ORIGIN AND FUNCTION OF CD8 T CELLS IN MHC CLASS Ia-DEFICIENT MICE

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Dedication

To Mom, Dad, Yonghe, and Ashley Liu

ORIGIN AND FUNCTION OF CD8 T CELLS IN MHC CLASS Ia-DEFICIENT MICE

by

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DISSERTATION

Presented to the Faculty of the Gaduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

August, 2005

Acknowledgements

I take this opportunity to thank my advisor, Dr. Forman, for his constant support and guidance. Through criticism and encouragement, through joy and laughter, he has led me into the wonderful research world. He teaches me how to think scientifically and how to become an independent researcher. Without his patience and persistence, I could not have come so far.

Many thanks are also given to the other committee members, Dr. Stroynowski, Dr. Takashima, Dr. Lu, and Dr. Bouwer. I thank them for their enthusiasm and guidance throughout my project. The stimulating discussions and countless constructive suggestions have been the driving force for the improvement of my work.

I would like to thank all the people that I interacted with who were once part of Forman lab. They are not only colleagues but also good friends. I especially thank Dr. Ailin Bai, Dr. Zoran Kurepa, Dr. Rance Berg for teaching me techniques and helping me by discussions. I thank the current members of the Forman Lab, Parisa Sinai, Sean Murray for their friendships and collaboration.

I would like to acknowledge several people who have helped me on various projects. I thank Jessie Mellon in Dr. Niederkorn's lab for teaching me thymectomy technique. Dr. Tabaczewski helped me refold and purify Qa-1b protein. Dr. Matsue helped me culture bone marrow derived dendritic cells. I thank them for their time and willingness to help.

I would like to give thanks to the members of the Immunology Graduate program and the Center for Immunology. Everyone I have interacted with had a positive impact on my graduate school experience.

I am deeply grateful to my mom and dad, my grandma and grandpa, and my sister for their endless support, encouragement, and prayers throughout my life. I would not have made it without their unwavering belief in me,

Finally, my deepest thanks and love to my husband, Yonghe Liu. Good days or bad days, he is always there with me. Without him, life would not have been so fulfilling.

ORIGIN AND FUNCTION OF CD8 T CELLS IN MHC CLASS Ia-DEFICIENT MICE

Publication No. _____

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The University of Texas Southwestern Medical Center at Dallas, 2005

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B6.H-2K$^{b-/-}$D$^{b-/-}$ (DKO) mice are devoid of class Ia but express normal levels of class Ib molecules. They have low levels of CD8 T cells in both the thymus as well as peripheral T cell compartments. Although the percentage of splenic CD8αα T cells is increased in these animals, approximately 90% of CD8 T cells are CD8αβTCRαβ. In contrast to B6 animals, most of the CD8 T cells from these mice have a memory phenotype (CD44^{hi}) including both CD8αβ and CD8αα subsets. In the thymus of DKO animals, there is a decrease in the percentage of single positive CD8 T cells, although most are CD44^{low}, similar to that seen in B6 mice. Our results indicated that the paucity of CD8 T cells in DKO mice might be in part due in reduced thymic export, lower basal proliferation, high apoptosis, and inability to undergo homeostatic expansion. DKO mice have greatly reduced numbers of mature CD8αβ T cells in their periphery. However,

these non class Ia selected CD8αβ cells are able to mediate immune responses to a number of pathogens. Approximately 60% of the CD8αβ T cells in the spleen and peripheral lymph nodes of naïve DKO mice display a memory ($CD44^{hi}$) phenotype. To investigate the origins of these non class Ia selected CD8αβ$CD44^{hi}$ cells, we traced the phenotype of recent thymic emigrants (RTEs) and found that most were $CD44^{lo}$. We also determined if their appearance was thymus-dependent and found that only a small percentage of non class Ia selected CD8αβ$CD44^{hi}$ cells develop in a thymus-independent pathway. Functionally, CD8αβ$CD44^{hi}$ cells from DKO mice are able to secrete IFN-γ in response to interleukin (IL)-12 and IL-18 in the absence of cognate antigen. When challenged with anti-CD3 in vivo, nearly half of these cells produce IFN-γ within 3 hours. When purified CD8αβCD44hi cells from Thy1.2.DKO mice were transferred into Thy1.1 DKO recipients and then challenged with *Listeria monocytogenes* (LM), an antigen specific anti-LM response was observed six days later. Our data suggest that non class Ia selected CD8αβ$CD44^{hi}$ cells in naïve animals can respond to antigen and play a role in the innate as well as the early phase of the acquired immune response.

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Prior publications

Su, J., R. E. Berg, S. Murry, and J. Forman. 2005. Thymus-dependent memory phenotype CD8 T cells in naïve B6.H-2K$^{b-/-}$D$^{b-/-}$ mice mediate an antigen specific response against *Listeria monocytogenes*. Submitted to Journal of Immunology.

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Cowley SC, Hamilton E, Frelinger JA, Su J, Forman J, Elkins KL. 2005. CD4$^-$CD8$^-$ T cells control intracellular bacterial infections both in vitro and in vivo. J Exp Med. 202(2):309-19.

Abstracts

Su, J., and J. Forman. CD8 T cells in MHC class Ia–deficient mice. AAI, Denver, CO, 2003.

Su, J., R. E. Berg, S. Murry, and J. Forman. Thymus dependent MHC non class Ia selected memory phenotype CD8 T cells from naïve mice provide rapid protection against infection. AAI, San Diego, CA, 2005.

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List of Abbrevations

The following list does not include standard abbreviation published in the first issue of each volume of *The Journal of Immunology*

bm	bone marrow
β_2m	β_2-microglobulin
DC	dendritic cell
DKO	B6.H-2K$^{b-/-}$D$^{b-/-}$
ER	endoplasmic reticulum
GATA-3	GATA-binding protein-3
HKLM	heat killed LM
HSP	heat shock protein
ICS	intracellular cytokine staining
IFNAR	type I IFNα/β receptor
iIEL	intestinal intraepithelial lymphocytes
IL-7Rα	interleukin 7 receptor α-chain
LCMV	lymphocytic choriomeningitis virus
LLO	Listeriolysin O
LM	*Listeria monocytogenes*
MIC	MHC class I chain related
MR1	MHC-related protein 1
ORF	open reading frame

PLP proteolipid protein

Qdm Qa-1 determinant modifier

Rag2 recombination activating gene 2

RTEs Recent thymic emigrants

T-bet transcription factors T-box expressed in T-cells

T_{CM} central memory T cells

T_{EM} Effector memory T cells

Thp naive T helper precursor

TipDCs TNF- and iNOS-producing DCs

TL Thymus Leukemia

Tx Thymectomized

ULBP unique long 16 (UL16)-binding protein

Introduction

I: MHC class Ia and non class Ia molecules

I-1 Overview of MHC class Ia molecules

The Major histocompatibility complex (MHC) is a genetic region, located on the short arm of chromosome 6 of the human, chromosome 17 of the mouse, chromosome 20 of the rat, and cluster I of the chicken. It has effects on tumor or skin transplantation and control of immune responsiveness and resistance to infection (Simpson 1988). It can be separated into two different classes, MHC class I and MHC class II, based on differences in function, structure, source of peptide ligands, and expression pattern. MHC class I molecules are expressed on the cell surface of all nucleated cells, although the expression level varies among different cell types. They are heterodimers, composed of a heavy chain, which is an Mhc-encoded type I integral membrane proteins, a β_2-microglobulin (β_2m) light chain, and a short peptide 8-10 amino acids in length, all of which are essential for the formation of a stable MHC class I protein. The heavy chain is comprised of three domains (α1, α2 and α3). The α1 and α2 domains fold to form two antiparallel α helices which lay over a platform of antiparallel β-sheet strands. This structure forms the peptide-binding site, which binds antigenic peptides of 8–10 amino acids in length derived from intracellular proteins. The α3 domain of the heavy chain is associated with β_2m and forms a structure important for interaction with the T cell accessory molecule CD8 (Bjorkman et al. 1987).

Those MHC class I proteins whose heavy chains are encoded by highly polymorphic genes (H-2K, D, and L in mice; HLA-A, B, and C in human) are termed "classical" or class I-

region associated (Ia) MHC (O'Callaghan et al. 1998). Cell-surface MHC class Ia proteins are essential for the thymic development of $CD8^+$ T cells and for providing protection against lysis by natural killer (NK) cells. The peptides that bind MHC class Ia molecules are normally derived from the degradation of intracellular proteins but also derived from ingested foreign molecules or dysregulated proteins, viruses, and other cellular pathogens. The degradation of cytosolic proteins, either self or pathogen derived, is a tightly regulated process that is primarily mediated by a multicatalytic protease complex called the proteasome (Brown et al. 1991). The resulting peptides must be able to translocate from the cytosol into the endoplasmic reticulum (ER) lumen to associate with immature MHC class I molecules (Neefjes et al. 1993). The transporter associated with antigen processing (TAP) plays an essential role in this process and TAP deficient cells express extremely low levels of stable MHC class I molecules on the cell surface (Van et al. 1992). When presented at the cell surface as ligands for recognition by the antigen specific receptor of CD8 T cells or stimulatory and inhibitory receptors expressed by NK cells, they control the response of these immunological effector cells.

I-2 Overview of non-MHC class Ia molecules

In addition to the highly polymorphic MHC class Ia genes, most vertebrates have many other genes which encode for MHC class I-like proteins (Trowsdale 1995). These genes are called non classical MHC class Ia genes or non class Ia genes. The non classical MHC class Ia genes include genes that fall into the murine MHC class Ib region which includes H2-Q, -T, and -M, as well as genes which lies outside the MHC region, such as CD1. Although the number of class I genes in the Q, T, and M regions of the murine MHC is large, only a few are known to encode cell membrane molecules that associate with β_2m and thus likely to present antigen to the TCR on CD8 T cells. Unlike class Ia molecules, MHC class Ib genes have

limited polymorphism and the expression of class Ib molecules is relatively low and sometimes limited in tissue distribution. The class Ia molecules, HLA-A, -B, and -C in human, and H2-K, -D, and -L in mouse bind a diverse array of peptide ligands. By contrast, it has been noted that some class Ib molecules normally bind a limited array of peptides (Qa-1^b) (DeCloux et al. 1997), formylated peptides (M3) (Lindahl et al. 1997), or no peptides (T10, T22) (Wingren et al. 2000), while others such as Q7 and Q9, which encode Qa-2 molecules, present a wide array of peptides (Joyce et al. 1994).

I-2-1 Murine non-classical MHC class I molecules

The mouse MHC contains approximately 30-40 class Ib genes which map in three distinct regions termed Q, T and M (Shawar et al. 1994). The Q region contains approximately 10 genes. Most Q region genes are expressed ubiquitously. Q10 is primarily synthesized and secreted by hepatocytes and binds a wide array of self peptides. The function of Q10 remains unknown (Lew et al. 1986;Zappacosta et al. 2000). The most well defined MHC class Ib molecules encoded within the H2-Q region are Qa-2 molecules. These molecules are relatively non-polymorphic. They are the combined product of four very similar genes (Q6-Q9) (Stroynowski 1995). The Qa-2 genes encode molecules that bound to the cell membrane via a glycophosphatidylinositol (GPI) anchor (Stroynowski et al. 1987). These Qa-2 molecules can be secreted by activated T cells. Expression of both membrane bound and secreted forms of Qa-2 requires β_2m and TAP (Tabaczewski and Stroynowski 1994). They are capable of binding a wide repertoire of peptide ligands (Tabaczewski et al. 1994). These peptides characterized by two major anchors: a His at position 7, and a hydrophobic residue at position 9. Membrane-bound Q9 is one of the most well characterized murine MHC class Ib proteins. Q9 binds a large array of TAP-dependent nonameric peptides and has many similarities to the classical class I

MHC. It is widely expressed in nearly all tissues. However, Q9 expression was found to be silenced or severely deficient in a large panel of in vivo-derived tumor cell lines. Recent reports demonstrated that Q-9 is able to mediate protective immunity against multiple disparate tumors (Chiang et al. 2002;Chiang et al. 2003).

Twenty-five class I-like genes have been described in the H2-T region of mice, but only some of them have an open reading frame (ORF) (Shawar et al. 1994). In mouse, both T3 and T18 encode TL (Thymus Leukemia) antigens expressed in gut (Hershberg et al. 1990). T18 also encodes TL in thymus. The expression of TL is β_2m-dependent and TAP-independent (Holcombe et al. 1995). TL is expressed on intestinal epithelial cells, suggesting their specialized role in mucosal immunity. The natural ligands of TL on intestinal intraepithelial lymphocytes (iIELs) are CD8αα molecules (Leishman et al. 2001). It has been demonstrated that both αβ T cells and γδ T cells can elicit cytolytic activity through recognition of TL (Tsujimura et al. 2001). TL-specific CTL recognized the α1/α2 domain of TL without any antigen peptides (Tsujimura et al. 2003).

The T region encoded genes T22 and T10 (which have 94% sequence identity) have been shown to act as ligands for γδ T cells (Shawar et al. 1994). T10 and T22 associated with β_2m, but do not appear to bind peptide ligands (Wingren et al. 2000;Crowley et al. 1997) While T 22 is ubiquitously expressed, T 10 is predominantly expressed by hematopoietic cells (Bonneville et al. 1989). A recent study indicates that activated lymphocytes up-regulate expression of T10 and T22, and that these molecules may interact with regulatory γδ T cells (Crowley et al. 2000).

Gene T23 encodes Qa-1, a molecule possibly orthologous to human HLA-E. Both molecules bind similar leader-derived peptides from other class I molecules and both are

ligands for CD94/NKG2 receptors on NK cells. Qa-1 exhibits a broad tissue expression, but cell surface levels are much lower than that of class Ia molecules. Qa-1 has 10 alleles identified by serological and CTL techniques (Soloski et al. 1995). Among them, Qa-1b is one of the most well defined molecules. Although Qa-1b is expressed on a variety of hematopoietic tissues, it is only minimally or not expressed at all on resting T cells and thymocytes and its expression is significantly increased following peripheral T cell activation by antigen (Tewarson et al. 1983;Jiang and Chess 2000). Qa-1b binds Qdm (Qa-1 determinant modifier), a nonameric peptide derived from the leader sequence of certain alleles of H-2 D and L MHC class Ia molecules in a TAP dependent way (Kurepa et al. 1998;Aldrich et al. 1992). Qa-1b/Qdm complexes bind to nonclonally distributed CD94/NKG2A, CD94/NKG2C and/or CD94/NKG2E receptors on natural killer (NK) cells. NK cell medicated lysis can be either inhibited or activated by interaction with Qa1b expressing cells, depending on whether NKG2A or NKG2C is complexed with CD94 (Vance et al. 1999;Vance et al. 1998). Qa-1b is also capable of presenting bacterial antigens to CD8 T cells. Qa-1b restricted CTL can be detected in mice that have been infected with facultative intracellular bacterial pathogens Listeria monocytogenes (LM) and Salmonella typhimurium (Bouwer et al. 1997;Lo et al. 2000). The salmonella derived epitope identified is derived from the Salmonella GroEL (HSP60) protein.

Another role that has been implicated for Qa-1 is linked to CD8 T cell dependent suppression (Sarantopoulos et al. 2004). Recent studies have suggested that a subpopulation of $CD8^+$ T cells might suppress the response of activated $CD4^+$ T cells and B cells through an interaction that depends on expression by target cells or antigen presenting cells (APCs) of the MHC class Ib molecule Qa-1 (Jensen et al. 2004). Qa-1–deficient animals develop exaggerated secondary $CD4^+$ T cell responses after viral infection or immunization with foreign or self

peptides. In addition, enhanced $CD4^+$ T cell responses of Qa-1–deficient mice to mouse proteolipid protein (PLP) self peptide are associated with increased susceptibility to recurrence of EAE; this phenotype is remedied by lentiviral-based expression of the correct Qa-1 allele (Hu et al. 2004). These data indicate Qa-1 is required for an appropriately decreased $CD4^+$ T cell response to infection and for resistance to autoimmunity.

In the M region, there are approximately 18 genes, but only M3 is well characterized. M3 encodes a molecule that present relatively short peptide antigens (five to seven residues) that possess an N-terminal formyl moiety (Lindahl et al. 1997). The endogenous natural ligands are derived from mitochondrial proteins (Loveland et al. 1990). In contrast to class Ia molecules, M3 surface expression is undetectable in most cell types except B cells due to the paucity of endogenous formylated methionine-containing peptides that could only arise from mitochondrial proteins (Vyas et al. 1994;Chiu et al. 1999). Since prokaryotes initiate protein synthesis with *N*-formylated methionine, M3 molecules can bound peptide antigens derived from intracellular bacteria and function as restriction elements for CD8 T cell. The majority of M3 is retained in the ER and traffics rapidly to the cell surface upon addition of exogenous peptide or presumably infection by intracellular bacteria. M3 can be induced by addition of *N*-formylated peptides from mitochondria or infection by intracellular bacteria on the surface of many cell types, most efficiently on APCs, i.e., B cells, macrophages, and dendritic cells (Chiu et al. 1999). Basal and inducible expression of M3 is TAP-dependent (Hermel et al. 1991;Attaya et al. 1992). The most well studied H2-M3 restricted cytolytic T lymphocyte (CTL) response is against LM infection. To date, there are three listerial antigens presented by M3 have also been identified: f-MIGWII is derived from the LemA transmembrane protein (Lenz et al. 1996), f-MIVIL is derived from an unknown protein source (Gulden et al. 1996;Princiotta et

al. 1998) , and f-MIVTLF is derived from the AttM transcriptional attenuator polypeptide (Princiotta et al. 1998). One interesting aspect about H2-M3-restricted $CD8^+$ T cells is that they can exhibit cross-reactivity to different bacterially derived *N*-formylmethionine peptides (Ploss et al. 2003). In addition to LM, M3 can also present N-formylated antigens derived from Mycobacteria tuberculosis to CD8 T cells (Chun et al. 2001).

Several murine nonclassical MHC class I molecules are mapped outside of the MHC region. CD1 molecules are a family of highly conserved antigen-presenting proteins that are similar in function to classic MHC molecules. Whereas classic MHC molecules present peptides, CD1 proteins bind and display a variety of lipids and glycolipids to T lymphocytes (Porcelli and Modlin 1999). The five known isoforms of CD1 are classified into two groups, group I (CD1a, CD1b, CD1c, and CD1e in humans) and group II (CD1d in humans and mice), based on similarities between nucleotide and amino acid sequences (Porcelli 1995). Murine CD1d is expressed by nearly all hematopoietic cells. Certain population of T cells, known as NK T cells, that express both NK cell receptors and a restricted set of αβ T-cell receptors, are activated by CD1d molecules (Burdin et al. 1998). Most human NKT cells have a restricted TCR repertoire which includes an invariant Vα24-JαQ rearrangement. In the mouse a similar situation occurs with Vα14-Jα281 rearrangement and the invariant TCRα chain combines with a Vβ8.2, Vβ2 or Vβ7 TCRβ chain (Schumann et al. 2003). These cells are important for suppressing autoimmunity and graft rejection, enabling resistance to infection, and promoting tumor immunity (Godfrey and Kronenberg 2004;Kronenberg and Gapin 2002). Several lipid molecules have been described that are presented by Group 1 molecules and are derived from bacterial pathogens including Mycobacterium Leprae and M. tuberculosis (Beckman et al. 1996;Sieling et al. 1995). Little is known about the specific endogenous antigens that NKT

cells recognize although a recent study has identified the lysosomal glycosphingolipid, isoglobotrihexosylceramide (iGb3), as a key endogenous NKT cell antigen (Zhou et al. 2004).

Like CD1, MHC-related protein (MR1) is encoded on chromosome 1 in both mice and humans. MR1 is the most highly conserved molecules of all MHC class I and class I-like molecules between mice and humans (Yamaguchi et al. 1997). It has been reported that mMR1 and hMR1 share 90% identity in their α1/α2 domains. MR1 expression is β2m-dependent and TAP-independent (Miley et al. 2003). MR1 is the activation and restriction element for a small population of T cells preferentially expressed in the gut lamina propria called Mucosal-associated invariant T (MAIT) cells (Treiner et al. 2003). Like NKT cells, MAIT cells express an invariant hVα7.2/mVα19-Jα33 TCRα chain paired with a restricted set of Vβ segments. The Vβ repertoire of MAIT cells is diverse, Vβ13 and Vβ2 segments are preferentially used in humans and Vβ8 and Vβ6 segments in mice. MAIT cells have a memory phenotype (Treiner et al. 2005). The function of MAIT cells is unknown.

I-2-2 Human non-classical MHC class I molecules

In the human MHC, there are six HLA-expressing MHC class I genes: HLA-A, B, C, E, F, and G. HLA-E, F, and G are oligomorphic non-classical MHC class Ia genes. HLA-E is the best defined of these molecules. HLA-E preferentially binds peptides derived from the leader sequence of certain HLA class Ia molecules and binds to the CD94/NKG2 heterodimer on NK cells, similar to murine Qa-1 (Braud et al. 1997;Braud et al. 1998). HLA-G is primarily localized to trophoblast, interacts with inhibitory receptors, such as CD94/NKG2A, ILT2 and KIR2DL4, on NK and other hematopoetic cells, and can generate several alternatively spliced forms (Rajagopalan and Long 1999). HLA-F is primarily an intracellular localized molecule

which may under certain conditions be induced to be expressed on the cell surface. It interacts with IL-T2 and -T4 receptors (Lepin et al. 2000).

The MIC (MHC class I chain-related) family is class I like genes mapped within the MHC. MICA and MICB are primarily expressed in the gastrointestinal epithelium (Groh et al. 1996). It was demonstrated that γδ T cells could lyse cells by recognition of MICA and MICB (Groh et al. 1998). It was also shown that the MIC molecules can also interact with the cell surface receptor NKG2D/DAP10, the activating receptor which is found on NK and most CD8 cells (Bauer et al. 1999). The unique long 16 (UL16)-binding protein (ULBP) is a noval family of MICs which are not located in the MHC but instead mapped to chromosome 6q25. They can bind to CMV glycoprotein UL16 and also stimulate NK cytotoxicity through the NKG2D receptor (Cosman et al. 2001;Sutherland et al. 2001). In the mouse the retinoic acid early transcript 1 (Rae-1) family and the minor histocompatibilty antigen H60 which are located on chromosome 10 and show weak homolog with MHC class I also function as ligands for the mouse NKG2D receptor (Cerwenka et al. 2002;Cerwenka et al. 2000;O'Callaghan et al. 2001).

II CD8 T cells

II-1 Development of CD8 T cells

CD8 T cells are an important population of lymphocytes which participate in adaptive immunity. The primary function of these cells in the immune response is to kill cells within the host which have been infected with pathogens, such as viruses or intracellular bacteria, or have been transformed. The antigen specific T cell receptor of CD8 T cells recognizes pathogen derived peptides which are presented on the surface of the infected cell by MHC class I molecules. CD8 T lymphocytes are derived from bone marrow hematopoietic stem cells.

Hematopoietic stem cells develop into a population of common lymphoid progenitors and from this cell population arises the pro-T cell lineage which are precursors committed towards T cell differentiation (Hozumi et al. 1994;Matsuzaki et al. 1993). These cells are double negative in that they lack expression of the cell surface markers CD4 and CD8. Through interaction with thymic stromal cells and the cytokines they produce these immature thymocytes undergo proliferation and begin the first steps of T cell receptor (TCR) gene rearrangement. Thymocytes begin assembling the TCR β chain first, and with successful rearrangement this protein is expressed on the cell surface in associated with the surrogate pTα chain. These cells undergo vigorous proliferation as the TCR α chain begins the rearrangement process. This population of cells becomes $CD4^{+}CD8^{+}$ double positive and make up over 80% of all thymocytes. The antigen specific receptor must be capable of recognizing and interacting with self MHC molecules. However, if the TCR interaction with MHC/self peptide exhibits a high avidity, this cell will be deleted by negative selection. Only thymocytes that pass positive and negative selection are allowed to successfully develop into mature cells which can exit the thymus and emigrate into the periphery. More than 90% immature thymocytes fail these selections and are deleted by progrogrammed cell death (Merkenschlager et al. 1997). With successful selection comes the lineage commitment of thymocytes to the $CD8^{+}$ or $CD4^{+}$ T cell subsets.

The CD8 molecules can be expressed either as an α/α homodimer or an α/β heterodimer. CD8α/α cells are different from CD8αβ and have been shown to be $CD44^{hi}$ and can be either MHC Class I or class II selected by agonist self-paptides in the thymus (Gangadharan and Cheroutre 2004;Leishman et al. 2002).

II-2 Activation of naïve CD8 T cells

Once CD8 T cells have completed their maturation in the thymus, they enter the bloodstream and are exported to the periphery. Naïve lymphocytes are in continuous circulation throughout the body, homing to specialized secondary lymphoid organs such as the spleen and lymph nodes. Naïve CD8 T cells are $CD44^{lo}CD122^{lo}Ly6C^{lo}CD69^{lo}CD25^{lo}CD62L^{hi}CCR7^{hi}$ (Kaech et al. 2002). The uptake and presentation of antigen by professional APCs is critical for activation of naïve T cells (Liu and Janeway, Jr. 1992). At least two signals are required to initiate the activation of naïve T cells. Signal 1 comes from antigen specific TCR interaction with its cognate ligand presented by MHC/peptide complex on the APCs and signal 2 is induced by interaction of APC co-stimulatory molecules (B7) with their receptors (CD28) expressed on naïve T cells. However, some immune responses remain intact in the absence of CD28, for example, it is not necessary for CTL priming in lymphocytic choriomeningitis virus (LCMV) infection, suggesting that alternative costimulatory pathways may exist (Shahinian et al. 1993). Upon encountering foreign microorganisms, naïve CD8 T cells proliferate and differentiate into effector CD8 T cells trigged by these two signals synergistically.

The role of CD4 T cells in providing help to CD8 T cell responses to infection is still unclear. In some situations, such as CD8 T cell responses to tumor antigens or peptide-pulsed DCs, CD4 T cell are required for the generation and/or maintenance of cytolytic CD8 T cells. However, for the response to infectious agents, the requirement for CD4 help can be replaced by direct infection of APCs or perhaps inflammatory mediators induced by infection. For example, the CD8 T cell responses to LCMV infection are largely CD4 T cell independent. However, The CD8 responses to HSV infection or influenza virus infection are CD4 T cell dependent. The LM-specific CD8 T cell response was CD4 T cell dependent although the anti-

listeria memory CD8 T cells generated in the absence of CD4 T cells were functional (Shedlock and Shen 2003;Sun and Bevan 2003).

II-3 Effector and memory CD8 T cells

As naïve $CD8^+$ T cells become effector cells, the activated CD8 cytotoxic T lymphocyte (CTL) can contribute to antimicrobial immunity primarily through two main effector functions: cytokine secretion and lysis of the infected cell. CTL can lyse infected targets cells by two independent pathways: granule (containing perforin and granzymes) exocytosis and initiating target cell death through the expression of Fas-ligand, a member of the TNF superfamily. After activation, CTLs up-regulate their cell surface markers such as CD44, CD69, CD122, and CD25. Chemokine receptors such as CCR5, CCR4, CCR2, CXCR4 and CXCR2 are expressed to assist the migration of effector cells to inflamed tissues (Kaech et al. 2002). Chemokines are principal regulators of lymphocyte and DC migration in immune system. Homeostatic chemokines such as CCL19 (Epstein-Barr virus-induced gene-1 ligand chemokine, macrophage inflammatory protein-3β) and CCL21 (secondary lymphoid tissue chemokine, 6Ckine) are constitutively expressed within secondary lymphoid organs and regulate lymphocyte and DC homing to these organs. CCL19 and CCL21 are ligands for the chemokine receptor CCR7 (Yoshida et al. 1997;Yoshida et al. 1998). Effector T cells dowregulate the expression of CD62L (L-selectin) and CCR7, thus their ability to migrate to the lymph nodes is compromised (Kaech et al. 2002). They are able to rapidly produce IFN-γ, which enhances the phagocytic function of the APC, causes the upregulation of MHC class I and class II molecules, promotes differentiation of CD4 T cells into Th1 cells, induces B cell class switching to IgG_{2a} antibody and stimulates CD8 T cell cytotoxicity. Effector CD8 T cells are highly cytotoxic directly ex vivo, and contain high level of Granzyme B, which enters the target cell via the pores formed

by perforin and initiates cell death by cleaving a variety of protein substrates that are linked to the induction of DNA fragmentation. The effector CD8 cell population is relatively short-lived. To re-establish cellular homeostasis after resolving the infection, most of the effector CD8 T cell population undergoes massive contraction by the process of activation-induced apoptosis (Green and Scott 1994). Cytokine withdrawal is considered as a main cause of effector cell contraction. Only a small population of effector T cells survives and differentiates into long-lived memory T cells, which can respond rapidly upon re-exposure to the pathogen. The process of memory CD8 T cell development is not fully understood. Recent studies suggested that increased expression of the interleukin 7 receptor α-chain (IL-7Rα) identifies the effector $CD8^+$ T cells that will differentiate into memory cells. IL-$7R^{hi}$ effector cells contained increased amounts of anti-apoptotic molecules, and adoptive transfer of IL-$7R^{hi}$ and IL-$7R^{lo}$ effector cells showed that IL-$7R^{hi}$ cells preferentially gave rise to memory cells that could persist and confer protective immunity (Kaech et al. 2003).

CD8 memory cells can be defined phenotypically as $CD44^{hi}$. However, they also up-regulate other cell surface markers such as Ly6C, CD122, CD43, CD132, ICAM-1, LFA-1 and IL-7Rα (CD127) (Wang et al. 2004;Sprent 1997;Schluns et al. 2000). CD8 memory cells are able to rapidly secret IFN-γ upon restimulation, but were not immediately cytotoxic ex vivo and did not contain high level of Granzyme B. The pattern of gene expression by memory CD8 T cell has been studied. Genes involved in signal transduction, such as members of the p38 and jun kinase (JNK) signaling pathways, cell migration, such as CCR2, and CXCR4, cell division, such as cyclin E1, and B1, and effector functions, such as IFN-γ, and fas ligand were found to be up-regulated (Kaech et al. 2002). Memory CD8 T cells require lower concentrations of antigen and less co-stimulation than naïve CD8 T cells for activation (Mullbacher and Flynn

1996). Unlike naïve CD8 T cells which require both TCR/self-MHC interaction and cytokine IL-7 for their survival, memory CD8 T cells are maintained in vivo in the absence of MHC class I molecules in the presence of cytokines IL-7 or IL-2/IL-15 (Bruno et al. 1996;Tanchot et al. 1997;Murali-Krishna et al. 1999). New experimental data supported that $CD4^+$ T-cell help is absolutely required for the generation of CD8 T cells capable of efficient recall responses to antigen (Rocha and Tanchot 2004). CD4 T-cell help was not required for the activation and differentiation of antigen-specific T cells in the primary immune response. By contrast, CD8 T cells primed in the absence of CD4 help proliferated very poorly when re-challenged with antigen. They also secreted low levels of cytokines, similar to those secreted by naïve cells, and were unable to compete with naïve cells of the same specificity in a secondary immune response (Janssen et al. 2003;Bourgeois et al. 2002;Shedlock and Shen 2003). They are less stable, showing diminished antibacterial protective immunity over time (Sun and Bevan 2003). Mice that lack CD4 T cells mount a primary CD8 response to *Listeria monocytogenes* equal to that of wild-type mice and rapidly clear the infection. However, protective memory to the LM challenge is gradually lost in the former animals (Sun and Bevan 2003).

Based upon anatomical location, expression of cell surface markers and functions, memory T cells have been divided further into two subsets: central memory (T_{CM}, predominantly migrating to lymphoid tissues) and effector memory (T_{EM}, predominantly migrating to extralymphoid tissue) subpopulations (Gupta et al. 2005). T_{CM} migrate efficiently to the spleen and peripheral lymph nodes, whereas T_{EM} cells can be found in other sites, such as lungs and liver. However, both T cell subsets are presented in the blood and spleen. The expression of CCR7, a chemokine receptor that controls homing to secondary lymphoid organs, is used to divide human memory T cells. $CCR7^-$ memory cells (T_{EM} cells) mostly $CD62L^-$ and

capable of producing effector cytokines immediately upon antigen recognition, whereas $CCR7^+$ memory cells (T_{CM} cells) are mostly $CD62L^+$ and have greater proliferative potential upon re-stimulation. It has been hypothesized that T_{EM} confer immediate protection, whereas T_{CM} functions as a reservoir for generating a large pool of secondary effectors for re-enforcement upon re-infection (Northrop and Shen 2004). T_{EM} cells have been suggested to be the end product of memory cell development (Sallusto et al. 1999;Champagne et al. 2001). However, other studies suggested that T_{EM} and T_{CM} are largely independent subpopulations (Baron et al. 2003). Studies using mouse model supported the existence of functionally distinct memory T cell subsets (Masopust et al. 2001). In contrast to human studies, $CD8^+$ T_{EM} and T_{CM} generated following LCMV infection in mice are similar in their effector functions and T_{CM} actually provide more effective protective immunity. Upon antigen recognition, both T_{EM} and T_{CM} are able to produce IFN-γ. They also have similar levels of the lytic molecule granzyme B in intracellular stores and both subsets are capable of direct *ex vivo* lysis of target cells. Moreover, T_{CM}, rather than T_{EM}, has been demonstrated to provide better protection against challenge, regardless of the type of pathogen and the route of infection (Barber et al. 2003;Wherry et al. 2003). It has been propose that T_{CM} and T_{EM} do not necessarily represent distinct subsets, but are part of a continuum in a linear naïve to effector to T_{EM} to T_{CM} differentiation pathway. T_{EM} is a transitory population representing an "intermediate" cell type in the effector-to-memory transition. T_{CM} are the true memory cells because it is only this population that exhibits both of the two hallmark characteristics of memory cells, long tem persistence in vivo by self-renewal and the ability to rapidly expand upon reencounter with pathogen (Northrop and Shen 2004;Wherry et al. 2003). In contrast to protection against systemic LCMV infection, T_{EM} has been found to be more effective than T_{CM} at providing immunity to pulmonary infection with

Sendai virus (Roberts and Woodland 2004). Therefore, more studies are needed to clarify the nature of these memory $CD8^+$ T cells.

Il-4 regulation of CD8 T cell responses

Immune responses are initiated when resting precursor CD4 T cells are triggered by MHC/peptide complexes with co-stimulatory molecules on the surface of APCs. Consequently the CD4 T cells proliferate, begin to secrete cytokines, such as IL-2, IFN-γ, IL-4, etc., and express important cell surface molecules including the α chain of IL-2 receptor (CD25), CTLA-4, and CD40 ligand (CD40L). One of the earliest antigen activation induced cell surface molecules expressed by T cells is CD40L. A critical consequence of the interaction of CD40L with CD40 expressed on APCs is the up-regulation of other key co-stimulatory molecules, including CD80 and CD86 (Yang and Wilson 1996;Grewal et al. 1996). These molecules interact with T cell CD28 or CTLA-4 molecules to determine whether the outcome of antigen triggering will be either functional T cell activation or, alternatively, T cell inhibition induction (Lane 1997). Thus, antigen triggering in the absence of CD28 triggering is known to induce anergic T cells. Similarly, blockade of the CD40L/CD40 pathway can lead to tolerance induction (Kurtz et al. 2004;Schwartz 2003).

The large majority of T-cell co-stimulators belong either to the CD28 or the TNF-receptor families of molecules (Sharpe and Freeman 2002;Greenwald et al. 2005;Croft 2003). These co-stimulatory molecules can be defined as cell-surface molecules that cannot functionally activate T cells by their own, but rather amplify or counteract signals provided by the TCR complex. Only a few (CD28, CD27, HVEM) are expressed on T cells constantly, whereas the majority of T-cell co-stimulators are induced only following cell activation. All of the constitutively expressed co-stimulators have positive amplifying effects, whereas the

inducible co-stimulators contains both positive (ICOS, CD30, OX40, 4-1BB, SLAM) and negative (CTLA-4, PD-1, BTLA) regulators. Almost all of their ligands are expressed on all professional antigen-presenting cells, such as dendritic cells, B cells and macrophages. However, CD80 and CD86, the common ligands for CD28 and CTLA-4, are largely restricted to professional APCs (at least in humans), whereas the ligands for other T-cell co-stimulators are broadly expressed on professional APCs and peripheral tissues (Kroczek et al. 2004).

After differentiation and emigration from the thymus to the peripheral immune organs, CD4 T helper cells are termed naive T helper precursor (Thp) cells. Thp cells are functionally immature and capable of secreting only IL-2 (Sad and Mosmann 1994). One of the consequence of MHC/peptide triggering of $CD4^+$ T cells is the further differentiation of the Thp cells into the functional distinct T helper 1 (Th1) and T helper 2 (Th2) subsets. Much work has been done to study the signals that drive these naive Thp cells, which secrete only IL-2, to become Th1 or Th2 effector cells. The Thp activation and differentiation in the periphery requires at least two separate signals. The first signal is delivered by the TCR/CD3, after its interaction with MHC/peptide. The second signal is produced by a number of co-stimulatory molecules typified by the CD28/B7, OX40, and LFA-1/ICAM receptor-ligand pairs. Whether an immune response will be dominated by Th1 versus Th2 cells is clearly influenced by the nature of these two signals. However, neither of these two signals is as potent a determinant of Th cell fate as the cytokine milieu itself (Seder and Paul 1994). The cytokines themselves play the most critical role in T helper cell polarization. The two critical cytokines that control Th1 and Th2 differentiation are IL-12 and IL-4, respectively (Parronchi et al. 1992;Manetti et al. 1993;Maggi et al. 1992;Hsieh et al. 1993) . These two cytokines enhance the generation of their own Th subset and simultaneously inhibit the generation of the opposing subset. These subsets,

Th1 and Th2 are responsible for cell-mediated/inflammatory immunity and humoral responses, respectively (Glimcher and Murphy 2000). This division is consistent with previous demonstrations that the host tends to mount either a cell-mediated or humoral response, but not both, in response to pathogens. The hallmark cytokine of Th1 cells is IFN-γ, and Th1 cells also produce IL-2, TNF, and LT, cytokines that mediate delayed type hypersensitivity (DTH) responses and macrophage activation. The signature cytokine of Th2 cells is interleukin-4 (IL-4), and Th2 cells also secrete IL-5, IL-9, IL-10, and IL-13, cytokines that provide help to B cells and are critical in the allergic response (Mosmann and Sad 1996). The elaboration of distinct cytokines by the Th subsets provides a second level of control intrinsic to the outgrowth and function of CD4 T cells. For example, IFN-γ secreted by Th1 cells is known to down-regulate the differentiation and function of Th2 cells, and conversely, IL-4 and IL-10 inhibit Th1 cell differentiation (Fiorentino et al. 1991;Mosmann and Coffman 1989). It has been reported that the differentiation of naive Th cells towards Th1 or Th2 cells is regulated by the transcription factors T-box expressed in T-cells (T-bet) and GATA-binding protein-3 (GATA-3) (Chakir et al. 2003). Upon IL-4 binding to its receptor, GATA-3 is induced through Stat6. GATA-3 can augment its own expression by autoactivation and activate the Th2 cytokine cluster. GATA-3 can also shut down Th1 development through the repression the IL-12Rβ2 chain expression.

In order to regulate the immune response and to decrease the potential for autoimmunity, the immune system has also evolved several mechanisms to down-regulate and control the outgrowth, differentiation, and function of peripheral antigen-activated CD4 T cells. The immune response can be inhibited by several T cell subsets, including NK T cells, $CD25^{+}CD4^{+}$ T cells, and a subpopulation of CD8 T cells. CD8 T cells regulate peripheral immune responses, in part, by specifically controlling the outgrowth of antigen-triggered CD4 T cells. This

regulatory function of $CD8^+$ T cells has been shown, in vivo, to control the emergence of autoreactive CD4 T cells as well as CD4 T cells reactive to conventional antigens, including alloantigens. The regulatory CD8 T cells recognize antigen-activated CD4 T cells in a TCR specific manner restricted by the MHC class Ib molecule, Qa-1 (Hu et al. 2004). $CD4^+CD25^+$ regulatory T cells (T_{reg}) have emerged as a unique population of suppressor T cells that maintain peripheral immune tolerance. They specially suppress the responses of other T cells. In some cases, suppression has been associated with the secretion of IL-10, transforming growth factor-β or both of these cytokines (Kursar et al. 2002).

CD8 T cells, similar to CD4 T cells, have been shown to be a source of a wider variety of cytokines, including both type 1 and type 2 cytokines. Depending on the cytokines present during primary stimulation, CD8 T cells can be polarized into type 1 and type 2 CD8 T (Cerwenka et al. 1998;Carter and Dutton 1996). Both the Tc1 (CTLs producing type 1 cytokines) and Tc2 subsets were shown to be cytolytic. Tc2 CD8 T cells preferentially secrete IL-4, IL-5, IL-6 and IL-10 and kill predominantly by the perforin pathway, whereas Tc1 CD8 T cells predominantly secrete IFN-γ and IL-2 and kill by either perforin- or Fas-mediated mechanisms (Dobrzanski et al. 2004;Mosmann et al. 1997).

II-5 Acquisition of memory phenotype by CD8 T cells independent of antigen priming

In addition to antigen priming, CD8 T cells can also acquire a memory phenotype in the absence of foreign antigens by several different mechanisms.

Peripheral T cell numbers are maintained at remarkably stable levels throughout the life cycle because the size of the lymphocyte pool is crucially important to the adaptive immune

system. In the absence of overt antigen proliferation, naïve T lymphocytes divide minimally in a full T cell compartment. They require signals delivered by MHC molecules for their survival (Tanchot et al. 1997). Both naïve and memory T cells undergo antigen-independent proliferation after transfer into a T cell-depleted environment, a process that has been termed homeostatic proliferation. Naive T cells require specific interactions with self-peptide/MHC complexes in order to undergo efficient homeostatic proliferation in lymphopenic conditions. In contrast to naïve T lymphocytes, memory T cells are continually undergoing "basal proliferation", the low level of antigen-independent division observed in a full T lymphocyte compartment. The proliferation of memory cells in response to lymphopenia is also MHC independent. The naïve and memory CD8 T cells have different cytokine requirements for homeostatic proliferation. IL-7-mediated signals, not IL-15-mediated signals were required for naïve CD8 homeostatic proliferation. However, memory CD8 T cells could use either IL-7- or IL-15- mediated signals for homeostatic proliferation (Goldrath et al. 2002). IL-7 also plays a key role in the survival and proliferation of thymocytes during early stages of T cell development and contributes to peripheral T lymphocyte survival. IL-15 is a T and NK cell stimulatory factor, similar in structure and function to IL-2. Both of their receptors share the same IL-2/IL-15Rβ and common γ chain.

When naïve CD8 T cells undergo homeostatic proliferation in a lymphopenic environment, it results in the up-regulation of expression of CD44, CD122, Ly6C as well as other memory cell surface markers. These acquired memory phenotype $CD8^+$ T cells under lymphopenic conditions appear indistinguishable from antigen-experienced memory T cells (Goldrath and Bevan 1999;Sprent 1997). They are capable of lysing target cells and secrete IFN-γ in response to MHC/peptide stimulation, the magnitude and kinetics of these responses

are similar to those of memory cells. The acquired memory phenotype is stable. Thus, the homeostatic proliferation does not replenish the naïve T-cell pool, which only can be replenished by thymic export (Ge et al. 2002).

Memory phenotype CD4 T cells are found in naïve mice maintained under germfree conditions, suggesting their development is not foreign antigen dependent. Since T cells from male anti-H-Y TCR transgenic mice can develop exytrathymically and display memory cell markers, it is possible that the development of memory phenotype CD8 T cells in naïve mice is driven by the interaction of the αβTCR with self-antigens and developed extrathymically. CD8 T cells that develop extrathymically in H-Y TCR transgenic mice have been shown to be $CD44^{hi}$ and express IL-2Rβ (CD122). In contrast to naïve intrathymically developed CD8 T cells, it has been reported that these extrathymically developed T cells are positively selected by self-antigens and proliferated in response to IL-2 or IL-15, independent of antigen stimulation (Yamada et al. 2000). They rapidly produced interferon gamma but not IL-4 after TCR cross-linking. They are unresponsive to antigenic stimulation. The memory phenotype CD8 T cells in naive normal mice showed the similar characteristics to the thymus-independent CD8 T cells in the H-Y TCR transgenic mice. Therefore, it has been proposed that a significant part of the $CD44^{hi}$ memory phenotype CD8 T cells in naive normal mice represents thymus-independent CD8 T cells (Yamada et al. 1998;Yamada et al. 2000;Yamada et al. 2001).

Except the above mechanisms, cells positively selected on hematopoietic cells are also shown to acquire memory cell markers, such as $CD44^{hi}$ at the site (Urdahl et al. 2002).

III Non classical MHC class I selected CD8 T cells in B6.H-2Kb$^{-/-}$Db$^{-/-}$ mice

III-1 Generation of B6.H-2Kb$^{-/-}$Db$^{-/-}$ mice

Mice deficient for β_2m or TAP do not express normal levels of cell-surface MHC class I proteins and consequently are defective in MHC class I-restricted antigen presentation and in thymic selection of CD8 T cells. These TAP$^{-/-}$ and β_2m$^{-/-}$ mice have been used widely as MHC class I-deficient models, but this deficiency is not complete (Perarnau et al. 1999). A small fraction of MHC class I heavy chains apparently folds normally and is transported to the cell surface even in the absence of β_2m (in particular, the H-2D^b gene product) or in the absence of TAP-dependent peptides. In addition, "empty" class I molecules are expressed on the cell surface of TAP$^{-/-}$ and β_2m$^{-/-}$ animals, so that total MHC class I cell-surface levels are decreased at most 10-fold. Although such empty class I molecules decay rather rapidly in *ex vivo* cultured cells, nothing is known about their half-life *in vivo*. TAP$^{-/-}$ and β_2m$^{-/-}$ animals also possess a limited repertoire of self-MHC class I-restricted CD8$^+$ T cells, which can be explained by their selection on the remaining low levels of MHC class I. These animals reject allogeneic skin grafts and tumors and give rise to viral and allogeneic CD8+ T cell-mediated responses, consistent with the continued presence of functional MHC class I proteins. In order to obtain complete MHC class Ia deficient mice, H-2Kb$^{-/-}$Db$^{-/-}$ mice were generated by crossing H-2K$^{b-/-}$ with H-2D$^{b-/-}$ mice and screening progeny for the rare intra-H-2 recombinant that carries both targeted genes on the same chromosome.

H-2K$^{b-/-}$and H-2D$^{b-/-}$ single knock out mice were generated from E14TG2a embryonic stem cells derived from the 129/Ola mouse strain of the H-2^{bc} haplotype (Perarnau et al. 1999). The MHC class Ia, class II and class III regions of the H-2^{bc} haplotype of 129 are considered identical to the H-2b haplotype of B6 mice, but the H-2^{bc} haplotype of 129 differs from H-2^b of B6 in the H-2 T region that contains genes encoding MHC class Ib molecules. However, they

express the same form of Qa-1 and no differences were noted between 129 and B6 in the H2-M region. The H2-Kb$^{-/-}$ and H2-Db$^{-/-}$ animals were crossed, and their offspring were screened for an intra-H-2 recombinant that carried both targeted loci on the same chromosome. Resultant DKO mice were backcrossed six times to C57BL/6 mice. Thus, C57BL/6 mice are considered as controls.

DKO mice have genomic deletions of the MHC class Ia genes, H2-Kb and H2-Db. Thus, they lack expression of class Ia K^b and D^b heavy chains but show normal expression of β_2m and class Ib molecules (Perarnau et al. 1999). DKO animals do not have reduced viability compared with their MHC class Ia-positive littermates, as has also been observed for TAP$^{-/-}$ and β_2m$^{-/-}$ animals. Thus, DKO mice, in which the responses dominated by classical MHC class I are absent, provide a unique system for studying the functions and immunological activities of non classical MHC class I molecules.

III-2 Characterization of the CD8 cells in DKO mice

In DKO mice, there is approximately 5- to 10-fold reduction in the number of peripheral CD8 T cells, with some compensatory increase in the CD4 T cell compartment. This reduction was comparable to that seen in β_2m$^{-/-}$ animals. One explanation for the small peripheral CD8 pool is a defect in thymic development caused by the lack of classical MHC class Ia molecules. Indeed, in the thymus of DKO mice, the thymic CD4$^-$CD8$^+$ single positive population was reduced around 5-fold (to 1% of total), whereas the numbers of CD4$^+$CD8$^-$ single positive cells were comparable to those in control animals. There is also approximately 7-fold reduction in the percentage of mature single positive CD8 (CD24$^-$CD4$^-$CD8$^+$) thymocytes.

Although DKO mice have greatly reduced numbers of mature $CD8^+$ T cells, these non class Ia selected $CD8^+$ T cells are able to function effectively to mediate immune responses against infectious agents. For example, CD8 cells from these DKO mice mount strong allo-responses against class Ia molecules including K^b and D^b (Vugmeyster et al. 1998). H2-M3 restricted CD8 T cells can play an important role in the clearance of *Listeria monocytogenes* (LM) infection (Seaman et al. 2000). H2-M3 restricted $CD8^+$ cells have also been found in *Mycobacteria tuberculosis*-infected mice (Urdahl et al. 2003). Qa-1 restricted CD8 T cells are found in Salmonella typhimurium or LM infected mice (Bouwer et al. 1997;Lo et al. 2000). Furthermore, the diversity of the $CD8^+$ T cell repertoire selected only on MHC class Ib molecules is very similar, with respect to the usage of various Vα and Vβ rearrangements, to the one selected on both MHC class Ia and class Ib molecules (Laouini et al. 2000). CD8 T cells selected by non classical MHC class I molecules in DKO mice are also capable of rapidly producing IFN-γ after in vivo stimulation with anti-CD3 and have been proposed to be an early source of IFN-γ that promotes Th1 priming (Das et al. 2001).

Unlike CD8 cells in the spleens of B6 mice, most CD8 T cells in the spleen of DKO mice have a $CD44^{hi}$ phenotype (Seaman et al. 1999). This phenotype is conventionally used to identify memory CD8 T cells and thus could reflect the possibility that most class Ib-restricted cells in adult mice have previously encountered antigens, either representing non-pathogens or self-antigens. The memory phenotype has also been noted on cells undergoing homeostatic expansion (Murali-Krishna and Ahmed 2000). It is a marker that once acquired is thought to remain stable on such cells. It has been noted that single positive cells in neonatal mice proliferate strongly and display a $CD44^{hi}$ phenotype, reflecting the lymphopenic environment that they are exposed to (Le et al. 2002). Since the number of peripheral CD8 cells is decreased

in DKO animals and thus presents a continued CD8 lymphopenic environment, this may in part account for this memory phenotype. To support this, spleen cells from day 1-old B6 mice displayed a relatively high ratio of $CD44^{hi}$:$CD44^{lo}$ CD8 cells that rapidly decreased with age. By day 28, the ratio was close to that seen in adult mice. In contrast, day 1 spleen cells from DKO mice showed a high ratio of $CD44^{high}$:$CD44^{low}$ CD8 cells which remained high not only at day 56 but continued that phenotype throughout adult life. Another marker for T cell memory, expression of CD62L, was also examined similiarly. Both B6 and DKO splenic CD8 cells are predominantly $CD62L^{low}$ at birth. By day 28, the ratio of $CD62L^{hi}$:$CD62L^{lo}$ cells had greatly increased in B6 animals but remained low in B6.$K^{b-}D^{b-}$ mice. By day 56, this difference was even greater (Kurepa et al. 2003). However, previous studies have shown that although there is a lymphoid compartment that regulates homeostatic proliferation, the CD4 and CD8 compartment are homeostatically co-regulated (Dummer et al. 2001;Gruber and Brocker 2005;Rocha et al. 1989). CD4 T cells have been shown to efficiently inhibit homeostatic proliferation of naive CD8 T cells. Thus, the CD8 T cell homeostatic proliferation in DKO mice might be inhibited by the normal level of CD4 T cells. The issue about CD4/CD8 competition is now being re-examined by the finding that homeostatic proliferation of T cells may be regulated by clonal competition for self ligands, because polyclonal CD4 and CD8 T cells proliferate when injected into TCR transgenic hosts, but not when injected into syngeneic polyclonal hosts (Troy and Shen 2003). Another study has shown that monoclonal T cells from TCR transgenic mice fail to proliferate when injected into the same strain, but divide not only in T-cell deficient $Rag^{-/-}$ hosts, but also in TCR transgenic $Rag^{-/-}$mice, whose T cells have a different specificity (Moses et al. 2003). Thus, T cells ignore large numbers of competitors as

long as their TCR specificity differs (Stockinger et al. 2004). The principles underlying T cell homeostasis need further investigations.

By studying radiation bone marrow-chimeric mice in which either B6 or $\beta_2m^{-/-}$ mice were lethally irradiated and reconstituted with C57BL/6 bone marrow (B6 to B6 or B6 to $\beta_2m^{-/-}$ chimeras, respectively), Urdahl et al found that CD8 T cells selected on hematopoietic cells (B6 to $\beta_2m^{-/-}$ chimeras) express a similar activated phenotype as those CD8 cells in DKO mice (Urdahl et al. 2002). To further study the capacity of non class Ia selected CD8 cells to be positively selected on hematopoietic cells, they constructed bone marrow-chimeric mice that expressed MHC class Ib molecules only on hematopoietic cells (DKO to β_2m chimeras) or only on non-hematopoietic cells (β_2m to DKO chimeras). Their results showed that MHC class Ib restricted CD8 T cells could be selected both in chimeras that expressed MHC class Ib only on hematopoietic cells and in chimeras that expressed these molecules only on non-hematopoietic cells. Those MHC class Ib restricted CD8 T cells that were positively selected on hematopoietic cells had a more activated phenotype than MHC class Ib-restricted CD8 T cells selected on non-hematopoietic cells. They proposed that the activated phenotype of MHC class Ib-restricted CD8 T cells in DKO mice might be associated with positive selection on hematopoietic cells, unlike the class Ia selected $CD8^+$ T cells which can only be selected on non-hematopoietic cells. A previous study investigated the phenotype and development of T cells from transgenic mice expressing a T cell receptor with specificity for insulin presented by Qa-1(b). They found the transgenic CD8 T cells did not express activated phenotype. However, unlike conventional T cells, their selection is at least as efficient when the selecting ligand is expressed only on hematopoietic lineage cells as compared to expression on epithelial cells in the thymus (Sullivan et al. 2002).

The origin of the activated phenotype of $CD8^+$ cells in DKO is still unclear. It could be the result of CD8 T cells being selected in the thymus on hematopoietic cells. Alternatively, the phenotype may be the result of an extrathymic pathway for their development or the result of a post-thymic encounter with factors in the periphery.

IV Immune responses to Listeria monocytogenes (LM)

IV-1 Overview of LM

Listeria are gram positive organisms which are non-spore forming and use flagella for motility. The genus Listeria consists of six species: monocytogenes, grayi, innocua, ivanovii, seeligeri, and welshimeri. Of these, Listeria monocytogenes (LM) is considered a major pathogem, capable of causing diseases in human and a wide variety of animals (Hof and Hefner 1988). LM is a facultative intracellular bacterium that has been used widely as a laboratory model to understand immune responses to intracellular bacterial infections (Gouin et al. 1994). The ability of LM to successfully infect and spread within mammalian cells requires the expression of many virulence factors which act at different stages of the infection cycle. Studies utilizing intravenous infection have shown that within 10min, approximately 90% of the bacteria are found in the liver, and the remaining 10% can be recovered from the spleen(Conlan and North 1991). The majority of bacteria are phagocytosed by resident macrophages within these target organs, such as Kupffer cells which line the sinusoids of the liver (Lepay et al. 1985). LM is still capable of infecting and replicating within non-phagocytic cells such as hepatocytes, fibroblasts, and intestinal eqithelial cells (Racz et al. 1972;Rosen et al. 1989). The majority of bacteria which were phagocytosed are initially trapped and killed in the phagosomal compartment (Mackaness 1962). A small percentage of LM is able to escape into the macrophage cytosol, replicate, and spread through infection of neighboring cells. Escape

from the phagosome largely depends on the production of a key virulence factor, the sulfhydryl-activated hemolysin, Listeriolysin O (LLO) (Cossart et al. 1989). LLO lysis of the internalized vacuole membrane allows LM to enter the host cell cytosol. LM mutants which lack expression of LLO fail to escape the phagocomal compartment, and are relatively non-virulent (Portnoy et al. 1988). Once in the cytosol, bacteria replication soon begins. Another key virulent determinant of LM is its ability to polymerize host cell derived actin filaments as a means of motility, which subsequently allows for the infection of neighboring cells (Dabiri et al. 1990). The LM ActA protein is required for this function (Kocks and Cossart 1993). LM can spread cell to cell without being exposed to any extracellular host defenses, such as antibodies and complement.

VI-2 Innate immunity to LM infection

In the murine model of infection, resistance to LM requires effector cells from both innate and adaptive immune system. Innate immunity lacks specificity and memory. However, it is able to respond rapidly following infection. It is critical for limiting the initial spread of LM and allowing time for acquired immune responses to develop and confer sterilizing immunity (Unanue 1997). Although resident tissue macrophages are important in the establishment of an LM infection through internalizing the bacteria, they are also key effectors of early resistance when activated (Mackaness 1962). Bacterial invasion of a macrophage can induce a stress response, which includes the upregulation of heat shock protein (HSP) 70, HSP60, and the MAP-kinase phosphatase (Schwan and Goebel 1994). The response to this cellular stress is the activation of NF-kB, which is believed to induce the cytokine production (Hauf et al. 1994). Two important cytokines secreted by LM infected macrophages are TNF-α and IL-12, which have been demonstrated to act synergistically in driving NK cells to produce

IFN-γ, a potent macrophage activating cytokine (Dunne et al. 1994;Tripp et al. 1994). An important role for IFN-γ in the induction of LM resistance has been further demonstrated in IFN-γ knockout mice, which exhibit increased susceptibility to infection (Harty and Bevan 1995). IFN-γ increases macrophages bactericidal ability most likely through the production of nitrogen and oxygen intermediates (Bermudez 1993).

Neutrophils are also a key innate mediator in early LM resistance. The IL-1 and IL-6 secreted by LM infected macrophages play an important role for the recruitment of neutrophils to sites of infection (Rogers et al. 1994;Dalrymple et al. 1995). Neutrophils may be able to kill hepatocytes and limit the spread of infection (Conlan et al. 1993). Neutrophils are also important for generating protection following a secondary LM infection, despite rapid T cell immunity (Rakhmilevich 1995). γδ T cells have also been implicated in the early response to LM (Ohga et al. 1990). Intraperitoneal infection of mice with LM results in an influx of γδ T cells which precedes the appearance of the more conventional αβ T cells. Macrophages, NK cells, neutrophils and γδ T cells all appear to have some function in resistance to this intracellular pathogen. IFN-γ and tumor-necrosis factor (TNF) are essential for the primary defense against infection with LM, and mice that lack these cytokines or their cognate receptors rapidly succumb to infection (White et al. 2000b;White et al. 2000a;Harty and Bevan 1995). However, Type I IFNs, which induce antiviral responses by binding a common receptor, the type I IFNα/β receptor (IFNAR), and protect the mammalian host, seen to impair anti-listeria immune defenses (Perry et al. 2005). $IFNAR^{-/-}$ mice which are unable to either produce or respond to type I IFNs, were more resistant to LM, having greater survival rates and lower LM titers in the liver and spleen than wild-type mice (Carrero et al. 2004;Auerbuch et al. 2004). Additionally, injection of mice with polyI:C enhanced LM-induced death in wild type but not

IFNAR$^{-/-}$ mice, further supporting that the response to type I IFNs is detrimental to the host during LM infection (O'Connell et al. 2004). A recent study indicated that the newly discovered population of TNF- and iNOS-producing DCs (known as TipDCs) is essential for the control of bacterial growth in vivo (Serbina et al. 2003b). Innate immune defenses are activated in a stepwise manner, with sequential triggering of receptors and downstream signaling pathways. MyD88 (myeloid differentiation primary-response protein 88), an intracellular adaptor protein that transmits many, but not all, Toll-like receptor (TLR) –mediated signals, is essential for innate defense against LM (Seki et al. 2002;Serbina et al. 2003a). While this rapid immunity serves to reduce the initial bacterial burden, it is not capable of generating full protection. This has been shown in severe combined immunodeficient (SCID) mice which have an intact innate response, but lack the adaptive immunity system. These animals are incapable of clearing an LM infection, and can remain chronically infected for several weeks or months (Bancroft et al. 1986).

IV-3 Adaptive immunity to LM infection

The adaptive immune system is comprised of T cells and B cells which confer antigen-specificity to an immune response, and long lived memory protection. Both CD8 and CD4 T cells respond to LM infection. Although CD4 T cells were thought to have less impact on LM clearance (Czuprynski and Brown 1990), CD4 T cells produce large amounts of Th1 cytokines that are thought to contribute to the clearance of LM during the course of LM infection (Hsieh et al. 1993). In mice that lack CD4 T cells, although primary CD8 T cell responses are normal, long-term maintenance of memory CD8 T cells is impaired and protective immunity against re-infection is gradually lost (Sun and Bevan 2003). At the same time, CD4CD25 regulatory T

cells inhibit LM specific memory CD8 T cell responses through playing a role in controlling CD8 T cell proliferation during a secondary response (Kursar et al. 2002).

The CD8 T cell population is important for generating rapid sterilizing immunity to both primary and secondary LM infection. On entering eukaryotic cells, LM invades the cytosol of the infected cell, where secreted bacterial proteins are processed for presentation by MHC class I molecules (both classical MHC class Ia and non-classical MHC class Ib) on the professional APC surface. A protective CTL response is mounted against LM antigens that are presented by those MHC class I molecules. CD11c expressing-DCs has been shown to be important in priming of naïve CD8 T cells specific for bacterial peptides presented by either classical or nonclassical MHC class I molecules (Jung et al. 2002). Sterilizing clearance of LM is mediated by CD8 T cells. The CD8 T cells that mediate protective immunity include two populations: one is restricted by MHC class Ia molecules and the other is restricted by MHC class Ib molecules. These two T cell populations respond to bacterial infections with different kinetics and potentially provide distinct contributions to the immune response. Although it has been suggested that anti-LM CD8 T cells may have been primed by prior exposure to commensal organisms in LM-naïve animals (Lenz and Bevan 1997), the phenotype of the CD8 T cells that respond to this infection is not known.

IV-2-a Antigen specific MHC class Ia restricted CD8 T cells.

LM-derived MHC class Ia restricted peptides are generated from secreted proteins, many of which contribute to bacteria virulence (Pamer et al. 1997). LLO is one of the most antigenic of the secreted proteins in terms of the induction of specific CD8 T cells. Another antigen that induces a substantial CD8 T cell response is p60, a hydrolase involved in bacteria septation. Both of them are degraded rapidly in the cytosol of the host cell by cytosolic

proteasomes (Pamer et al. 1997). The rapid proteasome-mediated degradation of toxic bacterial proteins not only enhances host-cell survival but also generates peptide fragments that enter the MHC class I antigen-processing pathway. After a threshold quantity of antigen has been presented, the magnitude of the T-cell response is unaffected by increased presentation of antigens. MHC class Ia restricted CD8 T cells response to LM (as determined using MHC class I tetramers) reach peak frequencies approximately 8 days after intravenous inoculation. And T–cell responses are independent of the quantity or duration of in vivo antigen presentation. Infusion of naïve CD8 T cells into mice at different time points after primary bacterial infection approved that in vivo priming of CD8 T cells occurred with optimal efficiency 1 day after bacterial inoculation and then progressively diminished. After 72 hours, naïve CD8 T cells were not primed, despite the persistence of viable bacteria. T cells that were primed following live infection underwent prolonged division and became cytolytic and produced IFN-γ. By contrast, T cells that were primed by heat killed LM (HKLM) underwent attenuated division and did not acquire effector functions (Pamer 2004).

IV-3-b Antigen specific non classical MHC class I restricted CD8 T cells

MHC class Ib restricted CD8 T cell responses are also induced by LM infection (Seaman et al. 2000). In fact, MHC class Ia-deficient mice generate CD8 T cell mediated protective immunity against LM infection. The best characterized MHC class Ib responses are H2-M3 restricted, which presents peptides that contain N-formyl methionine at the N-terminus-characteristic of peptides derived from bacteria. Listeria-encoded peptide epitopes for H2-M3 restricted CD8 T cells have been identified, f-MIGWII, f-MIVTLF, f-MIVIL (Princiotta et al. 1998;Gulden et al. 1996;Lenz et al. 1996). Although most studies have demonstrated peptide presentation, one study suggested that bacterial glycolipids may be presented by H2-M3 to LM-

specific $CD8^+$ T cells (Ploss et al. 2003). H2-M3 restricted CD8 T cells are intrinsically different from classical restricted CD8 T cells. During primary infection, their responses develop more rapidly than conventional MHC class Ia restricted responses (Seaman et al. 1999). A surprising feature of H2-M3 restricted T cells is their relatively "promiscuous" recognition of peptides, a feature that might be a function of the structural similarity of the defined peptides. Thus, unlike most MHC class Ia-restricted T cells which are specific to a single pathogen-derived epitope, H2-M3-restricted $CD8^+$ T cells display a much higher degree of peptide cross-reactivity (Nataraj et al. 1998;Ploss et al. 2003). It has been suggested that H2-M3-restricted $CD8^+$ T cell responses are primed by a complex mixture of *N*-formylated bacterial peptides that induce multiple cross-reactive T cell clones. Promiscuous recognition by H2-M3-restricted $CD8^+$ T cells of peptides sharing a similar molecular pattern is an example of the adaptive immune system pirating a fundamental innate immune strategy for antimicrobial defense (Ploss et al. 2003). H2-M3 restricted T cells rapidly clonally expand and achieve peak frequencies 5 to 6 days after primary infection with LM. Thus, the H2-M3 restricted T cell response precedes the MHC class Ia restricted response. H2-M3 restricted T cells are cytolytic and produce IFN-γ, and because they appear early in infection, they may have an important role in bacteria clearance. In contrast to their important role during the primary infection, H2-M3 restricted T cells surprisingly only make a minor contribution to memory responses following re-infection (Kerksiek et al. 1999). Compared with the extensive expansion observed by classically restricted CD8 T cells after secondary challenge, H2-M3 restricted cells undergo a very limited expansion upon re-infection. Recent work suggests that despite their limited ability to proliferate, a population of H2-M3-restricted memory T cells is generated. Cells in this population are indistinguishable from MHC class Ia restricted CD8 T cells with respect to

surface markers and longevity (Kerksiek et al. 2003). One explanation is that H2-M3 restricted T cells might not undergo clonal expansion because of competition from MHC class Ia restricted T cells. A recent study demonstrated that MHC class Ia-restricted memory CD8 T cells prevented the expansion of H2-M3-restricted memory T cell populations by limiting dendritic cell antigen presentation in LM immuned mice (Hamilton et al. 2004).

V CD8 T cell and Innate immunity

V-1 Non clonally derived receptors on CD8 T cells and their possible function in innate immunity

The cells of innate immune system primarily use germline-encoded receptors to defend against infected or transformed cells. NK cells are a part of innate immune system. They use combined activating and inhibitory receptors to perform their function, such as providing cytokines to activate other cells of the immune system and directly kill infected or transformed cells. The cells of the adative immune system generally use functionally rearranged receptors to recognize antigens. However, cells of the adaptive immune system can express some of the germline-encoded receptors. CD8 T cells belong to adaptive immune system and depend on TCR for antigen recognition. Even in naïve mice, there are approximately 20% of CD8 T cells from naïve B6 mice that display the memory marker CD44 and can proliferate in response to cytokines, such as IL-2, independent of TCR stimulation (Dhanji and Teh 2003). Both activating and inhibitory NK receptors are also found on these $CD8CD44^{hi}$ cells (Dhanji and Teh 2003).

All of the well-defined inhibitory NK cell receptors contain an immunoreceptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic domains. By contrast, several

activating NK receptors use adapter proteins containing an immunoreceptor tyrosine-based activation motif (ITAM). Activating receptor NKG2D is a type II transmembrane-anchored glycoprotein expressed as a homodimer on the surface of all mouse and human NK cells and most γδ TCR^+ T cell. NKG2D does not form dimers with CD94. Human ligands for NKG2D are MICA, MICB, ULBP and the mouse ligands are RAE-1, H60, and MULT1 (Bahram et al. 2005;Steinle et al. 2001;Cerwenka et al. 2002;Bacon et al. 2004). The adapter proteins associated with NKG2D are also different in human and mouse. In mouse, two isoforms of NKG2D that are generated by alternative splicing have been described. The longer protein (NKG2D-L) exclusively associates with the DAP10 adapter protein, whereas the short isoform (NKG2D-S) is promiscuous and can pair with either DAP12 or DAP10 (Gilfillan et al. 2002). By contrast, humans express only an NKG2D-L protein, which exclusively uses DAP10 for signaling (Billadeau et al. 2003). Differential use of DAP10 and DAP12 by NKG2D has functional consequences in that DAP10 seems to exert mainly costimulatory functions but DAP12 can induce a full signaling cascade resulting in cytolysis, cytokine secretion, and proliferation (Diefenbach et al. 2002). DAP 12 is normally expressed by activated NK cells, not naïve CD8 T cells. Although NKG2D-mediated responses are beneficial in immune responses against tumors and pathogens, evidence is emerging that this system may also be deleterious by contributing to autoimmunity (Groh et al. 2003).

CD94 and NKG2 encode type II transmembrane proteins of the C-type lectin-like family. CD94 can be expressed on the cell surface as a disulfide-linked homodimer or as a disulfide-linked heterodimer with NKG2A or NKG2C. NKG2A has an ITIM in its cytoplasmic domain and CD94/NKG2A heterodimers function as inhibitory receptors (Vance et al. 1998). By contrast, CD94/NKG2C heterodimers serve as activating receptors and require association

with the DAP12 adapter protein for signaling (Lanier et al. 1998). In mouse, staining of all antigen-specific $CD8^+$ T cells showed that CD94 almost exclusively dimerized with the inhibitory receptor NKG2A (Gunturi et al. 2004). Studies using OT-1 TCR transgenic mice showed that all antigen-specific $CD8^+$ T cells express high level of CD94 upon stimulation and maintain intermediate levels of CD94 throughout the memory stage. The inhibitory function of CD94/NKG2 on murine CD8 T cells is controversial. However, CD94/NKG2 receptors have been implicated in the protection from apoptosis and improvement of the survival of memory $CD8^+$ T cells (Gunturi et al. 2003).

Most of Ly49 genes encode inhibitory receptors with ITIMs in their cytoplasmic domains, whereas others (e.g., Ly49D and Ly49H in B6 mice) are activating receptors that associate with the ITAM-bearing DAP12 adapter molecule. Only inhibitory Ly49 receptors have been reported to be expressed on $CD8^+$ T cells (Coles et al. 2000). 2B4 (CD244) is a cell surface glycoprotein that is expressed by memory-phenotype $CD8^+$ T cells and all NK cells (Assarsson et al. 2005). It belongs to the CD2 subset of the IgG family of receptors. The CD2 family members are expressed predominantly on hematopoietic cells and have been shown to interact with other molecules of the same subfamily to modulate lymphocyte responses. The ligand for 2B4 is CD48 which is another member of this family and expressed widely on hematopoietic cells (Lee et al. 2005). 2B4 is conserved in humans and mice. It can be either activating or inhibitory (McNerney et al. 2005).

It has been found that IL-2 activated $CD8CD44^{hi}$ cells from naïve mice up-regulated their expression of NKG2D, CD94, and 2B4 receptors. They express not only DAP10 but also DAP12 adaptor molecules, which is normally expressed in activated NK cells. These activated cells demonstrate preferential killing of syngeneic tumor cells. Expression of the NKG2D

ligand Rae-1 on the tumor cells led to greatly enhanced killing of the target cells (Dhanji and Teh 2003).

Under conditions of dysregulated IL-15 expression in vivo in patients with celiac disease and in vitro in healthy individuals, multiple steps of the NKG2D/DAP10 signaling pathway leading to ERK and JNK activation are coordinatedly primed to activate direct cytolytic function independent of TCR specificity in effector CD8 T cells (Meresse et al. 2004). Celiac disease is elicited by gluten intolerance in HLA-DQ2 and DQ8 individuals. A key pathologic event is a massive cell death in the epithelial compartment infiltrated by IL-15 activated CTL (Green and Jabri 2003). The TCR-independent NK-like killing mediated by intraepithelial intestinal CTL, which expressed high levels of NK receptors and expanded massively under high exposure to IL-15 in the epithelial compartment, might lead to the tissue damage in celiac disease as well as other pathological processes (Meresse et al. 2004).

V-2 IFN-γ secretion by CD8 T cells independent of TCR

In addition to acquired immunity, CD8 T cells can also play a role in innate immunity by secreting IFN-γ in response to cytokines. IFN-γ is a type I cytokine that exhibits a wide array of proinflammatory activities. It is produced in most types of infections and plays a key role in amplifying innate and adative immune responses (Kambayashi et al. 2003). During the non-antigen specific early phase of infection, IFN-γ is believed to be primarily provided by NK and NKT cells in response to pathogen-derived inflammatory mediators (Kim et al. 2000;Ogasawara et al. 1998). However, a portion of $CD8CD44^{hi}$ memory cells have also been reported to be an early source of IFN-γ especially in the lymph nodes after LPS, type I IFN or poly(I:C) injection (Kambayashi et al. 2003). The CD8 T cell response to LPS stimulation is indirect through macrophage/DC-derived IFN-α/β, IL-12, and IL-18. Antigen primed memory

CD8 cells have been demonstrated to be able to secrete IFN-γ in response to IL-12 and IL-18 in the absence of cognate antigens (Berg et al. 2003). This finding suggested that CD8CD44^{hi} memory cells may contribute to innate immunity by providing an early non-Ag-specific source of IFN-γ. It also has been reported that IFN-γ is induced rapidly in a small subset of CD8 T cells after anti-CD3 injection (Das et al. 2001). By examining the cells from normal and various MHC-knockout mice, MHC class Ib-restricted and TAP-independent CD8^{+} cells have been proposed to be an early source of IFN-γ that has a role in polarizing CD4 T cells to become Th1 cells. Taken together, these results suggest that in response to bacterial or viral products, memory T cells may contribute to innate immunity by providing an early source of IFN-γ independent of TCR stimulation.

Chapter II: Objective

The murine MHC encodes both classical (class Ia) and non class Ia molecules. The generation and function of classical MHC class I restricted CD8 T cells is well characterized. In contrast, the phenotype and activation of non class Ia restricted CD8 T cells are not thoroughly studied. DKO mice lack expression of class Ia K^b and D^b heavy chains but show normal expression of non class Ia molecules. Therefore, DKO mice represent a powerful tool for investigating the potential of CD8 cells specific for non-class Ia antigens. The primary goal of my thesis is to use DKO mice as a model to elucidate the phenotypes and origins of non class Ia selected CD8 T cell and to understand their role in immune responses.

It has been known that most CD8 T cells in DKO mice express a memory phenotype. Little is known about the ontogeny of these non class Ia restricted $CD8CD44^{hi}$ cells. Thus, the first specific aim is to determine the origin of $CD8CD44^{hi}$ cells in DKO mice. There are several mechanisms that CD8 T cells can acquire a memory phenotype without encounter foreign antigens. Some $CD8CD44^{hi}$ cells in naïve B6 mice are thought to be developed extrathymically. Whether the non class Ia restricted $CD8CD44^{hi}$ cells are also developed in a thymus independent pathway will be examined. CD8 T cell can acquire memory phenotype through homeostatic proliferation. The greatly reduced CD8 cell number in the periphery of DKO mice produces a lymphopenic CD8 compartment. The ability of CD8 cells in DKO mice to undergo homeostatic proliferation will also be evaluated.

It is interesting to know whether these $CD8CD44^{hi}$ cells in DKO mice are capable of generating immune responses to pathogenic infection. Thus, the second specific aim is to examine the function of non class Ia selected $CD8CD44^{hi}$ cells in adaptive immunity. The

important role of non class Ia restricted CD8 T cells in primary response to Listeria monocytogenes (LM) infection have been well studied. However, little is know about which cell subsets contribute to these anti-listeria immunity. Purified CD8CD44^{hi} cells from DKO mice will be transferred and challenged by LM and their responses will be investigated.

The role of CD8CD44^{hi} cells in innate immunity has been reported recently. The third specific aim of this project will be determine whether CD8CD44^{hi} cells from DKO are capable of playing a role in innate immunity. Their ability to rapidly produce IFN-γ in response to anti-CD3 or pro-inflammatory cytokines will be assessed. More studies will be done to investigate whether these CD8CD44^{hi} cells express NK cell markers and whether they have NK-like cytotoxicity. Together, these experiments will allow for a better understanding the origin and function of non class Ia restricted CD8CD44^{hi} T cells.

Chapter III: Materials and Methods

Mice. C57BL/6 (B6) and C57BL/6.PL-Thy1^{a}/Cy (B6.Thy1.1) mice were purchased from Jackson Laboratory (Bar Harbor, ME). B6.K$^{b-/-}$D$^{b-/-}$, B6.K$^{b-/-}$, and B6.D$^{b-/-}$ mice were generated as previously described and were a generous gift from F. Lemonnier (Institute Pasteur, Paris, France). B6.K$^{b-/-}$D$^{b-/-}$$\beta_2$-M$^{-/-}$ animals were a kind gift from Dr. C. Surh (The Scripps Research Clinic, San Diego, CA). DKO were crossed to B6.Thy1.1 mice and the F_2 progeny were screened for Thy1.1$^{+/+}$DKO animals. All mice were bred and maintained in animal colonies at the University of Texas Southwestern Medical Center (Dallas, TX) under specific pathogen-free conditions and used at 8-16 weeks of age.

Bacteria. LM 10403 serotype 1 was originally provided by H. G. A. Bouwer (Veterans Affairs Medical Center, Portland, OR). Bacteria are grown on brain-heart infusion agar plates (Difco, Detroit, MI). Virulent stocks were maintained by repeated passage through B6 mice. The LD_{50} dose for B6 mice is approximately 2 x 10^4 bacteria. For infection, log phase cultures of LM grown in brain-heart infusion broth were washed twice and diluted in PBS before injection of 2 x 10^3 bacteria in the lateral tail vein. Injected bacteria numbers were more accurately determined by speading bacteria samples on brain-heart infusion plates. Spleens were harvested for analysis at day 6 after infection.

Cell lines, cell culture, and reagents. The J774 macrophage cell line (H-2^{d}) was grown in complete DMEM (Life Technologies) with 10% FCS (Atlanta Biologicals), 2mM L-glutamine, 100U/ml penicillin, and 100ug/ml streptomycin. For infection of J774 cells with LM, antibiotic-free medium was used. Log phase cultures of LM grown in BHI broth were harvested and washed twice with PBS. J774 cells that had been cultured in antibiotic-free

medium for 18 hours were infected at a multiplicity of infection (MOL) of 5:1 by LM. After 60 min, the infected J774 cells were washed twice and put into DMEM medium containing 100μg/ml gentamicin. Four hours after initial infection, J774 cells were harvested and counted.

Single cell suspensions were prepared from freshly isolated spleen. Red blood cells were lysed with 1mM Tris Ammonium Chloride. Culture of mouse splenocytes was carried out in RPMI (Life Technologies) supplemented with 10% FCS, 25mM HEPES, 2mM L-glutamine, vitamins, 100U/ml penicillin, 100ug/ml streptomycin, 1mM sodium pyruvate, 0.1 mM nonessential amino acids, 20uM 2-ME and 1.3 X 10^2 U/ml recombinant human IL-2. Cells were grown at 37ºC in humidified incubator containing 7% CO_2. Recombinant murine IL-12 (5 ng/ml final concentration) and IL-18 (10 ng/ml final concentration) were purchased from Peprotech Inc., Rocky Hill, NJ. Blocking antibodies against murine IL-12 and IL-18 were purchased from Peprotech Inc. and Medical and Biological Laboratories Co., LTD. (Nagoya, Japan), respectively, and used at 1μg/ml final concentration. RMAS, a TAP deficient murine T cell lymphoma cell line and its transfectants RMAS-Rae-1 and RMAS-H60 cell lines were a kind of gift of Dr. David H. Raulet (UC, Berkeley, CA) and grown in RPMI medium with 10% FCS.

Antibodies and flow cytometry. Spleen, thymic, and lymph node cells (2 x 10^6) were stained for 15 min at 4°C with the appropriate concentrations of mAbs in PBS containing 2% FCS and 0.1% NaN_3. The following antibodies from BD Biosciences (San Diego, CA) were used for flow cytometry: anti-CD24 (M1/69), anti-CD8α (53-6.7), anti-CD8ß (53-5.8), anti-CD44 (IM7), anti-IFN-γ (XMG1.2), anti-CD90.2 (Thy1.2) (53–2.1), anti-CD90.1 (Thy1.1) (OX-7), anti-TCR V_β segments (TCR $V_{\beta 2, 4, 5, 6, 7, 8.1/.2, 8.3, \text{and } 9, 10, 11, 12, 13, 14}$), anti-CD3ε (145-2C11), anti-CD4 (RM4-5), anti-CD122 (TM-β1), anti-CD62L (MEL-14), anti-CD16/CD32

(2.4G2), anti-Ly49A^{B6} (A1), anti-Ly49G2 (4D11), anti-Ly6C (AL-21), anti-NKG2A^{B6} (16a11), anti-CD94 (18d3), anti-NK1.1 (PK136) anti-CD244.2 (2B4 B6 alloantigen). Secondary streptavidin-conjugated reagents were used to reveal biotinylated primary antibodies. The following antibodies are from eBioscience (San Diego, CA): anti-Ly49 C/I/F/H 914B11), anti-NKG2D (C7). Purified anti-NKG2D (MI-6) blocking antibody and its isotype control antibody Rat IgG2a are also from eBioscience. Data were acquired using a FACSCalibur flow cytometer and analyzed using CellQuest software (BD Biosciences, Mountain View, CA). For IFN-γ detection by intracellular cytokine staining (ICS), the Cytofix-Cytoperm kit (BD Pharmingen) was used according to the manufacturer's protocol. Briefly, cultured cells were treated with Golgiplug containing brefeldin A (BD PharMingen), harvested, and stained for cell surface markers. The cells were then fixed, permeabilized, and stained for intracellular IFN-γ.

Intrathymic FITC injection. Intrathymic injections were performed according to published protocols (Berzins et al. 1999;Almeida et al. 2001). Briefly, 6-week old B6 or DKO mice were anesthetized and the chest cavities were opened by a ventral midline incision to reveal the thymic lobes. 10μl of fluorescein isothiocyanate (FITC) solution (1 mg/ml; Sigma-Aldrich, St. Louis, MO) were injected into each thymic lobe with a 30-gauge needle. The FITC solution typically resulted in random labeling of 30-60% of the thymocyte population. The wound was closed with surgical clips, and the mouse was warmed until fully recovered from anesthesia. Mice were killed by CO_2 asphyxiation around 16-20 hr post-injection, The thymus, spleens, and lymph nodes were taken for analysis. Instruments were washed between removal of each organ, and the FITC-injected thymus was always removed last to avoid cross-contamination of samples.

Thymectomy and bone marrow chimeras. Suction thymectomies were performed at 6-8 weeks of age as described (SJODIN et al. 1963;Dulude et al. 1999). Completeness of the procedure was confirmed by visual inspection at the time of sacrifice. Bone marrow chimeras were prepared by γ-irradiating (1200rad) thymectomized or normal B6.DKO recipient mice with a ^{137}Cs source followed by intravenously injection of 10^7 T-depleted bone marrow cells from Thy1.1.DKO donors. Before injection, T cell depletion was performed with CD4 and CD8 Dynal beads according to manufacturer's instructions and was >95% effective. Chimeras were infected with LM 6-8 weeks later.

BrdU labeling. For splenocyte BrdU labeling, groups of B6 and DKO mice were given freshly made 0.8mg/ml BrdU (Sigma-Aldrich, St. Louis, MO) solution in drinking water everyday for 10 days. Spleen cells were harvested and red blood cells were lysed with 1mM Tris Ammonium Chloride. After staining for surface markers, cells were incubated with cytofix/cytoperm buffer (BD Pharmingen) for 20min at 4ºC. The fixed and permeabilized cells were washed twice with Perm/Wash buffer. Then 500U/ml Deoxyribonuclease I (DNase I, Sigma-Aldrich) solution, which is in 1X PBS with 0.15M NaCl and 4.2mM MgCl and its PH value is 5.0, were used to treat the cells for 30 minutes at 37ºC. After washing, the DNase I treated cells were incubated with FITC-conjugated anti-BrdU antibody for 1 hour at room temperature. The stained cells were analyzed by FACSCalibur and CellQuest software. For thymocyte BrdU labeling, groups of B6 and DKO mice were give 500µl 2mg/ml BrdU solution in 1X PBS intraperitoneally. Four hours later, the mice were given the second 500µl 2mg/ml BrdU injection. One hour after the second injection, the thymus were harvested and staining were performed as mentioned above.

Detection of Apoptosis. The percentage of cells undergoing apoptosis was determined by staining 1 X 10^6 cells with Annexin V according to the manufacturer's instructions (BD PharMingen) and performing FACS analysis. Briefly, after cell surface marker staining, cells were washed by PBS twice and then resuspended in 1 x Annexin V Binding Buffer and incubated with Annexin V for 15 minutes at room temperature in the dark. The Annexin V stained cells were analyzed by flow cytometry within one hour.

Adoptive transfer of CFSE-labeled cells. For cell transfers, single-cell suspensions were prepared from freshly isolated spleens. After lysing RBCs using erythrocyte lysing buffer (R&D Systems, Minneapolis, MN), splenocytes were enriched for T cells by passing them through nylon wool columns. The cells were labeled with Carboxyfluorescein diacetate, succinimidyl ester (CFSE) by incubation with 1 μM CFSE (Molecular Probes, Eugene, OR) for 15 min at 37°C in balanced salt solution followed by quenching the unlabeled CFSE by adding excess amounts of FCS and washing. Recipient mice were injected intravenously with 10 x 10^6 CFSE-labeled cells. When necessary, recipient mice were subjected to gamma-irradiation with 650 rad 24 h before cell transfer. Donor CFSE-labeled cells were identified in normal and irradiated hosts and analyzed for CFSE expression to establish rounds of cell division in the host. Calculation of percent cells in each round was performed according to an established protocol.

In vitro anti-CD3 stimulation. For in vitro anti-CD3 stimulation, purified anti-CD3ε (145-2C11) or isotype control IgG (G235-2356) were coated on 96-well tissue culture plates at 5μg/well in 50μl PBS at 4ºC overnight. Just before adding cells, remove the antibody solution with a multichannel pipettor. Rinse each well with 200 μl of PBS and discard PBS to remove all unbound antibody from each well. $CD8^+$ slenocytes from B6 and DKO mice were selected

by CD8α beads and sorted into $CD44^{hi}$ and $CD44^{lo}$ populations by Moflo. The purified $CD8\alpha^{+}CD44^{hi}$ and $CD8\alpha^{+}CD44^{lo}$ cells were counted, labeled by CFSE, and added into the anti-CA3 and control antibody coated plates in 10^4/200μl/well. At day 2 and day 4, cells were harvested and performed intracellular IFN-γ staining. Four hours before harvest, Golgiplug were added to the culture. IFN-γ-producing CD8β T cells were detected by the Cytofix-Cytoperm kit (BD Pharmingen) as previously described. CFSE expression was analyzed to establish rounds of cell division in the culture.

CD8 T cell purification, adoptive transfer and stimulation. Single cell suspensions from the spleens of B6 and DKO mice were pooled and CD8α cells were purified with CD8α magnetic beads from BD PharMingen according to the manufacturer's instructions. For the separation of $CD8\alpha CD44^{hi}$ cells, CD8α magnetic bead-purified $CD8^{+}$ T cells were stained with anti-CD44-PE. After washing, the CD8α cell population was sorted on the basis of $CD44^{hi}$ using a MoFlo™ high speed sorter (Cytomation, Inc.). Purified $CD8\alpha CD44^{hi}$ cells ($2x10^5$) from DKO mice were injected intravenously into Thy1.1.DKO recipients and infected the next day with 2×10^3 LM. Six days later, the mice were sacrificed and the resulting splenocytes were cultured in 24-well plates at a concentration of 3×10^6/ well with 3×10^5 J774 cells, LM infected J774 cells and LM infected J774 cells with 1μg/ml anti-IL-12 and 1μg/ml anti-IL-18 antibodies overnight.

Detection of H2-M3 restricted anti-listerial $CD8CD44^{hi}$ cells. LM immunized splenocytes from $Thy1.2^{+/+}CD8\alpha CD44^{hi}$ cells transferred Thy1.1.DKO mice were harvested 6 days later. The splenocytes from naïve Thy1.1.DKO mice were served as negative controls. After lysis of red blood cells, the splenocytes were cultured in complete RPMI medium supplemented with 130U/ml recombinant human IL-2 without any peptide or with 10mM f-

MIGWII, 10mM f-MIVTLF, 10mM SIINFEKL peptides onvernight. GolgiPlug containing brefeldin A was added 4 h prior to the harvest of the cultures. IFN-γ-producing CD8 T cells were detected by the Cytofix-Cytoperm kit (BD Pharmingen) as previously described.

In vivo anti-CD3 stimulation. Mice were injected i.p. with 5 μg/mouse of NA/LE anti-CD3ε (145-2C11) or the same amount of control isotype-matched IgG (G235-2356) (BD PharMingen). Three hours later, splenocytes were harvested and cultured in 24-well plates at a concentration of 3×10^6/ well for 4 hours. GolgiPlug containing brefeldin A was added 2 h prior to the harvest of the cultures. IFN-γ-producing CD8 T cells were detected by the Cytofix-Cytoperm kit (BD Pharmingen) as previously described.

In vitro IL-12 and IL-18 stimulation. Splenocytes were cultured in 24-well plates at a concentration of 3×10^6/ well with 20U/ml recombinant human IL-2, or with 20U/ml recombinant human IL-2 and 5ng/ml IL-12 and 10ng/ml IL-18 overnight. GolgiPlug containing brefeldin A (BD PharMingen) was added 4 h prior to the harvest of the cultures. IFN-γ-producing CD8 T cells were detected by the Cytofix-Cytoperm kit (BD Pharmingen), according to the manufacturer's protocol were detected with as previously described.

In vitro high concentration of IL-2 stimulation. Single cell suspensions from freshly isolated spleens of B6 and DKO mice were labeled by CFSE and cultured in supplemented with 10% FCS, 25mM HEPES, 2mM L-glutamine, vitamins, 100U/ml penicillin, 100ug/ml streptomycin, 1mM sodium pyruvate, 0.1 mM nonessential amino acids, 20uM 2-ME and 200 U/ml recombinant human IL-2. At day 2 and day 4, cells were harvested and performed intracellular IFN-γ staining. Four hours before harvest, Golgiplug were added to the culture. IFN-γ-producing CD8β T cells were detected by the Cytofix-Cytoperm kit (BD Pharmingen) as

previously described. CFSE expression was analyzed to establish rounds of cell division in the culture.

CTL assay. Target cells (RMAS, RMAS-Rae-1, RMAS-H60) were labeled with ^{51}Cr (100μCi) for 1 h at 37ºC and then washed twice with RPMI. Labeled target cells (8000) were added to 96-well U-bottom plates. Freshly isolated splenocytes from DKO mice were deleted red blood cells and selected by anti-CD8α magnetic beads from BD pharmingen. Half of the beads purified CD8α T cells were incubated with isotype control antibody rat IgG2a at 50μg/ml for 30 min. By contrast, the other half beads purified CD8α T cells were incubated with anti-NKG2D blocking antibody MI-6 at 50μg/ml for 30 min. The antibody treated CD8α cells were added to the 96-well plate to mix with the target cells in different ratio (100:1, 50:1 and 1:1) in a final volume of 200 μl. The cytotoxic activity of freshly purified non-classical MHC class I selected CD8α was tested in standard ^{51}Cr –release assays. After a 4 h incubation, the supernatant were collected and counted. All assays were performed in duplicate. Targets incubated for maximum release and spontaneous release recieved 1% SDS or medium only, respectively. The percentage Specific lysis was calculated as 100 X ((experimental well cpm) – (spontaneous release cpm))/((maximum release cpm) – (spontaneous release cpm)).

Statistical analysis. In certain experiments, the significance of results was determined using the Student's *t*-test, as calculated with the GraphPad Prism programs.

Chapter IV: Results

Characterization of the phenotype, origin and proliferative properties of CD8 cells from DKO mice

1. Phenotype of CD8 T cells from MHC class Ia-deficient mice

We previously noted that the majority of CD8 T cells from naive DKO mice display a phenotype typical of memory cells with more than 60% of CD8 T cells being $CD44^{hi}$ (Seaman et al. 1999). It has been recently reported that most of the CD8 cells in $\beta_2m^{-/-}$ animals do not express CD8β, but express high levels of CD11c and CD11b, suggesting that they may be of DC lineage (Nesic et al. 2000). In addition, CD8αα T cells have been shown to be $CD44^{hi}$ (Ohtsuka et al. 1994). Therefore, we determined the percentage of $CD8^+$ T cells in DKO mice that are CD8αα by staining with both CD8α and CD8β Abs as well as for CD3 expression (Fig. 1a). In the CD8 T cell compartment, adult B6 mice have 2–3% CD8αα cells in their spleen. This percentage of CD8αα cells is elevated in the DKO mice to around 10% of $CD3^+CD8^+$ cells.

We also compared the level of CD44 expression of the CD8αα and CD8αβ cells. Although almost all CD8αα cells are $CD44^{hi}$ in both strains, most CD8αβ cells are $CD44^{lo}$ in B6 mice and $CD44^{hi}$ in DKO mice (Fig. 1, b and c). More than 98 and 92% of CD8αβ cells from B6 and DKO mice are $TCRV_\beta^+$, respectively (data not shown). Thus, although most non class Ia selected CD8 T cells express the $CD44^{hi}$ phenotype, they are conventional TCRαβCD8αβ T cells, similar to classical MHC class Ia selected CD8 T cells. On an absolute cell number basis, $CD8\alpha\beta CD44^{hi}$ cells ($0.58\pm0.05x10^6$) from DKO mice are still less than those

Figure 1

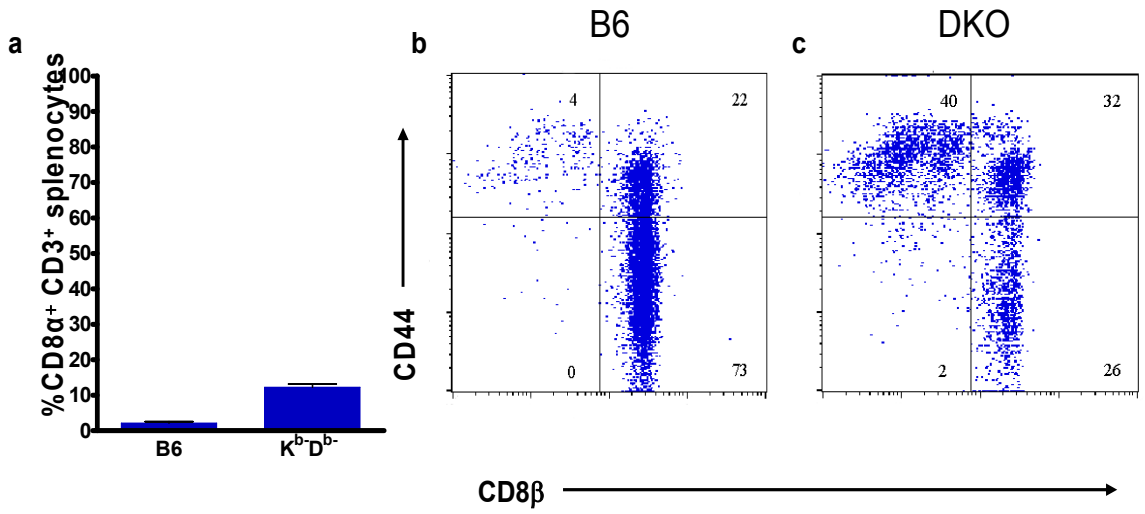

Figure 1: An elevated percentage of CD8 T cells in DKO are $CD8\alpha^{+}CD8\beta^{-}$. C57BL/6, and DKO spleen cells were quadruple stained with FITC-conjugated anti-CD3ε, PE-conjugated anti-CD8β, allophycocyanin-conjugated anti-CD44, and biotinylated anti-CD8α (followed by streptavidin-PerCP). *a*, The expression of CD8α,CD8β was analyzed after gating on $CD3^{+}$ cells. The percentages of $CD8\alpha^{+}\beta^{-}$ are shown. The mean ± SD from three mice per group is shown. *b*, B6 and *c*, DKO spleen cells were stained with FITC-conjugated anti-CD8α, PE-conjugated anti-CD8β, and APC-conjugated anti-CD44. Cells were gated on CD8α and analyzed for CD8β and CD44 staining. The numbers indicate the percentage of cells in each quadrant.

($1.66\pm0.1x10^6$) from B6 mice due to the paucity of total $CD8^+$ cells in DKO mice (Figure 2, a and b). Accordingly, CD8αα cells are not increased in the DKO animals. B6 and DKO mice have $1.24 \pm 1.10 \times 10^5$ and $0.85 \pm 0.20 \times 10^5$ ($n = 3$, P=0.58) CD8αα cells, respectively, in their spleen, which is consistent with the findings that these cells can be selected on class Ib antigens (Figure 2, c and d).

To further characterize the memory phenotype of $CD8\alpha\beta CD44^{hi}$ cells in DKO mice, we examined these cells for additional markers characteristic of memory cells. It has been reported that once formed, $CD8^+$ T cells can survive indefinitely but their survival requires contact with cytokines, in particular IL-15 (Berard et al. 2003). Consequently, we further analyzed these cells for the expression of the IL-15 receptor β-chain (CD122). As shown in Fig. 3, 46% of CD8 T cells from adult DKO mice are $CD122^+$. Virtually all of these cells are $CD44^{hi}$, although there are also 8% $CD44^{hi}CD122^-$ cells (Figure 3b). In contrast, only about 11% of CD8 T cells in B6 mice are $CD122^+CD44^{hi}$ (Fig. 3a). Ly-6C is a family member of GPI-linked proteins. It has been reported that had an accessory role in the activation of cytolysis and the production of IFN-γ (Johnson et al. 1993). However, its role in memory T cell function is still unclear and requires further study. Ly-6C is expressed at very high levels on memory CD8 T cells (Kaech et al. 2002). We found most CD8αβ cells in DKO mice express Ly6C (Figure 3d). In contrast, there are only less than 20% CD8αβ cells in B6 mice express Ly6C (Figure 3c). We also examined these cells for the expression of the lymph node-homing receptor CD62L which is decreased on activated memory cells. In DKO mice, 17% of $CD8\alpha\beta CD44^{high}$ splenocytes are $CD62L^{low}$, whereas in B6 splenocytes only 4% of $CD8\alpha\beta CD44^{high}$ T cells are $CD62L^{low}$ (Fig. 3, e and f).

Figure 2

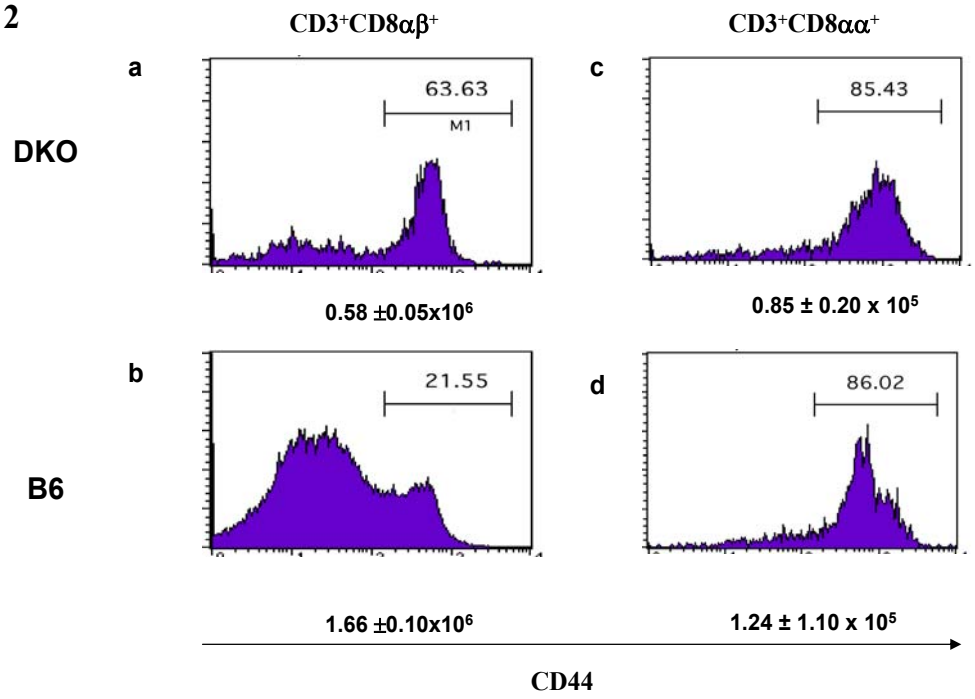

Figure 2: There are less CD8αβCD44^{hi} cells and similar CD8αα cells in DKO mice compared to B6 mice. Splenocytes from DKO and B6 mice were stained with FITC-conjugated anti-CD3, PE-conjugated anti-CD8β, PerCP-conjugated anti-CD8α, and APC-conjugated anti-CD44. The expression of CD44 was analyzed after gated on CD3$^+$CD8αβ$^+$ cells (a and b) and CD3$^+$CD8αα$^+$ cells (c and d). The numbers inside the histograms indicate the percentage of cells in the gate displayed. The numbers below the histograms show the absolute number of CD3$^+$CD8αβ$^+$CD44^{hi} cells (a and b) and CD3$^+$CD8αα$^+$ cells (c and d) in DKO and B6 mice.

Figure 3

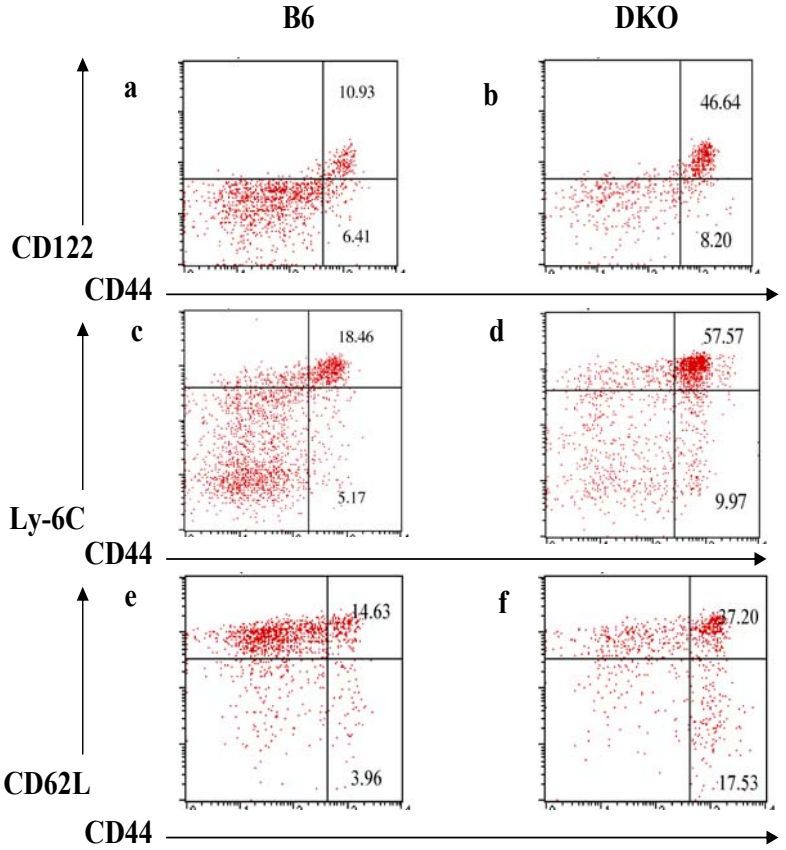

Figure 3: A significant portion of CD8αβ T cells in DKO mice is $CD44^{hi}CD122^{+}Ly\text{-}6C^{+}$ and $CD62L^{low}$. a and b, B6 and DKO spleen cells were stained with FITC-conjugated anti-CD8α, PE-conjugated anti-CD8β, APC-conjugated anti-CD44, and biotinylated anti-CD122 (followed by streptavidin-PerCP). The expression of CD122 and CD44 was analyzed after gating on $CD8\alpha\beta^{+}$ cells. c and d**,** B6 and DKO spleen cells were stained with FITC-conjugated anti-Ly-6C and PerCP conjugated anti-CD8α. e and f, B6 and DKO spleen cells were stained with biotinylated anti-CD62L (followed by streptavidin-PerCP). The numbers indicate the percentage of cells in each quadrant. Data are representative of four animals each.

It has been reported that CD8 T cells can express receptors characteristic of NK cells. Both activating and inhibitory NK receptors have been found on these cells. NK receptor expression by CD8 T cells is restricted to cells with an activated/memory phenotype. However, immediately ex vivo CD8CD44^{hi} cell from normal uninfected B6 mice do not express NK1.1 or significant levels of other NK receptors such as CD94, 2B4 or NKG2D (Dhanji and Teh 2003). To further characterize the phenotype of non class Ia restricted CD8αβCD44^{hi} cells, we stained CD8αβCD44^{hi} cells from naïve DKO and B6 mice for several NK receptors. Unlike NKT cells, non class Ia restricted CD8αβCD44^{hi} cells do not express NK1.1 (Figure 4 b). They also express a very low level of 2B4, similar to those cells from B6 mice (Figure 4 c and d). However, compared to those CD8αβ cells from B6 mice, non class Ia restricted CD8αβ cells from naïve DKO mice do express a higher level of Ly49 (7% vs 1%), NKG2D (10% vs 1%), CD94 (18% vs 1%) and NKG2A (17% vs 1%) (Figure 4 e-l). All the CD8αβ cells expressing NK cell receptors are CD44^{hi}, in agreement with previous reports.

It has been reported that no expression of activating Ly49 isoforms (Ly49D and Ly49H) was detected on T cells in contrast to NK cells. Only inhibitory Ly49 were expressed by CD8 cells (Coles et al. 2000). CD94 can dimerize with activating or inhibitory NKG2 isoforms. A previous study using transgenic OT-1 CD8 T cells indicated that CD94 is paired with the inhibitory receptor, NKG2A and not NKG2C/E (Gunturi et al. 2005). We further investigated the expression of NKG2A and NKG2D on CD8αβCD44^{hi}CD94^{hi} cells. More than 30% of CD8αβCD44^{hi} cells from naïve DKO mice express CD94 and co-express the inhibitory NKG2 receptor NKG2A (Figure 5 b). In contrast, only 5% of CD8αβCD44^{hi} cells from naïve DKO mice express CD94 and all of them also co-express the inhibitory NKG2 receptor NKG2A (Figure 5 a). More than 20% of CD8αβCD44^{hi} cells from naïve DKO mice express

Figure 4

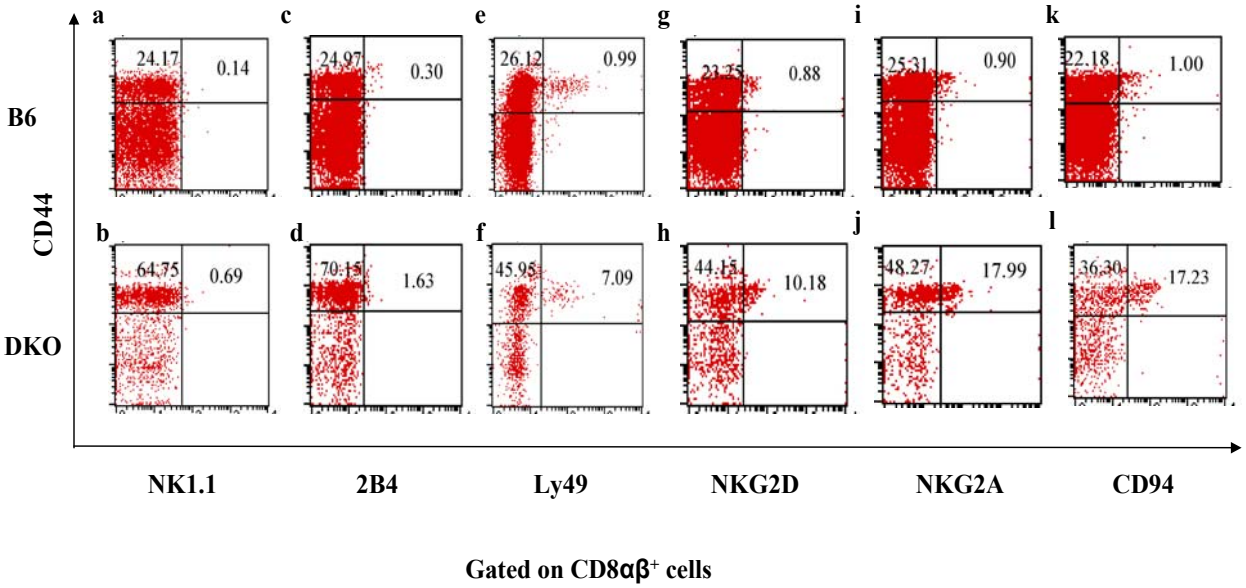

Figure 4: Expression of NK cell markers by CD8αβCD44^{hi} cells from naïve B6 and DKO mice. B6 and DKO spleen cells were stained with anti-CD8β,anti-CD44, and anti-NK1.1 (a and b), 2B4 (c and d), combination of Ly49A, Ly49G2, and Ly49 C/F/I/H (e and f), NKG2D (g and h), NKG2A (I and j) or CD94 (k and l). The co-expression of NK cell markers and CD44 was analyzed after gating on CD8αβ^{+} cells. The numbers indicate the percentage of cells in each quadrant. Data are representative of three animals in each group.

NKG2D. Most of these cells (76%) were also express CD94 (Figure 5 d). In contrast, only 5% of CD8αβCD44^{hi} cells from naïve B6 mice express NKG2D. Unlike cells from DKO mice, most (~70%) of these cells do not express CD94 (Figure 5 c).

2. Acquisition of the memory phenotype of CD8 T cells in DKO animals

All CD8^{+} T cells in a MHC class Ia-deficient host are presumably selected on molecules other than MHC class Ia. As a result, the CD8αβ T cell number in the periphery is reduced by 80–90% in DKO mice when compared with B6 mice. Therefore, naive T cells in these mice are circulating in a CD8 lymphopenic environment which might result in their expansion and acquisition of memory cell markers such as high level of CD44. The lymphopenic environment could in part be due to the reduced export from the thymus. Our data showed that a paucity of single positive CD8 cells present in the thymus as DKO mice have only about 1% of this cell population compared with 3–6% in B6 mice (Fig. 6, a and b). Furthermore, unlike CD8 T cells in the periphery, most of the CD8^{+}CD4^{-} cells in the DKO thymus are CD44^{lo}, similar to that seen in B6 mice. This is noted when the single positive cells are gated on either CD24^{-} or TCRVβ^{hi}, both markers for the mature phenotype of CD8 T cells that are ready to exit the thymus (Fig. 6, c–f).

To investigate the thymic export of B6 and DKO mature single positive CD8 T cells, we labeled the thymocytes with FITC by performing intrathymic injection. FITC, a fluorescent dye, injected into the thymus is taken up by dividing thymocytes, and retained after emigration to the periphery so that all peripheral FITC^{+} cells are recent thymic emigrants (RTEs) (Staton et al. 2004). Sixteen-20 hours after FITC injection, the spleens and lymph nodes were harvested and analyzed for the expression of CD8β and CD4. As expected, CD4 from both B6 and DKO mice showed a similar percentage of FITC^{+} cells which indicates a similar thymic

Figure 5

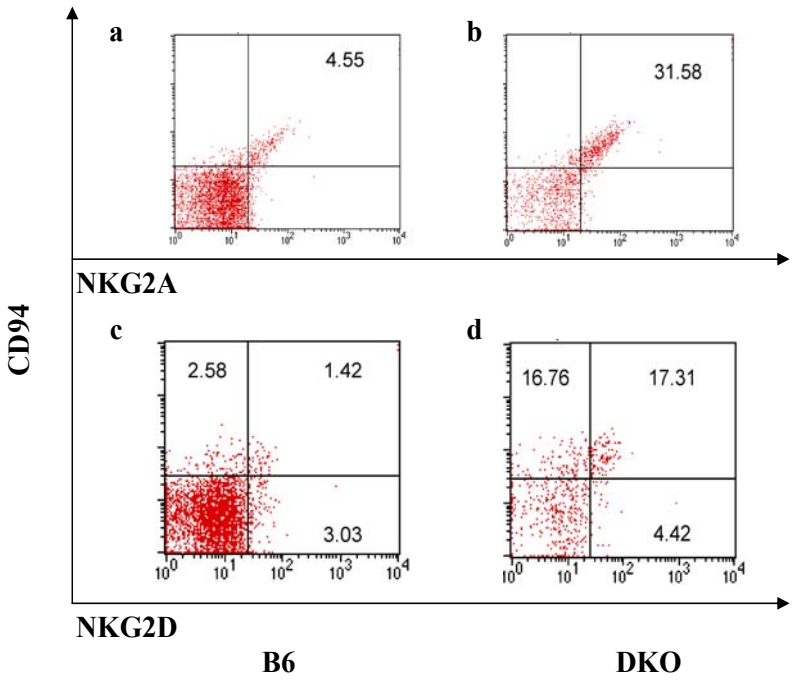

Figure 5: Co-expression of CD94 and NKG2A or NKG2D by CD8αβ^{+}CD44^{hi} cells from naïve B6 and DKO mice. a and b, B6 and DKO spleen cells were stained with FITC-conjugated anti-CD8β, PE-conjugated anti-NKG2A, PerCP-conjugated anti-CD94, and APC-conjugated anti-CD44. The expression of CD94 and NKG2A was analyzed after gating on CD8αβCD44^{hi} cells. c and d**,** B6 and DKO spleen cells were stained with FITC-conjugated anti-CD8β, PE-conjugated anti-NKG2D, PerCP-conjugated anti-CD94, and APC-conjugated anti-CD44. The expression of CD94 and NKG2D was analyzed after gating on CD8αβCD44^{hi} cells. The numbers indicate the percentage of cells in each quadrant. Data are representative of three animals each.

Figure 6

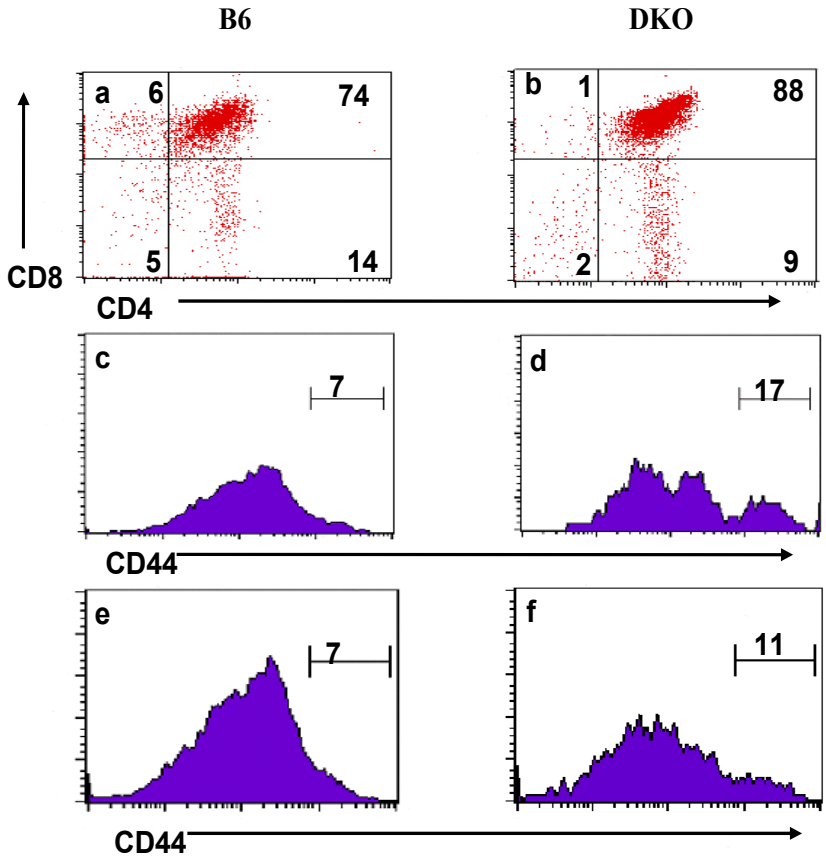

Figure 6: Paucity of $CD8^{+}CD4^{-}$ T cells in the thymus of DKO mice. a, B6 and b, DKO thymocytes were stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD8. c, B6 and d, DKO thymocytes were gated on $CD8^{+}CD4^{-}CD24^{-}$ and analyzed for CD44 expression. e, B6 and f, DKO thymocytes were gated on $CD8^{+}CD4^{-}TCRV\beta^{hi}$ and analyzed for CD44 expression. The numbers indicate the percentage of cells in the gate displayed.

emigration rate. However, a reduced percentage of $FITC^+$ cells in CD8 splenocytes were observed in DKO mice (Figure 7 a). In B6 mice, the CD4/CD8β ratio (4-5:1) was highest in thymocytes and decreased in $FITC^+$ RTEs whose CD4/CD8β ratio (3-4:1) was still a little bit higher than resident T cells (2:1). It is consistent with results that have been reported before (Boursalian et al. 2004). In DKO mice, we saw a high CD4/CD8β ratio (50:1) in the thymocytes which was much higher than those in B6 mice due to the paucity of CD8β thymocytes and normal development of CD4 thymocytes. Compared to B6 mice, the CD4/CD8β ratio (70:1) in RTEs was very high but was decreased a lot in resident T cells (30:1) (Figure 7 b).

Due to the low CD44 expression level of the mature single positive CD8αβ thymocytes in DKO mice, most non class Ia selected $CD8\alpha\beta CD44^{hi}$ cells in the periphery of DKO mice must be acquire their CD44 expression after they exit the thymus. In order to determine when these CD8αβ T cells acquired their memory phenotype, we examined CD44 expression on $FITC^+$ RTEs (after 16-20 h) in B6 and DKO mice after intrathymic FITC injection. As shown in Figure 7, approximately 12% of mature CD8αβ thymocytes, defined as $CD8\alpha\beta CD24^-$, are $CD44^{hi}$ in B6 mice. Similarly, around 15% of mature single positive $CD8\alpha\beta CD24^-$ thymocytes are $CD44^{hi}$ in DKO mice. Sixteen to 20 hours after intrathymic FITC injection, we found that around 13% of the $FITC^+$ CD8αβ RTEs in the spleens are $CD44^{hi}$ in B6 mice and about 23% of the $FITC^+$ CD8αβ RTEs in the spleens are $CD44^{hi}$ in DKO mice. This CD44 expression level of RTEs in B6 mice is lower than the resident CD8αβ splenocytes, about 25% of which are $CD44^{hi}$. The CD44 expression level of RTEs in DKO mice is even much lower than resident CD8αβ splenocytes, around 55% of which are $CD44^{hi}$. This indicates that the acquisition of a high expression of CD44 by CD8αβ cells in the periphery of DKO mice is a post-thymic event.

Figure 7

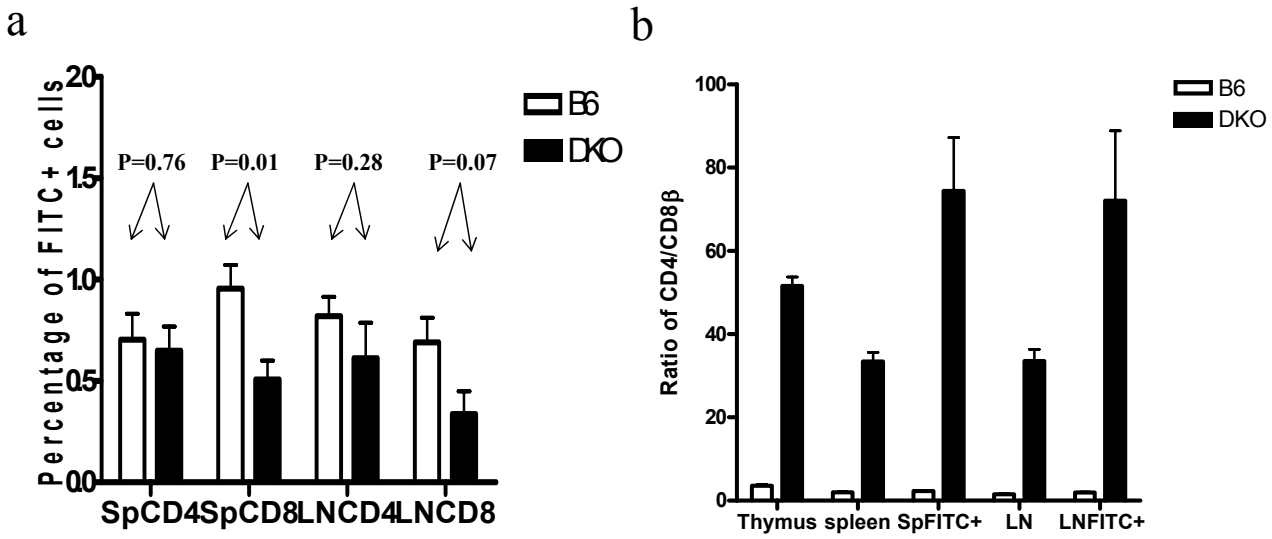

Figure 7. The thymic emigration rate of CD8αβ cell is reduced in DKO mice. Sixteen-20 hours after intrathymic FITC injection, thymocytes, splenocytes and lymph node cells were harvested and analyzed for CD4 and CD8β expression. a, Percentage of $FITC^+$ cells in $CD4^+$ and $CD8^+$ splenocytes and lymph node cells is shown. b, The ratio of CD4/CD8αβ in thymocytes, RTEs, and resident T cells in spleen and lymph nodes is shown. Thymocytes were gated on $CD8\beta^+CD24^-$ cells. Results are a summary of 4 independent experiments.

The result is consistent with previous reports that show that recent thymic emigrants are $CD44^{lo}$ (Boursalian et al. 2004). Similar results for RTEs are also observed in lymph nodes (Figure 8).

As a control, we examined the CD44 phenotype for CD4 RTEs and resident cells in both spleens and lymph nodes. The development of CD4 T cells in DKO mice is not affected by the deletion of class Ia heavy chains. We expected the same CD44 expression pattern of B6 and DKO mice. As shown in Figure 9, CD4 RTEs and resident cells from both B6 and DKO mice do have similar low levels of CD44 expression in both spleen and lymph nodes.

3. Few CD8 T cells arise in thymectomized DKO bone marrow chimeras

It has been reported that CD8 T cells that arise in a thymic-independent environment are $CD44^{hi}$. Some reports also suggest that the $CD8CD44^{hi}$ cell population in naïve normal mice is more similar to those of thymus-independently developed $CD8^{+}$ cells (Yamada et al. 2001). In order to test the possibility that $CD8\alpha\beta CD44^{hi}$ cells in naïve DKO mice developed from a thymic-independent pathway, we generated thymectomized bone marrow (Txbm) chimeras by repopulating lethally irradiated Tx DKO animals with T-cell depleted bone marrow from Thy1.1.DKO donors. Thymus intact bone marrow chimeras are used as control. As shown in Figure 10, there is approximately 1% CD8αβ splenocytes in normal DKO mice. There are about 0.40% donor derived $Thy1.1^{+}CD8\alpha\beta$ T cells that are resident in the spleen of a thymus intact bm chimera. In contrast, there are only about 0.10% donor derived $Thy1.1^{+}CD8\alpha\beta$ T cells that developed in the spleen of a Txbm chimera. Almost all of the $Thy1.1^{+}CD8\alpha\beta$ T cells in the Txbm chimera are $CD44^{hi}$ (Figure 11b), consistent with previous reports. However, less than 50% of the $Thy1.1^{+}CD8\alpha\beta$ T cells from thymus-intact bm chimera are $CD44^{hi}$ (Figure 11d), similar to that found in normal DKO animals. Taken together with the analysis of RTEs,

Figure 8

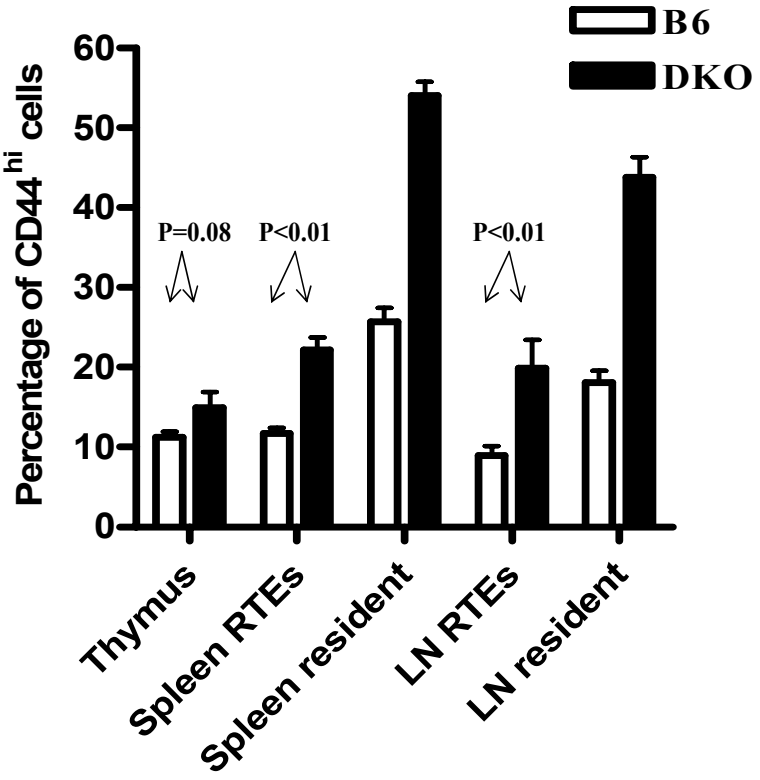

Figure 8: Most CD8αβ RTE in both B6 and DKO mice are $CD44^{lo}$. Sixteen-20 hours after intrathymic FITC injection, thymocytes, splenocytes and lymph node cells were harvested and analyzed for CD44 expression. Percentage of $CD8\alpha\beta CD44^{hi}$ thymocytes, RTEs, and resident T cells in spleen and lymph nodes is shown. Thymocytes were gated on $CD8\beta^{+}CD24^{-}$ cells. Results are a summary of 4 independent experiments.

Figure 9

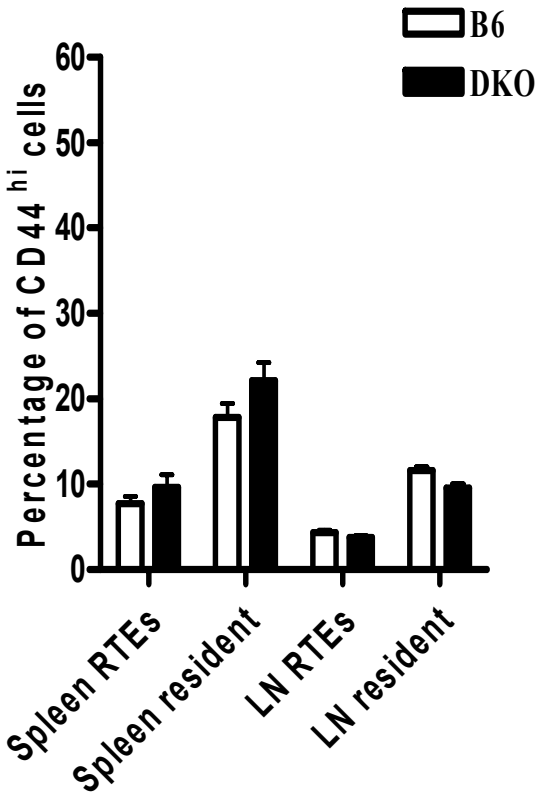

Figure 9: Most CD4 RTE in both B6 and DKO mice are $CD44^{lo}$. Sixteen-20 hours after intrathymic FITC injection, splenocytes and lymph node cells were harvested and analyzed for CD44 expression. Percentage of $CD4CD44^{hi}$ RTEs, and resident T cells in spleen and lymph nodes is shown. Results are a summary of 4 independent experiments.

Figure 10

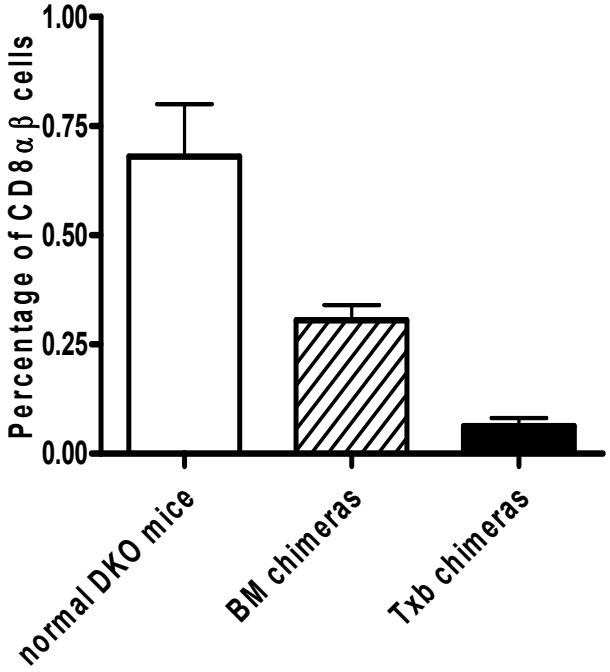

Figure 10: Most CD8αβ cells in DKO mice are thymus-dependent. Splenocytes from normal Thy1.1.DKO, Thy1.1.DKO bm→ DKO or Thy1.1.DKO bm⟶Tx DKO irradiated recipients were analyzed for expression of Thy1.1, CD8β and CD8α. The percentage of $Thy1.1^{+/+}CD8\alpha\beta^{+/+}$ cells in normal DKO, Thy1.1.DKO → DKO or Thy1.1.DKO ⟶ Tx DKO chimeric mice is summarized. The results are the mean value of 4 mice/ group.

Figure 11

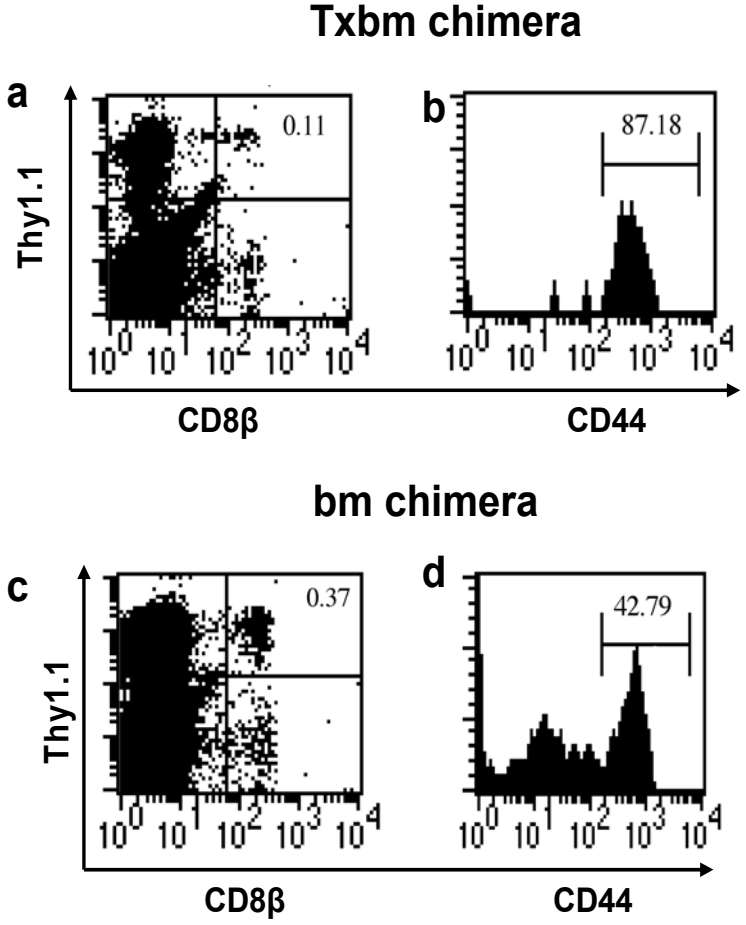

Figure 11. Most thymus independent CD8αβ cells in DKO mice are CD44^{hi}. Splenocytes from either Thy1.1.DKO bm→Tx DKO (a, b) or Thy1.1. DKO bm→DKO (c, d) irradiated recipients were analyzed for expression of Thy1.1, CD8β and CD44. The percentage of donor derived Thy1.1$^{+/+}$CD8αβ (a, c) and Thy1.1$^{+/+}$CD8αβ gated CD44^{hi} cells (b, d) is indicated by the numbers in the box. The results shown are representative of 3 independent

our data indicates that most CD8αβCD44^{hi} cells in DKO mice are thymus-dependent and acquire their phenotype in the periphery.

4. Lower proliferation and higher apoptosis of the non-classical MHC class Ia selected CD8αβ cells

It has been reported that the subsets of naïve- and memory-phenotype T cells in normal animals differ in their rate of turnover (Tough and Sprent 1994). Most T cells with a memory phenotype divide much more rapidly than naïve-phenotyped cells. However, a certain proportion of memory phenotype T cells remain in interphase for prolonged periods, similar to naïve T cells. Some naïve T cells appear to undergo cell division without losing their naïve phenotype even after many weeks (Tough and Sprent 1994). To examine the basal proliferation of subsets of CD8αβ T cells, groups of B6 and DKO mice were given 0.8mg/ml bromodeoxyuridine (BrdU), a thymidine analog, in their drinking water for 10 days. The BrdU incorporation by subsets of CD8αβ$^+$ T cells is shown in figure 12. Overall, the percentage of BrdU$^+$ CD8αβ$^+$ T cells from DKO mice was a little bit higher than those from B6 mice (15% vs 11%). However, when the percentage of BrdU$^+$ CD8αβcells was examined in CD44^{hi} and CD44^{lo} subsets, respectively, the results showed that both CD8αβ$^+$CD44^{hi} and CD8αβ$^+$CD44^{lo} cells from DKO mice actually has a lower proliferation than those from B6 mice (20% vs 30% for CD8αβCD44^{hi} and 4% vs 8% for CD8αβCD44^{lo} cells) (Figure 12 a). CD8αβCD44^{hi} cells from either DKO or B6 mice have a higher proliferation rate than CD8αβCD44^{lo} cells, consistent with previous reports (Tough and Sprent 1994). Due to the higher percentage of CD8αβCD44^{hi} cells in DKO mice, the overall proliferation rate of CD8αβ cells from DKO mice was not lower than those from B6 mice.

Figure 12

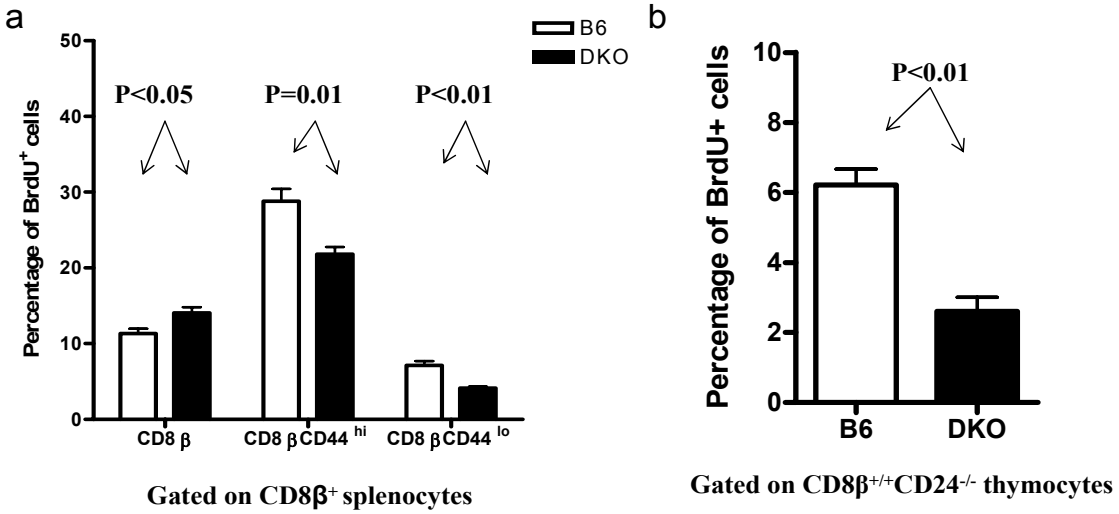

Figure 12: Lower proliferation rate of CD8β^{+}CD44^{hi} and CD8β^{+}CD44^{lo} T cells in DKO mice. Groups of B6 and DKO mice were given BrdU drinking water for 10 days. Freshly isolated splenocytes were stained with PE-comjugated anti-CD8β, APC conjugated anti-CD44. After fixation/permeation and DNase I treatment, the splenocytes were stained with FITC-conjugated anti-BrdU. a, The percentage of BrdU incorporating CD8 β^{+}, CD8 β^{+}CD44^{hi}, CD8 β^{+}CD44^{lo} splenocytes is shown. b. One hour after the second intraperitoneal BrdU injection, freshly isolated thymocytes were stained with PE-conjugated anti-CD24, biotinylated anti-CD8 β (followed by streptavidin-PerCP) and FITC conjugated anti-BrdU. The percentage of BrdU incorporating CD8 $\beta^{+/+}$CD24$^{-/-}$ thymocytes in B6 and DKO mice is shown.

To examine whether the lower basal proliferation of CD8αβCD44^{hi} and CD8αβ^{+}CD44^{lo} cells from DKO mice are intrinsic property of nonclassical class I selected CD8αβ cells, we studied the proliferation rate of mature single positive CD8αβ (gated on CD8αβ^{+}CD24^{-}) thymocytes. It is already known that thymocytes have a rapid rate of turnover. After 6-9 days, more than 98% of CD4^{+}CD8^{+} cells will be labeled by BrdU (Tough and Sprent 1994;Shortman et al. 1991). Therefore, a short BrdU labeling time will be enough to study the turnover rate of thymocytes. We labeled thymocytes by injecting 500µl 2mg/ml BrdU twice in a four hour interval intraperitoneally. Thymocytes were harvested one hour after the second injection. The mature single positive thymocytes from DKO mice had a lower proliferation rate than those from B6 mice (6% vs 3%) (Figure 12 b).

To study the apoptosis of CD8αβ cells from B6 and DKO mice, freshly isolated splenocytes and thymocytes were stained by Annexin V ex vivo. Apoptosis is characterized by a variety of morphological features such as loss of membrane asymmetry and attachment, condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA. One of the earliest indications of apoptosis is the translocation of the membrane phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. Once exposed to the extracellular environment, binding sites on PS become available for Annexin V. Overall, the percentage of Annexin V^{+} CD8αβ splenocytes cells from DKO mice was much higher than those from B6 mice (23% vs 8%) (Figure 12a). We further examined the percentage of Annexin V^{+} CD8αβ splenocytes in CD44^{hi} and CD44^{lo} subsets. Our results showed that both CD8αβCD44^{hi} and CD8αβCD44^{lo} splenocytes from DKO mice had a higher apoptosis rate than those from B6 mice (25% vs 16% for CD8αβ^{+}CD44^{hi} and 18% vs 6% for

CD8αβCD44^{lo} cells) (Figure 13 a). The much higher apoptosis rate of CD8αβ cells from DKO mice (48%) than those from B6 mice (18%) is also observed in the thymus (Figure 13b).

5. Ability of non class Ia selected CD8 cells to undergo homeostatic expansion in class Ia-deficient hosts

It has been reported that under conditions of low single positive T cell output from the thymus that peripheral single positive T cells can compensate by homeostatic expansion to fill their compartment (Almeida et al. 2001). Since the thymus from DKO mice has reduced export of CD8 T cells, it might therefore be expected that in the periphery these CD8 cells could expand by homeostatic proliferation to fill the CD8 compartment. Alternatively, non class Ia selected CD8 T cells might not be able to undergo homeostatic expansion and thus leave the CD8 compartment deficient in cell numbers. To examine the homeostatic proliferation ability of non class Ia selected CD8 T cells, we transferred CFSE-labeled T-cell enriched splenocytes from DKO mice into un-irradiated and sublethally irradiated DKO and B6.K^{b-}D^{b-}β_2m$^-$ mice. Since most non class Ia molecules are β_2m-dependent, B6.K^{b-}D^{b-}β_2m$^-$ mice served as the control without expression of any class I molecules (including both classical MHC class Ia and non class Ia molecules). When we tested the ability of CFSE-labeled CD8$^+$ T cells from DKO mice to proliferate in un-irradiated DKO animals, no proliferation was detected as expected (data not shown). Although DKO CD8 T cells transferred into irradiated DKO mice did undergo proliferation (Fig. 14 a), much of this proliferation was independent of class I molecules since proliferation was also observed in B6.K^{b-}D^{b-}β_2m$^-$ mice (Fig. 14 b). However, some expansion of the DKO cells occured on the non class Ia background since ~57.5% proliferated in the DKO vs ~26.5% in the B6.K^{b-}D^{b-}β_2m$^-$ recipients. Even though most of the donor cells are CD44^{hi} that are thought to be able to undergo expansion independent of class I

Figure 13

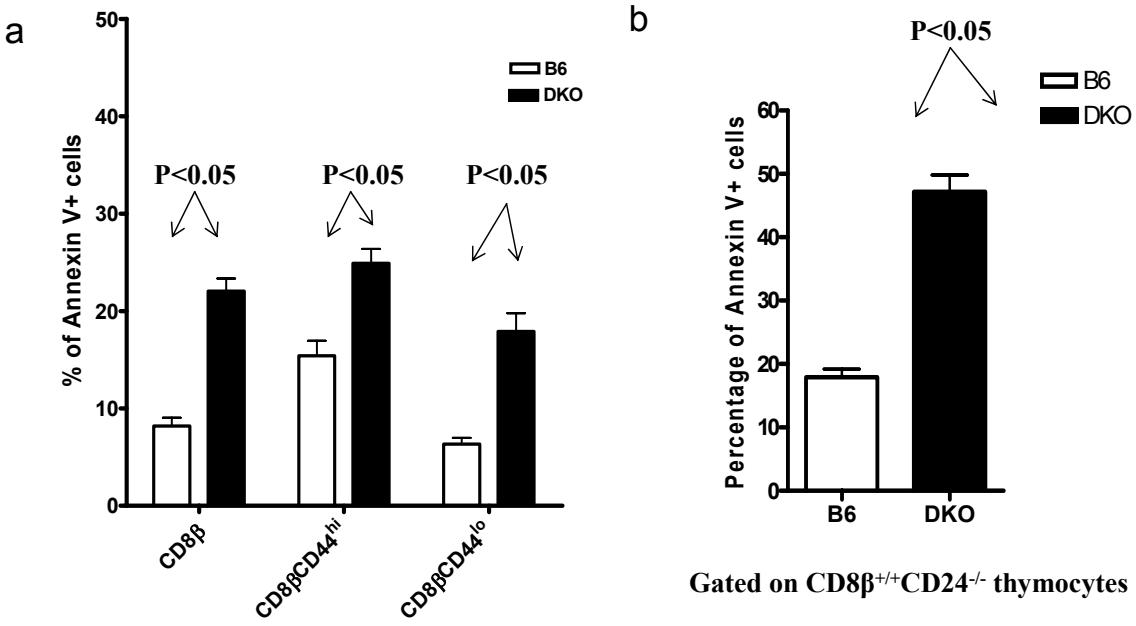

Figure 13: High apoptosis rate of CD8αβ T cells in DKO mice. Freshly isolated splenocytes from B6 and DKO mice were stained with FITC conjugated anti-CD8β and APC conjugated anti-CD44 and PE-conjugated Annexin V. a, The percentage of Annexin V positive $CD8\beta^{+}$, $CD8\beta^{+}CD44^{hi}$, $CD8\beta^{+}CD44^{lo}$ splenocytes is shown. Freshly isolated thymocytes were stained with PE-conjugated anti-CD24, biotinylated anti-CD8β (followed by streptavidin-PerCP) and FITC-conjugated Annexin V. b, The percentage of Annexin V positive $CD8\beta^{+/+}CD24^{-/-}$ thymocytes in B6 and DKO mice is shown. All flow cytometry is performed within one hour after Annexin V staining.

Figure 14

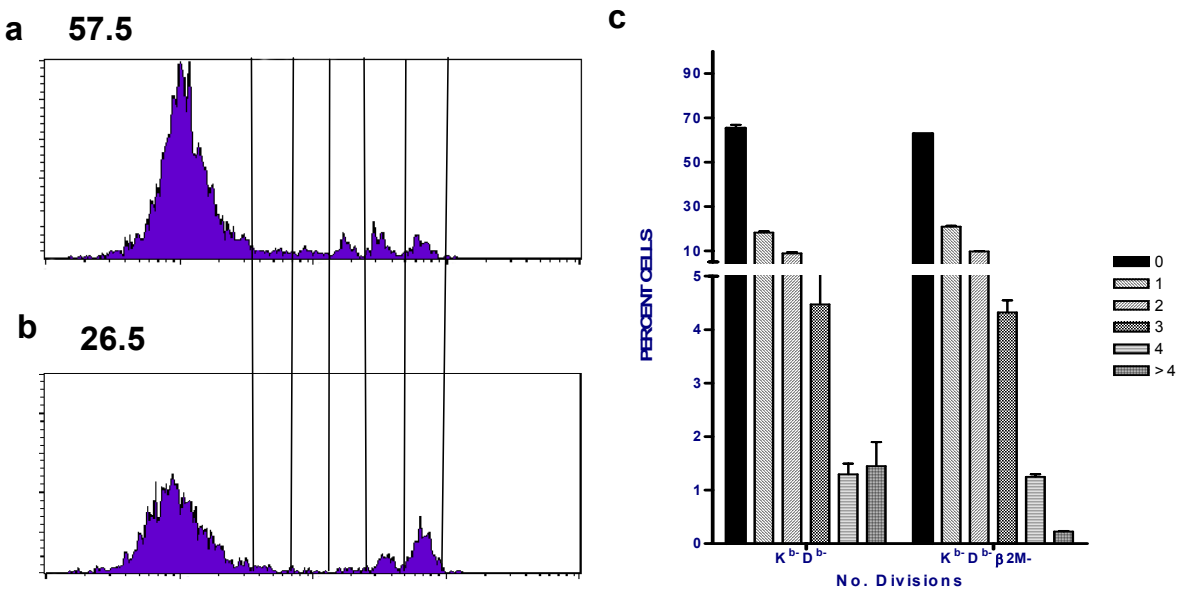

Figure 14: Homeostasis-driven proliferation of CD8 T cells in class Ia-deficient recipients. Ten x 10^6/mouse of CFSE-labeled DKO T cell-enriched spleen cells were intravenously injected into irradiated DKO (*a*) or B6.$K^{b-/-}D^{b-/-}\beta_2m^{-/-}$ (*b*) recipients. Host spleens were harvested 5 days later and stained for CD8. CFSE levels on gated donor cells are shown. Percentage of cells from two independent experiments (c) undergoing rounds of division was calculated. Zero divisions was established by the level of CFSE staining of donor cells injected into unirradiated recipients. The number above each figure shows the percentage of cells undergoing at least one round of dividion.

molecules, most of the non class Ia selected donor cells underwent a few rounds of divisions in either host. The low thymic export, the limited homeostatic proliferation ability combined with the lower proliferation rate and higher apoptosis rate might in part account for the paucity of CD8 cells in DKO mice.

6. Ability of CD8 cells from normal mice to undergo homeostatic expansion in MHC class Ia-deficient hosts

MHC Class Ia-deficient mice afford the opportunity to determine the ability of donor cells to undergo homeostatic proliferation when exposed to limited sets of antigens. Therefore, we analyzed the ability of B6 donor cells to undergo homeostatic proliferation in irradiated B6.$K^{b-/-}$ and B6.$D^{b-/-}$ and DKO recipients. Since naive CD8 T cells in the periphery actively engage in interactions with self-MHC molecules that they were selected on, it was expected that the percentage of transferred cells that proliferate in recipients would be higher in B6.$K^{b-/-}$ and B6.$D^{b-/-}$ than in DKO mice. Transfer of B6.Thy1.1 CD8 cells into irradiated B6 recipients resulted in all donor cells dividing by day 5 (Fig. 15). In contrast, transfer of these cells into irradiated B6.$K^{b-/-}$ and B6.$D^{b-/-}$ hosts resulted in 56% and 54% of cells not undergoing division, respectively, indicating that approximately half of CD8 T cells respond to either K^b or D^b plus non-class Ia molecules to undergo homeostatic expansion. Transfer of B6 CD8 cells into DKO hosts resulted in ~68% of cells undergoing no cell divisions (Fig. 16 a and c). A similar result is noted when these cells were transferred into B6.$K^{b-/-}D^{b-/-}\beta_2m^{-/-}$ mice (Fig. 16, b and c), suggesting that the percentage of non-class Ia-reactive CD8 T cells in normal B6 mice that undergo homeostatic expansion is very small. However, there was a difference between the ability of B6.Thy1.1 cells to proliferate in B6.$K^{b-}D^{b-}$ vs B6.$K^{b-/-}D^{b-/-}\beta_2m^{-/-}$ mice in that a minor

Figure 15

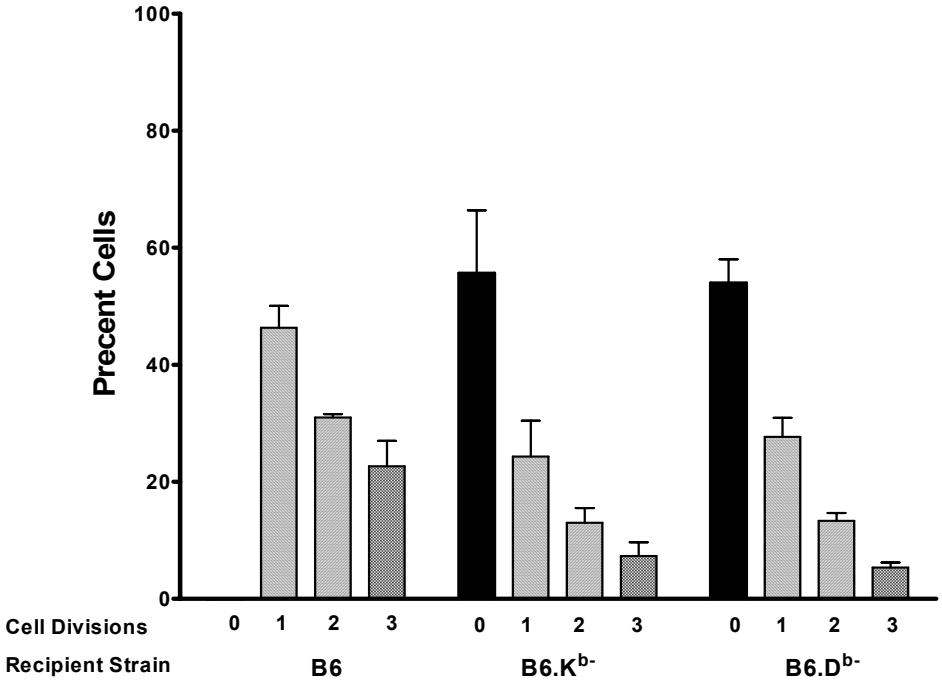

Figure 15: Homeostasis-driven proliferation of B6 CD8 T cells in recipients lacking a single class Ia molecule. B6-Thy-1.1 donor cells were inoculated into un-irradiated (data not shown) or irradiated B6, B6.Kb-, or B6.Db- recipients. Host spleens were harvested 5 days later and stained for Thy-1.1 and CD8 and analyzed for CFSE expression. Percentage of cells undergoing rounds of division was calculated. Zero divisions are established by the level of CFSE staining of donor cells injected into un-irradiated recipients. The data are representative of three mice per group.

Figure 16

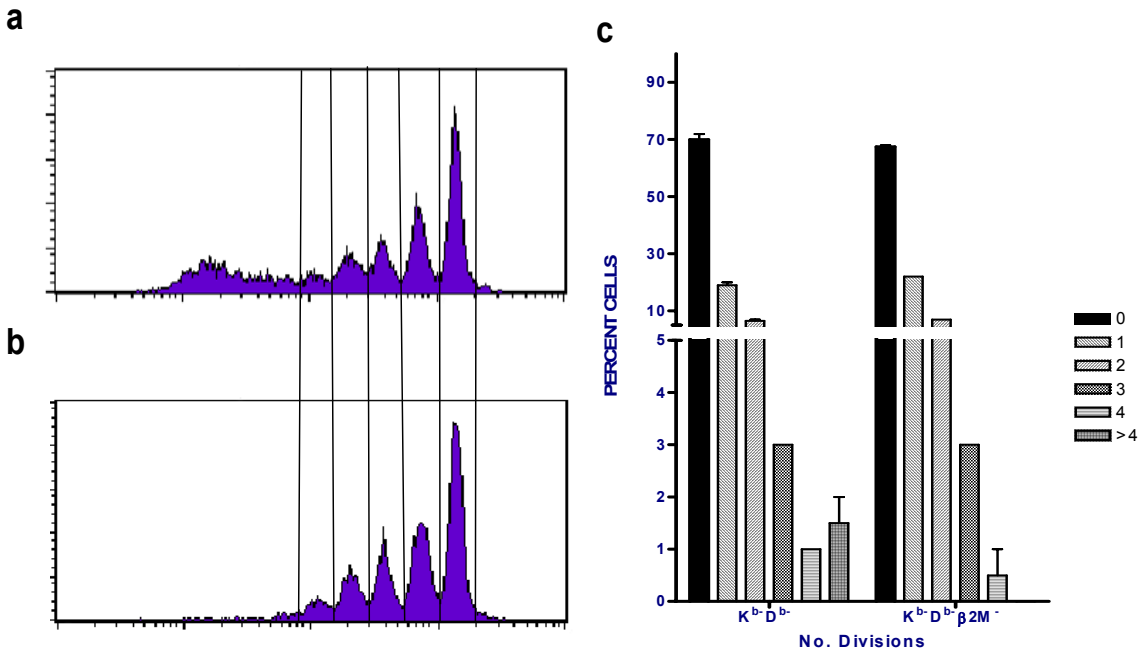

Figure 16: Homeostasis-driven proliferation of CD8 T cells from normal mice in class Ia-deficient recipients. Ten x 10^6/mouse of CFSE-labeled B6-Thy1.1 T cell-enriched spleen cells were intravenously injected into irradiated DKO (*a*) or B6.$K^{b-/-}D^{b-/-}\beta_2m^{-/-}$ (*b*) recipients. Host spleens were harvested 5 days later and stained for CD8. CFSE levels on gated donor cells are shown. Percentage of cells from two independent experiments (*c*) undergoing rounds of division was calculated. Zero divisions was established by the level of CFSE staining of donor cells injected into un-irradiated recipients.

population (1–2%) of cells underwent more than four divisions in the former but not latter hosts (Figure 16 a vs b and c).

Chapter V: Results

The role of non class Ia selected $CD8^+$ T cells in the innate and adaptive immune response

I. Ability of CD8 T cells from DKO mice to participate in the adaptive immune response

I-1 Ability of $CD8\alpha\beta CD44^{hi}$ cells from DKO animals respond to TCR dependent stimulation

There were four distinct polypeptides that are non-covalently associated with the TCRαβ heterodimer: CD3γ, CD3δ, and CD3ε chains and the disulfide-linked homodimer of the ζ chain. All of these associated proteins bear cytoplasmic tails with conserved tyrosine-based motifs that are phosphorylated upon receptor triggering and recruit a complex set of intracellular signaling molecules. The CD3 proteins participate in the assembly of the TCR-CD3 complex in the form of two different heterodimers, CD3 γε and CD3 δε (Sun et al. 2004). Activation of the TCR/CD3 complex-associated signal in a T cell can be accomplished by ligation of the T cell receptor TCR/CD3 complex. Anti-CD3 antibodies are directed to the nonpolymorphic part of the TCR/CD3 complex and crosslinking CD3 leads to a series of changes resulting in T cell proliferation and production of effector molecules including IL-2.

Our previous data indicated that non class Ia restricted CD8 T cells have a lower basal proliferation and homeostatic proliferation compared to CD8 T cells from B6 mice. To study the proliferative ability of these non class Ia selected $CD8\alpha\beta CD44^{hi}$ cells TCR-dependent stimulation, we stimulated purified and CFSE labeled $CD8CD44^{hi}$ cells from both B6 and DKO

mice with plate-bound anti-CD3 or isotype control antibody in complete RPMI medium with 20U/ml IL-2. At day 2 and day 4, the CD8CD44^{hi} cells were harvested and the anti-CD3 induced CD8αβCD44^{hi} cell proliferation was measured by CFSE dilution. The percentage of cultured CD8αβCD44^{hi} cells undergoing at least one round of division are shown in Figure 17. There were around 30% CD8αβCD44^{hi} cells undergoing proliferation after 2 days of anti-CD3 stimulation. Approximately 70% proliferated in response to anti-CD3 stimulation after 4 days. Thus, the non class Ia restricted CD8αβCD44^{hi} cells from DKO mice shows a similar proliferative ability in response to TCR engaged stimulation as those from B6 mice.

To further investigate non class Ia restricted CD8αβCD44^{hi} cells in response to TCR-dependent stimulation, we measured the percentage of IFN-γ secreting cells in the cultured anti-CD3 stimulated CD8αβ^{+}CD44^{hi} cells. As shown in Figure 18, around 15% CD8αβCD44^{hi} cells from B6 mice secrete IFN-γ after anti-CD3 stimulation for 2 days. In contrast, around 27% CD8αβCD44^{hi} cells from DKO mice secrete IFN-γ after anti-CD3 stimulation for 2 days. At day 4, a similar percentage of CD8αβCD44^{hi} cells from both B6 and DKO mice secreted IFN-γ. No IFN-γ secreting was observed in cells stimulated by isotype control antibody. Therefore, CD8αβCD44^{hi} cells from DKO mice are responding more rapidly than those cells from B6 mice to TCR-dependent stimulation.

I-2. Ability of thymus-independent CD8αβCD44^{hi} cells from DKO animals to respond to LM challenge.

It has been previously shown that CD8 T cells from DKO mice mount a specific response to LM (Seaman et al. 1999). The origin of these CD8 T cells is unknown. In order to test whether the responding CD8 cells arose from a thymus-dependent vs thymus-independent pathway we challenged Thy1.1.DKO reconstituted DKO Txbm chimeras and Thy1.1.DKO

Figure 17

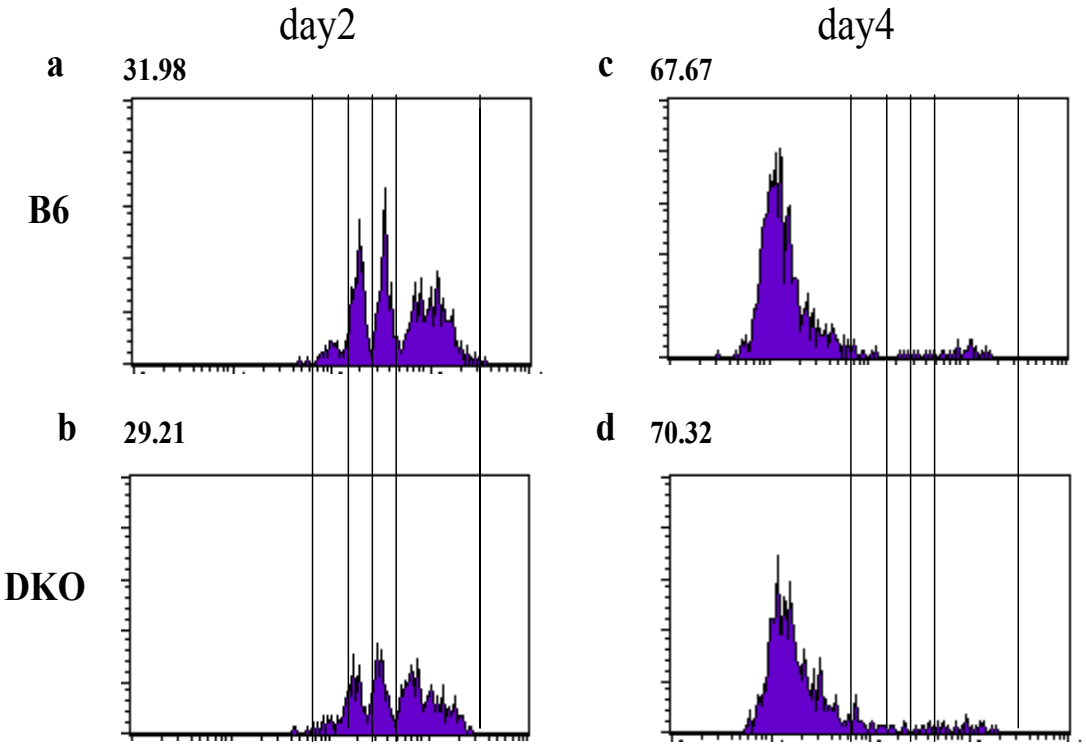

Figure 17: Extensive proliferation of CD8αβ^{+}CD44^{hi} cells after in vitro anti-CD3 culture. CD8^{+}CD44^{hi} splenocytes from both B6 and DKO mice were purified with CD8α magnetic beads and sorted by MoFlo^{TM} high speed sorter. They were labeled by CFSE. 1 X 10^{4}/200µl/well CD8^{+}CD44^{hi} cells in complete RPMI medium with 20U/ml IL-2 were added into the anti-CD3 bound 96-well plate. At day 2 and 4, the CD8^{+}CD44^{hi} cells were harvested and stained forCD8β. CFSE levels on gated CD8αβ^{+}CD44^{hi} cells are shown. The number above each figure shows the percentage of cells undergoing at least one round of dividion.

Figure 18

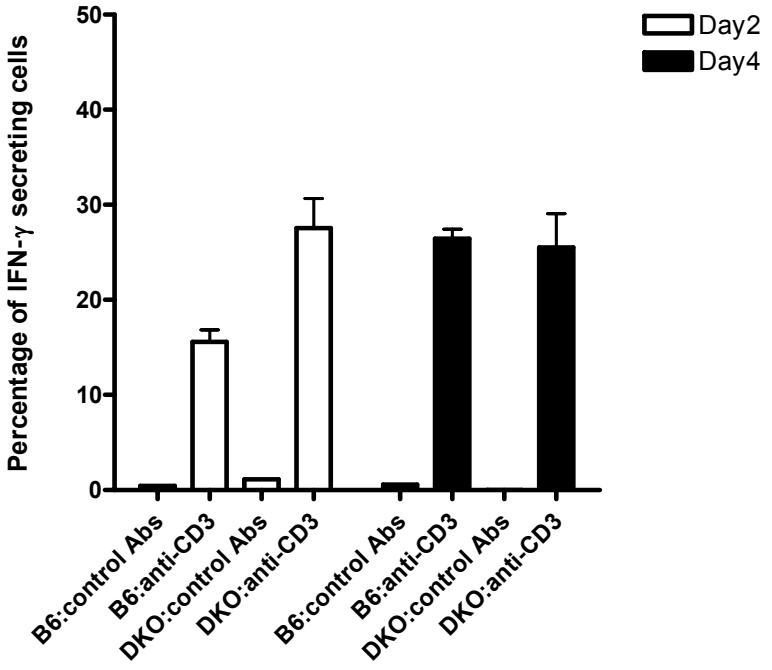

Figure 18: Rapid IFN-γ secretion by CD8αβCD44^{hi} cells after in vitro anti-CD3 stimulation. CD8^{+}CD44^{hi} splenocytes from both B6 and DKO mice were purified with CD8α magnetic beads and high speed cell sorter. 1 X 10^4/200μl/well CD8^{+}CD44^{hi} cells in complete RPMI medium supplemented with 20U/ml IL-2 were added into the antibody bound 96-well plate. At day 2 or day 4, Golgilug (Brefeldin A) was added into the culture for 4 hours and the CD8^{+}CD44^{hi} cells were harvested and stained for CD8β and IFN-γ. The percentage of IFN-γ secreting CD8β^{+}CD44^{hi} cells after the anti-CD3 or control antibody stimulation in both B6 and DKO mice was shown.

reconstituted DKO (thymus-intact) bm chimeras with LM. CD8αβ cells in the Txbm DKO chimera showed minimal expansion 7 days after challenge with LM (compare Figures 19 a with 11 a). By contrast, in the thymus-intact bm chimera, the CD8αβ T cells expanded in response to LM infection (compare Figure 19 b with 11 c), and more CD8αβ cells become activated and display the $CD44^{hi}$ phenotype (compare Figure 19 b with 11 d), similar to the CD8αβ T cells from normal DKO mice. The response stimulated by LM is summarized in Figure 20 where it is noted that there is a large increase in CD8αβ T cells in normal DKO and thymus-intact bm chimeras 7 days following infection with LM, whereas no increase is seen in the Txbm chimeras.

Although thymus-independent non class Ia selected CD8 cells did not show expansion after LM challenge, we investigated their ability to secrete IFN-γ in response to LM infection. This was done by culturing splenocytes from LM infected Txbm and thymus-intact bm chimeras overnight with LM infected J774 cells. As previously reported, LM infected J774 cells induce IFN-γ secretion in CD8 T cells by two pathways. One occurs when J774 cells present LM epitopes to CD8 T cells that induce antigen-specific IFN-γ secretion; the other is induced upon LM infection of J774 cells by secretion of IL-12 and IL-18 that induces non-antigen specific IFN-γ secretion (Berg et al. 2002). Approximately 50% of thymus-independent CD8αβ cells from a Txbm chimera secrete IFN-γ in response to LM infected J774 cells. However, this secretion is about completely blocked by including antibodies to IL-12 and IL-18 in the culture indicating that thymus-independent CD8 cells from DKO mice do not mount a specific primary immune response against this pathogen (Figure 21 a). Approximately 70% of the CD8 cells from a thymus intact bm chimera secrete IFN-γ in response to LM infected J774 macrophages. Unlike the result seen with the T cells from the Txbm animals, a

Figure 19

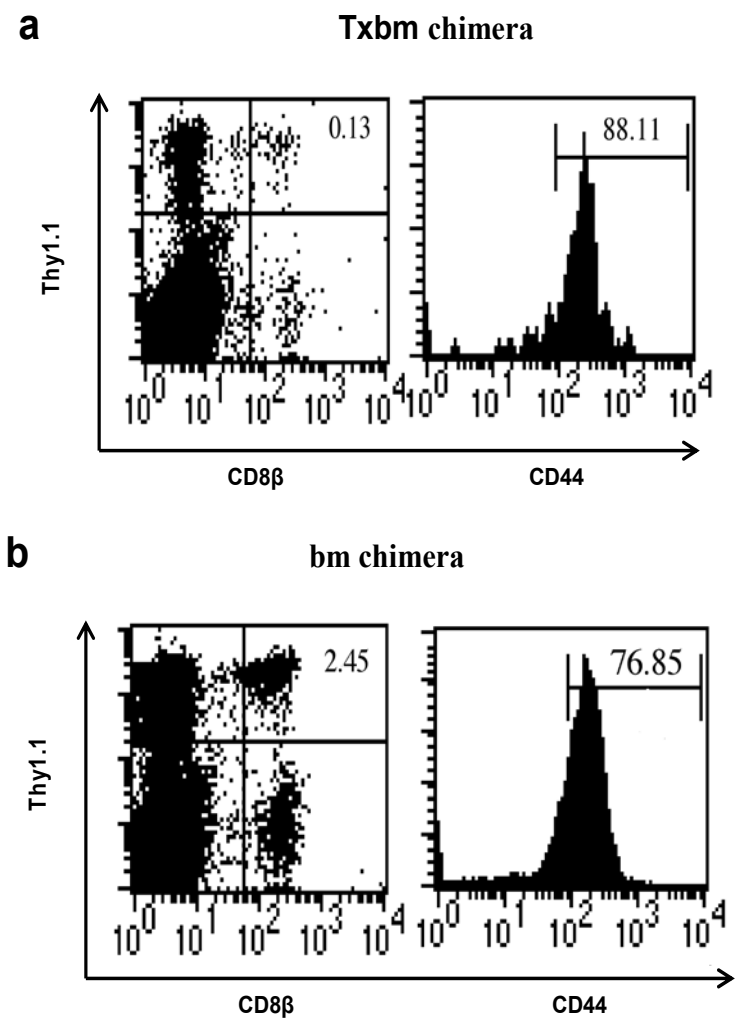

Figure 19: Thymus dependent CD8αβ cells expand and acquire CD44^{hi} expression in response to LM challenge. Thy1.1.DKO bm $\longrightarrow$ Tx DKO (a) or Thy1.1.DKO bm $\longrightarrow$ DKO (b) chimeras were challenged with LM. After 7d, the percentage of donor Thy1.1$^{+/+}$CD8β cells as well as the CD44 expression on the Thy1.1$^{+/+}$CD8β cells was examined (a, b). The numbers indicate the percentage

Figure 20

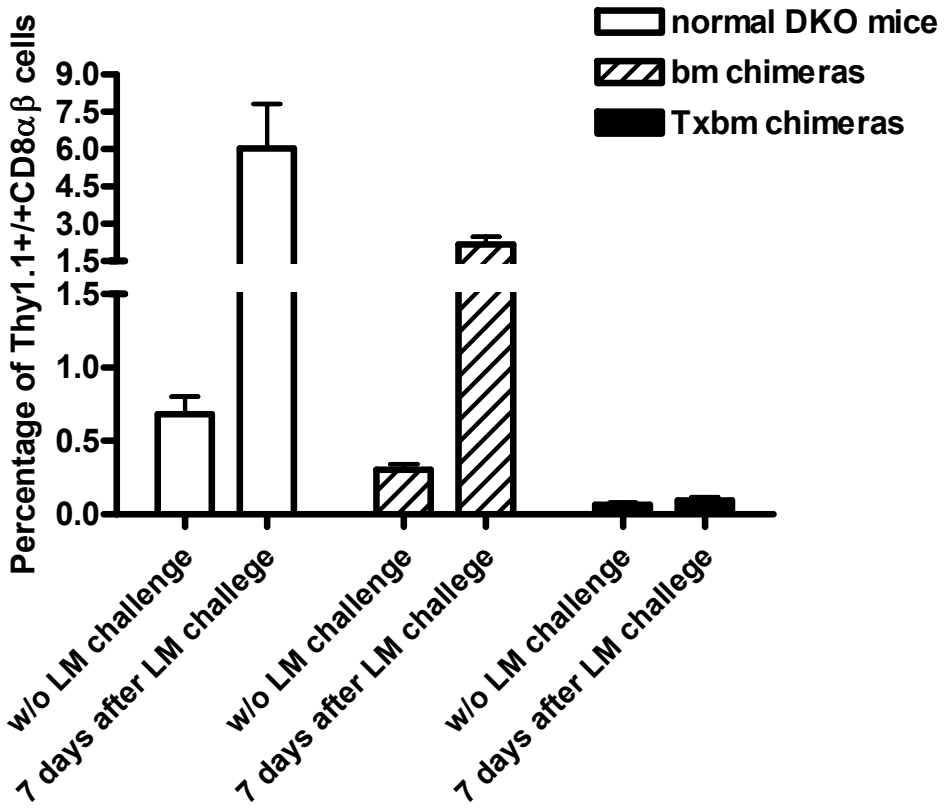

Figure 20: CD8αβ cells from Txbm animals do not respond to LM challenge. The percentage of Thy1.1$^{+/+}$CD8β cells from normal DKO mice, Thy1.1.DKO bm $\rightarrow$ Tx DKO or Thy1.1.DKO bm $\rightarrow$ DKO chimeras without LM challenge and 7 days after LM challenge was examined.

substantial proportion of CD8 T cells from the thymus-intact bm chimera secretes IFN-γ in response to LM infected J774 cells even in the presence of the cytokine blocking antibodies (Figure 21 b), similar to those CD8 T cells from normal DKO mice. This data is summarized in Figure 21 c and indicates that LM specific CD8 T cells in DKO mice are derived from a thymus-dependent pathway. Thymus-independent cells may only contribute to the innate response against LM infection by secreting IFN-γ in the presence of pro-inflammatory cytokines such as IL-12 and IL-18. However, they do not contribute to the antigen specific response to LM challenge.

I-3. Ability of CD8αβCD44^{hi} cells from DKO mice to mount an antigen specific response to LM

As previously reported, CD8^{+} T cells from naïve DKO mice play an important role in clearing a primary LM infection (Kerksiek et al. 1999). Although more than 50% of the CD8 T cells in DKO mice display a memory phenotype, whether these CD8^{+}CD44^{hi} cells are able to initiate an antigen-specific response against infection is not known. In order to determine whether these cells participate in this response, CD8 cells were purified with CD8α beads and then sorted for the CD44^{hi} phenotype (Figure 22 a-c). The sorted cells were then transferred into Thy1.1.DKO mice and challenged with LM. After 6 days we detected a small population of donor (Thy1.2$^{+/+}$) cells in the spleens of recipient animals and almost all detectable Thy1.2$^{+/+}$ donor cells are CD8αβ (Figure 22 d). The cells were cultured with LM infected J774 cells in vitro in the presence or absence of cytokine blocking antibodies (anti-IL-12 and anti-IL-18) to determine the potential of both donor and recipient cells to mount a specific response. Neither donor CD8αβCD44hi nor recipient CD8αβ cells secrete IFN-γ in response to uninfected J774 cells, as expected (Figure 23). However, approximately 80% of

Figure 21

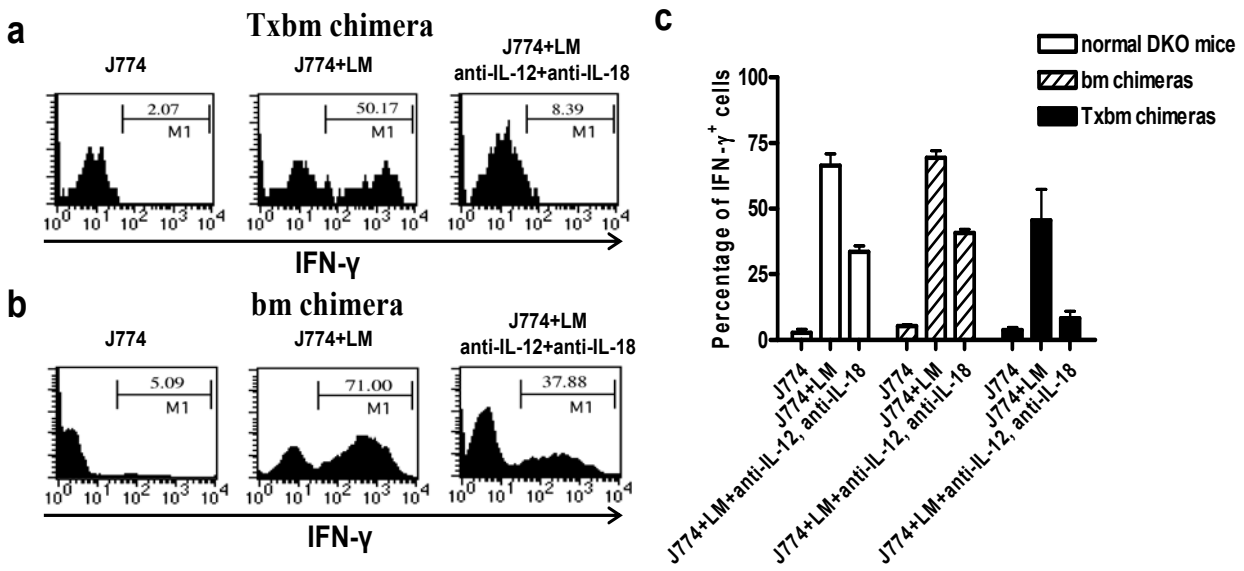

Figure 21: CD8αβ cells from Txbm animals do not mount an antigen specific response to LM challenge. Thy1.1.DKO bm⟶ Tx DKO (a) or Thy1.1.DKO bm→DKO (b) chimeras were challenged with LM. At day 7, splenocytes from the Txbm (a) and thymus intact bm chimeras(b) were cultured with uninfected J774, LM infected J774, and LM infected J774 with anti-IL-12 and anti-IL-18. IFN-γ ICS is shown for Thy1.1$^{+/+}$CD8β (donor) bone marrow derived cells. This data is summarized in panel c and represents the mean value from 4 mice/group.

Figure 22

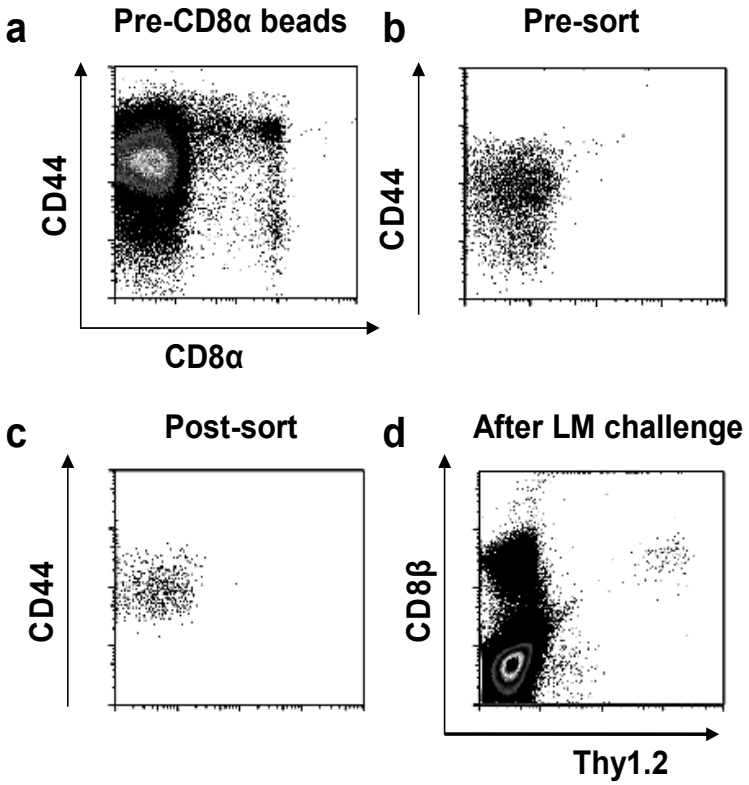

Figure 22: Transferred purified CD8αβCD44^{hi} T cells from naive DKO mice were detected in recipient mice after LM challenge. Cells were isolated from DKO spleens and stained with CD8α and CD44 (a). The CD8αCD44^{hi} cells were purified with anti-CD8α coated beads and then sorted for CD44hi expression (b, c). 2x10^5 of the purified and sorted CD8αβCD44^{hi} cells were transferred into Thy1.1.DKO recipients and then challenged the next day with LM. Six days later, splenocytes were harvested and the donor derived cells were detected by staining with Thy1.2 and CD8β (d).

these cells secrete IFN-γ in response to LM infected J774 cells. Inclusion of antibodies against IL-12 and IL-18 still resulted in a response to LM from both donor and recipient cells. Our data demonstrates that CD8CD44^{hi} cells from naïve DKO mice are able to initiate an antigen specific response against LM infection. Thus, the non class Ia selected CD8αβCD44^{hi} cell subset from these animals can contribute both to the innate and adaptive immune response to LM infection.

H2-M3 restricted anti-listerial immune response has been well characterized. Three H2-M3 restricted epitopes from LM have been identified: f-MIGWII, f-MIVIL, and f-MIVTLF. It is interesting to know whether non class Ia selected CD8αβCD44^{hi} cells which respond to LM infection are H2-M3 restricted. The splenocytes from LM immunized and Thy1.2$^{+/+}$CD8αCD44^{hi} cells transferred Thy1.1.DKO mice were harvested 6 days later and cultured without any peptide or with 10mM f-MIGWII, 10mM f-MIVTLF, 10mM SIINFEKL peptides overnight. There is almost no IFN-γ secretion by CD8 T cells from naïve DKO mice in response to any peptide stimulation. In contrast, more than 20% of both recipient-derived and donor-derived CD8 cells from LM immunized DKO mice secrete IFN-γ in response to either f-MIGWII or f-MIVTLF stimulation (Figure 24). Therefore, our data suggested that a significant portion of LM specific non class Ia restricted CD8αCD44^{hi} T cells are H2-M3 restricted.

II. Ability of CD8 T cells from DKO mice to participate in the innate immune responses

II-1. Ability of CD8 T cells from DKO mice to respond rapidly to anti-CD3 in vivo

MHC class Ib-restricted, TAP-independent CD8 cells have been proposed to be an early source of IFN-γ after anti-CD3 injection in vivo (Das et al. 2001). It is likely that the non class

Figure 23

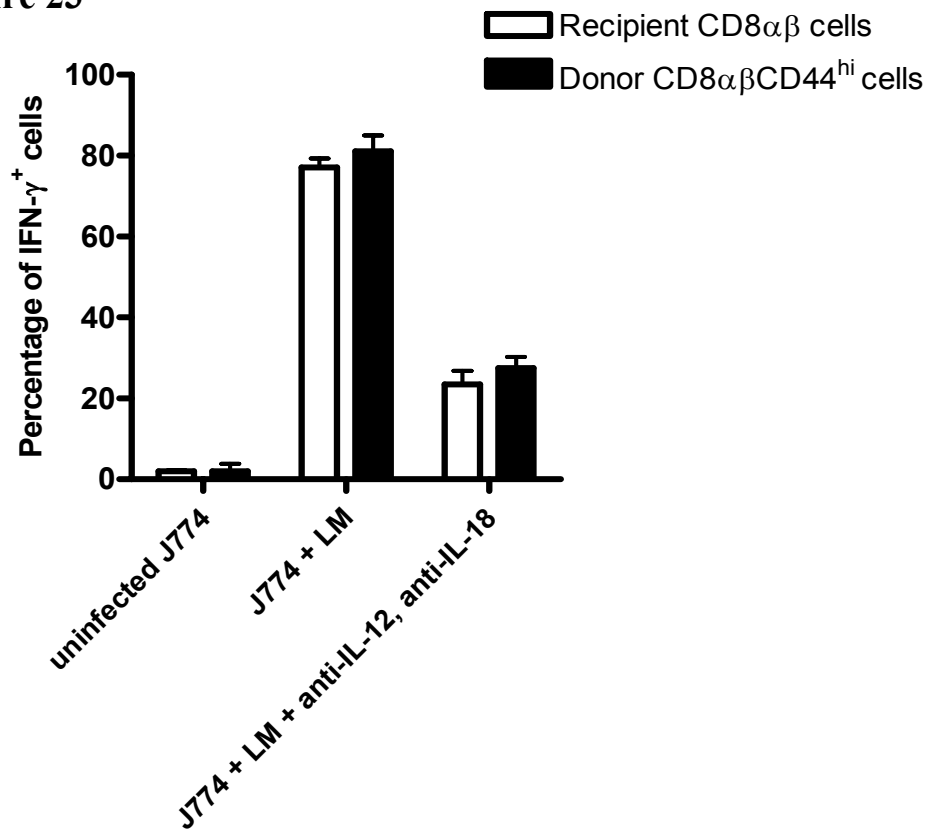

Figure 23: CD8αβCD44^{hi} T cells from naive DKO mice show an antigen-specific response to LM. 2 X 10^5 of purified and sorted CD8αβCD44hi cells were transferred into Thy1.1.DKO recipients and then challenged the next day with LM. Six days later, splenocytes were harvested and cultured with uninfected J774, LM infected J774 or LM infected J774 with anti-IL12 and anti-IL-18 mAbs overnight. The percentage of host-derived Thy1.1$^{+/+}$CD8αβ and donor derived Thy1.2$^{+/+}$CD8αβ cells that display ICS for IFN-γ is shown and represents the mean value of 4 mice/group.

Figure 24

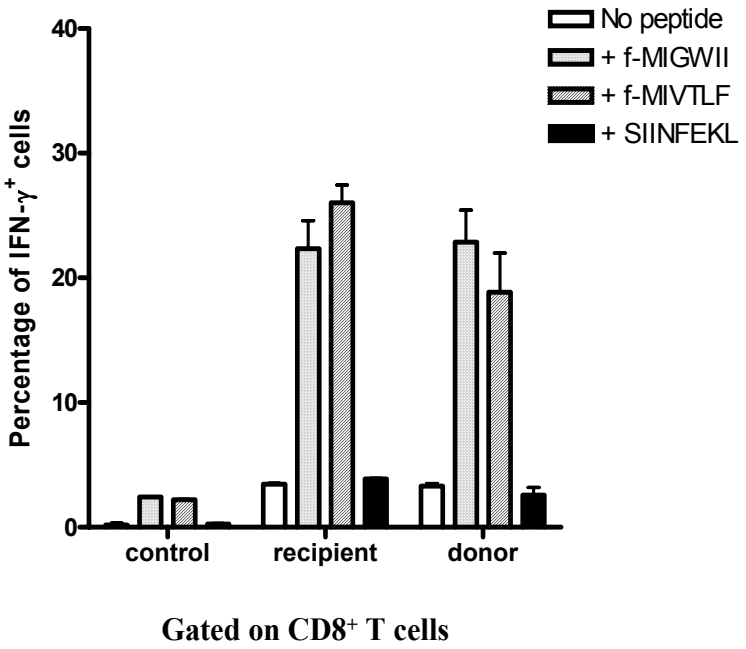

Figure 24: CD8αβCD44^{hi} T cells from naive DKO mice show an H2-M3 restricted antigen-specific response to LM. 2 X 10^5 of purified and sorted CD8αβCD44hi cells were transferred into Thy1.1.DKO recipients and then challenged the next day with LM. Six days later, splenocytes were harvested and cultured without any stimulation or with 10mM f-MIGWII, f-MIVTLF, or SIINFEKL peptides overnight. The splenocytes from naïve Thy1.1.DKO mice served as negative controls. The percentage of host-derived Thy1.2$^{-/-}$ CD8 cells and donor derived Thy1.2$^{+/+}$CD8 cells that display ICS for IFN-γ is shown and represents the mean value of 2 independent experiments.

Ia selected CD8αβCD44^{hi} cells are responsible for this rapid IFN-γ secretion within the first few hours after stimulation. After 3 h of anti-CD3 in vivo challenge, almost half of the CD8αβCD44^{hi} cells from DKO mice secrete IFN-γ compared to only about 20% of CD8αβCD44^{hi} from B6 mice (Figure 25). In contrast, only a very small percentage of CD8αβCD44^{lo} cells from both B6 and DKO animals secrete IFN-γ (Figure 24). These data indicate that one of the sources of the early burst of IFN-γ induced by anti-CD3 is from CD8αβCD44^{hi} cells restricted to a non class Ia MHC molecule(s).

Previous data shows a similar V_β gene usage between DKO and B6 CD8 T cells. We also noted that CD8αβCD44^{hi} cells in DKO mice have a similar V_β diversity as those in B6 animals (data not shown). To further investigate V_β usage by this population of IFN-γ secreting CD8αβCD44^{hi} cells, we used a panel of V_β antibodies to analyze their usage and compared this between DKO and B6 animals. As shown in Figure 26, all V_β expressing populations of the panel tested from CD8αβCD44^{hi} cells in both B6 and DKO mice showed a comparable ability to secrete IFN-γ after anti-CD3 stimulation. There was a higher percentage of IFN-γ secreting cells in each V_β subset in DKO vs B6 animals but there was no evidence of V_β repertoire skewing in this response.

II-2. Proliferation of CD8αβCD44^{hi} cells from DKO animals in response to IL-2 stimulation

The expression of CD122 (IL-2/IL-15Rβ) on CD8$^+$CD44^{hi} T cells suggests that they might be able to proliferate in response to cytokines such as IL-2 or IL-15. It has been reported that CD8$^+$CD44^{hi} cells from normal mice only require IL-2 for proliferation and the acquisition of cytolytic activity (Dhanji and Teh 2003). As shown before (Figure 3 a and b), more CD8$^+$CD44^{hi} cells from DKO mice express CD122 compared to those from normal B6 mice.

j

Figure 25

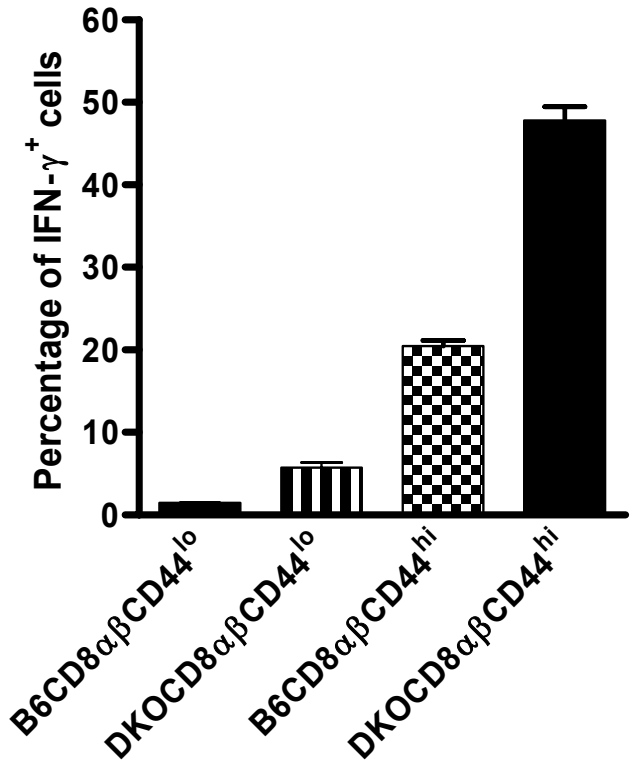

Figure 25: CD8αβCD44^{hi} cells from DKO mice secrete IFN-γ after anti-CD3 administration in vivo. B6 and DKO mice were injected with anti-CD3 mAb. After 3h their splenocytes were removed and cultured for 4 h with Golgiplug added to the cultures for the last 2 h. IFN-γ was detected by ICS. Percentage of IFN-γ secreting cells in CD8αβCD44^{lo} and CD8αβCD44^{hi} cell subsets from both B6 and DKO mice is shown. Results are the mean value of 6 mice/group.

Figure 26

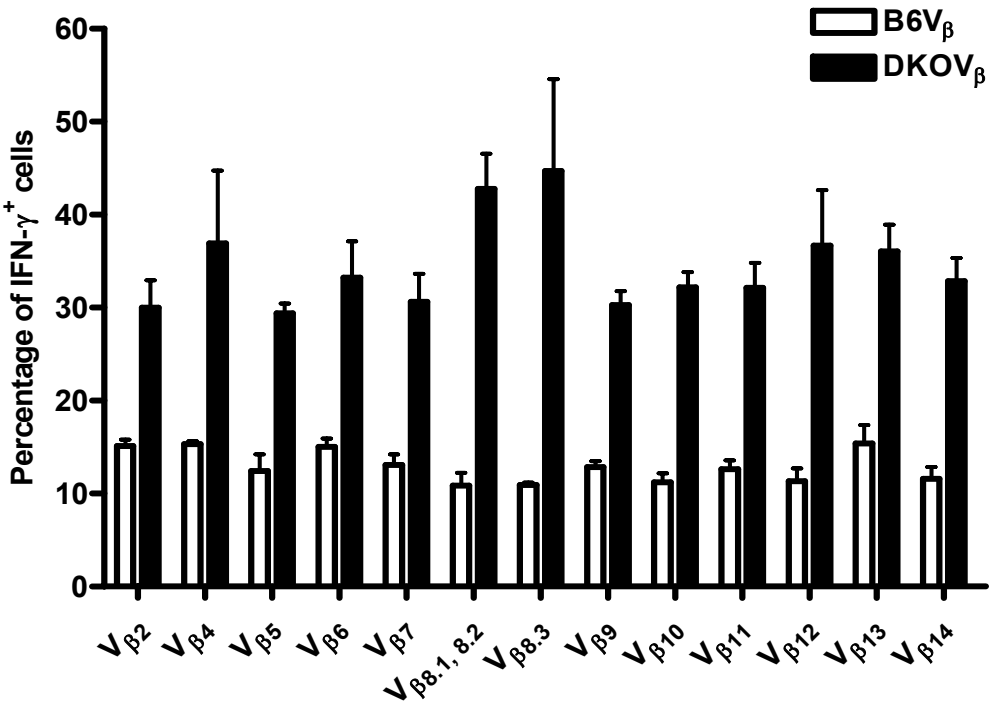

Figure 26: IFN-γ secreting CD8αβCD44^{hi} cells from DKO mice after anti-CD3 administration in vivo are not skewed torwards a particular Vβ expressing TCR. B6 and DKO mice were injected with anti-CD3 mAb. After 3h their splenocytes were removed and cultured for 4 h with Golgiplug added to the cultures for the last 2 h. IFN-γ was detected by ICS. The percentage of IFN-γ secreting CD8βCD44^{hi} cells that express individual TCR V_β chains in both B6 and DKO mice is shown. Results are the mean value of 3 mice/group.

To test the ability of these non class Ia restricted CD8CD44^{hi} cells to proliferate in response to IL-2, we purified CD8CD44^{hi} cells from DKO mice by CD8α magnetic bead selection and CD44^{hi} cell sorting, labeled these cells with CFSE and then cultured them with 200U/ml IL-2. The purified CD8CD44^{hi} cells from B6 mice were used as a control. As shown in Figure 26, almost no proliferation is observed in IL-2 stimulated CD8CD44^{hi} cells from both B6 and DKO mice on day 2 (Figure 27 a and b). However, there is about 38% CD8CD44^{hi} cells from DKO mice that have undergone at least five rounds of cell division by day 4, similar to those from B6 mice (36%; Figure 27 c and d). The result indicates that CD8CD44^{hi} cells are capable of proliferating in response to IL-2 and their proliferative ability is similar to those cells from B6 mice.

II-3. Ability of CD8αβCD44^{hi} cells from DKO animals to secrete IFN-γ in response to IL-12 and IL-18 stimulation

IL-12 and IL-18 are cytokines that are secreted following infection with LM and other pathogens and can activate cells to secrete IFN-γ during the innate phase of the immune response (Raue et al. 2004;Berg et al. 2002). Both memory and effector CD8 T cells express receptors for these cytokines and have been shown to secrete IFN-γ both in vitro and in vivo. To investigate the ability of non class Ia selected CD8αβCD44^{hi} cells to contribute to IFN-γ secretion during the innate response, CD8 T cells from naïve DKO mice were cultured overnight with IL-2 (control), or IL-2, IL-12 and IL-18 and tested for their ability to secrete IFN-γ by ICS. Culture of CD8 T cells with IL-2 alone did not induce IFN-γ secretion, as expected. However, more than 50% CD8αβCD44^{hi} cells secreted IFN-γ in response to IL-12 and IL-18 (Figure 28). Therefore, memory phenotype CD8 T cells in naïve DKO animals can

Figure 27

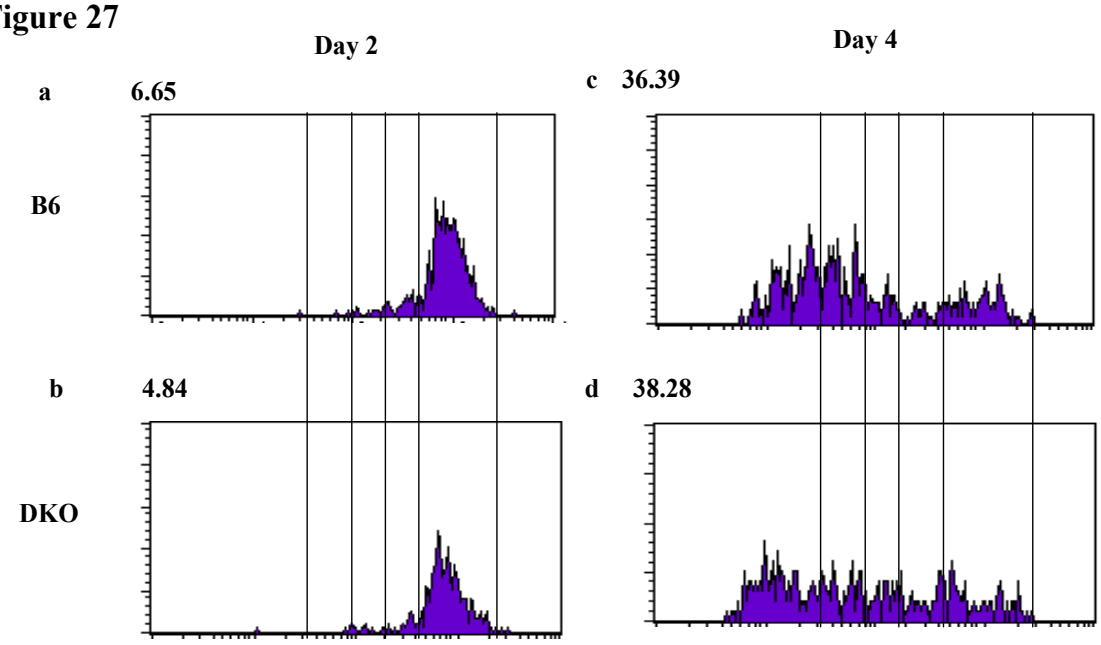

Figure 27: Proliferation of CD8αβCD44^{hi} cells in response to IL-2 stimulation. CD8^{+}CD44^{hi} splenocytes from both B6 and DKO mice were purified with CD8α magnetic beads and MoFlo™ high speed sorter. They were labeled with CFSE. 1 X 10^{4}/200µl/well CD8^{+}CD44^{hi} cells were cultured in complete RPMI medium supplemented with 200U/ml IL-2 in a 96-well plate. At day 2 (a and b) and day 4 (c and d), the CD8^{+}CD44^{hi} cells were harvested and stained for CD8β. CFSE levels on gated CD8αβ^{+}CD44^{hi} cells are shown. The numbers indicate the percentage of cellsundergoing at least one round of cell division.

Figure 28

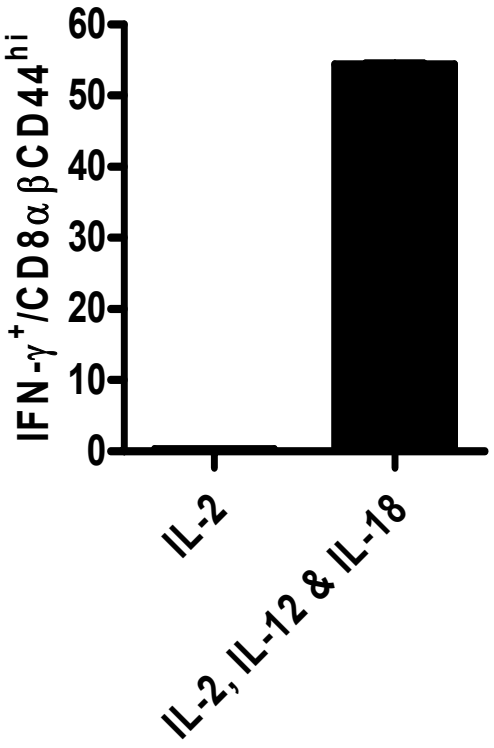

Figure 28: CD8αβCD44^{hi} cells from DKO mice secrete IFN-γ in response to IL-12 and IL-18 stimulation. Freshly isolated splenocytes from DKO mice were cultured overnight with IL-2 alone, or IL-2, IL-12 and IL-18 and tested for their ability to secrete IFN-γ by ICS. The percentage of IFN-γ secreting CD8αβCD44^{hi} cells under both culture conditions is shown.

participate in the innate response by being an early source of IFN-γ similar to that described for conventional memory cells.

II-4 CTL responses against target cells expressing NKG2D ligands by $CD8^+CD44^{hi}$ cells from naïve DKO mice.

Recent studies have shown that the activating NK cell receptor NKG2D plays a crucial role in the killing of syngeneic tumor cells. The activated NK cells express two alternative splice variants of NKG2D that associate differentially with DAP10 that provides a co-stimulatory signal, and DAP12 that provides a direct stimulatory signal. Unlike conventional CD8 T cells expressing DAP 10 only, IL-2 activated $CD8^+CD44^{hi}$ T cells from B6 mice express both DAP10 and DAP12. They preferentially kill syngeneic tumor target cells and this killing is greatly enhanced by the expression of the NKG2D ligand (Dhanji and Teh 2003).

In DKO mice, more than 10% CD8αβ cells express NKG2D without any cytokine stimulation. Therefore, it was of interest to determine whether these NKG2D expressing non class Ia restricted $CD8^+$ cells could mediate direct cytolysis through NKG2D. RMA tumor cells from B6 mice do not normally express NKG2D ligands. Purified $CD8^+$ cells from naïve DKO mice did not kill RMA target cells as expected (Figure 29 a). However, the NKG2D ligand (Rae-1 or H60) transfected RMA cells are also not killed by these non class Ia restricted $CD8^+$ T cells (Figure 29 b and c). Therefore, the non class Ia restricted $CD8\alpha\beta CD44^+$ cells did not induce NKG2D-dependent killing of target cells.

Figure 29

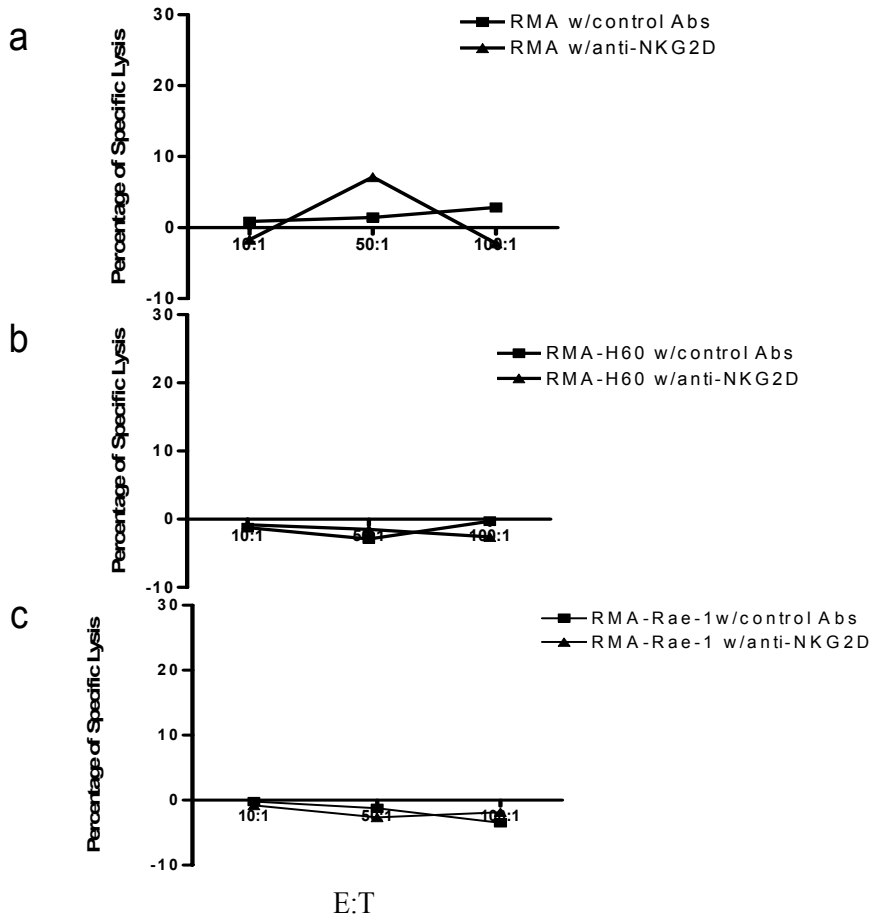

Figure 29: No CTL responses were detected against target cells expressing NKG2D ligands by $CD8^{+}CD44^{hi}$ cells from naïve DKO mice. Purified $CD8^{+}$ cells from DKO spleens were used as effectors in a 4 h standard ^{51}Cr release assay against RMA (a), RMA-H60 (b), and RMA-Rae-1 (c) target cells in the presence of anti-NKG2D (triangles) antibody or isotype control antibody (squares).

Chapter VI: Discussion

1. Phenotype and origin of non class Ia restricted CD8 T cells

DKO mice, which lack expression of MHC class Ia molecules K^b and D^b and retain normal expression of the repertoire of non class Ia molecules, are ideal models for analyzing $CD8^+$ T cells selected by non class Ia molecules. Both spleen and lymph node CD8 T cell populations in DKO mice exhibit an approximate 6-9 fold decrease compared to those in B6 mice (Perarnau et al. 1999). MHC class I-deficient $\beta_2m^{-/-}$ or TAP-$1^{-/-}$ animals have also greatly reduced numbers of CD8 T cells. Both of these strains have around 1% CD8 cells in their splenic compartment (Aldrich et al. 1994;Koller et al. 1990). This result may be expected since most CD8 T cells recognize β_2m-associated MHC class I molecules that present TAP-dependent peptides. In the case of DKO mice that lack MHC class Ia molecules, the deficiency in CD8 T cells likely reflects the reduced repertoire directed against non class Ia molecules. This is evident in the thymus of these mice which contain approximately 15-20% of single positive CD8 cells seen in B6 mice (Urdahl et al. 2002). This reduction in the number of thymic CD8 cells is further reflected in the periphery. Despite this reduction, DKO mice do generate and maintain a peripheral population of CD8 T cells that is selected by β_2m-associated non class Ia molecules(Vugmeyster et al. 1998;Perarnau et al. 1999). There are no CD8 T cell populations observed in B6.$K^{b-/-}D^{b-/-}\beta_2m^{-/-}$ mice (data not shown).

$CD44^{hi}$ is generally taken as a marker for CD8 memory (Sprent 1997). In naïve young B6 mice approximately 10-20% of CD8 T cells express this memory cell marker. This proportion of memory phenotype cells is even higher in naïve young DKO mice where more than 50% of CD8$\alpha\beta$ T cells are $CD44^{hi}$ (Seaman et al. 1999). Several reports have documented

that IL-7 and IL-15 promote the survival and homeostatic proliferation of memory CD8 T cells (Tan et al. 2002;Berard et al. 2003;Schluns et al. 2000). The expression of IL-15R (CD122) and IL-7R (CD127) was examined in these memory phenotype $CD8^+$ T cells. Our results showed that all $CD8\alpha\beta CD44^{hi}$ cells from either B6 or DKO mice express high level of IL-7R (CD127) (data not shown). However, More $CD8\alpha\beta CD44^{hi}$ cells from DKO mice express IL-15R (CD122). Whether these memory phenotype cells are conventional in that they have had prior exposure to extrinsic antigens is unclear. Although previous studies have provided several possible reasons for the appearance of these cells in both normal as well as DKO animals, their origin and function are still largely unknown (Yamada et al. 2001;Urdahl et al. 2002).

The high percentage of $CD8^+CD44^{hi}$ cells in the periphery of DKO mice could be due to a very high percentage of CD8αα T cells that are known to be $CD44^{hi}$. However, we noted that approximately 90% of $CD8^+$ T cells from these DKO mice are traditional $CD8\alpha\beta^+TCR\alpha\beta^+$. The reduction of the total number of $CD8\alpha\beta CD44^{hi}$ cells in DKO mice is much less than that of $CD8\alpha\beta CD44^{lo}$ cells. Therefore, DKO mice are more deficient in the naive cell subset. It would be interesting to know whether the high $CD8\alpha\beta CD44^{hi}/CD8\alpha\beta CD44^{lo}$ ratio in the periphery of DKO mice is due to high $CD8\alpha\beta CD44^{hi}/CD8\alpha\beta CD44^{lo}$ ratio in the mature thymocytes.

It has been reported that thymic education of some class Ib-restricted CD8 T cells can occur on radiosensitive cells in the thymus, unlike that generally observed for class Ia-restricted cells (Urdahl et al. 2002). Perhaps as a result of this, the single positive CD8 thymocytes acquire a $CD44^{hi}$ phenotype in situ. We examined mature single positive CD8αβ thymocytes based on the levels of CD24 and Vβ expression and found that most of these cells are actually $CD44^{lo}$. Thus, we conclude that the acquisition of the $CD44^{hi}$ phenotype is a post-thymic event similar to that seen in B6 animals.

By tracking FITC labeled RTEs, we are able to investigate the thymic emigration rate and the phenotype of these RTEs. Previous reports demonstrated that thymic export is the only way to replenish the naïve T cell compartment (Ge et al. 2002). Our data showed DKO mice had a lower percentage of $FITC^+$ cells in peripheral CD8 T cell population compared to B6 mice and a very high CD4/CD8β ratio in the RTEs. It suggested that there was a decreased thymic export in DKO mice. This may be due to the reduced CD8 thymocytes available for export in the DKO thymus. The low thymic export might partially account for the paucity of CD8 T cells in the periphery of DKO mice. However, most $CD8^+$ RTEs in DKO mice are $CD44^{lo}$, similar to that seen in B6 mice.

There are several possible explanations for the acquisition of the memory phenotype in post-thymic T cells from DKO mice. One possibility is that these non class Ia selected CD8 cells recognize nonpathogenic commensal bacteria and as a result become primed. A previous report demonstrated that animals housed in a conventional colony could mount CTL responses to H2-M3-restricted LM antigens without prior sentization (Lenz and Bevan 1997). In our studies, a small percentage of CD8 RTEs acquire the $CD44^{hi}$ phenotype very rapidly after exit from the thymus. This small percentage of $CD44^{hi}$ cells may represent the H2-M3 restricted cell population that recognized commensal antigens.

Although the appearance of memory cells in naïve mice could be because these animals have been exposed to environmental antigens derived from commensal organisms, memory phenotype CD4 cells are also found in naïve mice maintained under germfree conditions, suggesting that their development is not foreign antigen dependent (Dobber et al. 1992). Since T cells from male anti-H-Y TCR transgenic mice can develop extrathymically and display memory markers, it is possible that the development of memory phenotype CD8 T cells in

naïve mice is driven by the interaction of the αβTCR with self-antigens and developed extrathymically (Yamada et al. 1998;Yamada et al. 2000;Yamada et al. 2001). However, our finding that only a small percentage of CD8αβ cells develop in Txbm DKO mice compared to thymus-intact bm chimeras suggests that most non class Ia selected CD8αβ cells are from the thymus-dependent pathway. The thymus-independent CD8αβ cells only account for a small portion of CD8αβCD44^{hi} cells in DKO mice.

The size of the lymphocyte pool is very important for the adaptive immune system. Homeostatic proliferation functions to maintain peripheral T cell numbers so that if there is a decrease T cell numbers, naïve cells undergo proliferation to fill up the "space" (Goldrath 2002;Jameson 2002;Murali-Krishna and Ahmed 2000). It has been noted that single positive cells in neonatal mice proliferate strongly and display a CD44^{hi} phenotype, reflecting the lymphopenic environment that they are exposed to. Neonatal lymphopenia can allow CD8 thymic emigrants to undergo lymphopenia-induced proliferation during early neonatal life and thus equip the immune system with a set of pre-activated CD8 T cells before any infection. This might contribute to the rapid initiation of some immune responses in the adult (Le et al. 2002).

It has also been reported that under conditions of low single positive cell output from the thymus that peripheral single positive T cells can compensate by homeostatic expansion (Almeida et al. 2001). There is a 6-9 fold reduction of single positive CD8 cells in the thymus and few CD8^{+} cells in the periphery of DKO mice. It is possible that CD8 RTEs sense this space and undergo homeostatic proliferation to acquire the CD44^{hi} phenotype. This CD44^{hi} phenotype on CD8 T cells from naïve mice is also characteristic of other MHC class I-deficient

strains, such as $TAP^{-/-}$ and $\beta_2m^{-/-}$ mice, which have very few CD8 cells in their periphery (Kurepa et al. 2003).

2. Basal and homeostatic proliferation of non class Ia restricted CD8 T cells.

Without antigen stimulation, naïve CD8 T cells divide minimally in a full compartment. In contrast, memory phenotype CD8 T cells can renew themselves by undergoing "basal proliferation", the low level of antigen-independent division in a full compartment, in normal mice (Goldrath et al. 2002). Basal proliferation is important in maintaining the memory compartment. The lower basal proliferation rate of both $CD8\alpha\beta CD44^{hi}$ and $CD8\alpha\beta CD44^{lo}$ cells from DKO mice compared to these cells from B6 mice might contribute to the few CD8 T cells in the periphery of DKO mice. It has been reported before that there is an intrathymic expansion of mature single positive thymocytes before emigration to the periphery. There are approximately 6% $BrdU^+$ mature single positive CD8 cells in B6 mice, consistent with the previous report (Penit et al. 1995). However, there were only 3% $BrdU^+$ mature single positive CD8 cells in DKO mice. Therefore, the limited basal proliferation ability was an intrinsic character of non class Ia selected CD8αβ cells.

In addition to higher proliferation, we also found that non class Ia selected CD8αβ cells in both the spleens and thymus of DKO mice had a much higher apoptosis compared to these CD8αβ cells from B6 mice. Extensive cell apoptosis of CD8αβ cells in DKO mice may contribute to the lack of CD8 cells in DKO mice. Naïve T cells require signals delivered by MHC molecules for their survival. IL-7 is also required for the survival and proliferation of naïve cells (Marrack and Kappler 2004). In contrast, memory CD8 T cells can survive independent of TCR/MHC interactions (Murali-Krishna et al. 1999). Previous reports indicated that IL-7 plays an important role in supporting CD8 memory cell survival, whereas IL-15

drives their basal proliferation (Goldrath et al. 2002). As mentioned before, the expression of IL-7R (CD127) and IL-15R (CD122) by CD8αβCD44^{hi} cells in DKO mice is very high. Therefore, these cytokines are not the limiting factors for the proliferation and survival of non class Ia selected CD8αβCD44^{hi} cells. The low expression level of non class Ia molecules may not be able to provide the same strong signals for the proliferation and survival of naïve CD8αβ cells as MHC class Ia molecules. The different selection ability of non class Ia selected CD8αβ cells might also account for their different turnover rate.

Recent data using the model of homeostatic expansion have shown that although there is a lymphoid compartment that regulates this expansion, it is not compartmentalized into CD4 and CD8 subsets (Dummer et al. 2001). This issue is now being re-examined by the finding that TCR-transgenic T cells can expand in non-irradiated hosts that contain TCR-transgenic cells of another specificity (Troy and Shen 2003;Moses et al. 2003). However, it has been reported that no proliferation were observed when B6 CD8 cells were transferred into non-irradiated CD8-deficient recipients (Kurepa et al. 2003). Therefore, the existence of CD8 compartment is still unclear. It remains possible that CD8 cells sense CD8 "space" and attempt to fill this compartment. However, CD8 T cells homeostatic proliferation did not fill the CD8 compartment in DKO mice. It is not clear why the CD44^{hi} cells did not fill the CD8 compartment in these DKO mice.

One possibility is that cells specific for non class Ia molecules do not get proper signaling to allow for complete expansion. This could be a result of low expression of most of these molecules, as has been documented for Qa-1 and M3 (DeCloux et al. 1997;Chiu et al. 1999). Transfer of CD8 T cells from DKO mice into irradiated DKO recipients results in the ability of some of these cells to undergo expansion, although the extent of cell divisions was

much less than that observed by CD8 T cells responding to class Ia antigens. About half of the proliferation observed in irradiated DKO recipients was also seen in irradiated B6.$K^{b-/-}D^{b-/-}\beta_2m^{-/-}$ recipients which is depleted of MHC class I molecules, indicating that this half of proliferation is independent of MHC class I molecules. Another possibility, consistent with the recent data, is that CD8 T cell selection in the thymus, against at least some class Ib molecules, differs qualitatively from selection against class Ia molecules (Urdahl et al. 2002). Their positive selection by hematopoietic cells in the thymus may alter the ability of these non class Ia restricted CD8 T cells to fill the CD8 compartment or acquire their $CD44^{hi}$ phenotype.

B6 CD8 T cells displayed extensive homeostatic expansion in irradiated syngeneic hosts, as expected. Both B6.$K^{b-/-}$ and B6.$D^{b-/-}$ mice have around a two-fold reduction in peripheral CD8 T cells. When we transferred B6 CD8 T cells into irradiated B6.$K^{b-/-}$ and B6.$D^{b-/-}$ hosts, we noted that although homeostatic proliferation occurred, 50% of donor cells did not undergo cell division in either host. Therefore this is consistent with the decreased number of CD8 T cells and suggests that the CD8 repertoire in B6 mice is rather evenly distributed against both class Ia molecules. Transfer of B6 cells into DKO mice also resulted in homeostatic expansion of donor cells. However, in these hosts only about 20% of cells divided and the extent of division was similar when these cells were transferred into B6.$K^{b-/-}D^{b-/-}\beta_2m^{-/-}$ mice. The MHC class I-independent proliferation might come from $CD8CD44^{hi}$ cell dividing because $CD8CD44^{hi}$ cells don not require self-MHC/TCR interaction for their homeostatic proliferation (Tanchot et al. 1997;Murali-Krishna et al. 1999).

When comparing the difference in proliferation of B6 donor cells in DKO vs B6.$K^{b-/-}D^{b-/-}\beta_2m^{-/-}$ recipients, it was noted that only a very small percentage of cells underwent more than four divisions. This indicates that only a very small percentage of the TCR repertoire in B6

animals is non-class Ia restricted, based on the criteria of homeostatic expansion. It is also possible that at least some class Ia-selected T cells react against class Ib molecules as has been shown for the ability of the 2C T cell class Ia-reactive receptor to be selected in DKO mice (Maurice et al. 2001).

3. The function of non class Ia selected CD8αβCD44^{hi} cells

3.1 The function of non class Ia selected CD8αβCD44^{hi} cells in innate immunity.

Despite the limited basal and homeostatic proliferation ability of non class Ia selected CD8αβCD44^{hi} cells, they undergo the same strong proliferation in response to anti-CD3 or IL-2 stimulation as the CD8αβCD44^{hi} cells from B6 mice. Thus, these cells can be activated by either antigen-specific stimulation or antigen-independent cytokine stimulation. One characteristic of the memory phenotype is a rapid IFN-γ response following cell activation (Zimmermann et al. 1999). A rapid IFN-γ response is also characteristic of cells undergoing homeostatic expansion (Cho et al. 2000). Naïve cells that undergo homeostatic proliferation acquire a memory phenotype and produce more IFN-γ than naïve T cells in response to specific antigens. This suggests that the CD8αβCD44^{hi} cell in naïve DKO mice might represent an antigen-primed memory cell or a cell that has undergone homeostatic expansion. In either event, our current data indicate that most CD8 T cells that arise in an environment devoid of class Ia antigens acquire a CD44^{high} phenotype and are different from conventional CD44^{hi} class Ia-restricted memory cells.

It has been reported that IFN-γ is induced rapidly in a small subset of CD8 T cells following administration of anti-CD3 in vivo. This IFN-γ secreting subset is absent in mice that lack β_2m, but not in DKO mice, indicating that these CD8 T cells are dependent on non class Ia molecules (Das et al. 2001). We noted that there are more CD8αβCD44^{hi} cells from DKO mice

that secrete IFN-γ at a rapid rate after anti-CD3 stimulation in vivo compared to B6 mice. This might be due to the lower frequency of non class Ia restricted CD8αβCD44^{hi} cells in B6 mice. There is no IFN-γ secretion observed in CD8αβCD44^{lo} cells in both B6 and DKO mice. We proposed that non class Ia selected CD8αβCD44^{hi} cells could serve as one of the early sources of IFN-γ that may have direct role in polarizing Ag-primed naive CD4 cells toward becoming Th1 cells. The more rapid IFN-γ secretion was also observed in purified CD8αβCD44^{hi} cells from DKO mice stimulated by anti-CD3 antibody in vitro.

Non class Ia selected CD8αβCD44^{hi} cells can contribute to innate immunity by providing an early non-antigen specific source of IFN-γ by responding to inflammatory cytokine (IL-12 and IL-18) stimulation. We also observed that approximately 80% of the CD8 cells from LM infected DKO mice secrete IFN-γ after exposure to LM infected J774 macrophages. This response is in part due to signaling by IL-12 and IL-18 since anti-IL-12 and anti-IL-18 antibodies greatly reduced the response. Approximately 20-30% of CD8CD44^{hi} cells from naïve DKO mice are able to produce IFN-γ after culture with LM infected J774 macrophages. Here the secretion is completely blocked by anti-IL-12 and IL-18 (data not shown). Thus, this data suggests that non class Ia selected CD8CD44^{hi} cells are an early source of both antigen specific and cytokine induced IFN-γ secretion during infection and can potentially help bridge the gap between innate and adaptive immunity during intracellular bacterial infection. It is possible that these same non class Ia selected CD8CD44^{hi} cells play a role in normal B6 animals although it is possible that this population in normal mice can be selected on either class Ia or non class Ia antigens.

CD1d-restricted NK T cells, which are specific for glycolipid antigens, have a CD44^{hi} cell surface phenotype. They are selected upon interaction with hematopoietic cells, and have

been shown to exhibit rapid activation responses (Yoshimoto and Paul 1994). The majority of NK T cells express a semi-invariant TCR composed of an invariant $V_{\alpha 14}$-$J_{\alpha 18}$ chain, associated preferentially with $V_{\beta 8.2}$ or $V_{\beta 7}$ chains in mice (Schumann et al. 2003). However, we found that there was no preferential usage of particular V_{β} chains by these non class Ia selected CD8CD44^{hi} cells that secrete IFN-γ rapidly after anti-CD3 stimulation in vivo. Thus, non class Ia selected IFN-γ secreting CD8αβCD44^{hi} cells are heterogeneous cell populations and do not belong to a specific cell subset. This characteristic might be beneficial to the host by mounting a rapid IFN-γ response to a wide range of antigens which can be recognized by conserved non class Ia molecules.

Although initially described as NK cell specific, the activating and inhibitory NK cell receptors have also been found on γδ T cells, αβ T cells, B cells, monocytes, macrophages and dendritic cells . CD8 T cells in naive mice do not express significant levels of NK cell receptors. The expression of NK cell receptors was observed to be up-regulated in antigen stimulated CD8 T cells or cytokine (IL-2 or IL-15) activated CD8 T cells (Dhanji and Teh 2003;Dhanji et al. 2004). However, we noted an apparent elevated expression of CD94, NKG2A, and NKG2D expression on non class Ia selected CD8αβCD44^{hi} cells in naïve DKO mice. CD94$^{hi/int}$CD44^{hi} CD8 cells were indicated as the true effector and memory cells . Approximately 5% CD8αβCD44^{hi} cells in naïve B6 mice express CD94, whereas around 30% CD8αβCD44^{hi} cells in naïve DKO mice express CD94. CD94 exclusively dimerized with the inhibitory receptor NKG2A in both B6 and DKO mice. The expression of CD94/NKG2A has been implicated in inhibiting CD8 T cell cytotoxicity and providing protection from apoptosis (Gunturi et al. 2003). However, our data did not show a reduced level of apoptosis in CD8CD94^{hi} cell population compared to CD8CD94^{lo} cells in either B6 or DKO mice ex vivo (data not shown).

There are around 20% CD8αβCD44^{hi} cells in naïve DKO mice that express a high level of the activating NK receptor NKG2D. However, no NKG2D mediated cytotoxicity was observed against NKG2D ligand expressing target cells ex vivo. Their cytotoxity may be inhibited by inhibitory receptors because most NKG2D expressing CD8αβCD44^{hi} cells also express CD94/NKG2A. We proposed that these NKG2D expressing CD8αβCD44^{hi} cells must be activated by antigens or cytokines before they are able to lyse their target cells, similar to NK cells. This may serve as a protective mechanism to protect cell damage by autoimmunity.

3.2 The function of non class Ia selected CD8αβCD44^{hi} cells in adaptive immunity to LM infection

Non class Ia selected CD8 cells provide adaptive immune protection against LM infection. The antigen specific anti-LM response was mediated only by thymus dependent CD8 cells. There was no antigen specific proliferation and IFN-γ secretion observed in extrathymically developed CD8 cells. This is consistent with previous reports that thymus-independent CD8 cells are unresponsive to antigenic stimulation . However, they contribute to innate immunity by secreting IFN-γ in response to the inflammatory cytokines IL-12 and IL-18 secreted by LM infected macrophages.

While it is conventionally thought that naïve cells are the responding population to foreign antigens during the animals first encounter with a pathogen, it is possible that this non class Ia restricted memory phenotype population also has the potential to respond in an antigen specific manner. We showed that transfer of small numbers of naïve CD8CD44^{hi} cells from DKO donors into recipient animals resulted in an expansion of the transferred cells in vivo that mounted a specific response mediated by CD8αβ cells that arise in a thymus-intact environment.

H2-M3-restricted presentation of N-formyl methionine peptides to CD8 T cells provides a mechanism for selective recognition of bacterial infection (Kerksiek et al. 2001). It is known that the CD8 T cell response to LM derived antigens restricted by H2-M3 are quantitatively and qualitatively different from that observed against class Ia molecules. The H2-M3 restricted response which precedes that of the class Ia restricted response provides rapid and quantitatively substantial effector function during primary LM infection (Seaman et al. 2000). However, after a second LM infection, H2-M3-restricted memory T cell responses are limited in comparison to the much larger MHC class Ia-restricted responses although H2-M3-restricted memory T cells are generated, and are indistinguishable from classically restricted T cells in terms of cell surface memory markers and longevity (Kerksiek et al. 1999;Kerksiek et al. 2003). It has been indicated that MHC class Ia-restricted memory CD8 T cells prevented the expansion of H2-M3-restricted memory T cell populations by limiting dendritic cell antigen presentation (Hamilton et al. 2004). However, previous study showed that limited expansion of class Ib-restricted memory CD8 T cells in secondary anti-Listeria response was also observed in DKO mice which do not have class Ia restricted memory CD8 T cells (Seaman et al. 2000). Thus, this would imply that distinct class-I families influence different stages of the CD8 T cell response to infection, with H2-M3–restricted T cells playing a key role in controlling the early stages of LM infection. Our data showed that approximately 20% donor CD8CD44^{hi} cells secrete IFN-γ in response to H2-M3 restricted epitopes f-MIGWII and f-MIVTLF, respectively. Although it has also been reported that cross-recognition of N-formyl methionine peptides is a general characteristic of H2-M3-restricted CD8 T cells (Ploss et al. 2005), the actual total percentage of f-MIGWII and f-MIVTLF specific cells might be less than that shown in our result. However, the percentage of H2-M3 restricted LM specific CD8CD44^{hi} cells is still very

high. Taken together, this suggests that naïve DKO mice contain a population of memory cells, possibly sensitized by commensal organisms, which are specific for pathogenic epitopes including those displayed by LM. These memory phenotype CD8 T cells could account for the rapid primary response observed.

Other than H2-M3, Qa-1b has been demonstrated to be a restricting element for anti-Listerial CD8 T cells (Bouwer et al. 1997;Seaman et al. 1999). However, at this time the specific LM epitope(s) presented by Qa-1b has not been identified so the relative contribution of this effector population has not been examined.

4. Conclusions and Future Directions

The studies presented here have provided a better understanding of the murine non class Ia selected CD8αβ cells and their functions in the immune system. While previous studies have used DKO mice as a tool to study the phenotype, selection and function of non class Ia selected CD8 cells, our studies have extensively investigated their phenotypes, origins, and functions in innate and adaptive immunity. We clearly demonstrated that most of these non class Ia restricted CD8 cells are traditional CD8αβTCRαβ cells and express memory markers $CD44^{hi}CD122^{hi}Ly6C^{hi}CD127^{hi}$. By studying the CD44 expression in mature single positive CD8 T cells and FITC labeled RTEs, we demonstrated that the acquisition of CD44 expression by non class Ia selected CD8αβ cells was a post-thymic event. Furthermore, we noted that extrathymically developed CD8αβ cells only account for a small percentage of $CD8\alpha\beta CD44^{hi}$ cells in the periphery of DKO mice. Most non class Ia selected CD8 cells are thymus derived. The ability of CD8 cells from DKO mice to undergo basal and homeostatic proliferation is

Figure 30

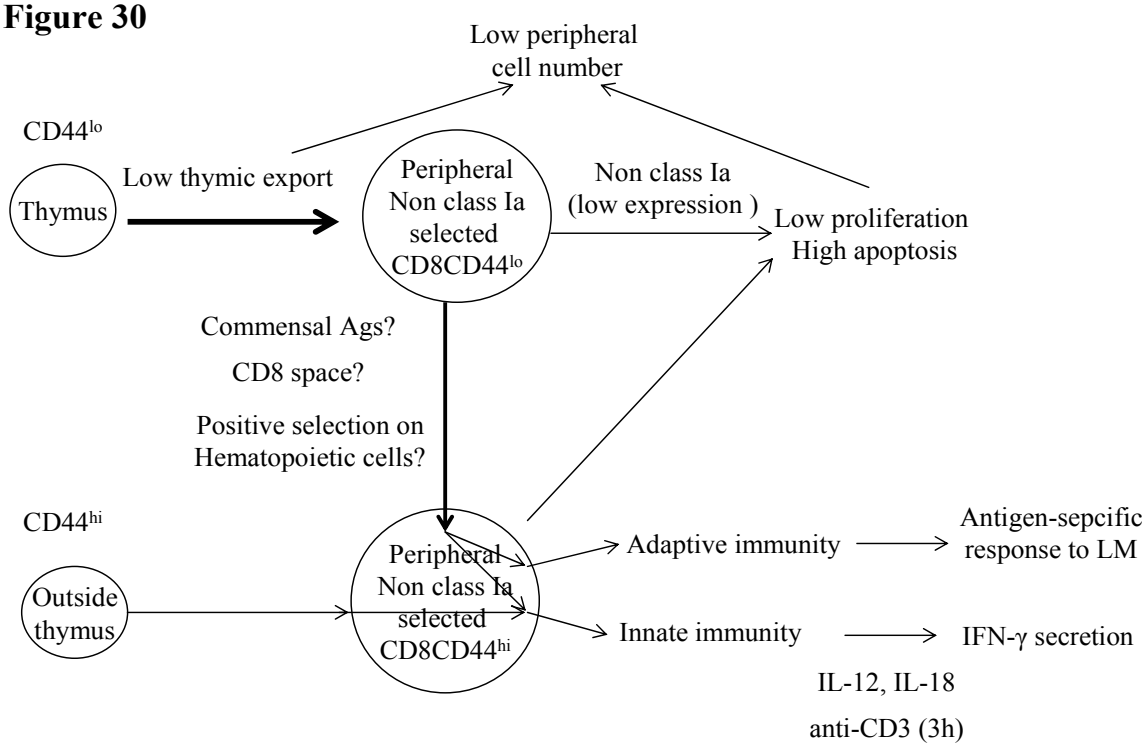

Figure 30. Model. In DKO mice, most mature CD8 thymocytes are $CD44^{lo}$. There is a reduced percentage of RTEs in DKO mice compared to B6 mice. The non class Ia selected RTEs acquire their $CD44^{hi}$ phenotype in the periphery. The reason why they acquire the memory phenotype is still not clear. Both $CD8CD44^{hi}$ and $CD8CD44^{lo}$ cells have a lower proliferation rate and higher apoptosis rate. The low thymic export, limited proliferation and extensive apoptosis may partially account for the low peripheral CD8 number in DKO mice. Only a small percentage of non class Ia selected $CD8CD44^{hi}$ cells are thymus-independent. They did not contribute to antigen specific response to LM infection. Only thymus derived non class Ia selected $CD8CD44^{hi}$ cells can participate in both innate and adaptive immunity.

investigated. Our data suggested that reduced thymic export, limited proliferation ability, and extensive apoptosis may responsible for that few CD8 cells in the periphery of DKO mice. These non class Ia selected CD8 cells play a role in both innate and adaptive immunity. Our model (Figure 30) summarized our current knowledge about the origin and function of non class Ia selected CD8 T cells.

To further investigate the origins of the $CD8CD44^{hi}$ cells in DKO mice, several questions need to be answered. One is about the proliferation rate of the RTEs. If homeostatic proliferation is the major mechanism for non class Ia selected CD8 cells to acquire $CD44^{hi}$ phenotype, RTEs should show a rapid proliferation after emigration into the lymphopenic CD8 compartment. Our preliminary experiments did not draw a conclusion due to the few RTEs we can harvest. The other question is when CD8 cells in DKO mice acquired their CD44 phenotype. After 16-20 hour intrathymic FITC injection, we found that a very small percentage of CD8 RTEs had already acquired the $CD44^{hi}$ phenotype in the periphery. It is interesting to know how fast these non class Ia selected $CD8CD44^{hi}$ cells acquired their phenotype. To trace the RTEs for a longer time period, the RAG2p-GFP transgenic mice can be used to cross into a DKO background. RAG2p-GFP transgenic mice are mice transgenic for green fluorescent protein (GFP) driven by the recombination activating gene 2 (Rag2) promoter (Boursalian et al. 2004). In RAG2p-GFP transgenic mice, the multicopy transgene generated a bright GFP signal whose induction occurred at the late $CD4^{-}CD8^{-}$ double-negative stage of intrathymic T cell development. The signal remained bright during the $CD4^{+}CD8^{+}$ double-positive stage. Although RAG expression was extinguished during the transition from the double-positive to $CD4^{+}CD8^{-}$ or $CD4^{-}CD8^{+}$ single-positive compartments, the GFP protein lingered in single-positive thymocytes and even in peripheral T cells, in which populations of GFP^{hi}, GFP^{lo} and

GFP^- cells could be defined. The GFP^{hi} peripheral T cells have left the thymus within one week. GFP^{lo} cells are 1-2 weeks older and GFP^- cells represent the mature peripheral T cells. This mouse model is also useful to answer the question about the proliferation of RTEs.

We demonstrate that $CD8CD44^{hi}$ T cells in DKO mice can secrete IFN-γ in an IL-12 and IL-18 dependent fashion in vitro and can be stimulated to produce IFN-γ in vivo and in vitro by administration of anti-CD3. We also generated bone marrow chimeras, using thymectomized or thymus-intact DKO mice as recipients of T cell-depleted DKO bone marrow. This approach allowed us to demonstrate that LM specific CD8 T cells must be selected in the thymus and that $CD8CD44^{hi}$ cells that were selected in the thymus can be Listeria specific. Since three Listeria derived, H2-M3 restricted epitopes have been defined, we performed ICS after peptide stimulation and found more than 20% $CD8CD44^{hi}$ cells are specific to H2-M3 restricted epitopes. Currently we only tested their responses to f-MIVTLF and f-MIGWII peptides. We could further test the responses to f-MIVIL peptide and perform tetramer stainings to further demonstrate their specificity.

Finally, we studied the expression of NK cell markers on the CD8αβ cells in the naïve DKO mice. We found CD94, NKG2A and NKG2D were up-regulated in these non class Ia selected $CD\alpha\beta CD44^{hi}$ cells. The ex vivo cytotoxic lysis of NKG2D ligand-expressing target cells by purified CD8 cells from naïve DKO mice did not show any specific killing. It suggests that these cells need activation to become cytotoxicity. It is interesting to know whether these NKG2D expressing $CD\alpha\beta CD44^{hi}$ cells from naïve DKO mice express DAP12 ex vivo. RT-PCR could be performed to answer this question.

In conclusion, we have characterized the phenotype and function of CD8 T cells resident in DKO animals along with the ontogeny of their CD44 phenotype. We further

investigate the proportion of CD8 T cells from normal mice that respond through homeostatic expansion in single class Ia-deficient as well as mice lacking both K^{b-} and D^{b-} molecules. Although our data suggested that only a very small percentage of CD8 T cells in normal mice is restricted by non MHC class Ia molecules, these non class Ia restricted CD8 T cells might respond to antigens and play a role in the innate as well as acquired immune responses, similar to that they do in DKO mice.

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