release varied from 8-15% of the maximum. All assays were performed in triplicate. Percent specific lysis was calculated as 100% x [cpm

(experimental) – cpm (spontaneous release)]/[(cpm (maximum release) – cpm (spontaneous release)].

RT-PCR

Sorted H-Y female T3.70^{+}CD8^{+} cells and male T3.70^{+}CD8^{+} cells (10^{6}) were activated with Tap-1$^{-/-}$ stimulators (10^{7}), H-Y peptide (1μM) and IL-2 (20U/ml) for 4 days. NK cells were activated with IL-2 (200U/ml) for 5 days. RNA isolation and PCR primers were described previously (14).

Immunoblotting

Purified CD8 T cells were stimulated for 10mins at 37°C with anti-CD3ε (2C11) or with PMA (25ng/ml) plus ionomycin (500ng/ml) and then pelleted and lysed in 10 mM Tris (pH 7.5), 150 mM NaCl, 1% TX-100, 0.1% SDS, and protease and phosphatase inhibitors. The lysates were separated on a 4-15% Tris-HCl polyacrylamide gel and transferred to a PVDF membrane. Blots were developed using ECL system (Amersham). Phospho-ZAP-70, ZAP-70, and phospho-ERK1/2 antibodies used for detection were from Cell Signaling Technologies (Beverly, MD). Anti-ERK mAb was from Santa Cruz biotechnology (Santa Cruz, CA).

Statistical Analysis

Student's t-test was used to determine p-values in CTL assays.

3.4 Results

Cell surface phenotype of CD8 T cells from self-antigen expressing H-Y TCR transgenic mice

The H-Y TCR is specific for a male peptide presented by H-2D^b (1). Fig. 3.1a shows the CD4/CD8 thymocyte profiles of H-2$^{b/b}$ H-Y male and female mice as well as H-2$^{b/d}$ H-Y male mice. It is clear that the presence of cognate antigen in H-2$^{b/b}$ male mice results in the deletion of the vast majority of DP thymocytes. In H-2$^{b/d}$ H-Y male mice, where there is half the number of the deleting H-Y/D^b complexes, there is incomplete deletion of DP thymocytes. Deletion of DP thymocytes in H-2$^{b/b}$ and H-2$^{b/d}$ male mice results in drastically reduced numbers of CD8 single positive (SP) thymocytes relative to H-2^b H-Y female mice (Fig.3.1a).

Although the majority of H-Y TCR$^+$ CD8 T cells are deleted in the male H-2$^{b/b}$ and H-2$^{b/d}$ mice, there is a large population of CD8$^+$ H-Y TCR$^+$ T cells in the spleen and lymph nodes of these mice. However, CD8$^+$ T cells from male H-Y mice differ from CD8$^+$ T cells from female H-Y mice in many aspects (Fig. 3.1b). Female H-2$^{b/b}$ CD8$^+$ T cells express the highest level of the CD8 co-receptor (referred to as CD8^{hi}); those from H-2$^{b/d}$ male mice express an intermediate level (referred to as CD8^{int}) and those from H-2$^{b/b}$ male cells express the lowest level (referred to as CD8^{lo}). There is also a correlation between the degree of cognate self-antigen exposure and the level of expression of memory markers in male H-

Y mice. In female mice, the lack of cognate self-antigen exposure results in $CD8^+$ T cells with a naïve phenotype ($CD44^{lo/int}$, IL-$2R\beta^-$, IL-$7R\alpha^{lo}$, $Ly6C^-$). In contrast, the $CD8^+$ T cells from H-Y male mice exhibit an activated/memory phenotype ($CD44^{int/hi}$, IL-$2R\beta^+$, IL-$7R\alpha^{hi}$, $Ly6C^+$). Notably, the $CD8^{lo}$ cells expressed higher levels of memory markers compared to $CD8^{int}$ cells.

Previous studies show that the $CD8^{hi}$ cells from female H-Y TCR transgenic mice can express both the transgenic as well as an endogenous α chain (18). In stark contrast, the CD8 T cells from H-Y male mice express exclusively the transgenic TCRα chain (Fig. 3.1b). Using the TCRα expression as a measure of TCR expression, it is clear that the $CD8^{lo}$ and $CD8^{int}$ cells expressed the same intermediate level of the $\alpha\beta$ TCR compared to $CD8^{hi}$ T cells, which express a high level (Fig. 3.1b). Corroborating results were obtained by analyzing expression of TCRβ and CD3ε on these cells (data not shown).

CD8 T cells from male H-Y mice possess an increased activation threshold

In agreement with a previous study (7) we found that the $CD8^{lo}$ cells proliferated poorly to antigen stimulation in the absence of IL-2 (Fig. 3.2a, upper panel). The $CD8^{int}$ cells also showed a similar proliferative defect. This proliferative defect was partially restored by exogenous IL-2 (Fig. 3.2a, lower panel).

The increased activation threshold of the $CD8^{int}$ and $CD8^{lo}$ cells may be due to a defect in TCR signal transduction. Signaling through the TCR leads to phosphorylation of CD3ζ, leading to the recruitment and subsequent phosphorylation of ZAP-70 (19). ZAP-70 activation eventually leads to the

activation of several important signaling pathways including the Ras/mitogen-activated protein kinase (MAPK) pathway. Figure 2b clearly shows that $CD8^{int}$ and $CD8^{lo}$ cells have a defect in early TCR signaling, as they do not efficiently phosphorylate ZAP-70 in response to TCR stimulation as compared to conventional, naïve, $CD8^{hi}$ cells. Correlated with diminished early TCR signaling, the cells also have a defect in downstream signaling events, leading to reduced p42/p44 (ERK1/2) MAPK phosphorylation. The inefficient activation of ZAP-70 correlates with the amount of cognate self-antigen the cells have encountered in vivo; the naïve phenotype $CD8^{hi}$ cells exhibit the strongest signaling, followed by the $CD8^{int}$ and then the $CD8^{lo}$ cells. This is in contrast to true memory CD8 T cells, which have a lower activation threshold, and more efficiently induce the phosphoryaltion of ZAP-70 and ERK1/2 relative to naïve cells (20). Interestingly, ERK 1/2 were efficiently phosphorylated in response to PMA and ionomycin (Fig. 3.1b), suggesting that there is no intrinsic defect in the ability of these cells to activate the ERK MAPK pathway.

IL-2 or IL-15 promotes the proliferation of $CD8^{lo}$ and $CD8^{int}$ cells

Since the $CD8^{lo}$ and $CD8^{int}$ cells are recovered from mice that express different levels of cognate self-antigen they offer an opportunity for determining whether prior antigen history affects their responsiveness to inflammatory cytokines. This was determined by culturing CFSE labeled $CD8^{lo}$ and $CD8^{int}$ cells with an exogenous source of IL-2 or IL-15. Proliferation at 48, 72, and 96 hours was determined by measuring the CFSE fluorescence level of the cultured $CD8^{+}$

cells. $CD8^{hi}$ cells from female H-Y mice do not proliferate in response to IL-2 or IL-15 (8) so we stimulated these cells with antigen + IL-2 to determine their proliferative potential. The results in Fig. 3.3a indicate that stimulation of $CD8^{hi}$, $CD8^{lo}$ or $CD8^{int}$ cells with a high antigen dose + IL-2 led to similar extent of cell division at 48, 72 or 96 hr. In contrast, only the $CD8^{lo}$ and $CD8^{int}$ cells could proliferate in response to cytokine alone (Fig. 3.3a). Interestingly, $CD8^{lo}$ cells proliferated much more rapidly than the $CD8^{int}$ cells in response to IL-2 at the 72 and 96 hr time points (Fig. 3.3a). IL-15 was more efficient than IL-2 in inducing the growth of $CD8^{lo}$ and $CD8^{int}$ cells, particularly at the 48 and 72 hr time points. These studies indicate that the prior antigen history of $CD8^{+}$ cells from male H-Y mice determine the qualitative and quantitative aspects of their responsiveness to inflammatory cytokines.

*Role of antigen and bacterial infection on the expansion of $CD8^{lo}$ cells in vivo*The antigenic requirements for the expansion of $CD8^{+}$ T cells from male H-Y mice in vivo was determined in adoptive transfer experiments where CFSE labeled $CD8^{hi}$, $CD8^{int}$, and $CD8^{lo}$ were injected into irradiated female or male B6 mice. Fig. 3.3b indicates that the $CD8^{int}$ and $CD8^{lo}$ cells homed to and expanded efficiently in the spleen of either cognate-antigen expressing male or non-antigen expressing female mice whereas the $CD8^{hi}$ cells could only expand in the presence of antigen. A recent report has also shown that $CD8^{lo}$ cells can in fact expand in MHC class I-deficient host (21). In our experiments we showed that $CD8^{lo}$ cells could expand in female recipients and they underwent one extra

division in male recipients. $CD8^{int}$ cells, on the other hand, were much more dependent on the male antigen for proliferation since they underwent about 3 fewer divisions in female recipients. In all cases, the presence of cognate antigen affected the clone sizes, suggesting that antigen does in fact play a role in the lymphopenia-induced expansion of these memory-phenotype CD8 T cells. This expansion in the absence of cognate self-antigen is likely mediated through interaction of IL-2Rβ and or IL-7Rα with IL-15 and IL-7 (22). By contrast, $CD8^{hi}$ cells could not expand in irradiated female mice as interactions between the H-Y TCR and female self-peptides/H-$2D^b$ alone are insufficient for the expansion of $CD8^{hi}$ cells in vivo (21, 23). $CD8^{hi}$ cells proliferated most efficiently in irradiated male mice.

Bystander proliferation of $CD8^{lo}$ cells in response to bacterial infection

The above studies suggest that $CD8^{int}$ and $CD8^{lo}$ cells are dependent on interaction with the self (male)-antigen for their development and likely to be of the same lineage. Since $CD8^{int}$ cells consistently showed an intermediate phenotype relative to $CD8^{lo}$ cells, we decided to focus the remainder of our experiments on $CD8^{lo}$ and $CD8^{hi}$ cells to further characterize the difference between these two cell types. Since $CD8^{lo}$ cells can expand in an antigen-independent manner in irradiated female mice, we determine if these cells could undergo bystander proliferation in response to bacterial infection. To this end we transferred $CD8^{lo}$ cells into non-irradiated B6 female and male mice and then infected the mice with a sublethal dose of Listeria monocytogenes (LM). As a

control, we also transferred $CD8^{hi}$ cells into non-irradiated B6 female mice and infected them with LM. Figure 3.4 shows that $CD8^{lo}$ cells expand in both male and female recipients during infection, although this expansion was greater in male mice. The $CD8^{hi}$ cells did not expand in either infected or uninfected recipients and in fact their numbers were depleted in the infected recipients. Consistent with this, a previous study has shown that nonspecific T cells are depleted early during infection with Listeria (24). Our results show that rather than being depleted, $CD8^{lo}$ cells expand in infected mice suggesting that inflammatory cytokines produced in response to infection could promote the growth of these cells in vivo.

$CD8^{lo}$ cells express NK receptors after activation

We recently showed that memory phenotype $CD8^{+}CD44^{hi}$ cells from normal mice express NK receptors after activation (14). Since $CD8^{lo}$ cells are similar to $CD8^{+}CD44^{hi}$ cells in terms of cell surface phenotype and cytokine responsiveness, we determined whether they also shared the ability to express NK receptors after activation. Immediately ex vivo, $CD8^{hi}$ and $CD8^{lo}$ cells are negative for all of the NK receptors tested, with the exception of a very low level of NKG2D and CD94 expression by the $CD8^{lo}$ cells (Fig. 3.5a). Since both $CD8^{hi}$ and $CD8^{lo}$ cells can be activated with antigen + IL-2, this protocol was used to activate both populations. Figure 3.5b shows that activated $CD8^{lo}$ T cells expressed several NK receptors especially 2B4, DX5, and CD94. By contrast, activated female $CD8^{hi}$ cells do not express DX5 or CD94. A high level of 2B4

was expressed by some of the activated female $CD8^{hi}$ cells. NKG2D is known to be expressed by all activated CD8 T cells (25) and was expressed equivalently by both activated $CD8^{hi}$ and $CD8^{lo}$ cells. Neither cell type expressed the B6 NK-T cell marker NK1.1.

Conventional antigen-activated CD8 T cells express NKG2D in association with the DAP10 adaptor molecule (26, 27). By contrast, NKG2D in activated murine NK cells is associated with both the DAP10 and DAP12 adaptor molecules (26, 27). Association of NKG2D with DAP10 provides a costimulatory signal whereas association with DAP12 confers a directly sitmulatory signal (26). We recently showed that $CD8^{+}CD44^{hi}$ cells from normal mice expressed both DAP10 and DAP12 after IL-2 activation (14). Fig. 3.5c showed that activated $CD8^{lo}$ cells from male mice also expressed both DAP10 and DAP12 whereas antigen-activated $CD8^{hi}$ cells from female mice expressed only DAP10. Similar results were obtained following activation of $CD8^{lo}$ cells with IL-2 (data not shown).

NKG2D is an activating receptor for $CD8^{lo}$ cells

We next determined if NKG2D could participate in the lysis of target cells expressing self-antigen. Activated $CD8^{hi}$ and $CD8^{lo}$ cells were used as effector cells in a CTL assay against peptide-pulsed RMA ($H\text{-}2^{b}$) cells or RMA cells transfected with Rae-1δ (RMA-Rae-1) (16), a ligand for NKG2D (25). It is clear that the NKG2D receptor participated in the lysis of RMA target cells by activated $CD8^{lo}$ T cells since RMA-Rae-1 target cells were lysed significantly better than

RMA target cells (Fig. 3.6a). This is particularly evident at low H-Y peptide concentrations, where additive effects between the TCR and NKG2D in the lysis of RMA-Rae-1 target cells was observed. However, at high concentrations of antigenic peptide, RMA and RMA-Rae-1 target cells were killed to the same extent. This result indicates that NKG2D contributes to killing when there is suboptimal stimulation of the TCR by the antigenic ligand. By contrast, there was no difference in the lysis of RMA and RMA-Rae-1 target cells by activated female $CD8^{hi}$cells at all concentrations of added H-Y peptide, consistent with the finding that NKG2D is not an activating receptor in conventional CD8 T cells.

We used a redirected killing assay to provide further evidence that NKG2D is an activating receptor in activated $CD8^{lo}$ cells. In this assay, killing of an Fc receptor expressing target (P815; H-2^d) ± an anti-NKG2D mAb was determined. We found that only anti-CD3ε, but not anti-NKG2D, was able to induce lysis of P815 target cells by activated $CD8^{hi}$ cells (Fig. 3.6b). This is consistent with the conclusion that NKG2D does not function as an activating receptor in conventional CD8 T cells (26, 27). By contrast, anti-NKG2D could enhance the lysis of P815 target cells by $CD8^{lo}$ cells in the redirected killing assay (Fig. 3.6b). It is noted that activated $CD8^{lo}$ cells exhibited significant killing of P815 targets even in the absence of any added antibody. This background killing of P815 targets was not due to NKG2D since we could not eliminate this background killing by using a blocking anti-NKG2D mAb (data not shown). It is conceivable that the non-antibody dependent killing of P815 targets by activated $CD8^{lo}$ cells

may be due to P815 ligands, which are recognized by other activating NK receptors.

Listeria infection induces expression of NKG2D on proliferating CD8^{lo} cells and a heightened ability to produce IFNγ.

Since CD8^{lo} cells could expand in response to LM infection (Fig. 3.4), we determine if LM infection induces expression of NKG2D on these cells. In addition, we wanted to determine the effect of bacterial infection on IFNγ production by CD8^{lo} cells. We transferred CFSE-labeled CD8^{lo} cells into non-irradiated B6 male mice and then infected them with LM. It is clear that NKG2D was expressed by the majority of cells that have divided in response to infection (Fig. 3.7a). Furthermore, cells that have undergone more cell divisions expressed the highest level of NKG2D. To test the production of IFNγ by the cells, we cultured the splenocytes from infected or uninfected mice for 6 hours in the presence or absence of H-Y peptide. The CD8^{lo} cells from both infected and uninfected mice did not produce any IFNγ without any TCR stimulation (Fig. 3.7b). However, upon a 6 hr stimulation with the antigenic peptide, CD8^{lo} cells from uninfected and LM infected mice produced high levels of IFNγ. By contrast, CD8^{hi} cells from uninfected or infected recipients did not produce any IFNγ in response to similar stimulation (data not shown). CD8^{lo} cells from LM-infected mice produced higher levels of IFNγ than those from uninfected mice. Furthermore, CD8^{lo} cells that have undergone the most number of cell divisions in response to LM infection produced the highest level of IFNγ (Fig. 3.7b). Our

observation that $CD8^{lo}$ cells from uninfected mice can produce IFNγ in response to antigen stimulation is consistent with the observation that $CD8^{lo}$ cells can produce IFNγ in response to TCR stimulation without the need for prior activation (7).

3.5 Discussion

In this report we have characterized an unusual population of self-antigen specific $CD8^+$ T cells in H-Y TCR transgenic mice. The development of these cells is dependent on interaction with cognate self-antigen and the intensity of interaction with cognate-antigen in vivo determines the expression level of CD8 and memory markers. However, in contrast to conventional memory cells, these cells have an increased threshold for activation relative to naïve $CD8^+$ T cells of the same antigen specificity. They proliferate in response to cytokines such as IL-2 and IL-15. When introduced into lymphopenic mice, these cells can expand in the absence of self-antigen. More interestingly, they proliferate in response to a bystander bacterial infection in normal mice. Immediately ex vivo, these cells are negative or express low levels of NK receptors but upon activation several NK receptors are induced, including NKG2D. Interestingly, activated male H-Y $CD8^{lo}$ cells express DAP12, an adaptor molecule normally expressed by NK cells (26, 27). Furthermore, the NKG2D receptor acts additively and also functions independently of the TCR in the killing of NKG2D ligand positive cells. We have recently reported the existence in normal mice of $CD8^+CD44^{hi}$ T cells that

possess similar cell-surface phenotype and functional properties as the male H-Y $CD8^{lo}$ T cells (14). These results support the hypothesis that the H-Y $CD8^{lo}$ cells are not a transgenic oddity but are a normal component of the murine immune system.

The H-Y TCR transgenic mouse provides a well-defined system for studying the developmental biology of this novel cell type. In this study we showed that they could be selected by a conventional antigen, i.e. the male peptide presented by MHC class Ia molecules. We also showed that the expression level of CD8 on this cell type is variable and reflects their prior antigen history. The degree of TCR/cognate self-antigen interaction in vivo also affects the response of these cells to IL-2 or IL-15 in vitro (Fig. 3.3). Proliferation in response to IL-2 occurred much more efficiently in $CD8^{lo}$ cells than $CD8^{int}$ cells presumably as a result of higher IL-2Rβ expression (Fig. 3.1).

$CD4^+$ or $CD4^-CD8^-$ NKT cells bearing an invariant TCRα chain (Vα14 in mice and Vα24 in humans) that are reactive to CD1d also share many similarities with $CD8^{lo}$ cells. NKT cells are an important component of the innate immune response to infection and can produce cytokines to activate other cells or can directly lyse some targets (reviewed in (28)). Although these NKT cells react strongly to α-galactosyl-ceramide (a sphingolipid from a marine sponge) presented by CD1d, it was recently shown that their activation in response to infection is dependent on CD1d/self-antigen and IL-12 (29). Both $CD8^{lo}$ cells and NKT cells exhibit an activated/memory phenotype and are thought to be self-reactive (30). Another striking similarity is that NKT cells are selected by high-

affinity interactions with self-antigen on cells other than thymic cortical epithelial cells (31) and this selection can occur in the absence of a thymus (32). MHC class Ib-restricted CD8 T cells are also thought to be selected by relatively high affinity interactions with self-antigens (33) and are also functionally similar to $CD8^{lo}$ and NKT cells in that they mount an early response against bacterial infection (34). It is tempting to speculate that cells such as NKT, MHC class Ib-restricted $CD8^{+}$ and $CD8^{lo}$ cells, which are selected by high affinity interactions with self-antigens, may be members of a family of T cells that evolved to provide an early defense mechanism against bacterial infection.

Our data showed that NK receptors such as NKG2D can function as an activating receptor in male $CD8^{lo}$ cells but not female $CD8^{hi}$ cells. NKG2D has recently been shown to be important for the recognition and destruction of tumor cells by NK cells (16, 25, 35). In vivo, the expression of the ligands for NKG2D, Rae-1 and H60, results in the rapid clearance of ligand-positive tumors with the generation of protective immunity against subsequent challenge with parental, ligand-negative, tumors (35). NKG2D has been demonstrated to associate with two different adaptor molecules, DAP10 and DAP12 (26, 27). In $CD8^{+}$ T cells, which only express DAP10, NKG2D engagement results in a costimulatory signal whereas in activated NK cells, the expression of DAP12 allows NKG2D to provide an activating signal (26, 27). In addition it has been shown that the ectopic expression of DAP12 in CD8 T cells results in the ability of NKG2D to transduce a directly stimulatory signal (27). Interestingly, we have found that

activated $CD8^{lo}$ cells express both DAP10 and DAP12, unlike $CD8^{hi}$ cells, making $CD8^{lo}$ cells similar to NK cells in this regard.

Despite the fact that H-Y $CD8^{lo}$ cells are self-reactive, the presence of large number of these self-reactive cells in male H-2^b H-Y mice did not lead to autoimmune disease. It is conceivable that the increased activation threshold of these cells is sufficient to prevent autoimmunity. We have shown that $CD8^{lo}$ and $CD8^{int}$ cells have a defect in TCR signaling which can be partially compensated for by the addition of exogenous IL-2. This defect stems from the inability of $CD8^{int}$ and $CD8^{lo}$ cells to efficiently phosphorylate signaling proteins such as ZAP-70 and ERK, in response to TCR stimulation (Fig 3.2b). A recent study supports the hypothesis that the lowering of T cell activation threshold can lead to autoimmune diseases in male H-Y TCR transgenic mice. Murga et al found that T cells with a null mutation in the E2F2 transcription factor have a lowered activation threshold (36). Interestingly, male H-2^b H-Y TCR transgenic mice with this null mutation develop an accelerated and much more severe lupus-like autoimmune syndrome than normal mice with this mutation. The unique combination of high activation threshold, ability to respond to inflammatory cytokines such as IL-15 and undergo bystander proliferation, and the expression of activating NKG2D receptor in response to activation, would render these self-specific $CD8^+$ T cells particularly adept at sensing infected and transformed cells. The observed cooperation between the TCR and NKG2D in the destruction of Rae-1^+ target cells would also allow these cells to focus on normal cells that express stress ligands in response to infection or transformation. These cells

would also differ from NK cells in their function since the expression of MHC class Ia antigens on target cells does not inhibit their function. For the $CD8^{lo}$ cells, MHC class Ia could in fact serve as an activating antigen. Thus, infected or transformed cells that are not susceptible to killing by NK cells, by virtue of high MHC class Ia expression, would be susceptible to lysis by activated self-specific $CD8^+$ T cells. The fact that a bystander infection results in the proliferation of $CD8^{lo}$ as well as the upregulation of NKG2D suggests that these cells could use this receptor to eliminate infected cells early during an infection. This non-MHC dependent function of $CD8^{lo}$ cells broadens the range of target cells and increases their versatility in surveillance against infected and transformed cells.

3.6 Acknowledgements

We thank Dr. Lewis Lanier (University of California, San Francisco) for providing us with the RMA-Rae-1δ cell line. We also thank Dr. Wayne Yokoyama for providing the anti-NKG2D mAb and Dr. Hao Shen (University of Pennsylvania) for the gift of wild type *Listeria monocytogenes* strain 10403s.

3.7 Figures

Figure 3.1 $CD8^+$ T cells from antigen-expressing H-Y TCR transgenic mice possess an activated/memory phenotype. a) The dot plots depict the CD4 and CD8 profile of thymocytes from H-2^b H-Y female, H-2^b H-Y male, and H-$2^{b/d}$ H-Y male TCR transgenic mice. The bar graph depicts the mean number of double positive (DP), double negative (DN) and CD4 and CD8 single positive (SP) cells recovered from these mice with the error bars representing 1 standard deviation. b) The expression of CD8 and the H-Y TCRα by lymph node cells from the mice in part (a). The histograms depict the expression of the indicated cell surface markers by gated H-Y TCR^+ $CD8^+$ T cells.

Figure 3.2 $CD8^+$ T cells from male H-Y mice possess a high activation threshold due to a defect in TCR signal transduction. (a) Purified $CD8^+$ T cells ($1x10^4$) from female H-2^b H-Y ($CD8^{hi}$), male H-2^b H-Y ($CD8^{lo}$), or male H-$2^{b/d}$ H-Y ($CD8^{int}$) mice were cultured with irradiated B6-Tap-$1^{-/-}$ splenocytes ($5x10^5$) $\pm$ IL-2 and the indicated concentration of H-Y peptide. Proliferation was determined after 3 days and the error bars represent the standard deviation of triplicate cultures. (b) Western blot analysis of $CD8^{hi}$, $CD8^{int}$, and $CD8^{lo}$ cells immediately ex vivo or after stimulation for 10mins with anti-CD3 (10μg/ml) or PMA (25ng/ml) and ionomycin (500ng/ml). Blots were probed with anti-phospho-ZAP-70 and phospho-ERK and then stripped and re-probed with unphosphorylated ZAP-70 and ERK2.

Figure 3.3 The extent of the memory-phenotype of H-Y CD8 T cells determines their ability to respond to cytokines in vitro as well as their ability to undergo homeostatic expansion in vivo. a) Purified $CD8^+$ T cells from female $H\text{-}2^b$ H-Y ($CD8^{hi}$), male $H\text{-}2^b$ H-Y ($CD8^{lo}$), or male $H\text{-}2^{b/d}$ H-Y ($CD8^{int}$) mice were labeled with CFSE and cultured with IL-2 (200U/ml), IL-15 (100ng/ml), or H-Y peptide (1μM), B6-$Tap1^{-/-}$ splenocytes ($1x10^7$), and IL-2 (20U/ml). Proliferation of gated H-Y $TCR\alpha^+$ $CD8^+$ cells was analyzed by FACS at 48 (filled histogram), 72 (dark line), and 96 hours (light line) with each CFSE peak representing one cell division. b) Purified $CD8^{hi}$, $CD8^{int}$ and $CD8^{lo}$ cells were CFSE-labeled and transferred into sublethally irradiated B6 female (filled histogram) or male (unfilled histogram) recipients. CFSE profiles of gated $CD8^+$ H-Y TCR^+ cells 7 days post-transfer are shown in the histograms.

Figure 3.4 Role of antigen and bacterial infection in the expansion of H-Y male $CD8^{lo}$ cells in vivo. Purified $CD8^{hi}$ and $CD8^{lo}$ cells were labeled with CFSE and injected into the indicated non-irradiated Thy1.1 congenic B6 male or female mice. The mice were then infected with Listeria monocytogenes (right column) or left uninfected (left column). On day 5 the expansion of labeled $CD8^{hi}$ and $CD8^{lo}$ cells in the spleens of infected or uninfected mice were analyzed by FACs. The numbers in the histograms represent the percentage of undivided cells.

Figure 3.5 Activated H-Y male $CD8^{lo}$ cells expressed NK receptors and DAP12 after activation. a) Lymph node cells from H-Y male and female mice were depleted of $CD4^{+}Ig^{+}$ cells and stained with the indicated antibodies. The histograms represent the expression of the indicated cell surface markers by gated female H-Y $TCR\alpha^{+}$ $CD8^{+}$, male H-Y $TCR\alpha^{+}$ $CD8^{+}$ or unstained cells. b) and c) Sorted $CD8^{+}$ H-Y $TCR\alpha^{+}$ male or female cells ($1x10^{6}$) were cultured with irradiated B6-$Tap^{-/-}$ ($1x10^{7}$) splenocytes + H-Y peptide (1μM) and IL-2 (20U/ml) for 4 days. b) Histograms represent the expression of the indicated cell surface markers by gated $CD8^{hi}$, $CD8^{lo}$ or unstained cells on day 4. c) PCR using primers specific for DAP10, DAP12 or β-Actin on cDNA from antigen + IL-2-activated $CD8^{hi}$ and $CD8^{lo}$ cells and IL-2-activated B6 NK cells.

Figure 3.6 NKG2D enhances the killing of target cells by male H-Y $CD8^{lo}$ cells in both an MHC restricted and non-MHC restricted fashion. Purified $CD8^{hi}$ and $CD8^{lo}$ T cells ($1x10^{6}$) were cultured with irradiated B6-$Tap^{-/-}$ ($1x10^{7}$) splenocytes + H-Y peptide (1μM) and IL-2 (20U/ml) for 4 days. a) Day 4 activated cells were used as effectors in a CTL assay against RMA (H-2^{b}, Rae-1δ^{-}) or RMA-Rae-1δ (H-2^{b}, Rae-1δ^{+}) cells at an effector to target ratio of 10 to 1 in the presence of the indicated concentrations of H-Y peptide. b) Day 4 activated cells were used in a redirected CTL assay against FcR^{+} P815 targets in the presence of the indicated mAbs. Part (a) was repeated 5 times with similar results. Error bars represent the standard deviation of triplicate cultures. * p-value < .002 ** p-value < .04

Figure 3.7 Bacterial infection primes $CD8^{lo}$ cells in vivo. Purified $CD8^{lo}$ cells were labeled with CFSE and injected into non-irradiated B6.Thy1.1 male recipients. 24h post-transfer the mice were infected with Listeria monocytogenes (LM) or left uninfected. a) The plots represent the CFSE and NKG2D profiles of gated $CD8^{lo}$ T cells on day 5 in either uninfected (left) or LM infected (right) mice. b) Spleens from day 5 infected (left) or uninfected (right) mice were cultured with a Golgi-inhibitor in the presence or absence of H-Y peptide (1μM). After 6h incubation the cells were fixed and stained with anti-IFNγ, anti-H-Y TCR, and anti-CD8 mAbs. The dot plots depict the CFSE and IFNγ profiles of gated H-Y TCR^{+} $CD8^{+}$ cells.

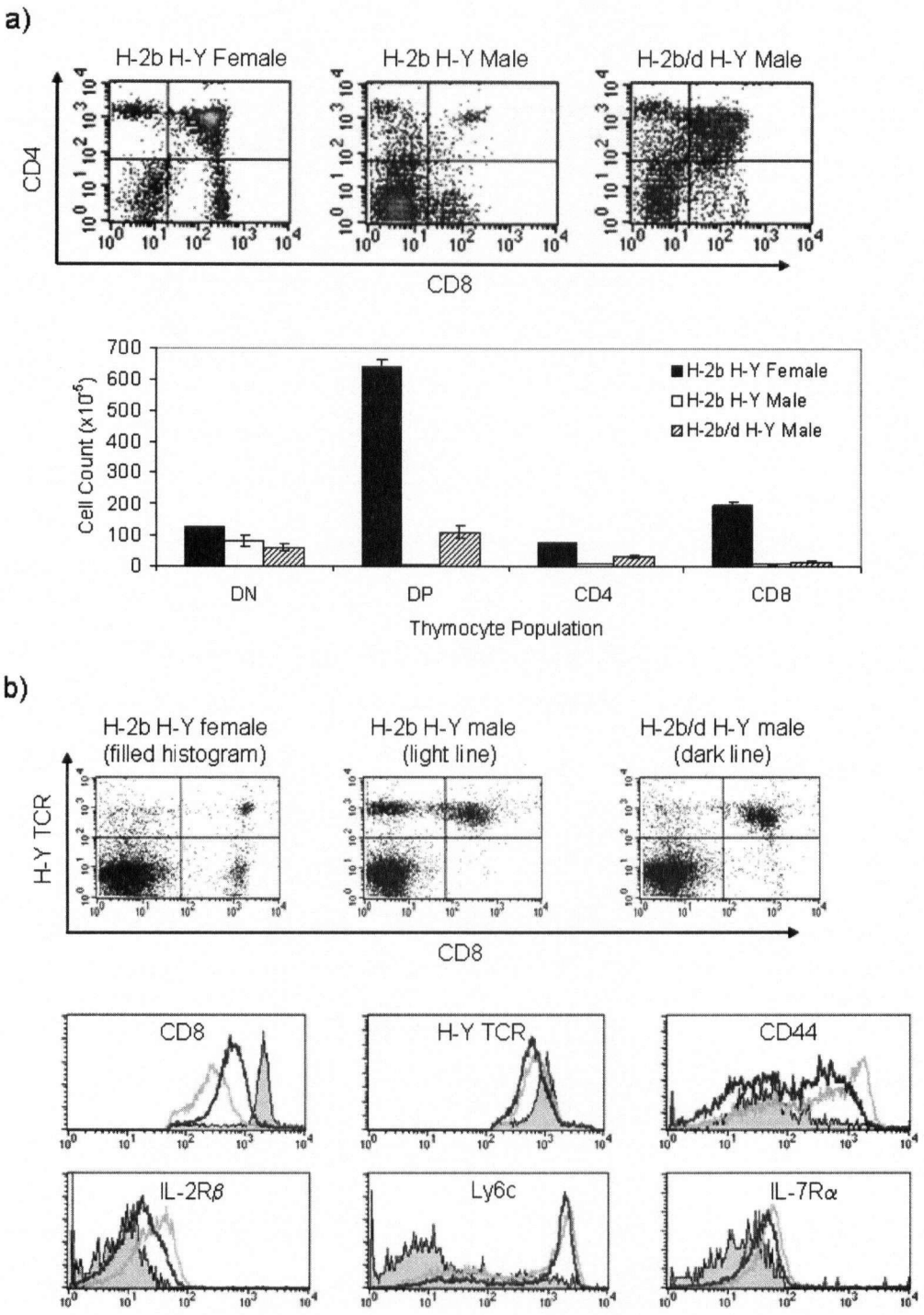
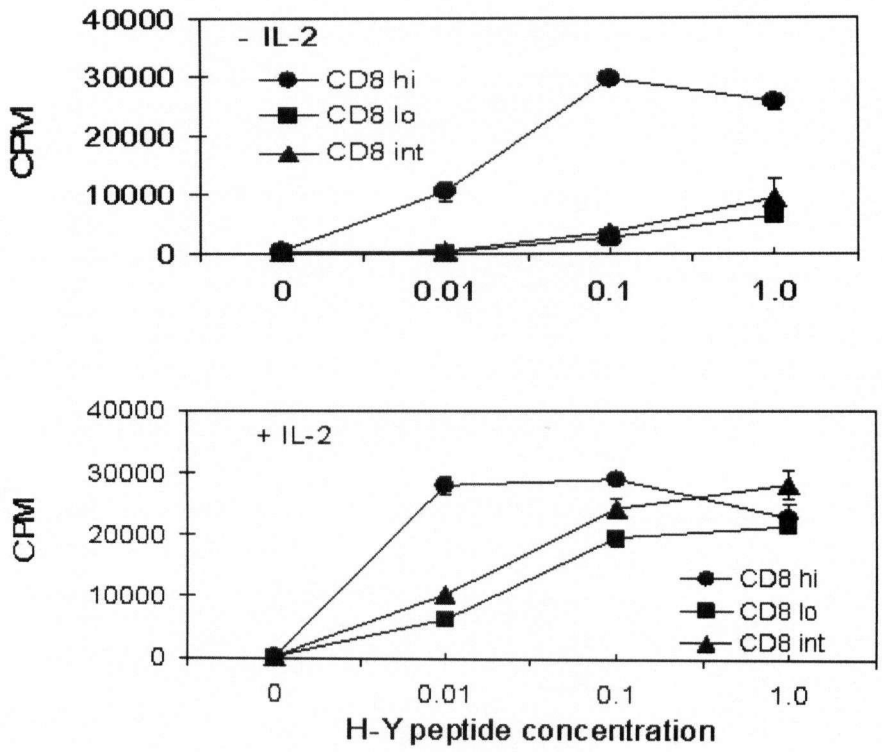


Figure 3.1 $CD8^+$ T cells from antigen-expressing H-Y TCR transgenic mice possess an activated/memory phenotype.

a)



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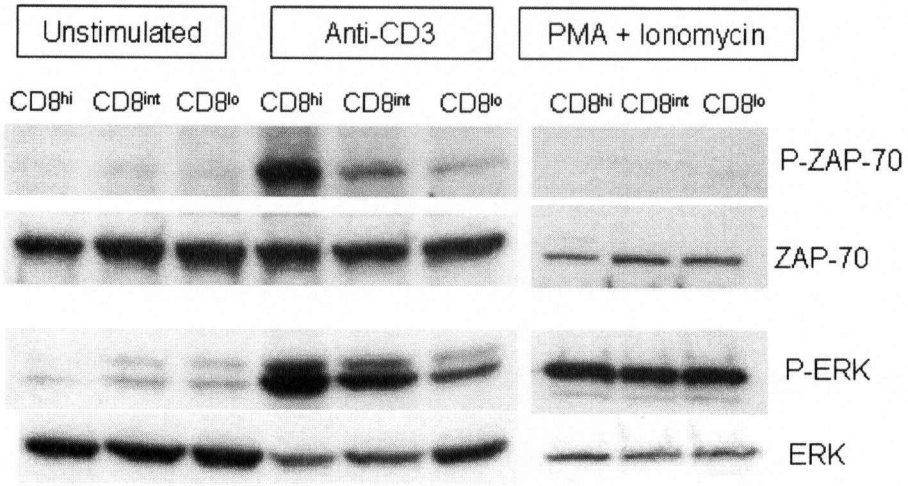


Figure 3.2 $CD8^+$ T cells from male H-Y mice possess a high activation threshold due to a defect in TCR signal transduction.

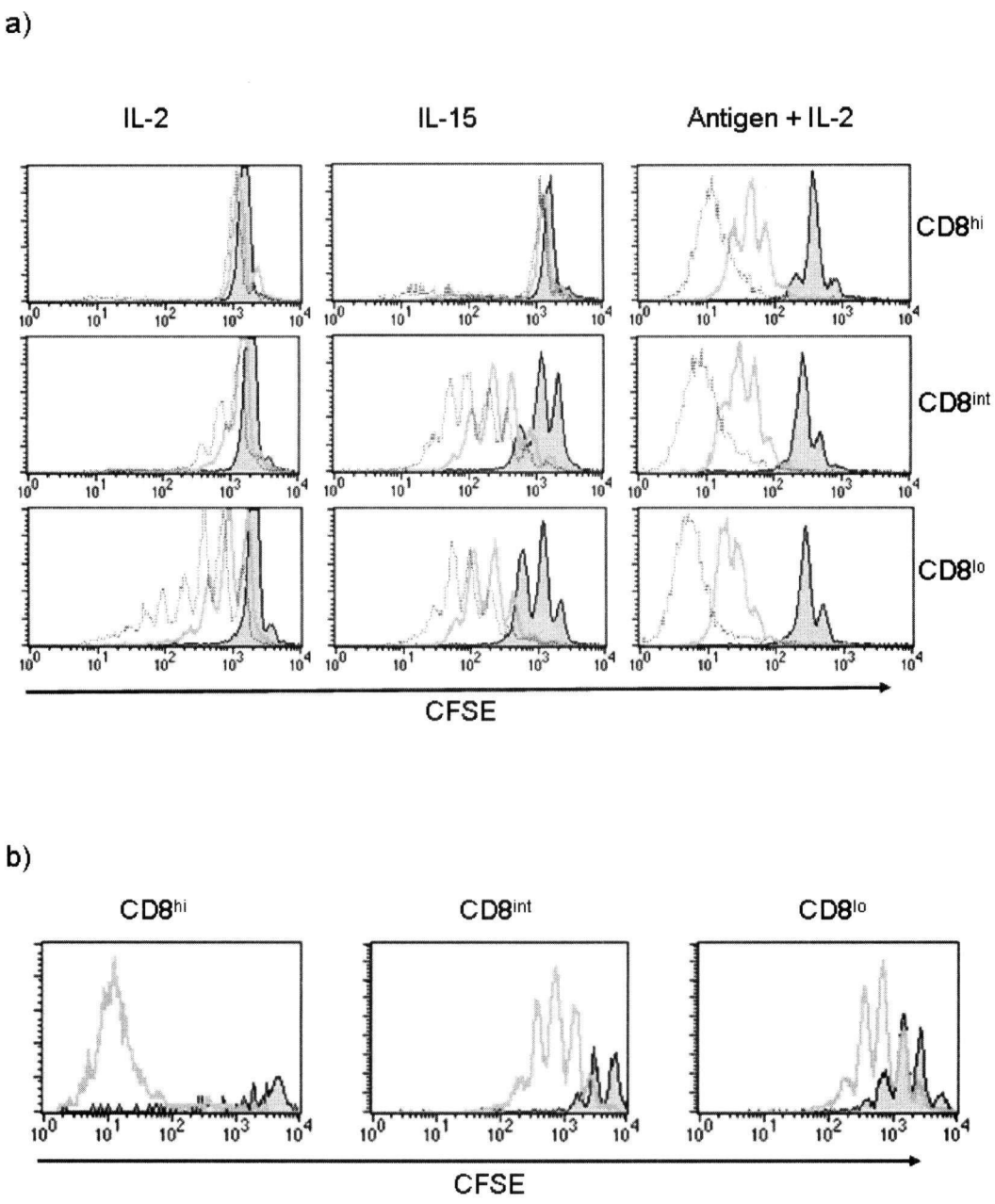


Figure 3.3 The extent of the memory-phenotype of H-Y CD8 T cells determines their ability to respond to cytokines in vitro as well as their ability to undergo homeostatic expansion in vivo.

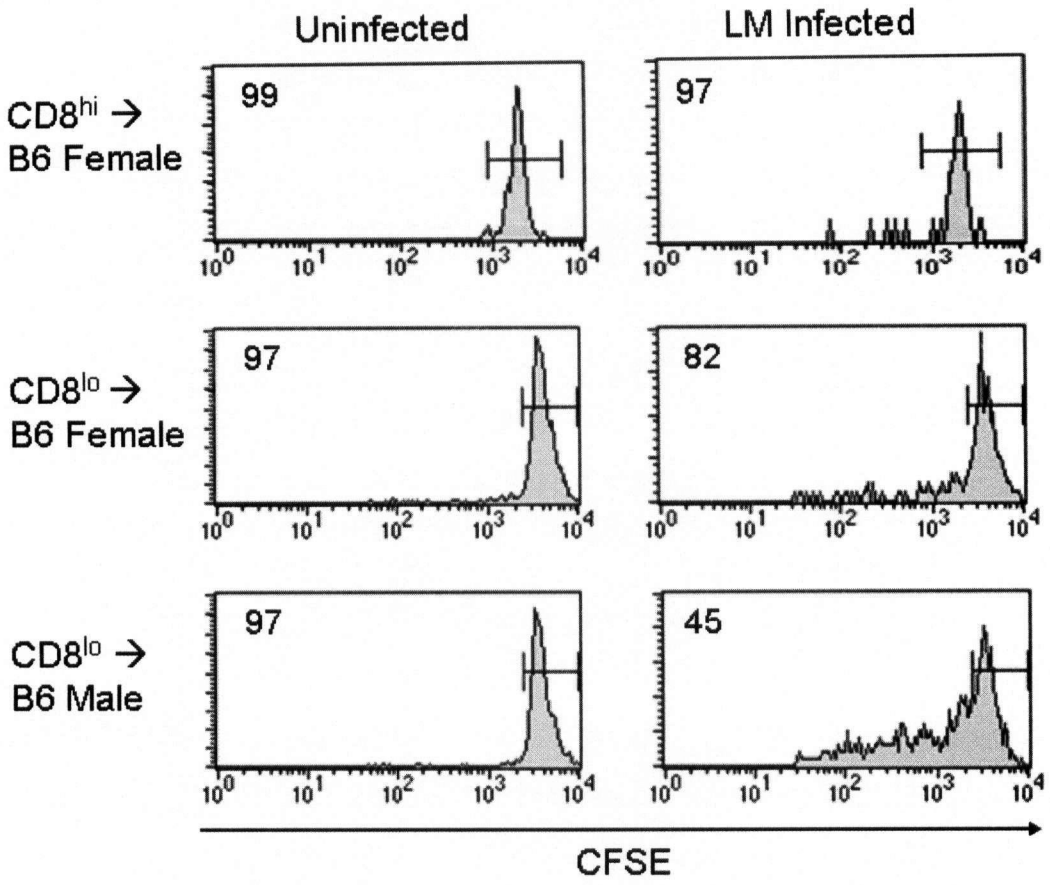


Figure 3.4 Role of antigen and bacterial infection in the expansion of H-Y male $CD8^{lo}$ cells in vivo.

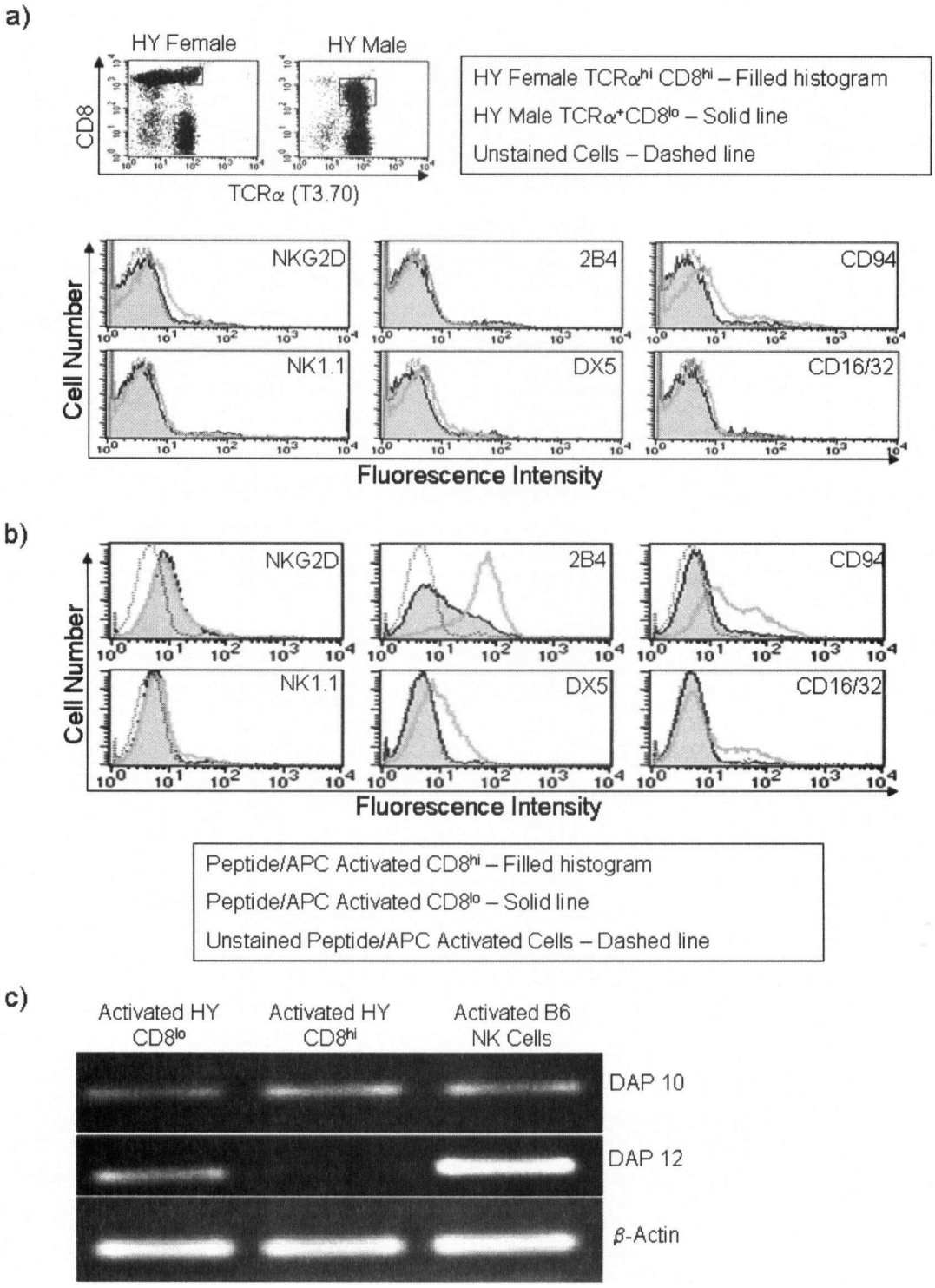


Figure 3.5 Activated H-Y male $CD8^{lo}$ cells expressed NK receptors and DAP12 after activation.

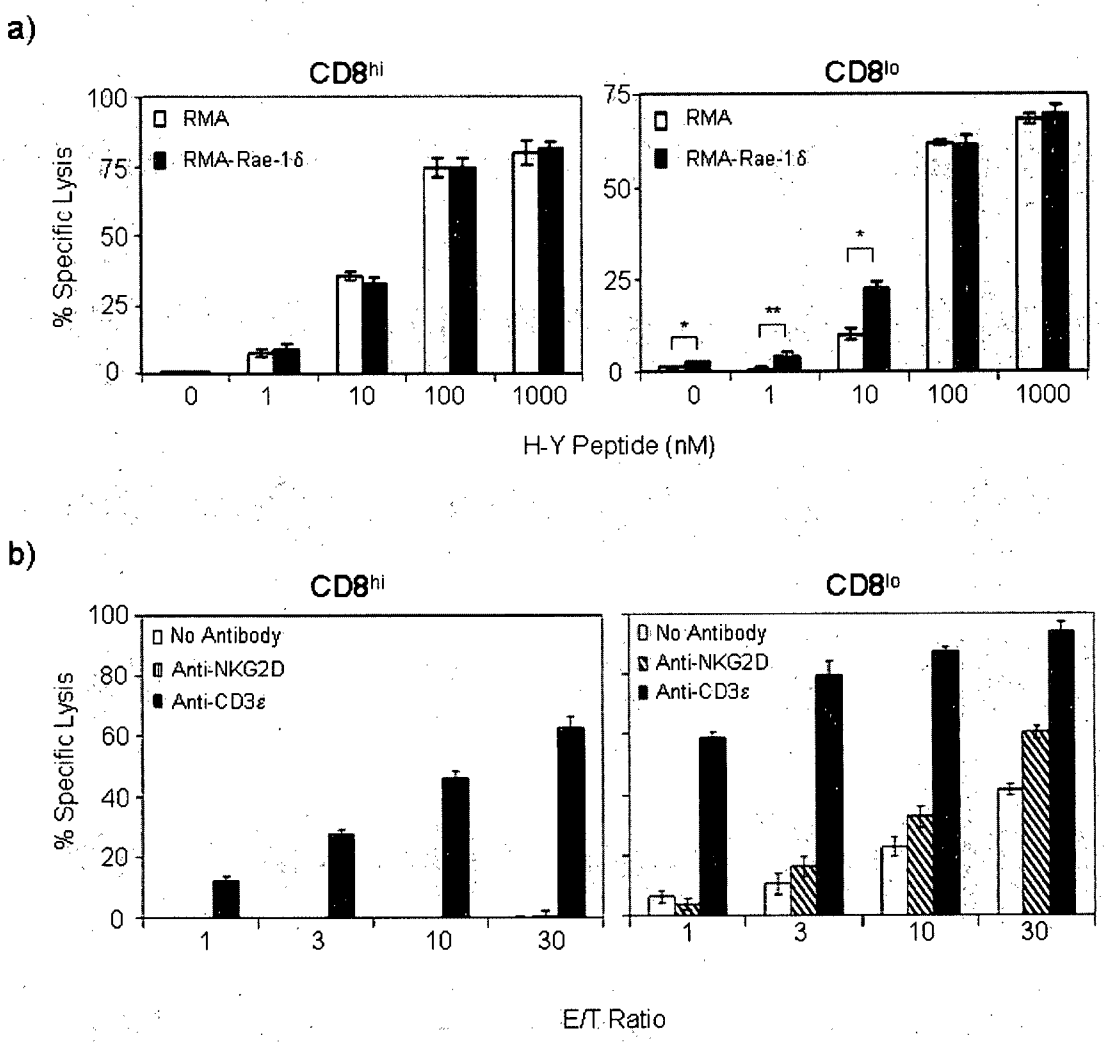
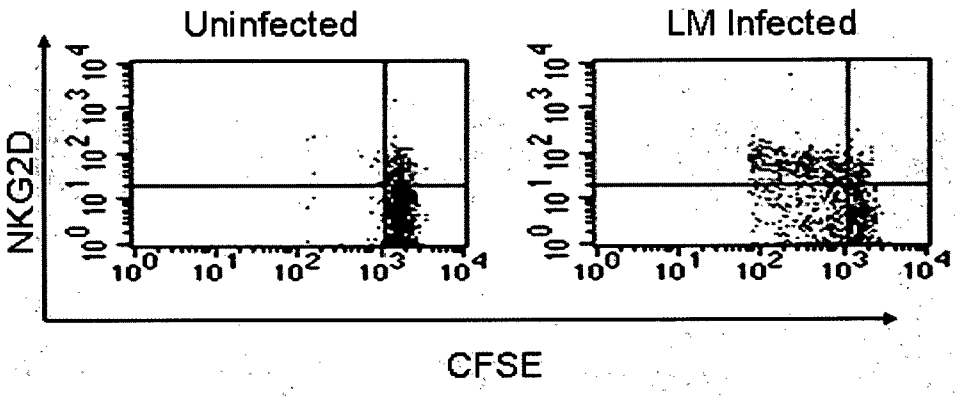


Figure 3.6 NKG2D enhances the killing of target cells by male H-Y $CD8^{lo}$ cells in both an MHC restricted and non-MHC restricted fashion.

a)



b)

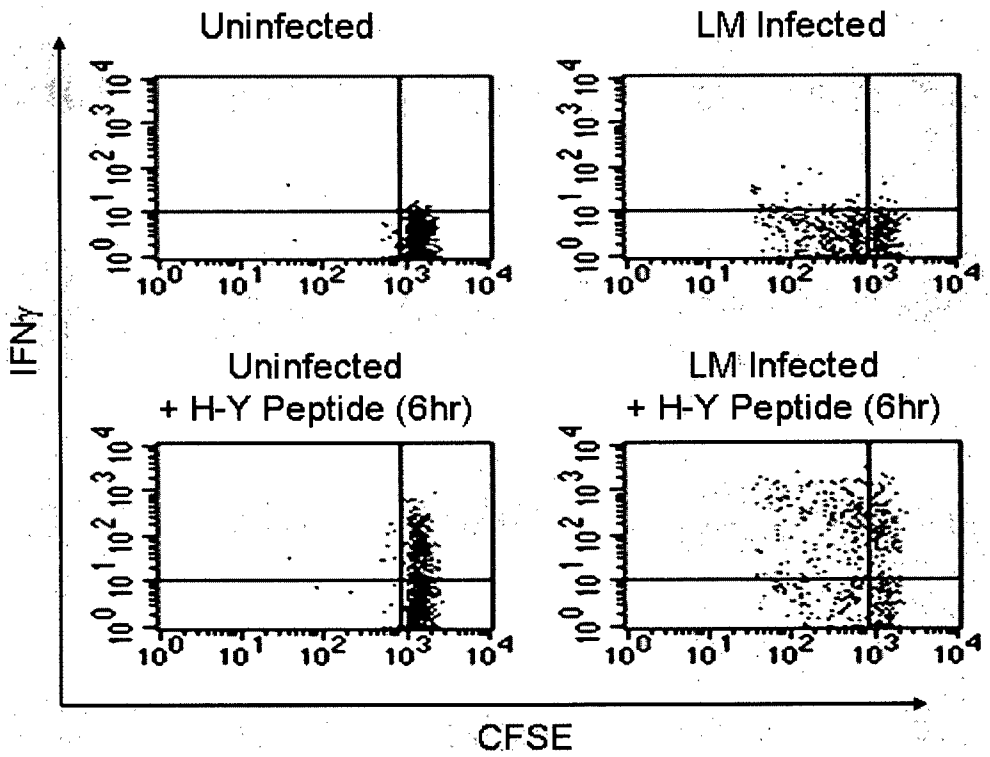


Figure 3.7 Bacterial infection primes $CD8^{lo}$ cells in vivo.

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Chapter 4 The Low Affinity Fc Receptor for IgG Functions as an Effective Cytolytic Receptor for Self-Specific CD8 T Cells[1]

4.1 Abstract

We have recently described a population of self-antigen specific murine $CD8^+$ T cells with a memory phenotype, which utilize receptors of both the adaptive and innate immune systems in the detection of transformed and infected cells. Here we show that upon activation with IL-2 ± Ag, between 10 and 20% of the activated self-specific $CD8^+$ T cells express the low affinity Fc receptor for IgG. By contrast, all IL-2-activated NK cells express high levels of this Fc receptor. The Fc receptor comprises the FcγRIIIα and FcRγ subunits. However, the FcRγ subunit also associates with the CD3 complex and this association likely contributes to the low expression of the Fc receptor in the activated cells. Although the Fc receptor is expressed at a low level on activated self-specific $CD8^+$ T cells, it functions very efficiently as a cytolytic receptor in ADCC. Fc receptor-dependent killing occurred in the absence of TCR stimulation but could be augmented by concurrent stimulation of the TCR. In addition to mediating ADCC, engagement of the FcR on self-specific $CD8^+$ T cells results in the production of both IFNγ and TNFα. This is the first report of an activating Fc receptor on self-specific murine $CD8^+\alpha\beta$ TCR^+ T cells and further establishes the importance of innate immune system receptors in the function of these self-specific $CD8^+$ T cells.

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[1] A version of this chapter has been published as:

Dhanji, S., K. Tse, and H. S. Teh. 2005. The low affinity Fc receptor for IgG functions as an effective cytolytic receptor for self-specific CD8 T cells. *J Immunol 174:1253*

4.2 Introduction

Natural killer (NK) cells represent a highly specialized lymphoid population characterized by potent cytolytic activity against tumor or virally infected cells. Their function is finely regulated by a series of inhibitory or activating receptors (1). The inhibitory receptors, specific for major histocompatibility complex (MHC) class I molecules, allow NK cells to discriminate between normal cells and cells that have lost the expression of MHC class I (e.g., tumor cells). Inhibitory receptors such as those belonging to the Ly49 family in mice and the killer Ig-like receptors in humans contain immunoreceptor tyrosine based inhibitory (ITIM) motifs in their cytoplasmic domains (2). Engagement of these receptors results in the recruitment of inhibitory phosphatases that prevent NK cell activation. The activating receptors responsible for NK cell triggering include NKp46, NKp30, NKp44 and NKG2D in humans and NKp46 and NKG2D in mice (3, 4). The activating receptors, some of which belong to the same families, do not contain activation motifs but rather associate with adaptor molecules or signaling partners important for signal transduction (5).

One of the first activating receptors described on NK cells is FcγRIIIα or CD16. CD16 is a low affinity Fc receptor (FcR) that binds to IgG and is involved in antibody dependent cell-mediated cytotoxicity (ADCC) in which an antibody coated target cell is destroyed by NK cells (6). Stimulation of CD16 on NK cells also results in the production of cytokines such as IFNγ, TNFα and GM-CSF (7). FcγRIIIα associates mainly with immunoreceptor tyrosine based activating motif

(ITAM)-containing homo or heterodimers of CD3ζ and FcεRIγ (FcRγ) in humans (8) or solely with FcRγ homodimers in mice (9). The binding of IgG to CD16 results in the phosphorylation of the ITAMs in the signaling chains leading to the recruitment of kinases such as ZAP-70 and Syk (10). These kinases initiate a signaling cascade resulting in the lysis of antibody-coated target cells. T cells also use ITAM containing receptors and Syk and ZAP-70 for their signal transduction [reviewed in (11)]. Thus the signals transduced by the engagement of CD16 on NK cells are very similar to those transduced via the engagement of the TCR on T cells.

CD16 expression is not limited to just NK cells as other cell types have been described which also express this receptor. γδ T cells have been shown to express CD16 as have a population of large granular lymphocytes (mostly $\alpha\beta TCR^+CD4^-CD8^-$ T cells) in humans (12, 13). In addition some memory-phenotype $\alpha\beta TCR^+CD8^+$ T cells in humans have been shown to express CD16 (14). We have previously described a population of $\alpha\beta TCR^+CD8\alpha\beta^+$ T cells in normal B6 mice that exhibit a memory phenotype characterized by the expression of high levels of CD44, IL-2Rβ, and Ly6C (15). These cells can be activated by cytokines such as IL-2 and IL-15 and upon activation they express several functional NK receptors including 2B4, CD94, and NKG2D as well as the NK adaptor protein DAP12. Using an H-Y TCR transgenic model, we have shown that the development of $CD8^+CD44^{hi}$ T cells is driven by the high affinity interaction of the αβTCR with cognate self-antigen (16). The H-Y TCR is specific for a male antigen (H-Y) presented by $H\text{-}2D^b$ and in $H\text{-}2^b$ H-Y female mice, the

lack of male antigen results in the development of H-Y CD8 T cells with a naïve phenotype ($CD44^{lo}$). In $H\text{-}2^b$ H-Y male mice, on the other hand, the presence of the cognate (H-Y) antigen results in the development of a population of $CD8^+$ T cells that are virtually identical in cell surface and functional phenotypes with the memory phenotype $CD8^+CD44^{hi}$ cells in normal B6 mice (16). Since memory phenotype $CD8^+$ T cells in normal and H-Y TCR transgenic mice are specific for self-antigens we will refer to these cells as self-specific $CD8^+$ T cells to distinguish them from conventional memory $CD8^+$ T cells. Self-specific $CD8^+$ T cells from both H-Y TCR transgenic (16) and non-transgenic (15) mice preferentially kill syngeneic tumor cells. This killing of syngenic tumors involves the MHC-restricted $\alpha\beta$ TCR as well as the activating NK receptor, NKG2D, which results in non-MHC-restricted lysis of target cells that express the NKG2D ligand, Rae-1 (16).

In this report we have described the expression and function of CD16 in self-specific $CD8^+$ cells from B6 and male H-Y TCR transgenic mice. We showed that this Fc receptor, comprising the $Fc\gamma RIII\alpha/FcR\gamma$ subunits, is similar in composition to the NK Fc receptor. Although this Fc receptor is expressed at a low level in self-antigen specific $CD8^+$ T cells, it is particularly efficient at initiating the destruction of antibody coated target cells and can induce the production of two key inflammatory cytokines, $IFN\gamma$ and $TNF\alpha$. These observations underscore the importance of innate immune system receptors in the function of these self-specific $CD8^+$ T cells.

4.3 Materials and Methods

Mice

Breeders for C57BL/6 (B6) mice were obtained from the Jackson Laboratories (Bar Harbor, ME). The H-Y TCR transgenic mice (17) were bred to the B6 background. Mice 6 to 12 weeks of age were used for the experiments described.

Abs and flow cytometry

The following antibodies were used: anti-CD4 (GK1.5), anti-CD8α (53.67), anti-CD8β (53.38), anti-NK1.1 (PK136), anti-CD3ε (2C11), anti-CD90 (T24, Rat IgG)(18), anti-CD16/32 (2.4G2, Rat IgG2b), anti-CD44 (PGP1), anti-FcRγ, anti-CD3ζ (19) and anti-H-Y TCRα (T3.70) (17). All antibodies were purchased from BD PharMingen (San Diego, CA) except anti-FcRγ (Upstate Biotechnology, Lake Placid, NY), anti-CD16/32 (American Type Tissue Culture Collection, Rockville, MD) and anti-H-Y TCRα (eBioscience, San Diego, CA). The CellQuest software program (Becton Dickinson, Mountain View, CA) was used for data acquisition and analysis.

Cell lines

Cell lines used were the RMA lymphoma (H-2^{b+}, Rae-$1\delta^{-}$) and Tap-deficient RMAS (H-2^{b-}, Rae-$1\delta^{-}$). The cell lines were cultured in IMEM (Life Technologies, Burlington, Canada), supplemented with 10% (v/v) FBS (Life Technologies), $5x10^5$ μM 2-ME and antibiotics (I-medium).

Cell purification and activation

$CD8^+CD44^{hi}$ T cells from B6 mice were purified and cultured in IL-2 (200U/ml) for 5 days as previously described (15). NK cells were enriched by depletion of $CD4^+CD8^+CD3^+Ig^+$ cells using Dynabeads and then cultured in IL-2 for 5 days resulting in a pure population of activated NK cells (15). Purified naive $CD8^+$ ($CD44^{lo}$) cells do not respond to IL-2 alone and were activated for 5 days on plate-bound anti-CD3ε (10μg/ml) and IL-2 (20U/ml). Purified H-Y TCR^+ $CD8^+$ T cells ($1x10^6$) were activated by culturing with $1x10^7$ B6 splenocytes, 1μM H-Y peptide and 20U/ml IL-2 for 5-6 days.

RT-PCR

RNA was extracted from activated cells and reverse-transcribed as previously described (15). PCR was preformed using previously described primers and reaction conditions (20).

CTL Assays

CTL assays against RMA and RMAS target cells were performed as previously described (15). For antibody-dependent cell-mediated cytoxicity (ADCC), the target cells were pre-treated with anti-CD90 mAb (10μg/ml) for 15min at room temperature prior to use. For the FcR-blocking experiment, anti-FcR mAb (2.4G2 used at 15μg/ml) was added to the effector cells 15 min prior to the addition of targets and was present throughout the assay. Spontaneous release varied from 8-15% of the maximum. All assays were performed in triplicate. Percent specific lysis was calculated as 100% x [cpm (experimental well) – cpm (spontaneous release)]/[(cpm (maximum release) – cpm (spontaneous release)].

Immunopreceipitation and immunoblot analysis

Cells were activated as described above and pelleted/lysed in 10 mM Tris (pH 7.5), 150 mM NaCl, 1% TX-100, 0.1% SDS, and protease and phosphatase inhibitors. The lysates were separated on a 4-15% Tris-HCl polyacrylamide gel and transferred to a PVDF membrane. Blots were developed using ECL system (Amersham). CD3 immunoprecipitation was preformed by treating lysates with pre-conjugated anti-CD3ε/Protein G-sepharose beads for 2 hours at 4°C followed by several washes. Complexes were then removed from the beads by resuspension in 2X protein sample buffer followed by boiling for 5 mins. The samples were then run in a 4-15% Tris-HCl gel and immunoblotted as described above.

4.4 Results

Expression of FcγRIIIα/FcRγ on activated self-specific CD8+ cells.

We have previously shown that self-specific $CD8^+$ T cells express an activated/memory phenotype (15, 16). Immediately ex vivo self-specific $CD8^+$ cells from B6 and male H-2^b H-Y mice do not express significant levels of CD16 (Fig. 4.1a and data not shown). However, upon activation with IL-2 alone (B6) or antigen and IL-2 (H-Y male) for 5-6 days, between 10 and 15% of the activated self-specific $CD8^+$ cells from B6 ($CD8^+CD44^{hi}$) and male H-2^b H-Y mice express CD16 (Fig. 4.1a). In contrast, all IL-2-activated NK cells express high levels of CD16 (Fig. 4.1a). Since naive ($CD8^+CD44^{lo}$) cells from B6 and female H-Y TCR transgenic mice do not proliferate in response to stimulation with IL-2 alone these cells were activated with anti-CD3 + IL-2 (B6) or antigen plus IL-2 (H-Y female) to induce activation and proliferation. It is clear that activated $CD8^+CD44^{lo}$ cells from B6 and female H-Y TCR transgenic mice do not express CD16 (Fig. 4.1a) consistent with the conclusion that conventional CD8 T cells do not express CD16 upon activation.

We have previously described the bystander expansion and activation of self-specific CD8 T cells in response to infection with the bacterial pathogen Listeria monocytogenes (LM) (15, 16). Therefore we wanted to determine whether the frequency of $CD16^+$ $CD8^+CD44^{hi}$ increased upon infection with LM. In uninfected B6 mice there is a small percentage of $CD8^+$ T cells that express CD16 but this number increases by about 3 fold upon infection with Listeria (Fig.

4.1b). In addition all of the $CD8^+CD16^+$ cells in both infected and uninfected mice express high levels of CD44.

The antibody used to detect CD16 expression also binds to CD32 and it was important to determine which Fc receptor subunits were actually expressed by the self-specific $CD8^+$ cells. RT-PCR with primers specific for various Fc receptor subunits was used to determine the composition of the expressed Fc receptor. It is clear from this analysis that activated self-specific $CD8^+$ cells from B6 and male H-2^b H-Y mice as well as NK cells from B6 mice express the mRNA for only FcγRIIIα (CD16) and FcRγ but not FcγRI or FcγRIIB (Fig.4.2a). A macrophage cell line was used as a positive control for the expression of FcγR1 and FcγRIIB (Fig. 4.2a).

In murine NK cells, CD16 can only pair with FcRγ homodimers (9). Furthermore, the forced expression of CD3ζ in murine NK cells actually interferes with the surface expression and function of CD16 through the formation of CD3ζ/FcRγ heterodimers, which cannot associate with CD16 (21).This finding suggests that the low cell-surface expression of CD16 on self-specific $CD8^+$ cells may be due to the high expression of CD3ζ in these cells. To address this possibility, we compared the total amount of CD3ζ and FcRγ in activated NK and self-specific $CD8^+$ cells by western blot. Figure 4.2b shows that activated NK cells express an undetectable level of CD3ζ and the most FcRγ when compared to self-specific $CD8^+$ cells from B6 ($CD8^+CD44^{hi}$) and H-Y male mice. By contrast, self-specific $CD8^+$ cells from B6 and H-Y male mice express both CD3ζ and FcRγ (Fig. 4.2b). As expected, activated conventional CD8 ($CD8^+CD44^{lo}$)

cells only express CD3ζ. These findings suggest that the low levels of CD16 surface expression in self-specific $CD8^+$ cells may be due to the expression of CD3ζ in these cells.

In conventional T cells the αβ TCR pairs with the CD3-family of signaling chains including CD3ε and CD3ζ (22). Since FcRγ is expressed in self-specific $CD8^+$ cells we wanted to determine if this signaling molecule could associate with the TCR/CD3 complex in these cells. To determine whether FcRγ associates with the CD3 complex, we immunoprecipitated the CD3 complex from IL-2-activated self-specific $CD8^+$ cells from B6 and male H-Y mice using an anti-CD3ε antibody and immunoblotted with either an anti-CD3ζ or anti-FcRγ antibody. Figure 4.2c clearly shows that anti-CD3ε precipitates both CD3ζ and FcRγ in self-specific $CD8^+$ cells from B6 and male H-Y mice. As expected, anti-CD3ε precipitates only CD3ζ in conventional $CD8^+CD44^{lo}$ cells. This association of FcRγ with the CD3 complex in self-specific $CD8^+$ cells likely interferes with the association of CD16 with FcRγ, resulting in low expression of CD16 in these cells.

IL-2-activated self-specific CD8+ cells can mediate ADCC.

After observing the expression of CD16 on IL-2-activated self-specific $CD8^+$ cells we determined whether this receptor could mediate ADCC. To this end, we activated self-specific $CD8^+$ cells and NK cells from B6 mice with IL-2 and then tested their ability to kill antibody-coated RMAS targets cells. Anti-CD3 + IL-2 activated naive CD8 ($CD8^+CD44^{lo}$) cells were included as a negative control. TAP-deficient RMAS cells were used as target cells to rule out contribution by

MHC class I molecules in the killing reaction. The RMAS cells were left untreated or pretreated with anti-CD90 (clone T24; 10μg/ml) mAb prior to use as target cells. Figure 4.3a clearly shows that IL-2-activated self-specific $CD8^+$ and NK cells can efficiently kill antibody-coated RMAS cells whereas anti-CD3 + IL-2-activated conventional CD8 ($CD8^+CD44^{lo}$) cells show absolutely no activity. We noted that anti-CD90 was more efficient in promoting the killing of RMAS targets by self-specific $CD8^+$ cells compared to NK cells. This finding is remarkable considering that only a small fraction of the self-specific $CD8^+$ cells express CD16 and the level of CD16 expressed per cell is significantly lower than for NK cells (Fig 4.1a). Since RMAS cells are killed efficiently by activated NK cells, the lack of killing of untreated RMAS cells by activated self-specific $CD8^+$ cells also indicates the lack of contaminating NK cells in the killing assay.

After observing that self-specific $CD8^+$ cells from B6 mice could efficiently lyse RMAS targets we determined if the presence of MHC class I had any effect on lysis by using TAP-sufficient RMA targets. In addition we wanted to determine whether blocking of the CD16 receptor with an anti-CD16 mAb on the activated self-specific $CD8^+$ cells could block killing of antibody-coated target cells. Figure 4.3b demonstrates that self-specific $CD8^+$ cells efficiently killed antibody-coated RMA cells. Furthermore, the killing of antibody-coated target cells was greatly reduced by blocking the CD16 receptor on self-specific $CD8^+$ cells prior to culturing with antibody-coated RMA targets. For NK cells the lysis of antibody-coated RMA cells was only partially inhibited by blocking CD16 on the NK cells. This is probably due to the high expression of CD16 on NK cells, which could not

be blocked completely by the anti-CD16 antibody treatment. An alternative explanation for the inefficient blocking of killing of antibody coated target cells by NK cells is that the anti-CD16 mAb functions as an agonistic mAb. However, this is unlikely since the treatment of NK cells with anti-CD16 mAb did not have any effect on the lysis of untreated RMA target cells (data not shown). These results clearly demonstrate that self-specific $CD8^+$ cells express a functional FcR, which can mediate ADCC. Furthermore, expression of MHC class I molecules on the target cells does not affect FcR-mediated killing.

The Fc receptor on self-specific CD8+ cells from H-Y male mice functions independently of the TCR.

Since self-specific $CD8^+$ cells from male H-Y mice express only the male-specific H-Y TCR we used these cells to determine whether the Fc receptor can function independently of the TCR. Self-specific $CD8^+$ cells from male H-Y mice and conventional $CD8^+$ cells from female H-Y mice were activated with antigen and IL-2 for 6 days. The activated cells were then assessed for cytolytic activity against untreated or anti-CD90-treated RMA ($H\text{-}2^b$) target cells. The killing of anti-CD90-coated target cells in the absence of exogenous H-Y peptide was used as a measure for the contribution of the Fc receptor in the killing reaction. Inclusion of the H-Y peptide in the assay allows an estimation of the contribution of the H-Y TCR in the killing reaction. It is clear from the data in Fig. 4.4 that self-specific $CD8^+$ cells from male H-Y mice killed antibody-coated RMA targets very efficiently even in the absence of the H-Y peptide. The killing of anti-CD90-

coated target cells was slightly enhanced by the addition of H-Y peptide (Fig. 4.4). The activated self-specific $CD8^+$ cells from male H-Y mice required almost 10nM exogenous H-Y peptide to attain the same level of killing as seen with anti-CD90-coated target cells in the absence of H-Y peptide. This is remarkable since the entire population of H-Y male cells expressed the H-Y TCR whereas at most 20 percent of the cells expressed CD16. By contrast, activated conventional $CD8^+$ T cells from female H-Y mice could only kill peptide-loaded target cells and the presence of antibody on the targets had no effect on this killing. These results suggest that CD16 functions independently of the TCR as an effective cytolytic receptor on self-specific $CD8^+$ cells. Furthermore, the Fc receptor and the TCR can act in an additive manner in the killing reaction.

Engagement of CD16 on self-specific CD8+ cells induces cytokine production. CD16 engagement on NK cells has been shown to induce the expression of several cytokines in addition to being able to induce ADCC (7). In order to test whether CD16 engagement on self-specific CD8 T cells could also mediate the production of cytokines we cultured antigen-activated H-Y male CD8 cells with RMA targets which had been pre-treated with anti-CD90 or left untreated. We found that these cells showed a significant increase in IFN-γ production in response to anti-CD90-coated RMA cells (7.2% IFN-γ^+) over untreated RMA cells (1.3%). Furthermore, this increase in IFN-γ production was reduced to near basal level by the inclusion of soluble anti-CD16 mAb in this assay (Fig. 4.5a). This

result suggests that the Fc portion of the bound anti-CD90 mAb on RMA cells induces the production of IFN-γ by H-Y male CD8 cells.

In order to obtain more direct evidence for the Fc receptor-mediated cytokine production by H-Y male CD8 cells, we used antibody cross-linking to stimulate CD16 directly. We cultured day 6 antigen-activated H-Y male CD8 T cells in wells coated with either no antibody (upper row), anti-CD16 (middle row), or anti-CD3ε (bottom row). After a 5 hr incubation period, we fixed and stained the cells for CD8 and IFN-γ (left column), TNF-α (middle column) or GM-CSF (right column). CD16 engagement resulted in a large increase in both IFN-γ and TNF-α production with the percentage of cytokine positive cells being similar to the percentage of $CD16^+$ cells in the sample; CD16 engagement did not induce the production of GM-CSF (Fig. 4.5b). We also found that soluble anti-CD16 is inefficient in inducing IFN-γ production by H-Y male CD8 cells (data not shown). It is likely that soluble anti-CD16 mAb is less efficient than plate-bound anti-CD16 mAb in aggregating Fc receptors and receptor aggregation is required for efficient activation. By contrast to Fc receptor stimulation, stimulation of the CD3 complex resulted in the majority of the cells expressing IFN-γ and TNF-α. In addition, anti-CD3 stimulated cells produced significant amount of GM-CSF (Fig. 4.5b). These results indicate that the Fc and the αβ TCR function as directly activating receptors and which transduce qualitatively and quantitatively distinct signals upon activation.

4.5 Discussion

In this study, we showed that upon activation with IL-2 or antigen plus IL-2, self-specific $CD8^+$ cells express an Fc receptor that is similar in composition to the low affinity Fc receptor for IgG that is found on NK cells. Even though these cells express only a relatively low level of this receptor when compared to NK cells, it functions very efficiently in the lysis of antibody-coated target cells. Fc receptor-mediated killing is independent of antigen expression on the target cells. Furthermore, expression of MHC class I molecules on the target cells did not affect the efficiency of Fc receptor-mediated killing. The Fc receptor can also act in an additive manner with the TCR in the lysis of the susceptible target cells. This Fc receptor not only mediates efficient lysis of susceptible targets, but it also induces the production of both IFNγ and TNFα. The combination of these properties would enable self-specific $CD8^+$ T cells to detect infected or transformed target cells which might not be detected by NK cells.

It is interesting to note that even though the H-Y male cells represent a clonal population in which all cells should have been activated equivalently, only a fraction of the activated cells express CD16. The low cell-surface expression of CD16 on self-specific $CD8^+$ cells is likely due to the high expression of CD3ζ in these cells. In murine NK cells, CD16 can only pair with FcRγ homodimers (9). Furthermore, the expression of CD3ζ in murine NK cells interferes with the surface expression and function of CD16 through the formation of CD3ζ/FcRγ heterodimers, which cannot associate with CD16 (21). We found that the FcRγ

chain in self-specific $CD8^+$ T cells is coprecipitated with CD3ζ by the anti-CD3ε mAb. It is likely that this association of FcRγ with the CD3ζ chain contributes to the low expression of the Fc receptor on IL-2-activated self-specific $CD8^+$ T cells. The association of the FcRγ chain with CD3ζ is not unique to self-specific $CD8^+$ T cells since in large granular lymphocytes and in T cells from tumor-bearing mice, the CD3 complex has been shown to associate with FcRγ (23, 24). The association of the αβ TCR with the FcRγ chain would enable the αβ TCR of these cells to be linked to additional signaling pathways.

We have previously shown that self-specific $CD8^+$ cells undergo bystander expansion in vivo in response to *Listeria* infection, likely as a consequence of the high expression of IL-2Rβ by these cells, which endowed these cells to proliferate in response to IL-2 or IL-15 (16). Furthermore, self-specific $CD8^+$ T cells that proliferate in response to bacterial infection exhibit a heightened ability to produce IFN-γ (16). These properties of self-specific $CD8^+$ T cells would enable them to detect infected cells and provide an early source of IFN-γ. The expression of a self-specific TCR and NKG2D on these cells would enable them to focus on host cells that expressed ligands induced by infection or transformation. Here we have provided evidence for the expression and functional significance of another activating NK receptor that adds to the arsenal of self-specific $CD8^+$ cells. We have shown that CD16 is expressed on a significant fraction of $CD8^+CD44^{hi}$ T cells upon *Listeria* infection in vivo or upon activation with IL-2 or antigen and IL-2 in vitro. Furthermore, engagement of CD16 on self-specific $CD8^+$ T cells results in efficient lysis of antibody-coated

targets as well as in the production of inflammatory cytokines. The possession of activating receptors of the innate as well as the adaptive immune system distinguished these cells from NK cells and suggests that this interesting cell type may be particularly adept in providing an early response to infected and transformed cells. These cells will also provide an early source of cytokines such as IFN-γ and TNFα, which would prime the adaptive immune system in the elimination of infected and transformed cells.

4.6 Acknowledgements

We thank Soo-Jeet Teh for excellent technical assistance and Dr. Hao Shen (University of Pennsylvania) for the kind gift of the wildtype *Listeria monocytogenes* strain 10403s.

4.7 Figures

Fig. 4.1 Activation of $CD8^+CD44^{hi}$ cells results in the expression of CD16. a) Purified $CD8^+CD44^{hi}$ cells from B6 and CD8 cells from male H-2^b H-Y TCR transgenic mice were used as a source of self-specific CD8 T cells. Purified $CD8^+CD44^{lo}$ cells from B6 and female H-2^b H-Y TCR transgenic mice were used as a source of naive CD8 T cells. Self-specific B6 $CD8^+$ T cells and NK cells were activated by culturing with IL-2 for 5 days. Naive CD8 T cells from B6 mice were activated by culturing with anti-CD3 + IL-2 for 5 days and CD8 cells from male and female H-Y mice were activated with Ag + IL-2 for 6 days. The activated cells were stained for the expression of CD16/CD32. The filled histograms represent the CD16/CD32 profile of the indicated cell type and the unfilled histograms represent unstained controls. b) Naïve B6 mice were infected with 10,000 CFU of *Listeria monocytogenes*. On day 5 the mice were sacrificed and the spleens removed and stained. The histograms depict the expression of CD8 and CD16 on gated CD8 T cells from infected (right) or uninfected (left) mice and the percentages of $CD16^+$ $CD8^+$ T cells are indicated. Three-color analysis revealed that the $CD8^+CD16^+$ cells also expressed high levels of CD44 (data not shown).

Fig. 4.2 Activated self-specific CD8 T cells express a low affinity Fc receptor similar to NK cells. Cells were activated as described in Fig. 4.1. a) RT-PCR analysis of FcγRIIIα, FcRγ, FcgRI, and FcgRIIB transcripts present in activated

cells. RNA from the J774 macrophage cell line was used as a positive control for FcγRI and FcγRIIB. b) Whole cell lysates of the activated cells were subjected to immunoblot analysis for the detection of FcRγ and CD3ζ protein. Blots were stripped and re-probed with anti-ERK2 as a loading control. c) Activated cells were lysed and the lysates were immunoprecipitated (IP) with anti-CD3ε antibody. The immunoprecipitates were then immunoblotted (IB) with anti-CD3ζ or anti-FcRγ mAbs. (N.D. – not determined)

Fig. 4.3 $CD8^+CD44^{hi}$ cells efficiently kill antibody-coated targets via an Fc-dependent mechanism. a) NK, self-specific CD8 ($CD8^+CD44^{hi}$) and naive CD8 ($CD8^+CD44^{lo}$) cells from B6 mice were activated as described in Fig. 4.1 and used as effectors in a standard chromium release assay against TAP-deficient RMAS target cells that had been treated with anti-CD90 mAb (10μg/ml) or left untreated. b) Activated NK and $CD8^+CD44^{hi}$ cells were used as effectors in a chromium release assay against MHC-sufficient RMA target cells that had been treated with anti-CD90 mAb (10μg/ml) or left untreated. The effector cells were also pretreated with anti-CD16 mAb (10μg/ml) or left untreated in order to block the Fc receptor. Error bars represent the standard deviation of triplicate cultures.

Fig. 4.4 CD16-mediated killing of target cells by self-specific CD8 T cells is TCR-independent. Self-specific CD8 T cells from H-2^b H-Y male mice and naive CD8 T cells from female H-2^b H-Y mice were activated as described in Fig. 4.1 and used in a standard chromium release assay against RMA targets either pre-

treated with anti-CD90 mAb (10μg/ml) or left untreated. The assay was done at a constant 10 to 1 effector to target ratio with the addition of the indicated concentrations of H-Y peptide. Error bars represent the standard deviation of triplicate cultures.

Fig. 4.5 Cytokine production by self-specific CD8 T cells in response to CD16 engagement. a) Day 6 antigen + IL-2 activated H-Y male CD8 cells were cultured with RMA cells treated with anti-CD90 (10μg/ml) or left untreated. Soluble anti-CD16 (10μg/ml) was added to some cultures to block Fc binding. IFN-γ production was assessed by intracellular staining after a 5 hr incubation period. b) Day 6 antigen + IL-2 activated H-Y male CD8 cells were cultured with plate bound anti-CD16 (10μg/ml), anti-CD3ε (10μg/ml), or without any antibody for 5 hours. The cells were then stained for intracellular IFN-γ, TNF-α, and GM-CSF. The numbers in the dot plots represent the percentage of cytokine-positive $CD8^+$ T cells.

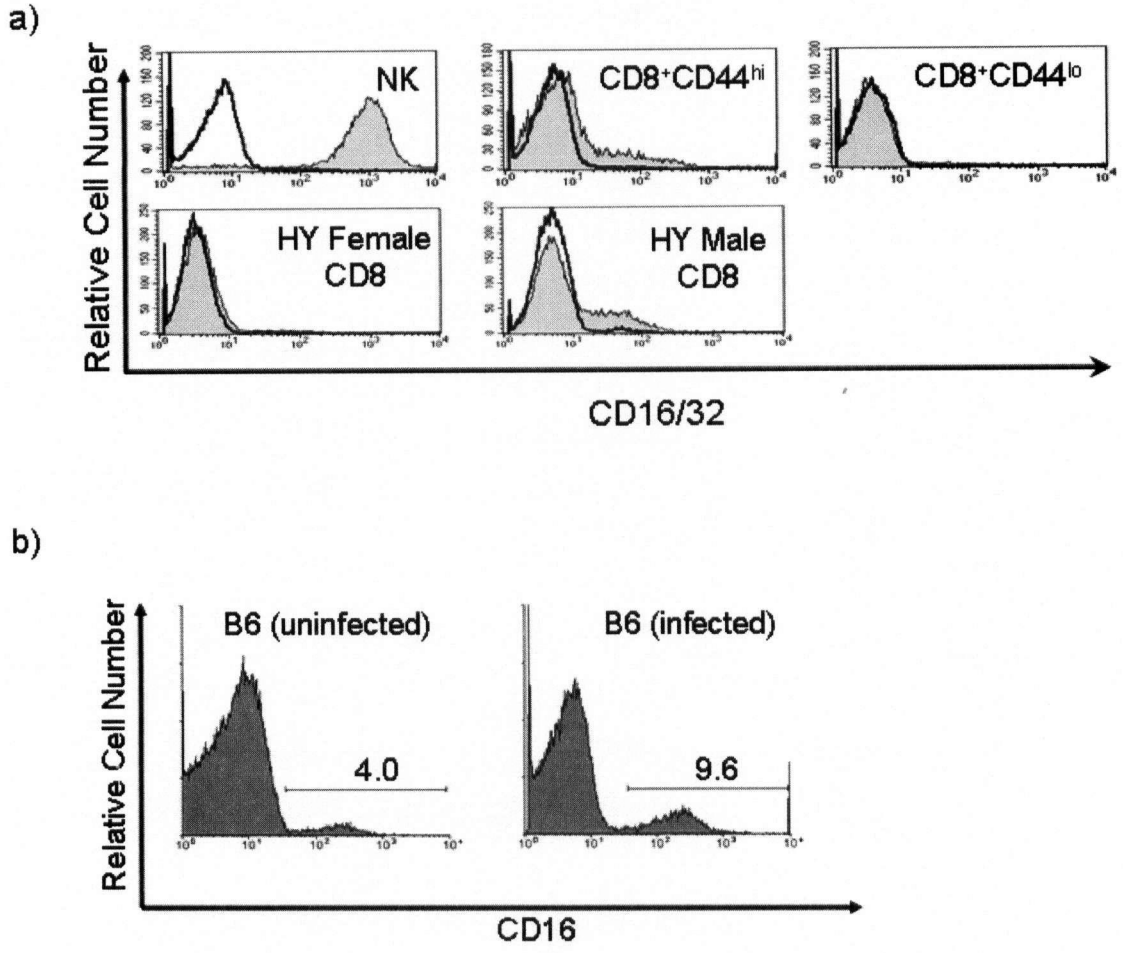

Fig. 4.1 Activation of $CD8^{+}CD44^{hi}$ cells results in the expression of CD16

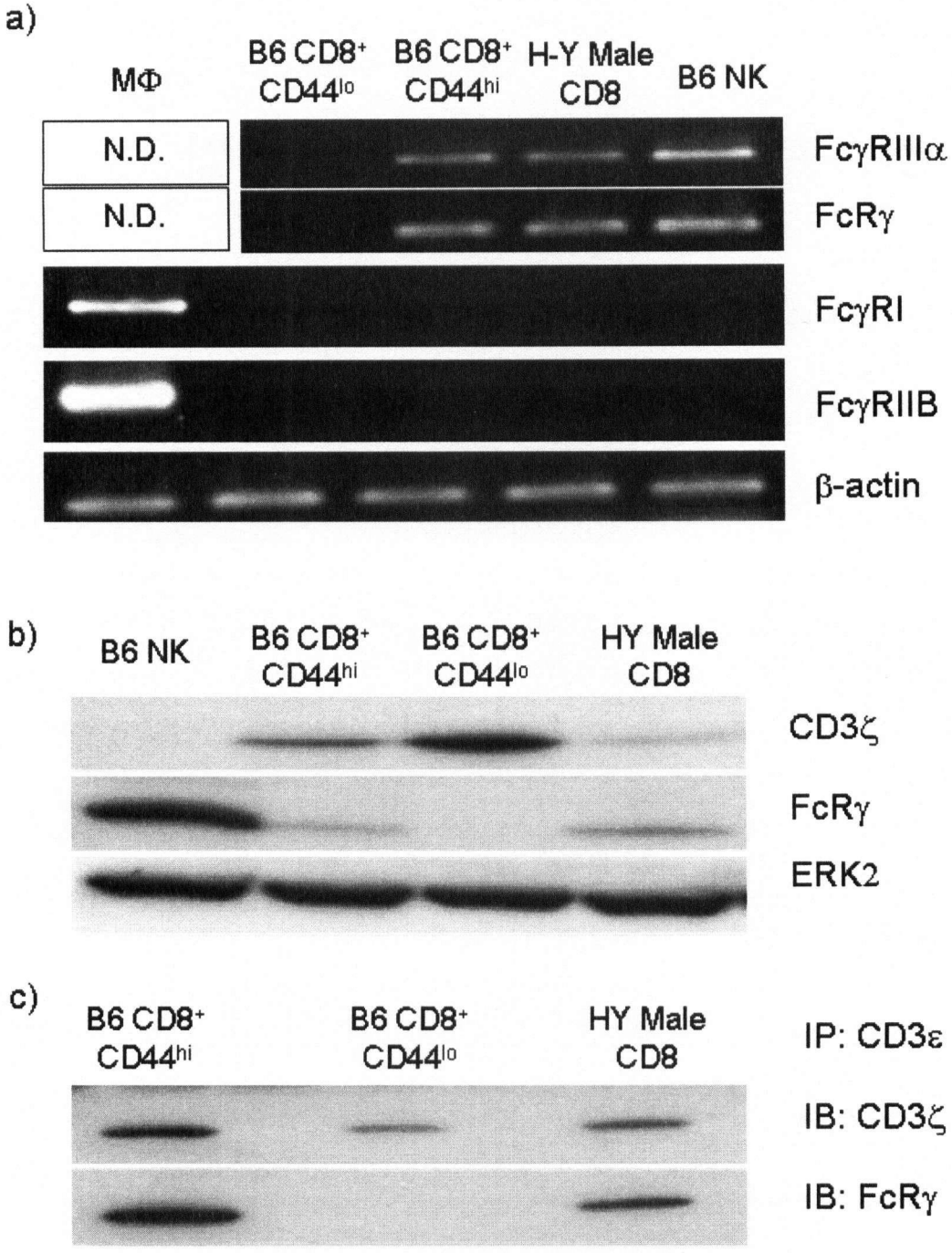

Fig. 4.2 Activated self-specific CD8 T cells express a low affinity Fc receptor similar to NK cells

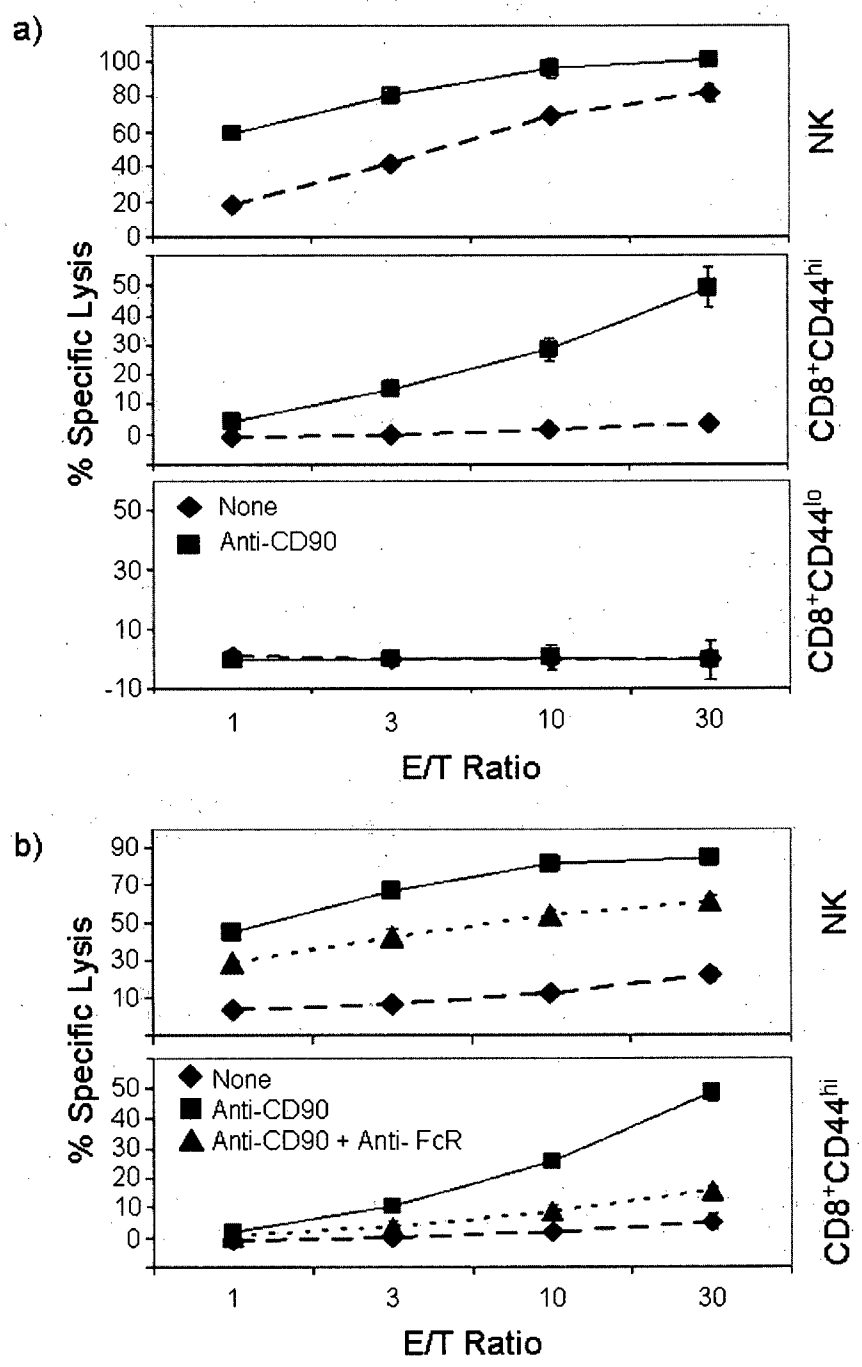

Fig. 4.3 $CD8^{+}CD44^{hi}$ cells efficiently kill antibody-coated targets via an Fc-dependent mechanism

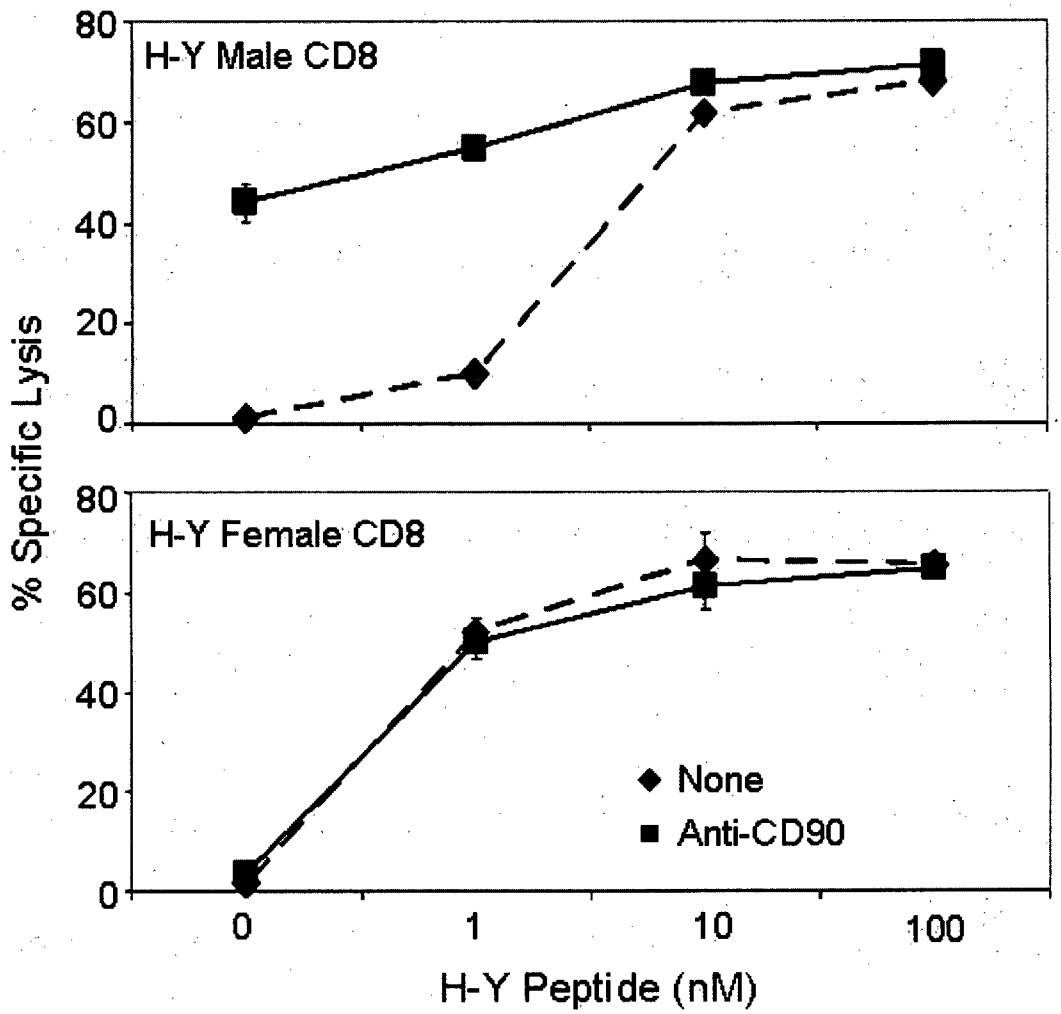

Fig. 4.4 CD16-mediated killing of target cells by self-specific CD8 T cells is TCR-independent

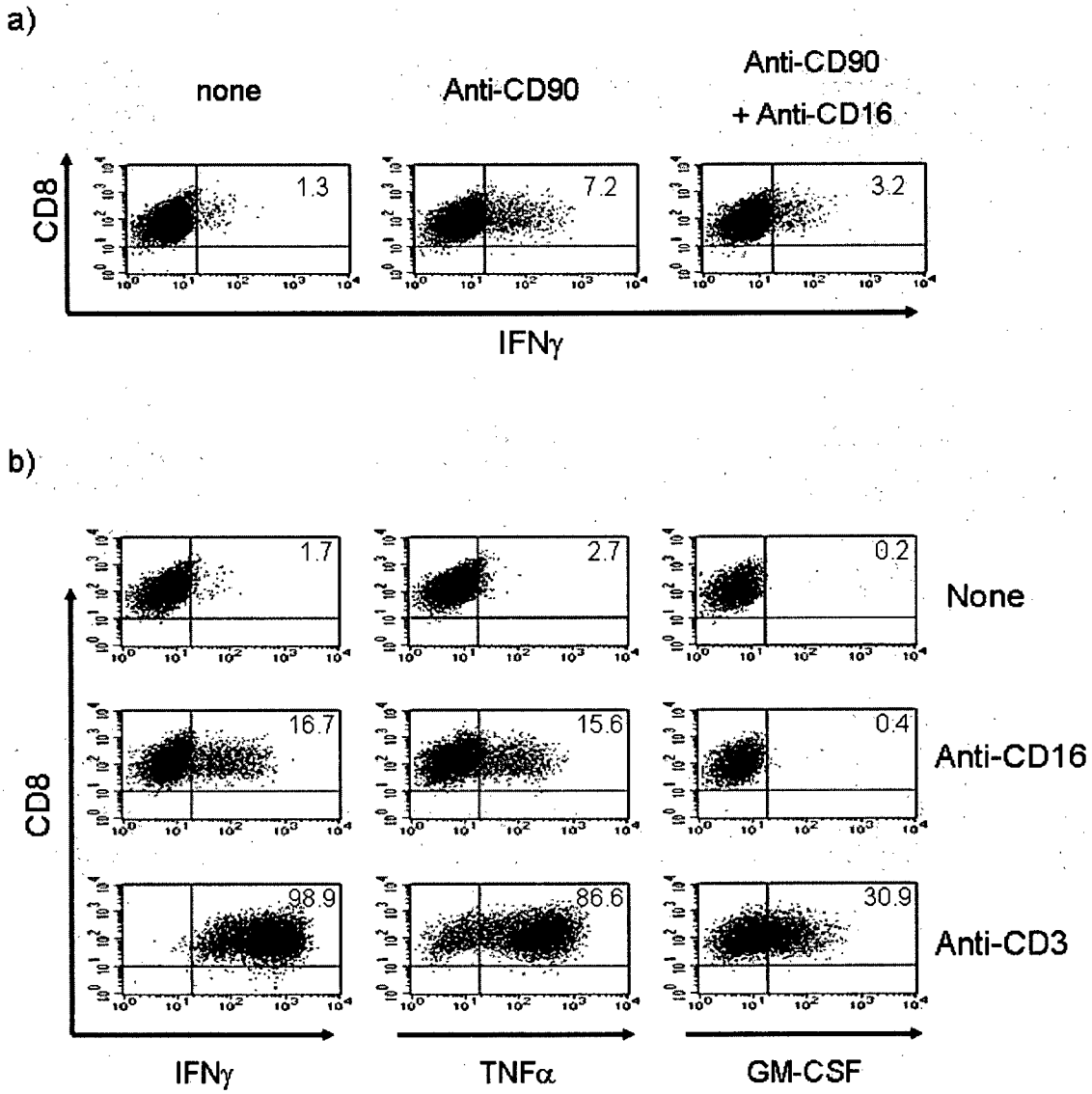

Fig. 4.5 Cytokine production by self-specific CD8 T cells in response to CD16 engagement

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Chapter 5 Self-Antigen Maintains the Innate Anti-Bacterial Function of Self-Specific CD8 T Cells in Vivo[1]

5.1 Abstract

Self-specific CD8 T cells, which are selected by high affinity interactions with self-antigens, develop into a lineage distinct from conventional CD8 T cells. We have previously shown that these self-specific cells acquire phenotypic and functional similarities to cells of the innate immune system including the expression of functional receptors associated with NK cells. Here we show that these self-specific cells have the ability to produce large amounts of IFNγ in response to infection with *Listeria monocytogenes* in a bystander fashion. The rapid production of IFNγ is associated with a dramatic reduction in the number of viable bacteria at the peak of infection. Self-specific CD8 T cells provide only marginal innate protection in the absence of self-Ag; however, the presence of self-Ag dramatically increases their protective ability. Exposure to self-Ag is necessary for the maintenance of the memory phenotype and responsiveness to inflammatory cytokines such as IL-15. Significantly, self-specific CD8 T cells are also more efficient in the production of IFNγ and TNFα, thus providing more cytokine-dependent protection against bacterial infection when compared to NK cells. These findings illustrate that self-reactive CD8 T cells can provide an important innate function in the early defense against bacterial infection.

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[1] A version of this chapter has been accepted for publication as:

Dhanji, S., M.T. Chow, and H. S. Teh. 2006. Self-Antigen Maintains the Innate Anti-Bacterial Function of Self-Specific CD8 T Cells in Vivo. *J Immunol.*

5.2 Introduction

Successful immune protection against pathogens relies on cooperation between the innate and adaptive arms of the immune system. Cells of the innate immune system provide the first line of defense against pathogens and are usually able to clear infections before they spread. Innate cells use germ line encoded receptors, which either recognize pathogens directly or recognize changes on host cells as a result of infection. In cases where the innate immune system is overwhelmed, the adaptive immune system is usually able to provide the additional immune responses that are required for the elimination of the pathogen. However, the response of adaptive immune cells is slow and requires the expansion of T cells and B cells that express rearranged receptors specific for pathogen derived epitopes. Another distinguishing feature of the adaptive immune system is the production of memory T and B lymphocytes, which are very effective in protection against subsequent infections by the same pathogen.

The distinction between innate and adaptive immunity is not absolute. Several adaptive lymphocytes have been shown to express antigen (Ag)-specific receptors of limited diversity in conjunction with the expression of receptors associated with innate cells. For instance, B1-B cells express rearranged B cell receptors (BCRs) that display limited diversity and show specificity for self-Ags such as oxidized phospholipids (1). T cells expressing $\gamma\delta$ T cell receptors (TCRs) also show limited diversity in their TCR rearrangements and some of these cells express innate system receptors which are specific for stress-induced Ags (2). Intraepithelial lymphocytes or iELs can express TCRs specific for self-Ags and

this interaction is crucial for their function (3, 4). Natural killer T cells (NKT) restricted to the non-classical MHC class I molecule, CD1d, also express invariant TCRs (Vα14*i* in mice) (5). This cell type is abundant in the spleen and liver where the cells are thought to become activated in response to inflammatory cytokines with concurrent recognition of lipids in the context of CD1d. They are then able to rapidly secrete large amounts of cytokines such as IFNγ or IL-4, which play a role in biasing the immune response towards the Th1 or Th2 lineage, respectively (6).

IFNγ is crucial for protection against various intracellular pathogens (7). Binding of IFNγ to its receptor on macrophages induces bactericidal activity in infected macrophages resulting in the production of reactive oxygen and nitrogen intermediates as well as the efficient fusion of lysosomes with phagosomes containing pathogens (8). IFNγ also has an impact on shaping the adaptive immune response and leads to the expression of host genes involved in Ag-processing and presentation (9). In addition, this cytokine plays an important role in the polarization of CD4 T cells to the Th1 lineage which can then activate macrophages (10). IFNγ signaling early during infection enhances CD4 and CD8 T cell responses (11, 12) and also programs contraction of Ag-specific $CD8^+$ T cells thus controlling the level of immunological memory (13).

Listeria monocytogenes (LM) is a gram-positive intracellular pathogen which is the cause of listeriosis in humans (14). Efficient protection against LM infection relies heavily on early IFN-γ production by innate cells (15). IFNγ production during infection with LM was thought to come mainly from natural killer (NK) cells

but recent studies suggest that memory-phenotype $CD8^+$ T cells are also a major contributor (16-20). Memory-phenotype $CD8^+$ T cells include those that are specific for foreign-Ags and which have been generated during a previous encounter with these Ags. These bona fide foreign-Ag specific memory $CD8^+$ T cells have been shown to provide an innate source of IFNγ in the absence of cognate Ag through IL-12 and IL-18 signaling (17). Interestingly, memory $CD8^+$ T cells have been shown to be more protective than NK cells during LM infection (18). However, not all memory-phenotype $CD8^+$ T cells are specific for foreign-Ags. $CD8^+$ T cells that are restricted to non-classical MHC class Ib molecules also have a memory-phenotype (21). Some MHC class Ib-restricted $CD8^+$ T cells have been shown to be selected by hematopoetic cells and can respond to LM infection in both an Ag-dependent and Ag-independent fashion by producing IFNγ (22, 23). Thus, memory-phenotype $CD8^+$ T cells have been shown to be a very significant source of early IFN-γ production during infections.

We have shown that memory-phenotype $CD8^+$ T cells in normal mice contain a subset of T cells that demonstrate a very high reactivity for self-peptide/MHC (24). By virtue of high expression of CD122 (IL-2Rβ), these cells respond to IL-2 and IL-15 both in vitro and in vivo. Upon activation, they express several NK receptors including CD16, NKG2D and the adaptor protein DAP12. CD16-engagement on these cells results in both the production of inflammatory cytokines and the lysis of antibody-coated target cells (25). NKG2D engagement also results in the lysis of NKG2D-ligand expressing target cells. These cells

comprise ~10% of peripheral $CD8^+$ T cells in normal mice and they demonstrate specificity for syngeneic tumor cells.

We have also characterized self-specific $CD8^+$ T cells in H-Y TCR transgenic mice (26). The H-Y TCR is specific for the male (H-Y) peptide presented by H-$2D^b$. In female H-2^b mice $CD8^+$ T cells expressing the H-Y TCR are positively selected. In male H-2^b H-Y TCR transgenic mice, the deletion of virtually all of the double positive thymocytes greatly affected the development of conventional CD8 as well as CD4 T cells (27, 28). However, large numbers of T cells which expressed low levels of CD8 and exclusively the H-Y TCR are present in the peripheral lymphoid organs of these male mice (29). More interestingly, these cells have a similar memory phenotype and functional properties as the self-specific $CD8^+$ T cells that are found in normal mice. They also express NK receptors that function in cooperation with the TCR. Most importantly, we have shown that these cells become activated in vivo in response to LM infection (26).

In this report we determined the role of self-specific $CD8^+$ T cells in protection against LM infection and assessed the role of self-Ag interactions in the maintenance and function of this cell type. We show that these cells are protective immediately ex vivo but more so after IL-15 activation. We also demonstrate that self-Ag interactions are crucial for the early protection of IFNγ-deficient mice from LM infection and for maintaining the memory-phenotype and cytokine responsiveness of these self-specific cells. Finally, we show that activated self-specific $CD8^+$ T cells produce more IFNγ and TNFα than NK cells and are more protective than NK cells during infection with LM.

5.3 Materials and Methods

Mice

Breeders for C57BL/6 (B6), B6.*foxn1*^{nu} (athymic nude), B6.Thy1.1, and B6.IFN$\gamma^{-/-}$ were obtained from the Jackson Laboratories (Bar Harbor, ME). The H-Y TCR transgenic (tg) mice were bred to the B6 or B6.*foxn1*^{nu} background. Mice 8 to 12 weeks of age were used for the experiments described.

Abs and flow cytometry

The following mAbs specific for the indicated molecule were used: CD8α (53-6.7), CD8β (53.58), CD44 (Pgp-1), H-Y TCRα (T3.70), CD94 (18D3), NK1.1 (PK136), NKG2D (CX5), CD122 (TM-β1), CD127 (A7R34), CD132 (4G3), IFNγ (XMG1.2), and TNFα (MP6-XT22). All mAbs were purchased from eBioscience (San Diego, CA) except anti-CD132 (BD PharMingen, San Diego, CA). For intracellular flow cytometry cells were stained for surface markers, washed, fixed with 2% paraformaldehyde and 0.2% Tween-20 in PBS for 20mins followed by one wash with PBS. The cells were then stained with mAbs specific for the intracellular cytokine in 0.2% Tween-20 in PBS. The CellQuest software program (Becton Dickinson, Mountain View, CA) was used for data acquisition and analysis.

CD8+ T cell purification

Single cell suspensions from the lymph nodes (LN) of H-Y male mice were treated with biotinylated anti-CD8β mAb followed by positive selection using the MiniMACS system (Miltenyi Biotech, Auburn, CA), according to the manufacturer's specifications. The resulting cells were >95% pure $CD8\alpha\beta^+$ H-Y $TCR\alpha^+$ T cells. For purification of B6 $CD8^+CD44^{hi}$ and $CD8^+CD44^{lo}$ T cells, $CD8\beta^+$ T cells from B6 lymph nodes were first enriched by MiniMACS. MiniMACS purified B6 $CD8^+$ T cells were then stained with anti-CD8α-FITC and anti-CD44-PE and sorted on a Becton Dickinson FACS Vantage SE Turbo sort cell sorter. Cell sorting was performed by Andrew Johnson (University of British Columbia) and the sorted $CD8^+CD44^{hi}$ or $CD8^+CD44^{lo}$ cells were >98% pure (24).

NK cell purification

Single cell suspensions from the spleens of B6 mice were treated with mAbs against CD4, CD8, and TCRβ, followed by depletion of Ab-coated cells using Dynabeads M-450 Sheep anti-mouse IgG (Dynal Biotech, Lake Success, NY), according to manufacturers instructions. The resulting cells were ~50-60% $CD3^-$ $NK1.1^+$ prior to culture and >95% $CD3^-NK1.1^+$ after culture in IL-15.

Adoptive transfers, infections, and bacterial load measurement

Purified H-Y CD8 T cells or sorted B6 CD8 T cells were injected into the lateral tail vein of IFN$\gamma^{-/-}$ or B6-Thy1.1 mice. For IL-2 or IL-15 cultured CD8 T cells and for IL-15 cultured NK cells, the cells were cultured in cytokine (100ng/ml) for 4 days prior to transfer. The number of cells adoptively transferred is stated in the figure legends. For infection experiments, mice were infected with ~10,000 CFU of wild type LM (strain 10403s) via the lateral tail vein one day after receiving adoptively transferred cells. On day 3 post-infection mice were sacrificed and their spleen and liver were homogenized in PBS. The resulting cell suspensions were mixed with an equal volume of 1% Triton X-100 in PBS and plated on brain-heart infusion agar plates in serial 10-fold dilutions and incubated at 37°C overnight prior to counting. Fold reduction was used to assess relative protection in some experiments and was calculated as (CFU control mouse/CFU experimental mouse).

5.4 Results

Self-specific CD8$^+$ T cells in H-Y TCR transgenic male mice can develop in the absence of a functional thymus

There is some controversy regarding whether the thymus is essential for the development of self-specific CD8 T cells in the H-Y model. In the first description

of the H-Y TCR^+ $CD8^+$ cells in H-Y male mice it was suggested that these cells were conventional $CD8^+$ T cells that had escaped negative selection by decreasing the expression of the $CD8^+$ coreceptor and thus lowering their avidity for self-peptide/MHC (27, 29). Consistent with the requirement for the thymus for their development was the observation that these cells failed to develop in athymic nude mice (29). However, this conclusion was challenged by a later report, which showed using adult thymectomy followed by bone marrow reconstitution that self-specific H-Y $CD8^+$ T cells can develop in the absence of a functional thymus (30). In view of our findings that these self-specific $CD8^+$ T cells are functionally distinct from conventional $CD8^+$ T cells, we decided to re-evaluate whether self-specific CD8 T cells can in fact develop in athymic nude ($foxn1^{nu}$) mice. We first compared the CD4 and CD8 populations in the lymph nodes of euthymic H-Y male, athymic H-Y male, euthymic H-Y female, and athymic H-Y female mice. The top panel in Fig. 5.1A shows that euthymic H-Y female mice have the largest population of both CD4 and CD8 cells making up 19.0% and 30.7% of the lymph nodes, respectively. Consistent with previous reports (27, 29) lymph nodes from euthymic H-Y male mice contain very few peripheral CD4 T cells (3.1%) but possess larger numbers of $CD8^{lo}$ cells (16.7%). By contrast, the lymph nodes of athymic H-Y male mice contain fewer numbers of these $CD8^{lo}$ cells (3.7%) and possess virtually no CD4 T cells (0.6%). Consistent with previous reports (27, 29) virtually all of the $CD8^{lo}$ cells (96.7%) in euthymic H-Y male mice express exclusively the H-Y TCR, as indicated by the same level of staining with either a mAb specific for the TCR β chain or the H-Y

TCR (Fig. 5.1A, lower panel). Also consistent with previous reports these $CD8^{lo}$ T cells express exclusively the CD8αβ heterodimer (29 and data not shown). By contrast, only 34.9% of the $CD8^+$ T cells in euthymic H-Y female mice express the H-Y TCR; this is consistent with previous reports that the majority of peripheral $CD8^+$ T cells from euthymic female H-Y TCR mice utilize endogenous TCR α chains for their positive selection (31). The majority (62.6%) of $CD8^{lo}$ cells in athymic male H-Y mice also express the H-Y TCR. It is noted that about one-third of the $CD8^{lo}$ cells from athymic H-Y male mice did not express the TCR β chain suggesting that these cells are unlikely to be of the TCRαβ lineage. In athymic H-Y female mice there is very poor development of $CD8^{lo}$ cells (1.0%) consistent with our previous observation that the presence of self-Ag is required for their development (26). In terms of absolute cell numbers, there is a 26-fold increase in the frequency of $CD8^{lo}$ $TCR\beta^+$ H-Y $TCR\alpha^+$ cells in athymic nude H-Y male mice compared to athymic nude H-Y female mice. There is also a 7-fold reduction in the recovery of $CD8^{lo}$ H-Y TCR^+ cells in athymic nude H-Y male mice relative to euthymic H-Y male mice. This suggests that additional cells of this lineage may develop in the thymus or that some thymus-dependent cell(s) may provide selection or survival cues for these cells. We also noted that the H-Y TCR^+ $CD8^{lo}$ T cells that developed extrathymically in athymic H-Y male mice expressed high levels of the memory markers CD44 and CD122 as well as the NK receptors NKG2D and CD94 (Fig. 5.1B). These observations support the following conclusions: (1) self-specific $CD8^{lo}$ H-Y TCR^+ T cells can develop in the absence of the thymus, (2) thymus-independent development of these cells is

relatively inefficient as indicated by a seven-fold reduction in absolute numbers relative to euthymic mice, (3) the development of self-specific CD8 T cells in athymic mice is also dependent on their interaction with self-Ags, and (4) the self-specific CD8 T cells that develop in athymic mice express high levels of memory markers (CD44 and CD122) as well as NK receptors (NKG2D and CD94).

Activated self-specific CD8 T cells produce pro-inflammatory cytokines

Vα14*i* NKT cells are an innate T cell type which has been shown to produce both Th1 (IFNγ) and Th2 (IL-4) cytokines upon activation (6). Since self-specific CD8 T cells can produce cytokines immediately ex vivo we determine if these cells were similar to NKT cells in their cytokine production profile. We compared the cytokine production of self-specific CD8 T cells from H-Y male mice to conventional Ag-specific CD8 T cells from H-Y female mice. $CD8^{lo}$ cells from euthymic H-Y male mice were used as a source of self-specific CD8 T cells since they provide a more convenient and larger source of these cells. We also included memory-phenotype (containing self-specific CD8 T cells of unknown specificity) and naive CD8 T cells from normal B6 mice in these analyses. Splenocytes from H-Y male, H-Y female and B6 mice were stimulated with PMA and ionomycin for 4 hours. We then measured the production of IFNγ, TNFα, IL-2, and IL-4 by intracellular flow cytometry after gating on the cell type of interest. It is clear that self-specific H-Y male CD8 T cells were only capable of producing the pro-inflammatory Th1 cytokines IFNγ, TNFα, and IL-2 but not IL-4. The naive H-Y female CD8 T cells were only capable of producing TNFα and a small

amount of IL-2. These results were mirrored by those obtained from the non-transgenic CD8 T cells from normal B6 mice where the memory-phenotype $CD8^+CD44^{hi}$ T cells were capable of producing IFNγ, TNFα, and IL-2 during a short stimulation and the naive $CD8^+CD44^{lo}$ T cells could only produce TNFα and IL-2. In addition, IFNγ-production by self-specific H-Y and memory-phenotype B6 $CD8^+CD44^{hi}$ T cells was maintained even at 24 hours post-stimulation whereas the naive H-Y female and naive B6 CD8 T cells still could not produce IFNγ (data not shown). IL-4 production by all the cell types was never detected even at 24 hours post stimulation. These results further emphasize the functional similarity of self-specific $CD8^{lo}$ T cells from H-Y male mice and the $CD8^+CD44^{hi}$ cells from B6 mice.

IFNγ production by self-specific CD8 T cell provides protection against bacterial infection

Memory-phenotype CD8 T cells have been shown to produce large amounts of IFNγ early during infection with Listeria and these cells have been shown to be protective during infection (16-20). To test the ability of self-specific CD8 T cells to produce IFNγ in vivo and to address whether this cytokine production has biological significance we measured the ability of this cell type to protect $IFN\gamma^{-/-}$ mice against LM infection. Two previous studies have used this approach to demonstrate that CD8 T cells and NK cells capable of producing IFNγ during infection can protect $IFN\gamma^{-/-}$ mice during LM infection due to their ability to provide

a source of IFNγ (17, 18). We transferred purified self-specific CD8 T cells into male IFNγ$^{-/-}$ mice, which were infected with 10^4 CFU of Listeria 1 day post transfer. At day 3 post-infection we sacrificed the animals and assessed cytokine production by the transferred cells in the absence of any restimulation. Figure 5.3A depicts IFNγ production by gated self-specific CD8 T cells recovered from the spleens of infected or uninfected IFNγ$^{-/-}$ mice. It is clear that a significant proportion (~15%) of the transferred cells was actively producing IFNγ and that this cytokine production was a consequence of infection. We also determined if IFNγ production by self-specific CD8$^+$ T cells was associated with a decrease in bacterial burden in the spleens of infected IFNγ$^{-/-}$ mice. Figure 5.3B clearly shows that IFNγ$^{-/-}$ mice that had received self-specific CD8 T cells prior to infection had at least a 10-fold reduction in bacterial load in the spleen relative to mice which did not. Therefore the ability of self-specific CD8 T cells to protect against Listeria infection is directly associated with their ability to produce IFNγ.

Memory-phenotype CD8$^+$CD44^{hi} cells and self-specific H-Y male CD8 T cells provide similar innate protection during infection

After determining the ability of self-specific CD8 T cells from H-Y male mice to protect IFNγ$^{-/-}$ mice from infection with LM we decided to test the relative efficacy of self-specific CD8 T cells from H-Y male mice, memory-phenotype CD8$^+$CD44^{hi} T cells from B6 mice and naive CD8$^+$CD44^{lo} T cells from B6 mice to confer protection against LM infection in IFNγ$^{-/-}$ mice. It is noted that memory-phenotype

CD8 T cells from B6 mice, like self-specific CD8 T cells from H-Y male mice, have the ability to produce IFNγ rapidly upon activation whereas naive CD8 T cells do not (Fig. 5.2). We sorted memory and naive-phenotype CD8 T cells from B6 mice and we purified self-specific CD8 T cells from H-Y male mice. We then transferred $9x10^5$ cells into male IFNγ$^{-/-}$ mice and infected the mice the following day with LM. The results in Fig. 5.4 demonstrate that both self-specific H-Y male CD8 T cells and memory-phenotype B6 CD8 T cells, but not naive B6 CD8 T cells, provide a small but significant degree of protection against LM infection in IFNγ$^{-/-}$ mice. This protection was observed in both the spleen and liver of IFNγ$^{-/-}$ that had received relatively few transferred cells ($9x10^5$), with both the H-Y male and B6 CD8$^+$CD44^{hi} cells reducing the bacterial load by more than 3-fold. This result indicates that self-specific CD8 T cells from H-Y male mice and CD8$^+$CD44^{hi} cells from B6 mice are functionally similar in terms of their ability to confer protection against LM infection in IFNγ$^{-/-}$ mice.

IL-15-activated cells provide protection against LM infection

Previous reports have shown that self-specific CD8 T cells can proliferate and become activated in vitro in response to IL-2 or IL-15 (32). The expression of IL-2Rβ (CD122) in conjunction with γc (CD132) is sufficient to confer lymphocytes with the ability to respond to both IL-2 and IL-15 (33, 34). Activation of NK cells or memory-phenotype CD8 T cells with either of these cytokines is able to enhance both the cytotoxicity as well as cytokine production by these cells (35-37). Since immediately ex vivo self-specific CD8 T cells provide protection against LM

infection in IFN$\gamma^{-/-}$ mice we wanted to see if activation of the cells with IL-2 or IL-15 could enhance this function. We cultured purified and CFSE-labeled self-specific CD8 T cells with either 100 ng/ml of IL-2 or IL-15 for 4 days. Both IL-2 and IL-15 induced the same amount of proliferation of these cells (Fig. 5.5A). We also determined the potential of cells cultured in either IL-2 or IL-15 to produce IFNγ, TNFα and the cytolytic marker, granzyme B. We found that PMA and ionomycin activated IL-2 and IL-15 cultured cells produced equivalent amounts of IFNγ. However, activated IL-2-cultured cells produced a greater amount of granzyme B than did IL-15-cultured cells. By contrast, IL-15-cultured cells produced more TNFα than IL-2 cultured cells. We next determined the ability of IL-2 or IL-15-cultured cells to protect IFN$\gamma^{-/-}$ mice from LM infection. IL-2 or IL-15 cultured cells were transferred into IFN$\gamma^{-/-}$ mice and the mice were infected 1 day later with LM. At day 3 post-infection we assessed the total numbers of transferred cells recovered as well as the bacterial load in the spleens of infected mice. Figure 5.5B clearly shows that self-specific CD8 T cells cultured in IL-2 were much less efficient at homing to and/or surviving in the spleens of infected animals as we recovered only about 10^5 IL-2 cultured cells 3 days post-infection. In addition the few IL-2 cultured cells that were present were completely ineffective in protecting IFN$\gamma^{-/-}$ mice from LM infection, causing no reduction in bacterial load (Fig. 5.5C). By contrast, IL-15 cultured cells did either home to and/or survive better in the spleen as we recovered about $7x10^5$ cells and the cells were extremely protective against infection, reducing the bacterial load by greater than 100-fold compared to control IFN$\gamma^{-/-}$ mice. The protection by IL-15

cultured cells was greater than that achieved with immediately ex vivo cells (Fig. 5.3B) even though protection was observed in both cases. Therefore consistent with results using IL-15 transgenic mice (38) culturing self-specific CD8 T cells with IL-15 provides a novel method for the expansion of cells with increase effectiveness in providing protection against bacterial infection.

Self-Ag interactions are important for innate protection by self-specific CD8 T cells

We next determined whether the effectiveness of self-specific CD8 T cells in controlling LM infection is dependent on interaction with self-Ag. IL-15 cultured cells were used since they were more protective than immediately ex vivo cells. We transferred 1 x 10^6 IL-15 cultured self-specific CD8 T cells into either male (self-Ag^+) or female (self-Ag^-) IFN$\gamma^{-/-}$ recipients and then infected the mice with 10^4 CFU of LM the following day. We then compared the relative reduction in bacterial load in mice that had either received or not received cells. This was done to eliminate any differences in bacterial load due to the sex of the recipient mice. Figure 5.6 shows that self-specific CD8 T cells were protective in both male and female recipients. However, protection offered by the transferred cells in female recipients was modest, resulting in only a 2-fold reduction in bacterial load in the spleen and liver of female IFN$\gamma^{-/-}$ mice receiving self-specific CD8 T cells relative to female IFN-$\gamma^{-/-}$ mice that did not. By contrast, the transferred cells offered much greater protection in male IFN$\gamma^{-/-}$ recipients, resulting in about a 10-fold reduction in bacteria in the spleen and about a 100-fold reduction in bacteria

in the liver relative to male IFN$\gamma^{-/-}$ mice that did not receive any cells. Protection conferred by self-specific CD8 cells from H-Y male mice against LM infection in female IFN$\gamma^{-/-}$ mice was increased by transferring more cells (data not shown). This result indicates that these cells can confer protection against LM infection in the absence of self-Ag. However, their protective function is greatly increased in the presence of self-Ag.

Self-Ag interactions are crucial for maintaining the memory phenotype and cytokine-responsiveness of self-specific CD8 T cells

Previous studies had shown that the expansion of self-specific CD8 T cells in response to either IL-2 or IL-15 in vitro was independent of self-Ag interactions (32). Our studies have suggested that self-Ag interactions played a role in the proliferation of self-specific CD8 T cells in vivo during LM infection (26). Since we had also shown that self-Ag interactions dictate the extent of the memory-phenotype in these self-specific CD8 T cells (26) we decided to test the idea that self-Ag interactions control the expansion of these cells in vivo in part by helping to maintain a memory phenotype and high levels of the associated cytokine receptors. To address this possibility we transferred self-specific CD8 T cells into congenic Thy-1.1 male or female recipient mice and determined their cell surface phenotype in the spleens of recipient mice 7 days post transfer. There was no difference in the frequency of self-specific CD8 T cells in the spleen or livers of male or female recipients (Fig. 5.7A) suggesting that survival and homing are unaffected by self-Ag 7 days post transfer. However, we noticed striking

differences in the expression of the TCR and CD44 as well as the cytokine receptors CD122 and CD127. Self-Ag interactions in male recipients led to lower TCR expression and higher expression of CD44 (Fig. 5.7B). CD122 and CD127 were also maintained at higher levels in male recipients over female recipients suggesting that self-Ag interaction is crucial for the high expression of these receptors in self-specific CD8 T cells. The lower expression of CD122 on self-specific CD8 T cells that are maintained in female recipients likely leads to their decreased ability to respond to IL-15. To test this possibility more directly we purified self-specific CD8 T cells which had been rested in either male or female recipients for 7 days, CFSE-labeled these cells and tested their ability to proliferate in response to IL-15 in vitro. Figure 5.7C clearly demonstrates that self-specific CD8 T cells maintained in Ag^- female recipients are less responsive to IL-15 as only 21% of the cells have undergone greater than 4 division events compared to about 51% of the cells maintained in male mice. These observations provide an explanation for the decreased proliferation of these cells in vivo in response to LM infection in female recipient mice.

Self-specific CD8 T cells provide greater IFNγ-dependent protection against LM infection than NK cells

Several recent reports have focused on the ability of memory-phenotype CD8 T cells to provide innate protection during infection. One report compared the ability of NK cells and foreign-Ag specific memory CD8 T cells to protect $IFN\gamma^{-/-}$ mice against infection with LM (18). This study found that memory CD8 T cells are

more protective than NK cells, in part due to their ability to localize to areas of the spleen bearing LM-infected macrophages. We compared the relative efficacy of self-specific CD8 T cells and NK cells in providing protection against LM infection by first comparing the ability of self-specific CD8 T cells and NK cells to produce IFNγ, TNFα and granzyme B. This was done by culturing both cell types in IL-15 for 4 days and re-stimulating the cells with PMA and ionomycin for 4 hours. We found that self-specific CD8 T cells were much more efficient producers of both IFNγ and TNFα than NK cells; however, NK cells were much more efficient in producing granzyme B (Fig. 5.8A). These differences seen after IL-15 activation were also true of these cell types immediately ex vivo (data not shown). To compare the ability of self-specific CD8 T cells and NK cells in conferring protection against LM infection in IFNγ$^{-/-}$ mice equal numbers of day 4 IL-15 cultured self-specific CD8 T cells and NK cells were transferred into male IFNγ$^{-/-}$ recipients. We then infected the mice with LM the next day and measured the reduction in bacterial load in the spleens and livers of mice receiving self-specific CD8 cells or NK cells. The results in Fig. 5.8B indicate that NK cells offered a small degree of protection against LM infection, reducing the bacterial load in the spleen and liver by ~two- and three-fold, respectively. By contrast, self-specific CD8 T cells were much more effective in reducing bacterial load in IFNγ$^{-/-}$ mice, reducing the bacterial load in spleen and liver by ~30- and 700-fold, respectively. These results indicate that self-specific CD8 T cells are more effective than NK cells in providing protection against LM infection in IFNγ$^{-/-}$ mice.

5.5 Discussion

In this report we have clearly demonstrated an important role for self-specific CD8 T cells in early protection of mice from LM infection. We have shown that these self-specific cells are potent producers of IFNγ and TNFα immediately ex vivo. We also showed that self-specific CD8 T cells offered significant protection against LM infection in IFNγ$^{-/-}$ mice, particularly in Ag-expressing mice. This protection was greatly enhanced by culturing self-specific CD8 T cells in IL-15 prior to transfer. We also demonstrate an important role for self-Ag interactions in maintaining the memory phenotype of these cells and their responsiveness to IL-15. Finally, we found that self-specific CD8 T cells were much more effective than NK cells in protecting IFNγ$^{-/-}$ mice against LM infection.

We have also confirmed in this study that self-specific CD8 T cells from H-Y male mice can develop in the absence of a functional thymus. We did however notice a significant reduction in the frequency of H-Y CD8 T cells in athymic male mice compared to euthymic mice (Fig. 5.1A). This suggests that thymus-dependent mechanisms or thymus-dependent T cells can improve the survival, selection, or expansion of this cell type. The fact that these self-specific CD8 T cells can be selected by cognate self-Ag outside the thymus indicates that these cells constitute a lineage that is distinct from conventional CD8 T cells (29, 30). The memory markers CD44 and CD122 as well as the NK receptors CD94 and NKG2D are significantly higher on self-specific CD8 T cells from athymic nude

mice relative to their euthymic counterparts. We have seen the expression of CD94 and NKG2D increase on self-specific CD8 T cells upon activation by Ag or IL-15 and thus the increased expression of these receptors on athymic CD8 T cells likely reflects their activation history (26). This may be due in part to increased availability of IL-15 due to the smaller numbers (one-seventh) of self-specific CD8 T cells in the athymic nude mice. Consistent with this hypothesis IL-15 has been shown to play a role in the induction of both NKG2D and CD94 in CD8 T cells (39, 40). The significance of the high level of CD94 expression in athymic CD8 T cells is unknown but it may play a role in their survival as one study showed that CD94 expression is associated with protection from activation induced cell death and correlates with increased survival in CD8 T cells (41). Whether athymic CD8 T cells preferentially express CD94 to enhance their survival remains to be determined.

Although this current study focused mainly on the role of a homogenous population of self-specific CD8 T cells from male H-Y TCR transgenic mice we have previously shown that memory-phenotype CD8 T cells from normal B6 mice share many similarities to this cell type (24). In this study, we have provided further confirmation that memory-phenotype CD8 T cells are very similar to self-specific H-Y CD8 T cells in their cytokine production profiles as well as in their ability to provide innate protection to IFN$\gamma^{-/-}$ mice against LM infection. The main problem with non-TCR transgenic mice for these studies is the heterogeneity of the memory-phenotype population, which makes it impossible to dissect the relative contributions of conventional memory CD8 T cells from self-specific CD8

T cells. The H-Y model is not unique when it comes to the development of memory-phenotype CD8 T cells specific for a self-Ag. For instance, in doubly transgenic mice, which express a transgenic TCR specific for the gag protein from Friend murine leukemia virus (FMuLVgag) as well as the transgenic cognate Ag, FMuLVgag, CD8 T cells expressing the transgenic TCR still develop. Furthermore, these cells exhibit a memory-phenotype similar to the self-specific CD8 T cells from H-Y male mice; these mice also did not develop detectable autoimmune diseases even though the self-specific CD8 T cells retain effector function and are specific for the self-Ag (43). A recent report on these cells also demonstrated their ability to proliferate in response to IL-2 or IL-15 alone (42). Another TCR transgenic model that shares similarities to ours is the P14 TCR transgenic in which CD8 T cells are specific for a peptide derived from the lymphocytic choriomeningitis virus gp (LCMV-gp). When P14 TCR transgenic mice are crossed to mice ubiquitously expressing cognate antigen the developing P14 CD8 T cells share a nearly identical cell surface phenotype with the self-specific CD8 T cells from H-Y male mice (Pamela S. Ohashi, personal communication). Whether the CD8 T cells expressing these self-specific TCR's behave the same way as those from H-Y male mice remains to be determined although based on their phenotype it is reasonable to believe that they will also share similarities in function. Together, these observations emphasize the importance of TCR/cognate self-Ag interactions for the development of self-specific CD8 T cells.

Self-reactivity is also characteristic of some other cell types of the adaptive immune cells. For instance NK-T cells exhibit a high degree of self-reactivity and function through the recognition of self-lipid in the context of CD1d (44). These cells also have an unusual requirement for positive selection in the thymus in that they are selected by bone marrow derived double positive thymocytes rather than cortical epithelial cells which select conventional T cells (5). CD8αα iELs are also selected by agonist self-peptides (3). Studies using H-Y TCR transgenic mice demonstrate strong interaction with self-Ag is required for the development of CD8αα iELs whereas weaker selection leads to the development of CD8αβ T cells (45). Interestingly, the strength of selection also correlates with the expression of genes associated with innate immune cells. Although the self-specific CD8 T cells are unrelated to CD8αα iIELs, it is conceivable that their unconventional functions may also be a consequence of their positive selection by agonist self-Ags.

One phenotypic similarity between self-specific CD8 T cells and other innate T cells is their memory phenotype. This phenotype is associated with expression of high levels of CD122, which confers IL-2 and IL-15 responsiveness. IL-15 responsiveness seems to be crucial for the maintenance of NK, NK-T, and memory phenotype CD8 T cells (which include self-specific CD8 T cells) as all of these cell types are either drastically reduced in number or virtually absent in mice lacking either IL-15 or its receptor (46, 47). An interesting finding of the present study is that self-specific CD8 T cells require self-Ag for the maintenance of high levels of CD122, which then makes them more responsive to cytokine

stimulation. Two T-box family transcription factors T-bet and eomesodermin have recently been shown to be crucial for the maintenance of IL-15-dependent cell types including NK, NK-T and memory phenotype CD8 T cells (48). Notably, these transcription factors act directly on the CD122 promoter, inducing transcription and expression of CD122. It remains to be determined whether T-bet or eomesodermin expression by self-specific CD8 T cells is induced or maintained through self-Ag interactions.

We have compared the ability of IL-2 or IL-15 cultured self-specific $CD8^+$ T cells to protect $INF\gamma^{-/-}$ mice from LM infection. The ineffectiveness of IL-2 cultured cells in conferring protection could be due to their inability to home to and/or survive in the spleen since IL-2 is limiting in vivo. IL-15 cultured cells, which are smaller and look more like naive cells compared to IL-2 cultured cells, either home more efficiently or survive better in the spleen of recipient mice. Furthermore, these cells offered superior protective function against LM infection, presumably as a consequence of their ability to respond to IL-15 produced in vivo upon infection. The ability of self-specific CD8 T cells to respond to IL-15 in vivo allows the cells to rapidly become activated during infection or inflammation and results in the production of cytokines such as IFNγ and TNFα. We have demonstrated in this report that these self-specific CD8 T cells do produce IFNγ early during infection and provide protection against LM infection whether or not the cells were pretreated with IL-15. The mechanism for the induction of IFNγ by these cells likely involves responsiveness to IL-12 produced by infected macrophages. IL-12 in conjunction with IL-2, IL-15, or IL-18 has been shown to

be sufficient to induce large amounts of IFNγ production from self-specific CD8 T cells (32). There is also the possibility that NKG2D may play a role in inducing IFNγ production by self-specific CD8 T cells. We have shown previously that these cells do in fact increase expression of NKG2D upon infection (26) and reports suggest that infected macrophages express the ligands for NKG2D (49). It is quite possible that NKG2D stimulation may play an important role in inducing IFNγ by these cells.

The requirement for interaction of self-specific CD8 T cells with their cognate Ag in conferring better protection against LM infection indicates that TCR interactions play a role in the function of these cells. We have shown in this study that continuous interaction with self-Ag in vivo is required for maintaining the high responsiveness of these cells to IL-15. Thus, the increased protective function of self-specific CD8 T cells in cognate Ag expressing mice is likely due to the requirement for self-Ag in maintaining high expression of cytokine receptors such as CD122, which allows them to sense bacterial infection more effectively by responding to physiological levels of IL-15. The self-specific CD8 cells are still protective in the absence of self-Ag (Fig. 5.6) and still retain function after being in an Ag^- female recipient for 7 days. It is clear however, that self-specific CD8 T cells are less efficient at responding to IL-15 in Ag^- female recipients but this inefficiency can be partially overcome by increasing the numbers of these cells.

NK cells are a source of early IFNγ and TNFα that are involved in protection from pathogens as well as tumor cells (50). Our study clearly demonstrates that self-specific CD8 T cells are much more potent producers of both IFNγ and TNFα

when compared to NK cells. Consequently self-specific CD8 T cells provide more protection than NK cells to IFN$\gamma^{-/-}$ mice during infection with LM. Another difference between self-specific CD8 T cells and NK cells is their localization. NK cells are relatively abundant in the spleen yet are virtually absent in the lymph nodes of mice (51). Self-specific CD8 T cells on the other hand are present in larger numbers in both the spleen and lymph nodes and are more efficient than NK cells in homing to either location (our unpublished observations). NK cells have been shown to be involved in many steps of T cell priming either by acting on dendritic cells (52) or by producing IFNγ that can participate in CD4 T cell priming (51). Since T cell priming occurs primarily in secondary lymphoid organs where self-specific CD8 T cells are abundant it remains possible that these cells may also be involved in this aspect of immunity. Notably, memory phenotype CD8 T cells, which serve as a source of early IFNγ, were shown to polarize CD4 T cells to the Th1 lineage (38, 53). It remains to be determined whether priming of CD4 T cells towards the Th1 lineage is another function of self-specific CD8 T cells.

5.6 Acknowledgements

We thank Soo-Jeet Teh for excellent technical assistance and John Priatel for helpful discussion.

5.7 Figures

Figure 5.1 Limited development of self-specific CD8 T cells in athymic (nude) H-Y TCR transgenic male mice. A) Lymph node cells from age-matched male and female H-Y TCR transgenic mice on either a wildtype B6 or athymic nude B6 background were stained with mAbs against CD4, CD8, TCRβ and the H-Y TCRα. The TCRβ vs. H-Y TCRα plots in the lower panel are for gated $CD8^+CD4^-$ cells from the upper panel. The cell recovery was similar for all of the mice and the data shown is representative of at least 4 mice per group. B) Expression of memory markers and NK receptors on gated self-specific male H-Y TCR^+ $CD8^+$ cells from euthymic (black histogram) or athymic H-Y male mice (grey line).

Figure 5.2 Self-specific CD8 T cells rapidly produce inflammatory cytokines upon activation. Splenocytes from euthymic H-Y male, H-Y female, or B6 mice were stimulated with PMA (10 ng/ml) and ionomycin (500 ng/ml) for 4 hours in the presence of a Golgi inhibitor. The histograms depict intracellular cytokine staining on gated $CD8^+$ H-Y $TCR\alpha^+$ cells from H-Y male and female mice and from $CD8^+CD44^{hi}$ and $CD8^+CD44^{lo}$ T cells from B6 mice. Cytokine production by unstimulated cells (black line) was compared to PMA and ionomycin stimulated cells (grey line) with the numbers in the plots representing the percentage of cytokine-positive cells after subtracting the background cytokine production.

Figure 5.3 Self-specific CD8 T cells provide an innate source of IFN-γ during infection. $3x10^6$ purified self-specific CD8 T cells were transferred into B6 male $IFN\gamma^{-/-}$ recipients, which were infected the following day with wildtype LM. A) Intracellular IFNγ staining in the absence of restimulation on gated donor cells ($CD8^+$ H-Y $TCR\alpha^+$) isolated 3 days post infection from $IFN\gamma^{-/-}$ recipients infected with LM or left uninfected. The cells were cultured for 5 hours in vitro in the presence of a golgi inhibitor prior to staining. B) Bacterial load in the spleens of $IFN\gamma^{-/-}$ mice on day 3 post infection that had received $3x10^6$ self-specific CD8 cells (black) or that did not receive any cells (white). Error bars represent the standard deviation from 3-4 mice per group.

Figure 5.4 Self-specific H-Y male CD8 T cells and memory-phenotype CD8 T cells from normal B6 mice provide similar protection against LM infection. $9x10^5$ sorted B6 $CD8^+CD44^{hi}$, B6 $CD8^+CD44^{lo}$, and male H-Y $TCR\alpha^+$ $CD8^+$ cells were transferred into male $IFN\gamma^{-/-}$ mice one day prior to infection with wildtype LM (10^4 CFU). Bacterial load in the spleen and liver of infected mice that received either no cells (black bar), B6 $CD8^+CD44^{lo}$ (white bar), B6 $CD8^+CD44^{hi}$ (dark grey bar) or male H-Y $TCR\alpha^+$ $CD8^+$ (light grey) cells is shown. The error bars represent the standard deviation from 3 mice per group.

Figure 5.5 Self-specific CD8 T cells expanded in IL-15 are protective in vivo whereas IL-2 expanded cells are not. (A) Purified self-specific CD8 T cells were CFSE-labeled and cultured in either IL-2 (100 ng/ml) or IL-15 (100 ng/ml) for 4

days. The CFSE profiles of the cultured cells on day 4 are as indicated. The CFSE-labeled cells cultured in IL-2 (filled histogram) or IL-15 (black line) were stimulated with PMA (10 ng/ml) and ionomycin (500 ng/ml) for 4 hours in the presence of a Golgi inhibitor. The cells were then stained intracellularly for IFNγ, TNFα, and granzyme. (B) $3x10^6$ day 4 IL-2 or IL-15 cultured cells were transferred into male IFNγ$^{-/-}$ mice that were infected the next day with LM. Absolute numbers of transferred cells previously expanded in IL-2 (white) or IL-15 (black) and recovered from the spleen of recipient mice at day 3 post infection are indicated. C) Bacterial load on day 3 post infection in spleen of IFNγ$^{-/-}$ mice that had received IL-2 (white) or IL-15 (black) cultured cells 1 day prior to infection compared to IFNγ$^{-/-}$ mice that did not receive any cells (gray). Error bars represent the standard deviation from 3-4 mice per group.

Figure 5.6 Self-Ag interactions enhance the protection mediated by self-specific CD8 T cells during infection. Purified self-specific CD8 T cells were cultured with IL-15 (100 ng/ml) for 4 days and then 10^6 cells were transferred into either male or female IFNγ$^{-/-}$ mice which were infected the next day with 10^4 CFU wild type LM. The data represent the relative reduction in bacterial load on day 3 post-infection in the spleens and livers of male (black) and female (white) IFNγ$^{-/-}$ mice that had received self-specific CD8 cells relative to male and female IFN-γ$^{-/-}$ mice not receiving any cells. Error bars represent the standard deviation from 3-4 mice per group.

Figure 5.7 Self-Ag interactions maintain the memory phenotype and cytokine responsiveness of self-specific CD8 T cells in vivo. $3x10^6$ purified self-specific CD8 T cells (Thy-1.2^+) were transferred into male and female B6-Thy-1.1 congenic mice. A) 7 days post transfer the frequency of donor cells (Thy-1.2^+) was quantified in the spleen and liver of recipient mice. B) Surface marker expression on gated transferred CD8 cells (Thy-1.2^+) 7 days post-transfer into male (black line) or female (filled histogram) mice. The numbers in the plots represent the MFI of cells from male (M) or female (F) mice. C) 7 days post transfer into male or female mice self-specific CD8 T cells were enriched for, labeled with CFSE, and cultured in IL-15 (100 ng/ml) in vitro for 3 days. The histograms represent the cell division of donor self-specific CD8 T cells from male (black line) or female (filled histogram) recipients in response to IL-15.

Figure 5.8 Self-specific CD8 T cells provide more IFN-γ-dependent protection in vivo against LM infection than NK cells. Purified self-specific CD8 T cells and NK cells were cultured in IL-15 (100 ng/ml) for 4 days. A) Intracellular staining for IFNγ, TNFα and granzyme B of self-specific CD8 (black line) and NK (filled histogram) cells stimulated with PMA (10 ng/ml) and ionomycin (500 ng/ml) for 4 hours. Numbers in the plots are the MFI. B) Bacterial load reduction day 3 post infection in the spleen and liver of IFN$\gamma^{-/-}$ mice receiving $1x10^6$ activated self-specific CD8 (black) or NK (white) cells one day prior to infection relative to control IFN$\gamma^{-/-}$ mice not receiving any cells. Error bars represent the standard deviation of data from 3-4 mice per group.

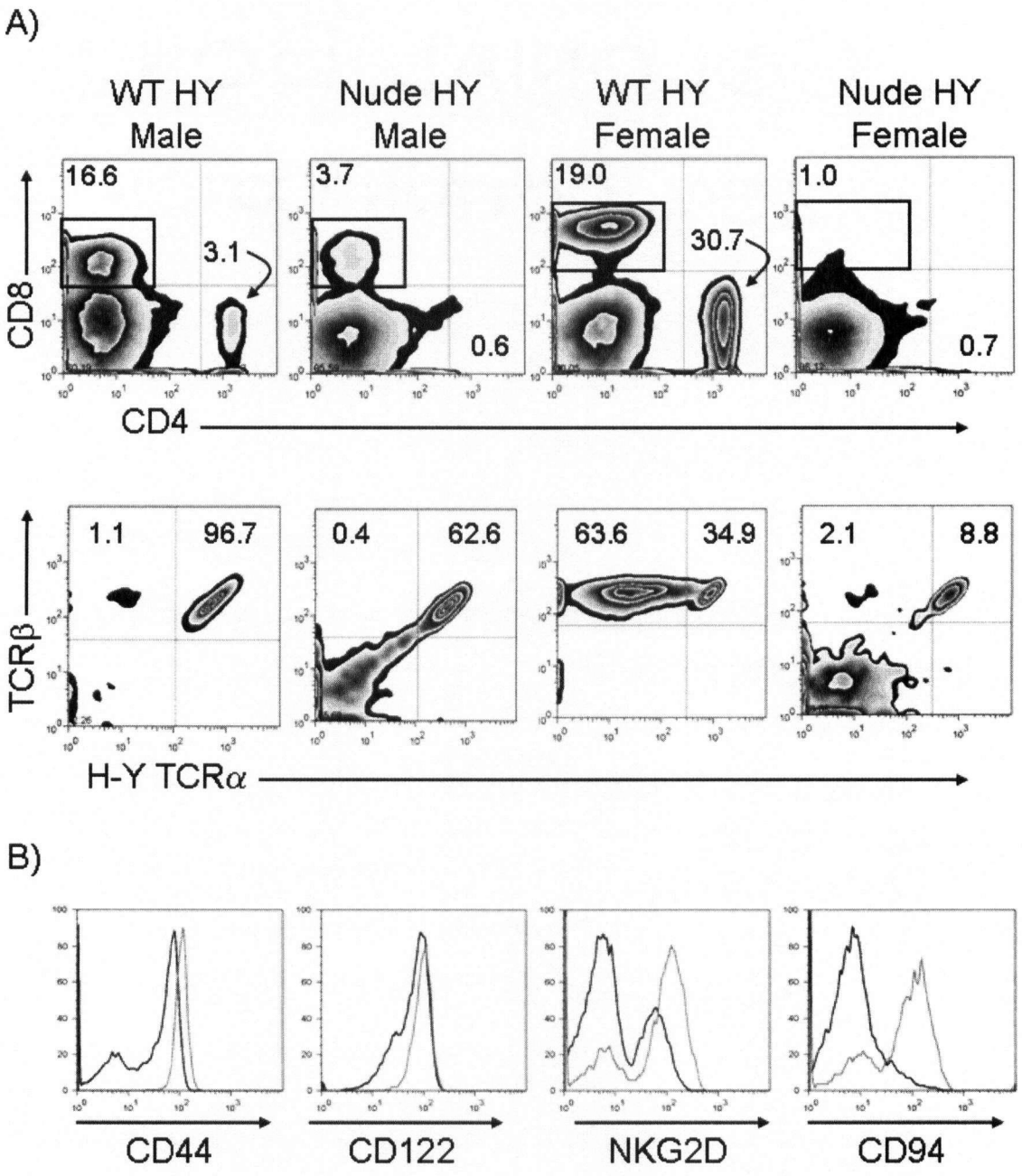

Figure 5.1 Limited development of self-specific CD8 T cells in athymic (nude) H-Y TCR transgenic male mice

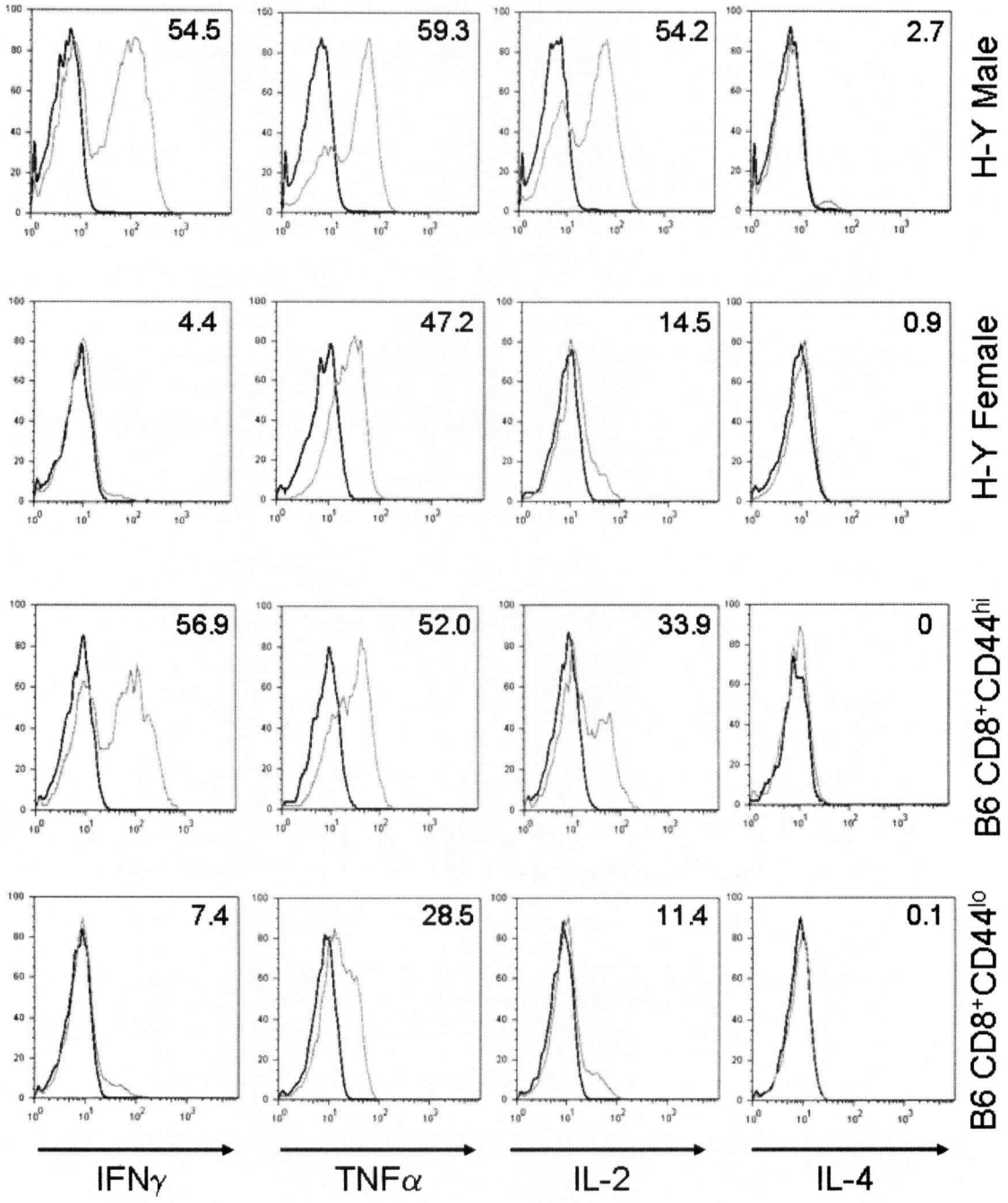

Figure 5.2 Self-specific CD8 T cells rapidly produce inflammatory cytokines upon activation

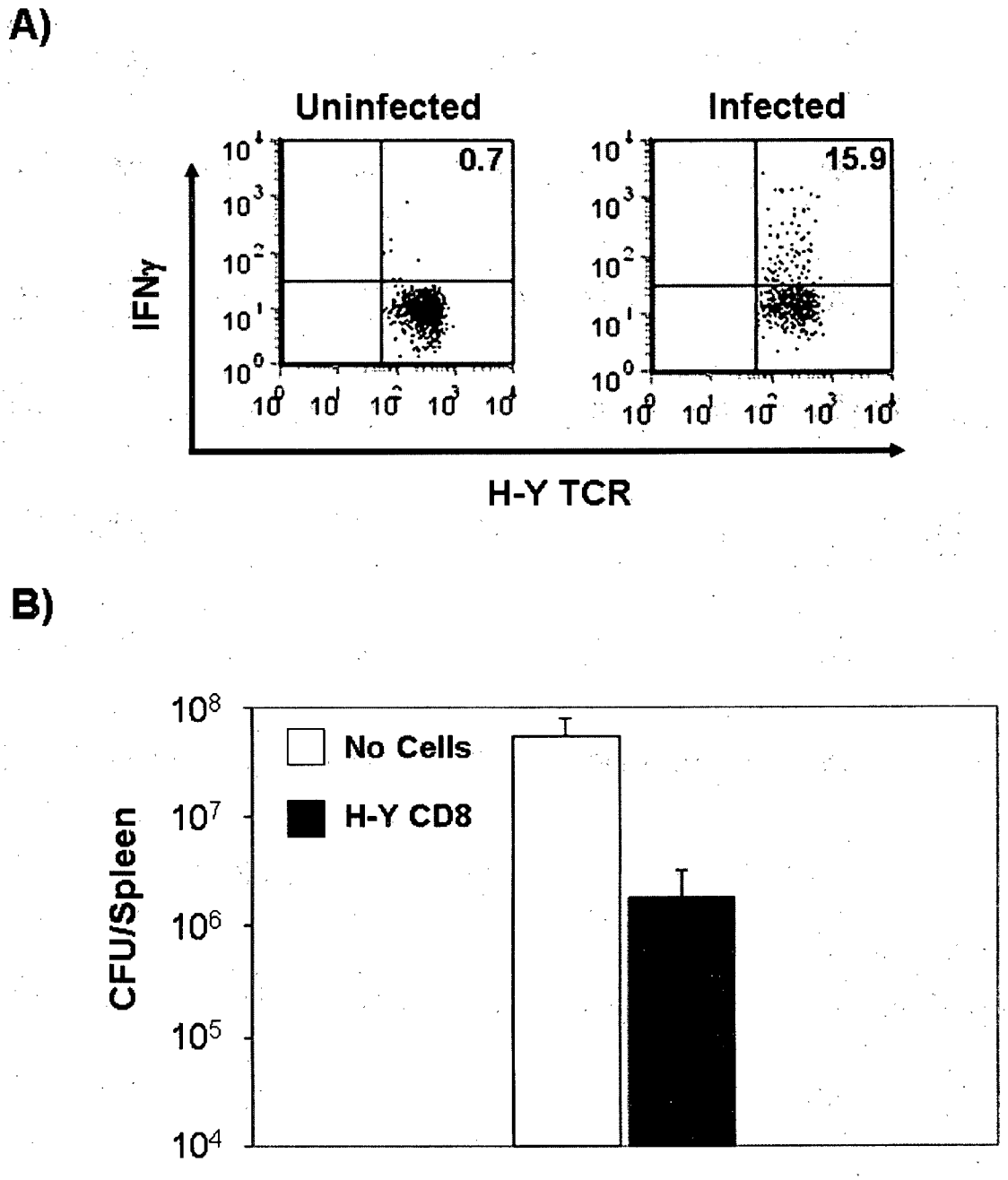

Figure 5.3 Self-specific CD8 T cells provide an innate source of IFN-γ during infection

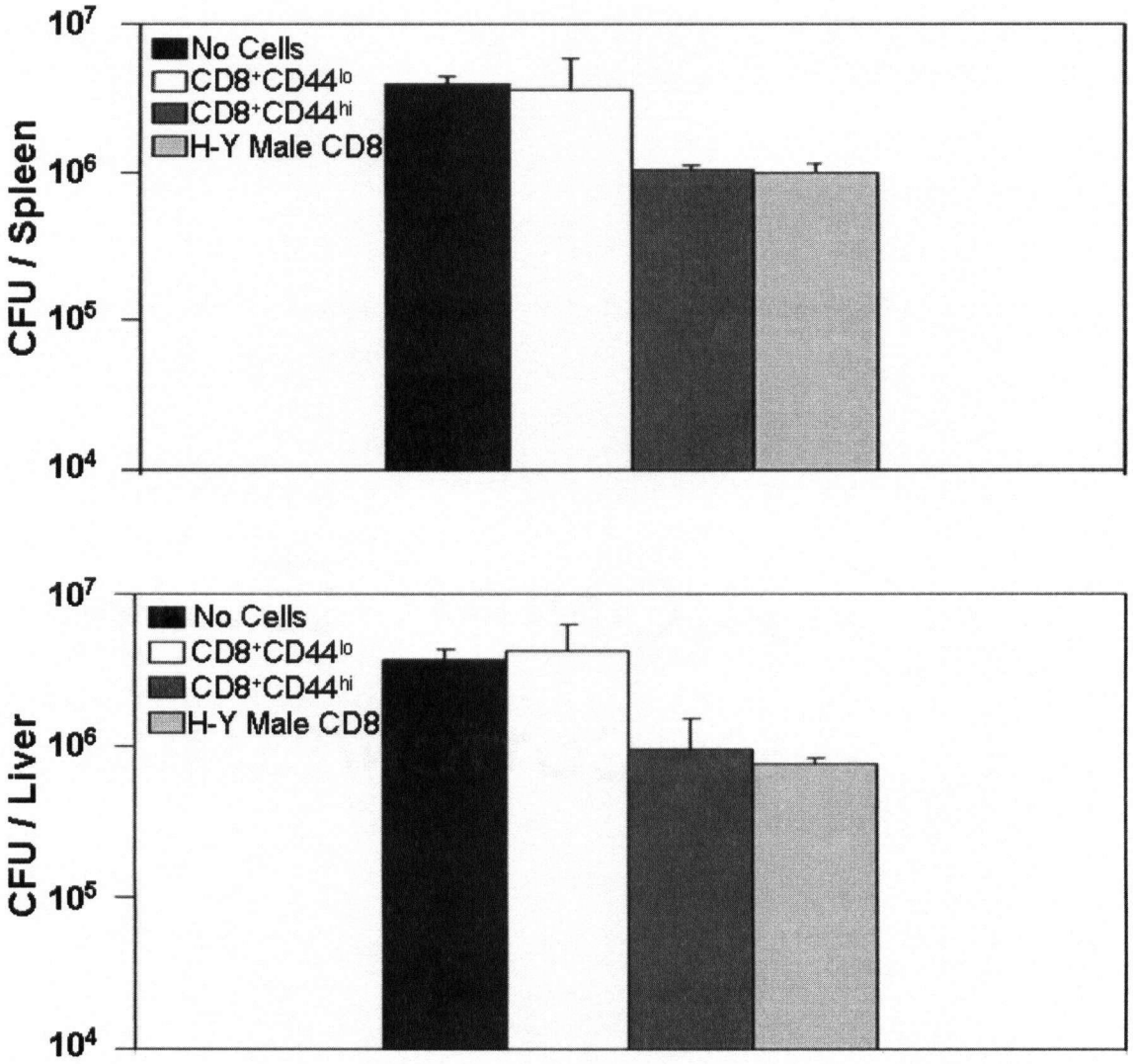

Figure 5.4 Self-specific H-Y male CD8 T cells and memory-phenotype CD8 T cells from normal B6 mice provide similar protection against LM infection

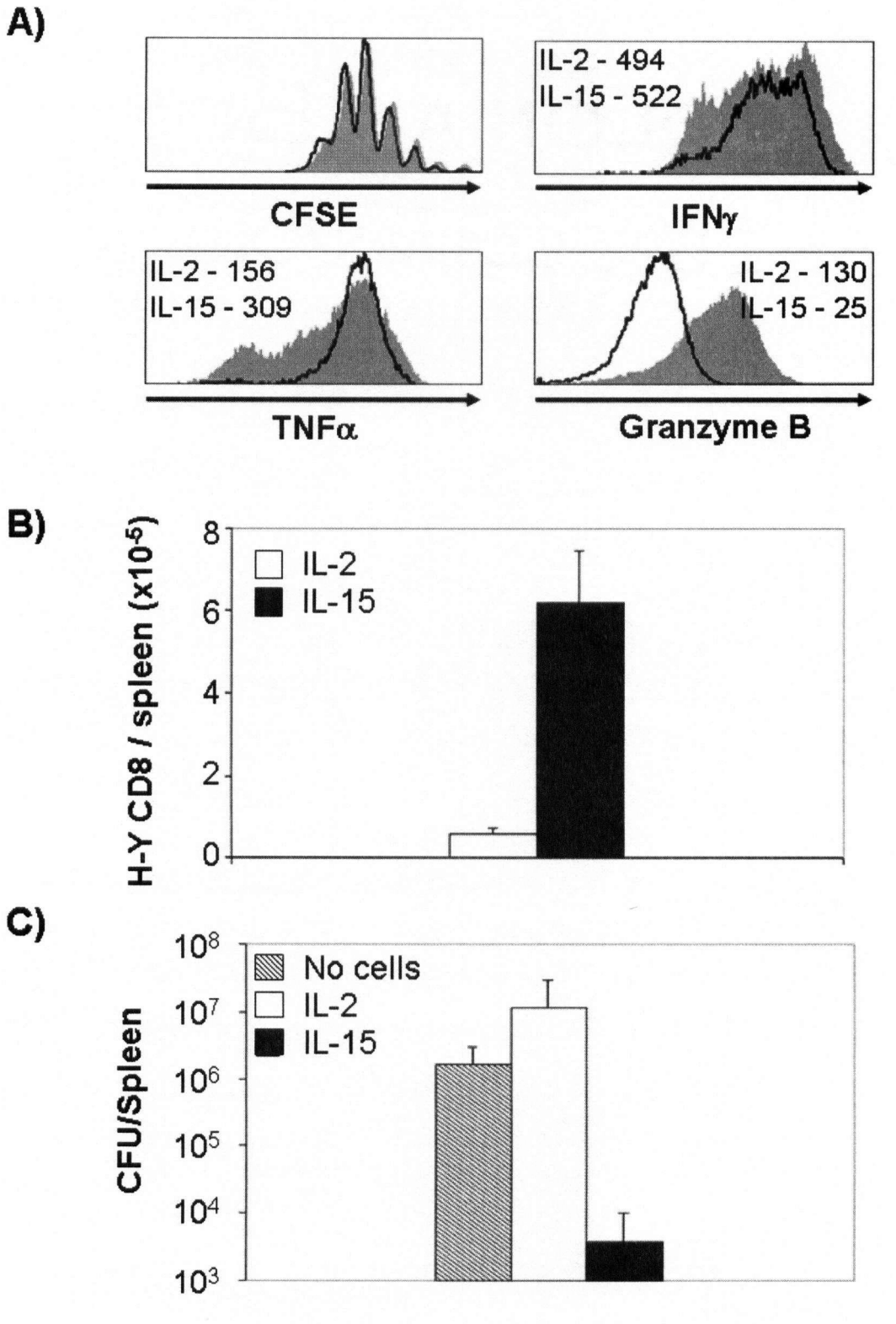

Figure 5.5 Self-specific CD8 T cells expanded in IL-15 are protective in vivo whereas IL-2 expanded cells are not

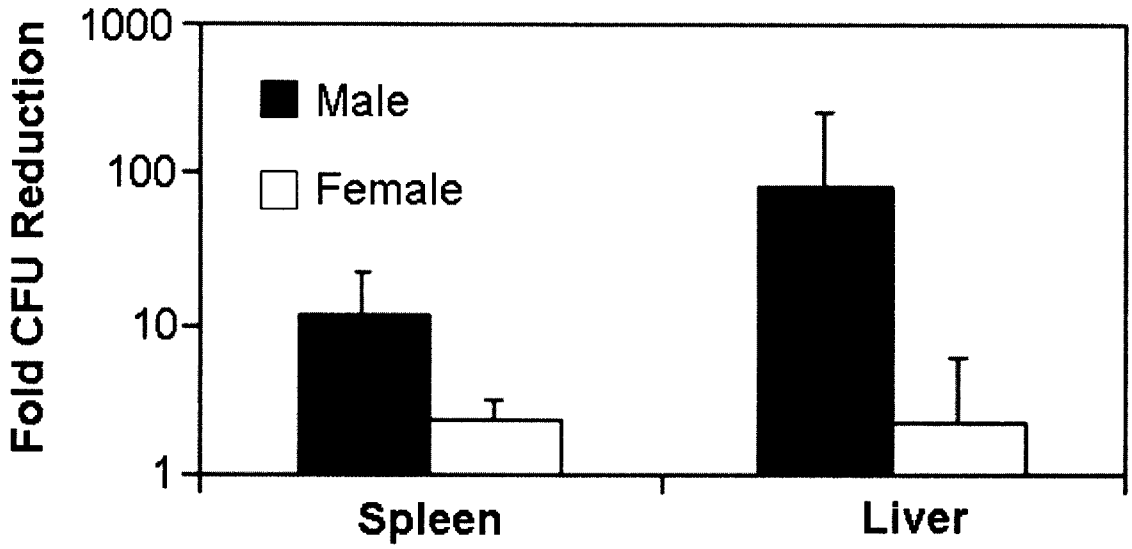

Figure 5.6 Self-Ag interactions enhance the protection mediated by self-specific CD8 T cells during infection

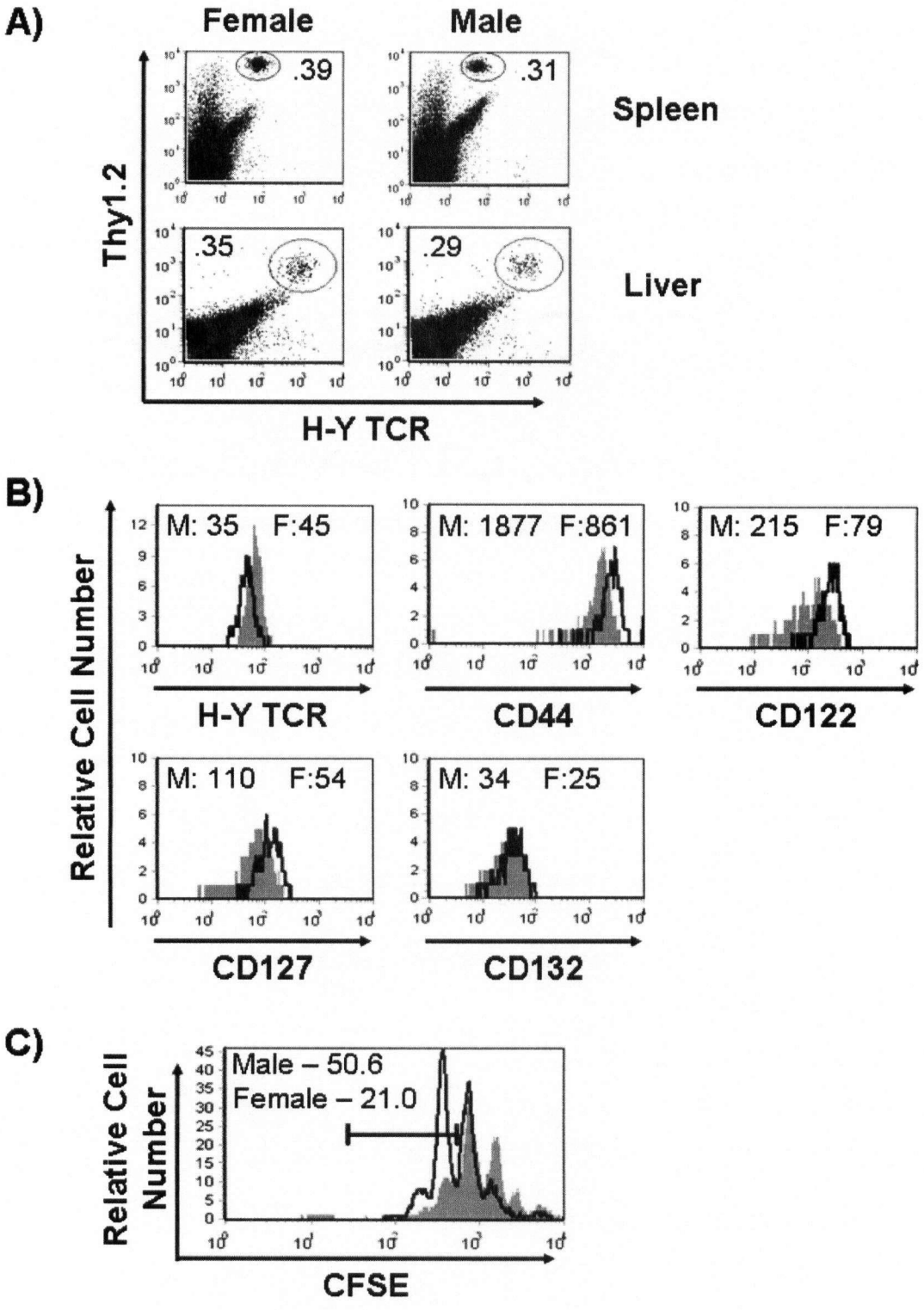

Figure 5.7 Self-Ag interactions maintain the memory phenotype and cytokine responsiveness of self-specific CD8 T cells in vivo

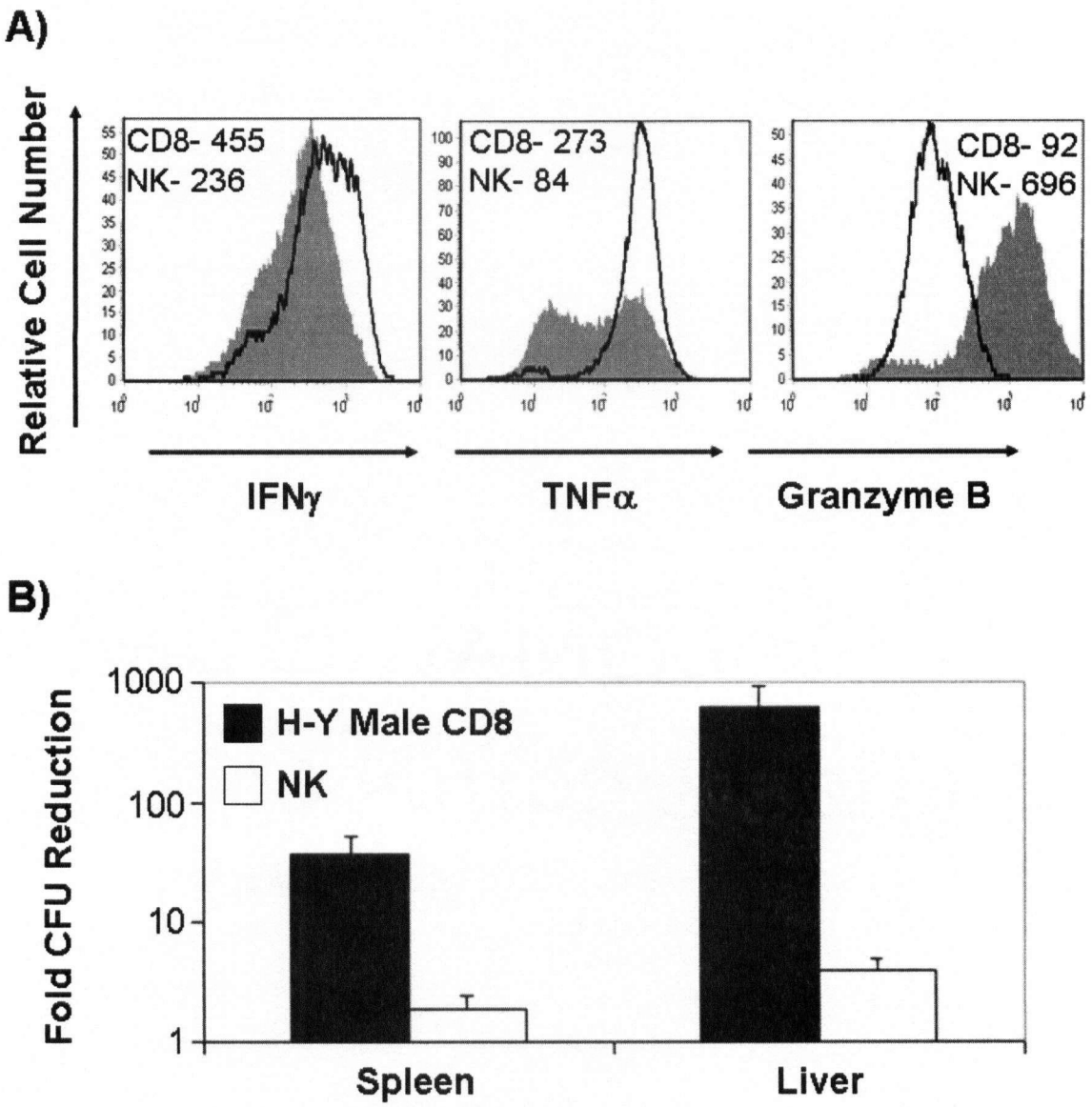

Figure 5.8 Self-specific CD8 T cells provide more IFN-γ-dependent protection in vivo against LM infection than NK cells

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Chapter 6 General discussion and perspectives

6.1 Self-specific CD8 T cells in non-TCR transgenic mice

This thesis has focused on the unusual properties of self-specific CD8 T cells from both normal and TCR transgenic mice. Chapter 2 was focused on the description of CD8 T cells in normal mice that demonstrated a significant degree of self-reactivity (1). These cells all had a memory-phenotype in naïve animals characterized by the expression of high levels of CD44 and CD122 and upon activation the cells expressed several NK receptors. The function of one of these NK receptors, NKG2D, was assessed in more detail and shown to be involved in the recognition of syngeneic tumor cells which expressed a ligand for this receptor. Chapter 3 went on to show that the self-specific $CD8^{lo}$ T cells found in male H-Y TCR transgenic mice shared a similar cell surface phenotype, NK receptor expression, and functional properties with self-specific CD8 T cells from non-TCR transgenic mice (2). The results in that chapter made it clear that the $CD8^{lo}$ cells found in male H-Y TCR transgenic mice were in fact a good model system to study the properties of self-specific CD8 T cells in non-TCR transgenic mice. As such, the H-Y model system was used to determine the role of self-antigen in the development and homeostasis of self-specific CD8 T cells. The use of $CD8^{lo}$ T cells from male H-Y TCR transgenic mice made it possible to determine the relative contributions of the self-specific TCR and NK receptors for target recognition by self-specific CD8 T cells. The experiments from chapter 4

showed that self-specific CD8 T cells from both H-Y male mice and from normal B6 mice could use the activating Fc receptor, CD16, for both cytokine production as well as the killing of antibody-coated target cells (3). In addition, this chapter uncovered another unusual feature of self-specific CD8 T cells that differentiate them from conventional CD8 T cells; the expression of FcRγ as part of the TCR complex. Chapter 5 was perhaps the most exciting chapter as it provided in vivo relevance for self-specific CD8 T cells by showing that they could provide innate protection during infection with LM. It also confirmed that the development of these cells was unconventional in that it could occur outside of the thymus. Perhaps most importantly this chapter showed that the expression of a self-reactive TCR allowed the cells to respond more vigorously to infections through the maintenance of CD122 expression.

Many of the findings presented in this thesis have been based on results obtained from using a homogenous population of self-specific $CD8^{lo}$ T cells from male H-Y TCR transgenic mice. This system was used because it provides a great degree of control during experiments since the TCR has a known specificity. The work in chapter two was aimed at describing the self-specificity of memory-phenotype CD8 T cells in normal non-TCR transgenic mice. This work clearly demonstrated that a significant proportion of $CD8^{+}CD44^{hi}$ T cells in the B6, Balb/c, and DBA/2 strains of mice demonstrate self-reactivity. More importantly these cells share an almost identical cell surface phenotype and functional properties with the self-specific $CD8^{lo}$ cells from male H-Y TCR transgenic mice. The biggest problem with the use of non-TCR transgenic mice

for these studies is the fact that the $CD8^{+}CD44^{hi}$ population in these mice is heterogeneous and consists of self-specific CD8 T cells, true foreign-antigen specific memory CD8 T cells, and MHC class Ib-restricted CD8 T cells. Thus, it was necessary to complement studies in normal mice with studies in TCR transgenic mice to gain novel insight regarding their developmental biology and function.

6.1.1 The relationship between foreign-antigen specific memory CD8 T cells and self-specific CD8 T cells

When naïve CD8 T cells encounter their cognate antigen for the first time they undergo several rounds of division and differentiation in order to generate a large number of effector cells capable of eliminating pathogens for which they are specific. The majority of these effector cells will then undergo a contraction phase leaving a small population of memory CD8 T cells specific for the original antigen. These memory CD8 T cells will then persist for several years at a greater precursor frequency and these cells will be capable of expanding more rapidly than the original naïve CD8 T cell (4). The maintenance of these memory T cells is independent of antigen but is heavily dependent on cytokines, especially IL-15 (5). Aside from their cell-surface phenotype there are other similarities between foreign-antigen specific memory CD8 T cells and self-specific CD8 T cells including their requirement for IL-15 and their ability to rapidly provide effector functions. There are also differences between foreign-

antigen specific memory CD8 T cells and self-specific CD8 T cells the biggest of which is their responsiveness to TCR stimulation.

One of the hallmark characteristics of foreign-antigen specific memory CD8 T cells is their ability to respond more rapidly during a reencounter with antigen. Memory CD8 T cells have been shown to have a decreased TCR signaling threshold and thus can activate signaling pathways downstream of their TCR much more efficiently than naïve T cells (6). This decreased TCR threshold is in stark contrast to the memory-phenotype self-specific CD8 T cells described in this thesis. In fact self-specific CD8 T cells actually have an increased signaling threshold relative to naïve CD8 T cells with the same specificity (2). When Yamada et al compared the phenotype of self-specific H-Y male CD8 T cells to foreign-antigen specific H-Y female memory CD8 T cells they found that the self-specific male cells were much less responsive to TCR stimulation (7). In addition, the self-specific H-Y male CD8 T cells were less efficient producers of IL-2 even though they could produce IFNγ. This difference in TCR signaling threshold differentiates self-specific CD8 T cells from foreign-antigen specific memory CD8 T cells and is probably in place to prevent the autoimmune potential of CD8 T cells bearing self-specific TCRs.

Memory CD8 T cells and self specific CD8 T cells both rely heavily on IL-15 for their maintenance and homeostasis. In fact mice deficient in either IL-15 or its receptor IL-15Rα have a large reduction in memory-phenotype CD8 T cells (8, 9). Homeostatic expansion of memory-phenotype CD8 T cells in response to lymphodepletion is heavily dependent on the availability of IL-15 (10, 11). IL-15 is

produced by a wide array of cell types (12) and is rapidly produced during infection resulting in the expansion of memory-phenotype CD8 T cells which express CD122. Both self-specific CD8 T cells and true foreign-antigen specific memory CD8 T cells can respond to IL-15 both in vitro and in vivo. In fact IL-15 can allow the survival of memory CD8 T cells in the complete absence of any peptide/MHC interactions (10). Self-specific H-Y male CD8 T cells are very responsive to IL-15 both in vitro and in vivo and this responsiveness is maintained by their interactions with self-antigen. It is interesting to note that foreign-antigen specific memory CD8 T cells from H-Y female mice, bearing the same TCR as self-specific H-Y male $CD8^{lo}$ T cells, are relatively unresponsive to IL-15 (7). It appears that this difference in IL-15 responsiveness is likely due to lower expression of CD122 by H-Y female memory CD8 T cells relative to self-specific male cells.

Foreign-antigen specific memory CD8 T cells have the ability to rapidly mediate effector functions such as target cell killing and cytokine production without the need for prior restimulation (13). Self-specific CD8 T cells can also kill target cells and produce cytokines without prior restimulation (14). In terms of cytokine production both foreign-antigen specific memory CD8 T cells and self-specific CD8 T cells can rapidly produce IFNγ upon TCR stimulation or upon stimulation with cytokines like IL-12, IL-15, and IL-18 and both of these memory-phenotype CD8 T cell populations can provide innate protection during bacterial infections (15-19). Whether differences exist in the amount of IFNγ production or

the stimuli that induce IFNγ production will need to be addressed more directly in the future.

It is clear from the studies presented in this thesis that foreign-antigen specific memory CD8 T cells and self-specific CD8 T cells share some similarities and it is also clear that these two cell types are also different. It would be of great interest to test the phenotype and function of these two cell types directly by comparing foreign-antigen specific memory CD8 T cells with several known specificities to self-specific CD8 T cells from H-Y male mice. In addition it would be interesting if one could determine the relative proportions of foreign antigen-specific versus self-specific CD8 T cells in memory phenotype CD8 T cells in normal mice.

6.1.2 The potential involvement of MHC class Ib molecules in the selection of self-specific CD8 T cells

The vast majority of CD8 T cells are selected by the highly polymorphic MHC class Ia molecules. However, CD8 T cells can also be selected by the less polymorphic MHC class Ib family of molecules. There are several different MHC class Ib molecules in mice (reviewed in (20)). Two of these MHC class Ib molecules, H2-M3 and Qa-1, are known to be involved in the activation of CD8 T cells during infection (21-23). There are several characteristics of MHC class Ib-restricted CD8 T cells that are similar to self-specific CD8 T cells and it remains possible that at least some self-specific CD8 T cells in non-TCR transgenic mice

are in fact restricted to MHC class Ib molecules. Similar to self-specific CD8 T cells, MHC class Ib-restricted CD8 T cells have a memory-phenotype even in naïve mice which allows them to become activated very rapidly during infections (16, 24). The cells can respond to LM infection in either an antigen-dependent or antigen-independent manner through the production of IFNγ (16). MHC class Ib-restricted CD8 T cells can be selected by hematopoetic cells and some of these cells can develop in the absence of a functional thymus (16, 24). Thus, there are many similarities between MHC class Ib-restricted CD8 T cells and self-specific CD8 T cells.

H2-M3 is an MHC class Ib molecule that binds and presents formylated peptides found in prokaryotic cells. The only formylated peptides found in eukaryotic hosts are those derived from mitochondrial proteins and thus the peptides capable of selecting H2-M3-restricted T cells is limited (20). One of the peptides involved in the positive selection of H2-M3-restricted CD8 T cells, ND1, is actually a weak agonist for mature H2-M3-restricted T cells (25). H2-M3-restricted CD8 T cells are very unusual in the sense that they have a memory-phenotype even in naïve mice and they can be selected by hematopoietic cells in the thymus (24).

Qa-1b-is another MHC class Ib molecule capable of selecting CD8 T cells. Qa-1b normally binds to the leader sequence of several MHC class Ia molecules (26) and acts as a ligand for the NKG2A/C/E NK receptors (27). Qa-1b is also capable of presenting other unconventional peptides including those derived from bacterial heat shock proteins like the GroEL peptide from several Gram-positive

and Gram-negative bacteria (28), peptides derived from the TCR chains of CD4 T cells (29), peptides from mouse heat shock protein 60 (HSP60) (30), as well as a peptide derived from insulin (31). Qa-1b-restricted CD8 T cells that recognize peptides from the TCR chains of CD4 T cells play an important role in regulating the CD4 response (32). Qa-1b restricted CD8 T cells specific for GroEL have been shown to expand during infection with Salmonella. Interestingly, these GroEL-specific CD8 T cells can also recognize self-derived HSP60 peptides presented by Qa-1b providing a potential link between Qa-1b, infection, and autoimmunity (30).

It is very tempting to speculate that there is in fact a relationship between both H2-M3 and Qa-1b-restricted CD8 T cells and self-specific CD8 T cells. Both cell types exhibit a memory-phenotype and both subsets respond rapidly during infections. Perhaps the reason that MHC class Ib molecules are so highly conserved is that they act similarly to the pattern recognition receptors (PRRs) such as the Toll-like receptors (TLRs) found on several innate cells. In fact the literature is consistent with the hypothesis that MHC class Ib-restricted CD8 T cells can recognize self-peptides that are induced or upregulated on infected or transformed cells. Consistent with this idea is the recognition of self-HSP60 on stressed cells by GroEL-specific CD8 T cells (30). Also consistent with this idea is the fact that mice lacking H2-M3-molecules are more susceptible to infection with LM (33) yet mice infected with a mutant strain of LM incapable of producing formylated peptides clear infection normally (34). These two potentially conflicting results can be reconciled by the fact that H2-M3 molecules present self-derived

formylated peptides to H2-M3-restricted CD8 T cells during infection with LM and that this process plays a key role in controlling bacterial infection. This hypothesis, if true, would confirm that MHC class Ib-restricted CD8 T cells are in fact self-specific and may behave similarly to the self-specific CD8 T cells described in this thesis. It will be of great interest to determine whether there is in fact a relationship between these seemingly unrelated cell types and perhaps the first place to look would be at the CD8 T cells found in MHC class Ia-deficient mice.

6.2 The in vivo significance of self-specific CD8 T cells

6.2.1 The autoimmune potential of self-specific CD8 T cells

Self-specific CD8 T cells express self-reactive TCRs and as such they have the potential to damage normal cells if they are not properly controlled. One of the main characteristics of self-specific CD8 T cells is their inability to proliferate and become activated in response to normal physiological levels of their cognate self-antigens (2). This antigen-unresponsiveness was shown in chapter 3 to be due to TCR signaling defects in the cells leading to an inability to efficiently activate pathways downstream of the TCR. If this high TCR signaling threshold is in effect

reversed, then self-specific CD8 T cells can cause autoimmunity as seen in E2F2-deficient mice (35).

The E2F family of transcription factors is important for regulating many aspects of T cell function including proliferation, apoptosis, and differentiation. A report by Murga et al (2001) described the autoimmune phenotype of mice lacking E2F2. These mice develop a late-onset autoimmune disease characterized by the expansion of memory-phenotype CD8 T cells leading to widespread inflammation and immunocomplex deposition. These $CD8^{+}CD44^{hi}$ cells displayed overt self-reactivity proliferating in response to syngeneic stimulator cells. This study went on to show that when E2F2-deficient mice transgenically expressed the H-Y TCR, male mice developed autoimmunity faster and with a more severe phenotype compared to non-TCR transgenic mice. The cause of the disease was the large population self-specific H-Y male $CD8^{lo}$ cells described in this thesis. The lack of E2F2 had lowered the TCR threshold of the cells allowing them to proliferate and become activated leading to disease. Thus it is clear that self-specific CD8 T cells have the capability of causing disease but they are normally held in check by their inability to efficiently become activated by TCR stimulation alone.

Self-specific CD8 T cells can become activated in response to cytokines such as IL-15 and this activation results in the expression of NK receptors (1, 2). IL-15-responsiveness is tightly controlled through the action of members of the suppressor of cytokine signaling (SOCS) family which dampen cytokine signaling (36). It remains possible however, that uncontrolled cytokine signaling may lead

to autoimmunity through the activation of self-specific CD8 T cells. In fact SOCS-1-deficient mice display a partially T cell-dependent autoimmune disease mediated by overproduction of IFNγ (37). IL-15-hypersensitivty in these animals plays a key role in the activation of the memory-phenotype CD8 T cells and these animals have an increase in the proportion of autoreactive CD8 T cells (38). The development of disease in SOCS-1-deficient mice depends heavily on the ability of the CD8 T cells to undergo homeostatic expansion suggesting that self-specific CD8 T cells, which display a remarkable ability to homeostatically expand, may play a role in disease in SOCS-1-deficient mice.

NK receptor expression by self-specific CD8 T cells confers the cells with the ability to recognize target cells in the absence of TCR stimulation. This generates cells which are no longer MHC-restricted and can thus potentially destroy host cells expressing the appropriate ligands. NKG2D was one of the NK receptors, which is central to the findings in this thesis. Self-specific CD8 T cells express both NKG2D and the adaptor DAP12 when activated with either antigen or cytokine alone (1, 2). Thus the NKG2D receptor on these cells has the potential to cause damage to host cells if they overexpress any NKG2D ligand. There are several reports in the literature which suggest that the uncontrolled expression of NKG2D coupled with expression of NKG2D-ligands does play a role in autoimmunity. In fact, the non-obese diabetic (NOD) strain of mice, was shown to over-express the Rae-1-family of NKG2D-ligands in pancreatic islet cells and that NKG2D expression on auto-reactive CD8 T cells was key in the progression towards diabetes (39). In this system the blocking of NKG2D was

sufficient to prevent the expansion of autoreactive CD8 T cells and could prevent disease. There may also be other conditions where NKG2D signaling is out of control. Bana Jabri's group showed that TCR-independent killing mediated by NKG2D may play a role in celiac disease (40). They found that IL-15 produced in celiac patients was sufficient to convert NKG2D on CD8 T cells into a directly activating receptor. This conversion led to MHC-independent killing activity by CD8 T cells from celiac patients. Thus it is possible that even in their system that self-specific CD8 T cells may cause disease if NK receptor expression or signaling is altered.

6.2.3 The potential role for self-specific CD8 T cells in anti-tumor immunity

It is clear that self-specific CD8 T cells have the potential to be harmful if left uncontrolled. On the flip side, self-specific CD8 T cells may actually be beneficial if they can be used to eliminate tumor cells which often express normal self-antigens. It is possible that self-specific CD8 T cells may in fact respond to tumor cells if they over-express particular self antigens or if they express ligands for activating NK receptors like NKG2D. For example if self-specific CD8 T cells exist for peptides from a particular tumor oncogene, then over-expression of this oncogene in transformed cells may be reflected by over-expression of peptides capable of activating self-specific CD8 T cells. Since self-specific CD8 T cells have an increased activation threshold they would require over-expression of cognate antigen for them to be activated. Similarly tumor cells may express one

or more NKG2D ligands allowing self-specific CD8 T cells to recognize and destroy these "stressed" cells in the absence of strong TCR stimulation.

There are several reports in the literature regarding the expression of NK receptors on melanoma-specific CD8 T cells, even those recovered from healthy donors (41, 42). It is possible that inhibitory receptors may prevent autoimmunity but allow a response if the target lacks the ligand for the receptor. In fact, inhibitory NK receptor expression by human melanoma-specific CD8 T cells has been shown to play a role in the killing of tumor cells that have lost expression of the MHC class I ligand for the receptor (43).

In mice, one group has shown that CD8 T cells specific for a non-mutated melanoma antigen, tyrosinase-related protein 2 (TRP-2), have a natural memory-phenotype identical to that of the self-specific CD8 T cells discussed in this thesis (44). In fact, these TRP-2-specific CD8 T cells can develop extrathymically suggesting that perhaps self-specific CD8 T cells described in this thesis can be specific for tumors.

It would be of great interest to determine if self-specific CD8 T cells specific for tumor antigens did behave the same way as self-specific H-Y male $CD8^{lo}$ T cells. There is one system that may be of particular interest. In doubly transgenic mice, which express a transgenic TCR specific for the gag protein from Friend murine leukemia virus (FMuLVgag) as well as the transgenic cognate Ag, FMuLVgag, CD8 T cells expressing the transgenic TCR still develop (45). Furthermore, these cells exhibit a memory-phenotype similar to the self-specific $CD8^{lo}$ T cells from H-Y male mice; these mice also did not develop detectable

autoimmune diseases even though the self-specific CD8 T cells retain effector function and are specific for the self-Ag (46). A recent report on these cells also demonstrated their ability to proliferate in response to IL-2 or IL-15 alone and more importantly showed that these self-specific CD8 T cells could eliminate tumor cells that expressed the self-antigen (47). Whether or not these cells express any NK receptors or function similarly to the self-specific CD8 T cells in this thesis is unclear although it seems likely that the cells will share some similarities.

6.2.3 Other potential roles for self-specific CD8 T cells

It is very tempting to speculate that self-specific CD8 T cells play many other roles in the immune response other than the innate production of cytokines to impede bacterial growth. NK cells have been shown to be involved in the production of cytokines such as IFNγ that can help to polarize the CD4 response (48). I have shown that self-specific CD8 T cells can actually produce significantly more IFNγ than NK cells. It is therefore conceivable that they can also be involved in this aspect of immunity. The literature is consistent with the idea that memory-phenotype CD8 T cells can actually provide a source of IFNγ for the priming of CD4 T cells (49). It should be relatively simple to test the ability of self-specific CD8 T cells to provide this function.

6.3 Concluding remarks

This thesis defines the developmental and functional properties of a novel subset of CD8 T cells specific for self-antigens. These cells express receptors characteristic of both T cells and NK cells and this property allows the cells to rapidly focus on infected, stressed or transformed cells. The results presented demonstrate a potential role for self-specific CD8 T cells in various important aspects of immunity ranging from innate immune protection, to tumor immunology, and autoimmunity.

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