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The Role of CD40 in Naïve and Memory CD8+ T Cell Responses: a Dissertation

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THE ROLE OF CD40 IN NAÏVE AND MEMORY $CD8^+$ T CELL RESPONSES

A Dissertation Presented

By

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ABSTRACT

Stimulation of CD40 on APCs through CD40L expressed on helper $CD4^+$ T cells activates and "licenses" the APCs to prime $CD8^+$ T cell responses. While other stimuli, such as TLR agonists, can also activate APCs, it is unclear to what extent they can replace the signals provided by CD40-CD40L interactions. In this study, we used an adoptive transfer system to re-examine the role of CD40 in the priming of naïve $CD8^+$ T cells. We find an approximately 50% reduction in expansion and cytokine production of TCR-transgenic T cells in the absence of CD40 on all APCs, and on dendritic cells in particular. Moreover, CD40-deficient and CD40L-deficient mice fail to develop endogenous CTL responses after immunization and are not protected from a tumor challenge. Surprisingly, the role for CD40 and CD40L are observed even in the absence of $CD4^+$ T cells; in this situation, the $CD8^+$ T cell itself provides CD40L. Furthermore, we show that although TLR stimulation improves T cell responses, it cannot fully substitute for CD40.

We also investigated whether CD40-CD40L interactions are involved in the generation, maintenance, and function of memory $CD8^+$ T cells. Using a virus infection system as well as a dendritic cell immunization system, we show that the presence of CD40 on DCs and other host APCs influences the survival of activated effector cells and directly affects the number of memory $CD8^+$ T cells that are formed. In addition, memory $CD8^+$ T cell persistence is slightly impaired in the absence of CD40. However, CD40 is not required for reactivation of memory $CD8^+$ T cells. It seems that CD40 signals during

priming also contribute to memory $CD8^+$ T cell programming but this function can be independent of CD4+ T cells, similar to what we showed for primary responses.

Altogether, these results reveal a direct and unique role for CD40L on $CD8^+$ T cells interacting with CD40 on APCs that affects the magnitude and quality of primary as well as memory $CD8^+$ T cell responses.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Ab	Antibody
ACK	Ammonium chloride/potassium
Ag	Antigen
APC	Antigen presenting cell
APC	Allophycocyanin
BCR	B cell receptor
CD	Cluster of differentiation
CFA	Complete Freund's adjuvant
CFSE	Carboxyfluorescein succinimidyl ester
OVA	Ovalbumin
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DN	Double negative
DP	Double positive
E:T ratio	Effector:Target ratio
EAE	Experimental autoimmune encephalomyelitis
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorter
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte/macrophage colony stimulating factor

gp33	LCMV glycoprotein epitope 33-41
HBSS	Hank's buffered saline solution
Flt3L	Fms-related tyrosine kinase 3 ligand
HIGM	hyper-IgM
IFN	Interferon
IL	Interleukin
IL-7R	IL-7 receptor
IRAK	IL-1R associated kinase
LCMV	Lymphocytic choriomeningitis virus
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
mAb	monoclonal antibody
M-CSF	Macrophage colony stimulating factor
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
MΦ	Macrophage
NK cells	Natural killer cells
NKT cells	Natural killer T cells
PAMPs	Pathogen associated molecular patterns
PDC	Plasmacytoid DCs
PE	Phycoerythrin
PerCP	Peridin chlorophyll-a protein

polyI:C	Polyriboinosinic-polyribocytidylic acid
PRR	Pattern recognition receptors
SP	Single positive
STAT	Signal transducer and activator of transcription
TAP	Transporter associated with antigen processing
TCR	T cell receptor
Tg	Transgenic
T_H cell	T helper cell
TIR domain	Toll/IL-1R domain
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRAF	TNF receptor associated factor
VSV	Vesicular stomatitis virus

CHAPTER I.

INTRODUCTION

Immunity refers to the ability of an individual to detect the presence of foreign antigens (Ag) and mount a protective response against them. The immune system is an organized mechanism of proteins, cells, tissues, and organs and it is divided into two arms. Innate immunity is immediate and non-specific; it functions as the first line of defense during an infection. In contrast, adaptive immunity takes a longer time to develop but is characterized by having specificity, diversity, and memory. Its principal mediators are lymphocytes, which express receptors that are specific for virtually any antigen. Adaptive immunity is made up of humoral and cell mediated components. Humoral immunity is mediated by B cells, which primarily make antibodies that bind to intact extracellular antigens including whole bacteria, viruses, and cells and their products. Cell mediated immunity is mediated by T cells, which only recognize processed peptide antigens that are bound to cell surface glycoproteins called major histocompatibility complex (MHC) molecules.

T cells can be further sub-divided based on their expression of the CD4 or CD8 co-receptors. $CD4^+$ T cells mainly secrete cytokines that activate other cells and induce B cells to produce antibodies and undergo class switching; hence they are also called helper T (T_H) cells. T_H1 cells primarily secrete IFN−γ and induce class switching to IgG2 and IgG3 isotypes. They are also involved in macrophage activation and clearance of intracellular pathogens as well as delayed-type hypersensitivity reactions. T_H2 cells on

the other hand secrete IL-4, IL-5, and IL-13, which promote class switching to IgG1, IgE, and IgA. These cells play an important role in the immune response to helminth infections and are also involved in allergy and asthma. Recently, T_H17 cells, which secrete IL-17 were identified. They constitute a separate lineage of T_H cells and they are involved in the pathogenesis of some autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) and type II collagen-induced arthritis (Harrington et al., 2005; Park et al., 2005). $CD4^+$ T cells can also differentiate into $CD25^+$ regulatory T cells (T_{REG} cells), whose main function is to inhibit the response of other T cells (Kronenberg and Rudensky, 2005).

$CD8^+$ T cells are primarily distinguished by their cytotoxic activity. These cells are able to directly kill target cells by secreting lytic substances such as perforin and granzymes or by expressing Fas ligand, which induces apoptosis in cells expressing the Fas receptor. $CD8^+$ T cells also secrete IFN–γ and TNF–α, which promote antigen presentation, macrophage activation, and target cell killing. They are therefore critical players in the immune response against infectious agents as well as tumors.

A. T cell development

In the bone marrow and fetal liver, pluripotent hematopoietic stem cells give rise to common lymphoid progenitors, which in turn give rise to T cells (Shortman and Wu, 1996). These progenitors subsequently migrate to the thymus, which is the primary site of T cell development (Carlyle and Zuniga-Pflucker, 1998). The progenitors start out as being negative for the T cell marker CD3 as well as the CD4 and CD8 co-receptors (Petrie et al., 1990). These cells, which are also referred to as double-negative (DN) cells,

can be further divided into four groups based on their expression of the cell surface molecules CD44 and CD25 (Shortman and Wu, 1996). DN1 cells are $CD44^{+}CD25^{-}$, DN2 cells are $CD44^{+}CD25^{+}$, DN3 cells are $CD44^{-/lo}CD25^{+}$, and DN4 cells are $CD44^{-/lo}CD25^{-}$. Rearrangement of the T cell receptor (TCR) β chain starts at the DN3 stage. Upon successful rearrangement, the β chain pairs with a surrogate α chain and forms a pre-TCR, which is expressed on the cell surface (von Boehmer and Fehling, 1997). The cells are called pre-T cells at this stage and they are positive for CD3 but they are still double negative for CD4 and CD8. The cells then differentiate into pro-T cells, which are double positive (DP) for CD4 and CD8. Two important processes occur at this stage of development (Starr et al., 2003). Positive selection ensures that only those T cells that recognize self-antigen and self-MHC survive (Guidos, 1996; Starr et al., 2003). A large number of T cells are not able to recognize self-antigen/MHC and these die by apoptosis (Huesmann et al., 1991). Negative selection in turn removes T cells that have an extremely high affinity for self-antigens and which could potentially cause autoimmunity (Nossal, 1994; Starr et al., 2003). The latter constitutes the major mechanism of establishment of central tolerance. T cells that have successfully undergone both positive and negative selection subsequently mature into single positive (SP) $CD4^{+}$ or $CD8^{+}$ T cells and migrate into the periphery as naïve or antigen-inexperienced cells (Singer et al., 1999). $CD4^{+}$ T cells recognize peptides bound to MHC class II molecules while $CD8^{+}$ T cells recognize peptides bound to MHC class I molecules (Germain, 1994). These co-receptors are important because they associate with the tyrosine kinase Lck, which

increases the sensitivity of the TCR antigen stimulation and is also involved in signal transduction (Collins et al., 1993).

B. MHC class I molecules

As mentioned above, $CD8^+$ T cells recognize a complex formed by peptide antigens bound to MHC class I molecules. MHC class I molecules are polygenic; they are encoded by several genes that are located within the major histocompatibility complex locus found in chromosome 6 in humans and chromosome 17 in mice (Janeway and Travers, 1997). MHC class I molecules are also polymorphic and furthermore, they are codominantly expressed. MHC class I molecules are constitutively expressed by almost all nucleated cells, but they are most highly expressed on lymphocytes and specialized antigen presenting cells. In addition, MHC class I molecule expression can be upregulated by cytokines such as Type I and Type II interferons (IFNs) (York and Rock, 1996).

MHC class I molecules are made up of a heterodimer consisting of a large transmembrane α chain and invariant $\beta 2$ microblobulin, which are non-covalently associated. The α chain has three domains, $\alpha 1$, $\alpha 2$, and $\alpha 3$. The polymorphisms in MHC class I molecules are confined mainly to the $\alpha 1$ and $\alpha 2$ domains, which hold or bind the peptides. These domains fold into a pocket-shaped structure called peptide binding groove or cleft that consists of two α helices lying on a sheet of eight antiparallel β strands (Garcia et al., 1998). It is closed at both ends and therefore can only accommodate short peptides generally 8-10 amino acids long (Bouvier and Wiley, 1994). Meanwhile, the $\alpha 3$ domain, which is responsible for binding to CD8, is relatively

constant and associates with β2 microglobulin, forming a structure that is similar to the constant region of immunoglobulins. In the absence of β2 microglobulin, the α chain is not stabilized and no MHC class I molecules are expressed on the cell surface.

C. Antigen processing and presentation to $CD8^+$ T cells

In general, the antigenic peptides that are bound to MHC class I molecules and presented to $CD8^+$ T cells are derived from intracellular protein antigens, which are endogenously synthesized in the cytoplasm (Rock et al., 2002). These can include self-proteins undergoing normal turnover, mutated proteins from tumor cells, and viral or bacterial products. Recently, it has been hypothesized that defective ribosomal products (DRiPs) are the major source of antigens for MHC class I presentation. DRiPS are defined as prematurely terminated polypeptides and misfolded polypeptides produced from translation of bona fide mRNAs in the proper reading frame (Yewdell et al., 1996).

Protein degradation takes place in the cytoplasm through the proteasome (York et al., 1999). It is a large barrel-shaped multicatalytic protease complex composed of 28 subunits arranged in four stacked rings, each containing seven subunits. Interferons can induce the expression of three other subunits that can displace constitutively expressed subunits and change the specificity of the proteasome. Two of these, LMP2 and LMP7, are encoded within the MHC locus while the third, MECL-1, is not. Proteasomes containing these subunits are referred to as immunoproteasomes. They preferentially cleave after hydrophobic and basic residues while reducing cleavage after acidic residues.

Proteins targeted for degradation are polyubiquitinated in the cytoplasm. After ubiquitination, the proteins are unfolded and the ubiquitin is removed. The proteins are

then threaded through the hollow core of the proteasome, which contains the active sites, and are subsequently chopped into products 6-30 amino acids long (York et al., 1999). Proteasomal cleavage results in the generation of carboxyl-terminal residues containing the preferred anchor residues for transport to the endoplasmic reticulum (ER) and binding to MHC class I molecules (Craiu et al., 1997). In contrast, it is thought that the generation of appropriate amino-terminal residues requires the activity of cytosolic or ER aminopeptidases (Craiu et al., 1997). In fact, it has recently been discovered that an ER-localized aminopeptidase, ERAP1, acts as a molecular ruler by trimming peptides in the ER into 8-9-mers (Saric et al., 2002; York et al., 2002). Peptide trimming by ERAP1 has been shown to be important in establishment of immunodominance during a viral infection (York et al., 2006).

Peptides generated in the cytosol are translocated into the ER by a specialized transporter called Transporter associated with Antigen Processing (TAP) (Uebel and Tampe, 1999). TAP is a heterodimer composed of TAP1 and TAP2 subunits, which are also encoded within the MHC locus and upregulated by interferons. Newly synthesized MHC class I molecules are unstable and cannot be transported to the cell surface until bound by a peptide. During translation, the α chain is first associated with a membrane-bound protein called calnexin. Upon binding to $\beta 2$ microglobulin, it dissociates from calnexin and binds to a complex formed by the TAP-associated protein tapasin and the chaperones calreticulin and Erp57 . When peptide enters the ER via TAP and binds to the peptide binding groove, the folding of the MHC class I molecule is stabilized and the

peptide-MHC complex exits the ER and is transported to the cell surface through the Golgi complex and exocytic vesicles (York et al., 1999).

Exogenous or extracellular antigens such as soluble protein, particulate Ag, and even whole cells can also be presented on MHC class I molecules through a process called cross-presentation (Shen and Rock, 2006). These antigens are acquired mainly through phagocytosis and peptides are generated through several different pathways. In the phagosome-to-cytosol pathway, the protein antigens in the phagosome are transported to the cytoplasm through a mechanism that is yet to be defined (Guermonprez and Amigorena, 2005). The proteins then get degraded by the proteasome and the peptides are transported to the ER via TAP, in a manner similar to that for endogenous antigens. In the vacuolar pathway, the phagocytosed protein antigens are targeted to endosomes, where they are degraded by the cysteine protease cathepsin S (Rock and Shen, 2005). This pathway is proteasome-independent and TAP-independent; it is still unclear how the peptides generated through this mechanism are able to bind MHC class I molecules (Shen et al., 2004). Cross-presentation is a function that is uniquely performed by a specialized subset of cells called professional antigen presenting cells (APCs).

D. Antigen presenting cells

Naïve $CD8^+$ T cells require at least three signals in order to be fully activated. Signal 1 is the antigenic signal and it is mediated by the peptide-MHC class I complex binding to the cognate T cell receptor (TCR) (Janeway and Bottomly, 1994). Signal 2 is the costimulatory signal and it is mediated by the B7 family molecules CD80 and CD86 (also called B7-1 and B7-2, respectively) binding to the CD28 receptor (Bugeon and

Dallman, 2000; Sharpe and Freeman, 2002). It is important for enhancing T cell responsiveness and survival. Signal 3 is the inflammatory signal and it is mediated by the cytokines IFN-α/β or IL-12 (Curtsinger et al., 2005; Kolumam et al., 2005; Valenzuela et al., 2002). It promotes the full differentiation of naïve T cells into effector cells and memory cells.

Almost all cells express MHC class I molecules and can present signal 1 to $CD8^+$ T cells. However, the ability to provide signals 2 and 3 is restricted to the so-called professional antigen presenting cells (APCs) (Garza et al., 2000; Sprent and Schaefer, 1990; Steinman and Young, 1991). These specialized cells are derived from the bone marrow and they are highly efficient at capturing extracellular antigens through phagocytosis or endocytosis. APCs secrete chemokine as well as chemokine receptors that enable them to migrate to lymphoid organs and interact with naïve T cells. They express adhesion as well as costimulatory molecules constitutively and they can upregulate the expression of these molecules upon further activation. In addition, they can also secrete inflammatory cytokines upon receipt of appropriate stimulation. APCs are primarily defined by their expression of MHC class II molecules. As such, they are also the only cells that are capable of presenting antigens to $CD4^+$ T cells (Sprent, 1995).

The term professional APC was originally used to describe dendritic cells (DCs). However, two other cells are now also considered as professional APCs, B cells and macrophages (MΦ) (Sprent, 1995). B cells are very effective at presenting antigens recognized by their B cell receptor (BCR) however they are relatively inefficient at phagocytosing and presenting other antigens (Rodriguez-Pinto, 2005). MΦ are abundant

in peripheral as well as lymphoid tissues and they are extremely proficient in phagocytosing extracellular pathogens as well as apoptotic cells (Underhill et al., 1999). However, they are also relatively inefficient at presenting antigens to $CD8^+$ T cells. Nevertheless, they have recently been shown to prime naïve $CD8^+$ T cells *in vivo* (Pozzi et al., 2005). Dendritic cells (DCs) are considered as the most potent APCs for priming of naïve $CD8^+$ and $CD4^+$ T cells (Banchereau and Steinman, 1998). This is mainly because of their high levels of expression of costimulatory molecules and their ability to acquire antigens from the environment and migrate to lymphoid organs. Since naïve T cells only recirculate through the blood and lymphatic system, they are largely unable to detect infections or tumors in parenchymal tissues. Therefore, the ability of APCs to transport antigens from peripheral tissues into lymphoid organs is crucial to the initiation of immune responses.

E. Dendritic cells

Dendritic cells (DCs) are bone marrow derived cells with branched or dendritic morphology that are located in lymphoid and non-lymphoid tissues (Steinman and Cohn, 1973). Their main function is the acquisition, processing, and presentation of antigens to T cells (Banchereau and Steinman, 1998). They also interact with other immune cells such as NK cells, NK cells, B cells, monocytes, and MΦ in order to elicit effective T cell responses (Degli-Esposti and Smyth, 2005). DCs continuously sample the local environment for foreign antigens but they also present self-antigens. This latter function is important for the establishment and maintenance of tolerance (Smits et al., 2005). DCs were first described in the late nineteenth century by Paul Langerhans (Langerhans cells)

but their antigen presenting function was only recognized in the 1970s (Steinman and Witmer, 1978).

There are several subsets of DCs, which can be distinguished based on expression of cell surface markers, anatomical location, or function (Adams et al., 2005; Ardavin, 2003; Shortman and Liu, 2002). In mice, DCs express CD11c (integrin–α chain) and there are at least six different subsets based on the expression of CD4, CD8, CD205 (multilectin domain molecule DEC205), and CD11b (integrin αM chain of Mac-1). These are the "myeloid-like" $CD4^{+}CD8^{-}CD205^{-}CD11b^{+}$, $CD4^{-}CD8^{-}$, $CD205^{-}CD11b^{+}$, and $CD4^{-}CD8^{-}CD205^{+}CD11b^{+}$, the "lymphoid-like" $CD4^{-}CD8^{hi}CD205^{hi}CD11b^{-}$, the Langerhans $CD4^{-}CD8^{lo}CD205^{hi}CD11b^{+}$, and the plasmacytoid $B220^{+}$. In humans, DC precursors express CD34 but do not express CD8. There are least four different subsets: Langerhans DCs, interstitial DCs, monocyte-derived DCs, and plasmacytoid DCs. Mouse and human plasmacytoid DCs serve a unique function in that they are the major producers of type I interferons upon virus infection. The generation of these functionally distinct DCs has been proposed to occur through two alternative models (Ardavin, 2003; Shortman and Liu, 2002). In the functional plasticity model, it is thought that all DCs arise from a single hematopoietic lineage and the different subsets develop according to local environmental cues. In the specialized lineage model, it is thought that the different DC subtypes derive from multiple hematopoietic precursors.

Some of the earliest studies on antigen presenting ability by DCs showed that they are very potent stimulators of mixed lymphocyte reactions (MLR) and further, compared to B cells and MΦ, they are the only cells that can induce significant allogeneic T cell

proliferation *in vitro* (Reis e Sousa, 2006). Subsequently, it was shown that DCs pulsed with peptides, proteins, RNA, or DNA can be used to prime cytotoxic $CD8^+$ T cells (CTLs) *in vivo* (Hamilton and Harty, 2002; Porgador and Gilboa, 1995). DCs pulsed with or constitutively expressing epitopes from viruses such as influenza, Sendai virus, lymphocytic choriomeningitis virus (LCMV), hepatitis B virus (HBV), and human immunodeficiency virus (HIV) have been shown to induce virus-specific CTLs and protective immunity (Ludewig et al., 1998). Furthermore, immunization with DCs pulsed with tumor-associated antigens has been shown to result in the induction of tumor-specific $CD8^+$ T cells and protection against tumorigenesis (Celluzzi et al., 1996; Paglia et al., 1996). Finally, there are a number of studies that show that DCs are the only cells capable of cross-presenting exogenous antigens (Melief, 2003). One study in particular made use of transgenic mice in which the diphteria toxin receptor (DTR) was expressed under the control of the CD11c promoter (Jung et al., 2002). Administration of diphtheria toxin resulted in the depletion of $CD11c^+$ DCs and failure to generate $CD8^+$ T cell responses to cell-associated antigens and intracellular pathogens. The highly potent antigen presenting ability by DCs has made them attractive immunotherapeutic candidates. DC vaccination has proven to be an effective strategy in inducing anti-tumor immunity, control of autoimmunity, and prevention of transplant rejection (Figdor et al., 2004).

F. Role of DC maturation in $CD8^+$ T cell responses

It is now well established that antigen presentation by DCs is important not just for the induction of immune responses to infectious agents but also for the establishment

of tolerance to self-antigens (Garza et al., 2000). For a long time, this dichotomy was attributed to functional differences in distinct DC subsets. For example CD8- DCs have been shown to direct T_H2 responses while $CD8^+$ DCs have been implicated in cross-presentation of MHC class I-restricted peptides as well as suppression of T cell responses (Miller et al., 2003). However, there is also evidence that whether the outcome of antigen presentation will be immunity or tolerance is largely determined by the maturation or activation state of DCs (den Boer et al., 2001; Reis e Sousa, 2006).

Under steady-state conditions or in the absence of infection or inflammation, most DCs exist in an immature or quiescent state (Adams et al., 2005; Reis e Sousa, 2004a; Tan and O'Neill, 2005). Immature DCs are highly endocytic; they are very active in sampling antigens from the environment through micropinocytosis, receptor-mediated endocytosis, or phagocytosis. However, they express relatively low levels of MHC class I and class II molecules as well as the costimulatory molecules CD80 and CD86. Moreover, they can only present antigens bound to MHC class I molecules and are unable to process antigens for MHC class II presentation. It is worth noting that since there is no infection, immature or quiescent DCs mostly present self-antigens that come from apoptotic cells undergoing physiologic turnover. They are somehow able to migrate to lymphoid organs but because they present only signal 1 and very little or no signals 2, and 3, they fail to induce productive immunity and instead induce peripheral tolerance (Redmond and Sherman, 2005). T cell activation in the presence of signal 1 but the absence of signals 2 and 3 only results in a brief period of proliferation and suboptimal development of effector function. Ultimately, this leads to either deletion of the activated

T cells or the induction of anergy, which is a state of functional unresponsiveness (Tan and O'Neill, 2005). In some cases, antigen presentation by immature or quiescent DCs also induces the development of regulatory T cells (Smits et al., 2005).

Upon maturation, there is a reduction in the ability of DCs to capture antigen but the efficiency of antigen processing and presentation on both MHC class I and class I molecules is increased, leading to enhancement of cross-presentation (Adams et al., 2005; Tan and O'Neill, 2005). In addition, there is also increased expression of MHC molecules, cell adhesion molecules such as lymphocyte function-associated antigen 1 (LFA-1) and costimulatory molecules CD80 and CD86. Mature DCs produce chemokines such as TARC, MDC, IP-10, RANTES, MIP-1α, and MIP-1β, which recruit other cells including monocytes, DCs, and T cells into the local environment. They also downregulate expression of the chemokine receptors CCR1 and CCR5 and upregulate the expression of CCR7 (Adams et al., 2005). This enables them to migrate to lymphoid organs through the chemokines CCL19 and CCL21. DC maturation is likewise accompanied by secretion of cytokines such as IL-2, IL-12, and type I IFNs (Morelli et al., 2001). Finally, DC maturation is associated with increased survival through the induction of anti-apoptotic proteins belonging to the Bcl-2 family (Hou and Van Parijs, 2004). Because mature DCs express the complete repertoire of signals 1, 2, and 3, they are able to efficiently induce productive immune responses (Reis e Sousa, 2006).

DC maturation can be induced by inflammatory cytokines such as TNF−α, microbial products such as lipopolysaccharide (LPS) that bind to Toll-like receptors, endogenous adjuvants such as uric acid (Gallucci et al., 1999; Shi et al., 2003), or T cell-

derived signals such as CD40 ligand that bind to CD40. The specific cytokine and chemokine profiles and the levels of costimulatory molecules induced are all dependent on the nature of maturation stimulus.

G. CD40 and CD40 Ligand (CD40L)

CD40 is a 48 kDa type I transmembrane glycoprotein cell surface receptor. It belongs to the TNF receptor family, which includes TNFRI, TNFRII, CD95, CD27, CD30, OX40, and 4-1BB (Croft, 2003). CD40 was first discovered as the receptor responsible for the full activation, differentiation, and survival of B cells upon interaction with activated $CD4^+$ T cells (van Kooten and Banchereau, 1997). Subsequently, it was shown to be constitutively expressed on all APCs, and it is upregulated upon infection or inflammation. It is also expressed in T cells, basophils, eosinophils as well as non-hematopoietic cells such as thymic epithelial cells, vascular endothelial cells, smooth muscle cells, keratinocytes, and fibroblasts. (Quezada et al., 2004; Schonbeck and Libby, 2001; van Kooten and Banchereau, 2000).

CD40 binds to CD40 ligand (CD40L/CD154/gp39), a 32-39 kDa type II transmembrane glycoprotein. It is a member of the TNF family, which includes the cytokines tumor necrosis factor-α (TNF-α) and lymphotoxin-α/β (LTα and LTβ), and the ligands for Fas (FasL), CD30 (CD30L), CD27 (CD27/CD70), OX40 (OX40L), and 4-1BB (4-1BBL) (Croft, 2003). CD40L was first identified in activated $CD4^+$ T cells and its expression is very tightly regulated unlike that of its receptor (Ford et al., 1999; Hermann et al., 1993; Roy et al., 1993). Surface expression is absent in naïve T cells but is induced as early as five minutes and reaches peak levels six hours post-activation with

antigen, anti-CD3, pro-inflammatory cytokines, or PMA and ionomycin (Casamayor-Palleja et al., 1995; Quezada et al., 2004; Schonbeck and Libby, 2001; van Kooten and Banchereau, 2000). Although CD40L is primarily expressed in $CD4^+$ T cells, it can also be found in $CD8^+$ T cells, B cells, NK and NKT cells, peripheral blood monocytes and phagocytes, platelets, epithelial cells, endothelial cells, and smooth muscle cells (Schonbeck and Libby, 2001).

The regulation of CD40 and CD40L gene expression is not well understood. There is evidence that transcription of CD40 and CD40L genes is regulated by the AT-hook transcription factor AKNA in human B cells, T cells, NK cells and DCs (Siddiqa et al., 2001). In mice, CD40 gene transcription has been shown to be mediated by STAT1 (signal transducer and activator of transcription 1) and NF-kB (Schonbeck and Libby, 2001). On the other hand, transcription of the CD40L gene has been shown to involve nuclear factor of activated T cells (NFAT) and is inhibited by cyclosporin (Fuleihan et al., 1994; Tsytsykova et al., 1996). CD40L is predicted to exist as a homotrimer and it also triggers trimerization of CD40 upon binding (Peitsch and Jongeneel, 1993). This leads to the recruitment of adaptor proteins belonging to the TNF receptor-associated factor (TRAF) family, which contain a conserved C-terminal TRAF domain (Pullen et al., 1998). There are six members of the TRAF family and all of them have been shown to associate with the cytoplasmic domain of CD40 upon stimulation (Pullen et al., 1998; Schonbeck and Libby, 2001; van Kooten and Banchereau, 2000). However the specific TRAFs that are recruited are dependent on the cell type expressing CD40 (Mukundan et al., 2005; Nguyen et al., 1999). For example, TRAF2 and TRAF3 mediate

phosphorylation of JNK and p38 kinases in B cells and play a role in class switching (Jabara et al., 2002). In DCs, phosphorylation of JNK and p38 kinases and subsequent production of the IL-12 p40 subunit require TRAF6 (Mackey et al., 2003). TRAFs regulate gene transcription through the MAPK and NF-κB pathways. Some of the target genes induced upon CD40 stimulation include inflammatory cytokines and the anti-apoptotic proteins Bcl-2 and BcL-x_L (Dallman et al., 2003; Quezada et al., 2004). In human monocyte-derived DCs, it has also been shown that CD40 stimulation induces Janus kinase 3 (JAK3) phosphorylation and signal transducer and activator of transcription 5a (STAT5) transactivation (Saemann et al., 2002).

H. Role of CD40-CD40L interactions in immune responses

The pivotal role of CD40-CD40L interactions in the generation of productive immune responses is highlighted by mutations in either the CD40 or CD40L genes, which result in a primary immunodeficiency in humans called hyper IgM syndrome (HIGM) (Allen et al., 1993; DiSanto et al., 1993; Ferrari et al., 2001). HIGM patients have defective antibody production and are susceptible to recurrent opportunistic infections with pathogens such as *Cryptosporidium* and *Pneumocystis carinii*. CD40-deficient and CD40L-deficient mice have also been generated and they likewise exhibit defects in both humoral and cellular immunity (Borrow et al., 1996; Castigli et al., 1994; Cayabyab et al., 1994; Grewal et al., 1996; Grewal et al., 1995; Kawabe et al., 1994; Oxenius et al., 1996; Renshaw et al., 1994; van Kooten and Banchereau, 2000; Xu et al., 1994). CD40-CD40L interactions have been shown to be required in the control of intracellular pathogens such as *Leishmania*, *Toxoplasma gondii*, and *Salmonella*, which

mostly rely on T_H1 responses (al-Ramadi et al., 2006; Grewal et al., 1997; Noelle, 1996; Soong et al., 1996). They are also important in the induction of $CD8^+$ T cell responses to non-inflammatory antigens such as soluble proteins and tumors (Mackey et al., 1998a; Mackey et al., 1997; Toes et al., 1998).

Administration of agonistic anti-CD40 Ab can increase the clonal expansion and delay the deletion of $CD4^+$ and $CD8^+$ T cells (Maxwell et al., 1999). This has been shown to result in enhancement of tumor protection, conversion of steady-state tolerance into immunity, and generation of memory T cells (Bonifaz et al., 2002; Clarke, 2000; Diehl et al., 1999; French et al., 1999; Lefrancois et al., 2000; Mackey et al., 1998a; Staveley-O'Carroll et al., 2003; Toes et al., 1998; van Mierlo et al., 2002). However, ligation of CD40 can also induce severe immunopathology or autoimmunity in the presence of self-reactive $CD8^+$ T cells (Cheng and Schoenberger, 2002; Ichikawa et al., 2002; Roth et al., 2002). Conversely, blockade of CD40 signaling, mainly through anti-CD40L Ab, results in inhibition of T cell activation, deletion of alloreactive $CD8^+$ T cells, or induction of anergy (Iwakoshi et al., 2000; Quezada et al., 2005; Wells et al., 1999). CD40L blockade has been shown to be an effective immunointervention strategy for inducing peripheral tolerance, e.g. to transplants, and control of some autoimmune diseases (Diehl et al., 2000; Hanninen et al., 2002; Homann et al., 2002; Phillips et al., 2003).

I. Role of CD40 stimulation in DCs and $CD8^+$ T cell responses

CD40 stimulation mediates one of the most effective signals for inducing DC activation and priming of $CD8^+$ T cell responses (Clarke, 2000; Diehl et al., 2000; Mackey et al., 1998a; Toes et al., 1998). Stimulation of CD40 can be achieved through

activated $CD4^+$ T cells, soluble CD40L or activating anti-CD40 antibody (Ab). This upregulates expression of costimulatory molecules CD80 and CD86, enhances production of cytokines (most notably IL-12), and promotes cross-priming to exogenous antigens (Ag) (Cella et al., 1996; Ridge et al., 1998; Schuurhuis et al., 2000; Yang and Wilson, 1996). It has been shown that the generation of protective tumor-specific $CD8^+$ T cell responses requires CD40 stimulation on DCs (Mackey et al., 1998a; Mackey et al., 1998b; van Mierlo et al., 2004). CD40 stimulation also converts tolerogenic $CD8^+$ DCs into immunogenic DCs (Grohmann et al., 2003; Grohmann et al., 2001). It can also restore the cross-priming ability of Rel-deficient DCs (Mintern et al., 2002a). There is evidence that ligation of CD40 on immature DCs renders them resistant from suppression by $CD4^+CD25^+$ T_{REG} cells (Serra et al., 2003). CD40-CD40L interactions between DCs and NKT cells have been implicated in the priming of naïve $CD8^+$ T cells (Fujii et al., 2004; Nishimura et al., 2000).

The generation of primary $CD8^+$ T cell responses to non-inflammatory antigens such as peptides, soluble proteins, particulate Ag, and cell-associated Ag, including peptide-pulsed DCs are largely dependent on help by $CD4^+$ T cells (Behrens et al., 2004; Clarke, 2000). $CD4^+$ T cells are important for "licensing" of DCs and this process is mediated by CD40 (Guerder and Matzinger, 1992; Smith et al., 2004; Sporri and Reis e Sousa, 2003). There are two models for how $CD4^+$ T cell help occurs. One model involves a sequential two-cell interaction, first, between CD40L-expressing $CD4^+$ T cells and CD40-expressing DCs leading to DC activation, and then between the activated DCs and $CD8^+$ T cells (Bennett et al., 1998; Prilliman et al., 2002; Ridge et al., 1998;

Schoenberger et al., 1998). An alternative model involves a direct interaction between CD40L-expressing $CD4^+$ T cells and CD40-expressing $CD8^+$ T cells (Bourgeois et al., 2002). In either case, $CD4^+$ T cell help and CD40 activity are often considered to be identical because CD40 stimulation can restore $CD8^+$ T cell responses primed in the absence of $CD4^+$ T cell help (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998). However there is also evidence that $CD4^+$ T cells can provide help to $CD8^+$ T cells through CD40-independent pathways (Lu et al., 2000).

J. Toll-like Receptors (TLRs)

Toll-like receptors (TLRs) comprise a subset of germline-encoded pattern-recognition receptors (PRRs) that recognize conserved structural motifs derived from microbial products (Janeway and Medzhitov, 2002; Medzhitov and Janeway, 2000). There are at least 11 TLRs in mice and 10 in humans, each with a different specificity. The ligands they recognize include molecules such as lipopeptides (TLR1 and TLR6), zymosan (TLR2), double-stranded RNA (TLR3), lipopolysaccharide (LPS) (TLR4), flagellin (TLR5), single-stranded RNA (TLR7 and TLR8), bacterial DNA (CpG) (TLR9), and a profilin-like protein (TLR11) (Akira and Takeda, 2004; Yarovinsky et al., 2005). These molecules are also collectively referred to as pathogen associated molecular patterns (PAMPs). However there is also evidence that TLRs may recognize endogenous ligands released by damaged or stressed cells (Akira et al., 2001). TLRs are differentially expressed in various cell types, but more importantly, they are expressed on APCs particularly DCs (Degli-Esposti and Smyth, 2005; Reis e Sousa, 2004b). The distribution of TLRs in the different subsets of human and mouse DCs is shown in Table 1. Aside

Table 1

Expression of TLRs among primary human and murine DC subsets

	Human blood		Mouse spleen			
	$CD11c^+$	PDC	CD4+	$CD8\alpha^+$	DN	PDC
TLR1	+++	++	++	++	++	++
TLR2	++	+ / –	++	++	++	++
TLR3	+++	–	+	+++	++	+ / –
TLR4	++	–	+	+	+	+
TLR5	++	+ / –	+++	+	++	++
TLR6	++	+	+++	++	++	++
TLR7	+	++	++	–	+	+++
TLR8	+	–	++	++	++	++
TLR9	–	+++	++	++	++	+++
TLR10	++	+ / –	N/A	N/A	N/A	N/A

Adapted from Reis e Sousa, 2004b

from differential cellular expression, the sub-cellular localization of TLRs also varies. While TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on the cell surface, TLR3, TLR7, and TLR 9 are expressed mainly on endosomes (Takeda and Akira, 2005).

TLRs are Type I integral membrane glycoproteins with an extracellular region containing leucine-rich repeat (LRR) motifs. Their cytoplasmic domain is homologous to that of the IL-1 receptor and they share a conserved region called the Toll/IL-IR (TIR) domain. TLR stimulation induces the recruitment of the TIR-containing cytoplasmic adaptor molecule MyD88, which, through its death domain recruits the serine-threonine kinases IRAK4 and IRAK1. This leads to the association of another adaptor molecule, TRAF6, which activates MAPK kinases (MKKs) and the IKK complex. Ultimately, NF-kb is activated and translocated to the nucleus, where it induces the expression of target genes such as TNF, IL-6, and IL-1β (Akira and Takeda, 2004). The MyD88-dependent pathway is used by all TLRs except for TLR3. Additionally, TLR2 and TLR4 use another TIR-containing adaptor, TIRAP, in the MyD88-dependent pathway (Horng et al., 2002). TLR3 uses the adaptor molecule TRIF in the MyD88-independent induction of IFN-β (Hoebe et al., 2003). Meanwhile, TLR4 uses a fourth adaptor molecule, TRAM, in the MyD88-independent/TRIF-dependent induction of IFN-β (Oshiumi et al., 2003). Both TRIF and TRAM also contain a TIR domain.

TLRs directly mediate innate immune responses by stimulating the release of inflammatory cytokines, phagocytosis and direct microbicidal effects (Akira et al., 2001). However, they also serve as a crucial bridge between innate and adaptive immunity. This is mainly because TLR stimulation directly activates APCs, particularly DCs, and

enhances their ability to induce T cell activation and differentiation (Pasare and Medzhitov, 2005; Reis e Sousa, 2004b; Schwarz et al., 2003). TLR stimulation has been shown to induce the rapid differentiation of monocytes into MΦ and DCs (Krutzik et al., 2005). It also rapidly upregulates the levels of CD80 and CD86, as well as CD40 on DCs (Manickasingham and Reis e Sousa, 2000; Reis e Sousa, 2004b). In addition, TLR stimulation induces the secretion of cytokines such as IL-6, IFN–α, TNF–α, and IL-12 (Edwards et al., 2002). Moreover, TLR stimulation facilitates DC migration by inducing secretion of chemokines and expression of chemokine receptors such as CCR2, CCR5, and CCR7 (Janeway and Medzhitov, 2002; Pasare and Medzhitov, 2005; Reis e Sousa, 2004b). Finally, it promotes cross-priming to exogenous antigens as well as DC cell survival (Datta and Raz, 2005; Datta et al., 2003; Wilson et al., 2006). TLR stimulation also reverses $CD8^+$ T cell tolerance induced by T_{REG} cells (Yang et al., 2004). These effects are similar to those that are induced upon CD40 stimulation.

K. Primary $CD8^+$ T cell responses

After exiting the thymus, naïve $CD8^+$ T cells circulate in peripheral lymphoid organs where they sample antigens presented by DCs. In uninfected mice, the precursor frequency of naïve antigen-specific $CD8^+$ T cells is estimated to be about 1 in 2 x 10^5 cells (Blattman et al., 2002). This translates to about 100-200 cells, and they have a spectrum of affinities for cognate antigen owing to expression of different TCR chains. These cells have a relatively slow turnover rate and they have a half-life of about 30 days (McDonagh and Bell, 1995; Tough and Sprent, 1994).

The generation of a highly effective $CD8^+$ T cell response is largely determined by its interaction with appropriately activated DCs, which present all the signals that are necessary for full activation and differentiation of T cells (Banchereau and Steinman, 1998). Some of the parameters that have been shown to be important in regulating $CD8^+$ T cell activation include the strength and duration of TCR stimulation and the presence of costimulation and inflammation. Upon encounter with antigen, $CD8^+$ T cell responses can be divided into four phases: activation, expansion, contraction, and memory generation. *In vitro*, it has been shown that a short stimulation period (two hours) can induce naïve $CD8^+$ T cells to undergo autonomous clonal expansion and develop into functional effectors (van Stipdonk et al., 2001). However, a four hour stimulation led to abortive $CD8^+$ T cell responses *in vivo* and a longer stimulation (20 hours) was required for full T cell differentiation (van Stipdonk et al., 2003). Using two-photon intravital microscopy, it has been shown that the interaction between antigen-bearing DCs and specific $CD8^+$ T cells in lymph nodes occurs in three distinct stages. The first stage is characterized by multiple transient encounters leading to T cell activation, the second stage is characterized by stable long-lasting contacts that result in cytokine production, and the third stage is characterized by resumption of rapid T cell migration and brief DC contacts coinciding with T cell proliferation (Cahalan and Parker, 2005; Hugues et al., 2004; Mempel et al., 2004).

A number of studies have demonstrated that the generation of primary $CD8^+$ T cell responses requires $CD4^+$T cell help (Behrens et al., 2004; Clarke, 2000; Kalams and Walker, 1998; Wang and Livingstone, 2003). Initially, $CD4^+$ T cells were thought to

provide IL-2 for stimulating $CD8^+$ T cell expansion. However, it is now well established that the $CD4^+$ T cells are important for activation or licensing of APCs so they can efficiently stimulate naïve $CD8^+$ T cells (Smith et al., 2004). APC licensing occurs when CD40L expressed by activated $CD4^+$ T cells binds to and stimulates CD40 on APCs (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998). Nonetheless, the primary $CD8^+$ T cell response against some pathogens including lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus (VSV), and *Listeria monocytogenes* are unimpaired in the absence of $CD4^+$ T cells or CD40 (Andreasen et al., 2000; Clarke, 2000; Hamilton et al., 2001; Ruedl et al., 1999; Shedlock et al., 2003; Whitmire et al., 1999). These pathogens are thought to bypass the need for $CD4^+$ T cell help in part because of their ability to directly activate DCs.

$CD8^+$ T cell activation is accompanied by marked changes in expression of several cell surface receptors. For example, naïve $CD8^+$ T cells have high levels of TCR and CD62L (leukocyte adhesion molecule), have low levels of CD44 (adhesion molecule), and do not express CD25 (IL-2R a chain), CD69 (early activation marker), and CD43 (anti-adhesion molecule). Upon activation, they downregulate TCR and CD62L and upregulate CD25, CD69, and CD43; the timing of expression of the above markers has been shown to correlate with cell division and acquisition of effector function (Oehen and Brduscha-Riem, 1998). Activated $CD8^+$ T cells undergo an intense period of proliferation or clonal expansion wherein their numbers increase up to 10^5-fold (Butz and Bevan, 1998). It has been shown that there is an upper limit to the size of the

effector $CD8^+$ T cell pool and it is controlled by clone-specific regulatory mechanisms (Kemp et al., 2004).

$CD8^+$ T cell expansion is also accompanied by the differentiation of $CD8^+$ T cells into cytotoxic effectors (CTLs) that are able to migrate into non-lymphoid sites of infection or inflammation (Westermann et al., 2001). CTLs are characterized by their ability to secrete IFN–γ and TNF–α, which have direct anti-viral activity and are also able to activate other immune cells. More importantly, they express FasL and secrete lytic granules containing perforin and granzymes. All of these molecules are critical for inducing target cell apoptosis (Griffiths, 1995; Janeway and Travers, 1997; Watanabe-Fukunaga et al., 1992). Perforin forms pores in target cell membranes while granzymes and FasL induce apoptosis through activation of caspases (Henkart and Catalfamo, 2004). The release of cytotoxic molecules by CTLs occurs in a directed manner; therefore CTL killing is very specific (Westermann et al., 2001). Depending on the infection or priming model, the expansion phase of $CD8^+$ T cell responses can last for 7-12 days. Once the antigen has been cleared, the vast majority (> 95%) of effector $CD8^+$ T cells undergo apoptosis (Badovinac and Harty, 2006). This contraction phase is important for preventing immunopathology brought about by excessive T cell activation. It is also important for restoring homeostasis of different T cell populations.

L. $CD8^+$ T cell memory

Those effector $CD8^+$ T cells that survive the contraction phase subsequently give rise to a stable pool of memory cells. Memory T cells are characterized by having the ability to mount a faster and stronger response upon secondary challenge (Farber, 2003).

This is mostly because they have a higher frequency and affinity to their cognate antigen compared to naïve T cells. In addition, they are able to secrete effector cytokines much faster compared to naïve $CD8^+$ T cells (Badovinac et al., 2003; Liu and Whitton, 2005; Zimmermann et al., 1999). Memory $CD8^+$ T cells have a longer lifespan as well as a faster turnover rate compared to naïve T cells (Surh et al., 2006; Tough and Sprent, 1994; Tuma and Pamer, 2002). They are also capable of antigen-independent survival (Murali-Krishna et al., 1999). In general, the number of memory $CD8^+$ T cells that are generated is directly proportional to the number of primary effector cells (Whitmire et al., 2000).

Recently, it was demonstrated that there are two types of memory $CD8^+$ T cells based on phenotype, function, and anatomical location (Jabbari and Harty, 2006; Roberts et al., 2005). Central memory T cells (TCM) are CD62Lhi and CCR7hi and they mainly reside in lymphoid tissues as well as the bone marrow (Mazo et al., 2005). TCM are able to rapidly proliferate upon secondary encounter with antigen however they lack immediate effector functions. On the other hand, Effector memory T cells (TEM) are CD62Llo and CCR7lo and they preferentially home to non-lymphoid tissues. Upon reencounter with antigen, TEM are able to rapidly secrete cytokines and exert cytotoxic function, however they are unable to proliferate extensively. The lineage relationship between TCM and TEM and the exact signals that induce their development are still unclear (Huster et al., 2006; Tough, 2003; Wherry et al., 2003). It has been shown that the strength of stimulation as well as the frequency of antigen specific cells can influence the commitment of memory cells into the TCM or TEM subtypes (Marzo et al., 2005; van Faassen et al., 2005). The initial encounter of naïve $CD8^+$ T cells with antigen has

been demonstrated to induce an instructional program for memory development (Badovinac and Harty, 2006; Kaech and Ahmed, 2001; Wherry and Ahmed, 2004). However full memory differentiation is a gradual process that is accompanied by stable changes in gene expression and takes several weeks to complete (Kaech et al., 2002).

It has long been known that $CD4^+$ T cells are required for the generation of effective $CD8^+$ T cell memory (Bevan, 2004; Bourgeois and Tanchot, 2003). The absence of memory $CD8^+$ T cell activity in mice lacking functional $CD4^+$ T cells was attributed to inefficient priming of naïve $CD8^+$ T cells. However, several studies have demonstrated that $CD4^+$ T cell help is required for the long-term maintenance and secondary expansion of memory $CD8^+$ T cells (Janssen et al., 2003; Masopust et al., 2004; Shedlock and Shen, 2003; Sun and Bevan, 2003; Sun et al., 2004; Williams et al., 2006a). The exact mechanism by which $CD4^+$ T cells promote memory generation and maintenance is still unknown. It is thought that it involves direct CD40-CD40L interactions between $CD4^+$ T cells and $CD8^+$ T cells as well as the induction of antigen-specific Ab in the case of acute virus infections (Andreasen et al., 2000; Bachmann et al., 2004; Borrow et al., 1998; Bourgeois et al., 2002; Thomsen et al., 1998). However, the requirement for $CD4^+$ T cells in long-lasting $CD8^+$ T cell memory is not absolute, as fully functional memory $CD8^+$ T cells can be detected in some systems despite the absence of $CD4^+$ T cells or B cells (Di Rosa and Matzinger, 1996; Marzo et al., 2004).

Various cytokines have been implicated in generation and maintenance of memory $CD8^+$ T cells. For example, inflammatory cytokines such as IL-12 and Type I IFNs have been shown to increase the expansion of effector $CD8^+$ T cells and also

enhance their differentiation into memory cells (Curtsinger et al., 2005; Kolumam et al., 2005; Mescher et al., 2006; Valenzuela et al., 2002). Three members of the γ-chain family of cytokines, IL-2, IL-7, and IL-15 are also important for $CD8^+$ T cell memory. IL-2 signals both during priming and reactivation are required for secondary expansion of memory $CD8^+$ T cells (Blachere et al., 2006; Williams et al., 2006b). Meanwhile, IL-15 is critical in the generation as well as homeostasis of memory $CD8^+$ T cells (Prlic et al., 2002). Finally, the maintenance of memory $CD8^+$ T cells is also dependent on IL-7 (Fry and Mackall, 2005; Prlic et al., 2002). In connection with this, it has been shown that IL-7 receptor (IL-7R) is expressed in memory $CD8^+$ T cell precursors as well as long-lived memory $CD8^+$ T cells (Bachmann et al., 2005; Huster et al., 2004; Kaech et al., 2003).

M. Rationale

Naïve T cells require contact with appropriately activated APCs in order to be primed (Banchereau and Steinman, 1998; Reis e Sousa, 2006; Schuurhuis et al., 2000). CD40-CD40L interactions mediate one of the most effective APC activating signals. Stimulation of CD40 on APCs, particularly on DCs, upregulates expression of costimulatory molecules CD80 and CD86, enhances production of cytokines (most notably IL-12), and promotes cross-priming to exogenous Ag (Cella et al., 1996; Ridge et al., 1998; Schuurhuis et al., 2000). CD40 stimulation is also the mechanism by which $CD4^+$ T cells provide help for the generation of $CD8^+$ T cell responses in a process called APC licensing (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998).

However, APCs can be activated by a number of other stimuli. Recent studies show that DCs and other APCs express TLRs that are able to bind microbial components

such as LPS, CpG, double-stranded RNA and some viral proteins, e.g. respiratory syncytial virus (RSV) fusion protein (Kurt-Jones et al., 2000; Reis e Sousa, 2004b; Takeda and Akira, 2005). Ligation of TLRs with these pathogen associated molecular patterns (PAMPs) induces similar effects as that of CD40 stimulation, e.g., activation of NF-κB, up-regulation of costimulatory molecules, production of cytokines, and promotion of cross-priming (Janeway and Medzhitov, 2002; Pasare and Medzhitov, 2005; Reis e Sousa, 2004b). It has even been shown recently that TLR agonists can abrogate tolerance induced by CD40L-blockade (Thornley et al., 2006). However, it is still not clear whether CD40- stimulated or TLR-stimulated DCs have identical $CD8^+$ T cell priming capability *in vivo*.

Given the different pathways by which DCs can be activated, we re-examined the requirement for CD40-CD40L interaction *in vivo.* We asked what is its natural role in the priming of naïve $CD8^+$ T cell responses in the absence or presence of microbial PAMPs. We also asked in what cells is CD40 expression important and what cells provide CD40L for CD40 stimulation. Finally, we asked whether CD40-CD40L interactions are involved in the generation, maintenance, and recall responses of memory $CD8^+$ T cells.

CHAPTER II.

MATERIALS AND METHODS

A. Mice

C57BL/6J (WT), B6.129P2-*Cd40*^{tm1Kik}/J (CD40$^{-/-}$), and B6.129S2-*Cd40lg*^{tm1Imx}/J (CD40L$^{-/-}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used from 5-10 weeks of age. CD40$^{-/-}$ mice were also obtained from Dr. Dale Greiner (UMass Medical School, Worcester, MA). P-14 and OT-I TCR-Tg breeders were originally obtained from Dr. Raymond Welsh (UMass Medical School) and Dr. Stephen Jameson (University of Minnesota, Minneapolis, MN), respectively. These were bred with C57BL/6-Igh^{a} thy1^{a} Gpi1^{a} mice (Jackson Laboratory) to yield TCR-Tg Thy1.1^{+} T cells. Additionally, P-14 mice were bred with B6.SJL-Ptprc^{a} Pep3^{b}/Boy mice to yield TCR-Tg CD45.1^{+} T cells. Lastly, P-14/Thy1.1^{+} mice were bred with CD40L$^{-/-}$ mice to yield CD40L-deficient, Thy1.1^{+} P-14 T cells. P-14 mice were typed by staining blood cells with Vα2 and Vβ8.1/8.2 Ab while OT-I mice were typed using Vα2 and Vβ5.1/5.2 Ab; samples were analyzed by flow cytometry. All mice were bred and housed in specific pathogen-free conditions at the UMass Medical School animal facility.

B. Generation of Dendritic cells and Macrophages

Bone marrow cells were flushed from the femurs and tibias of the indicated mice and depleted of red blood cells using ACK lysis buffer. The cells were re-suspended in complete media (RPMI-1640 containing 10% FCS, 2mM L-glutamine, 100mM HEPES, 100μM nonessential amino acids, 1X antibiotic/antimycotic and β-mercaptoethanol;

Gibco, Grand Island, NY) and plated in non-tissue culture treated Petri dishes. After an overnight incubation at 37°C in a 5% CO_2 incubator, the non-adherent progenitor cells were collected, washed, and re-suspended in complete media at a concentration of 0.5 x 10^6 cells/mL.

To make DCs, 7.5 x 10^6 progenitors were cultured in complete media containing 10 ng/mL GM-CSF and 5 ng/mL IL-4 (Corixa, Seattle, WA). To make macrophages (MΦ), the same number of progenitors were instead cultured in complete media containing 10% M-CSF. Supernatant from confluent DAP cell cultures was used as the source of M-CSF. The cells were cultured in 100 mm tissue culture dishes and fresh cytokines were added on day 4. Cells were harvested on day 7; by this time the DC plates contained adherent and non-adherent cells while the MΦ plates contained only adherent cells.

In vivo generated DCs were obtained by first injecting the indicated mice s.c. with B16 tumor cells that secrete FLt-3 ligand (Flt-3L). After 10-14 days, spleens were harvested and at this point, their cellularity had increased up to 7-fold and they contained 25-50% $CD11c^+$ cells (N418; BD Pharmingen, San Diego, CA). To determine the DC subset composition, the cells were stained with Ab against CD11b (M1/70), CD8 (53-6.7), B220 (RA3-6B2), and a pan-NK marker (DX5).

DCs and MΦ were pulsed with the minimal immunodominant MHC class I epitope from LCMV glycoprotein (KAVYNFATC; gp33 peptide) (Hudrisier et al., 1997) or chicken ovalbumin (SIINFEKL; OVA peptide) (Rotzschke et al., 1991) at a concentration of 1 μg per 5 x 10^6 cells for 2-4 hours at 37°C. When wild type and CD40-

deficient DCs were pulsed with OVA peptide under these conditions they had the same levels of SIINFEKL-K^b complexes as quantified with the 25D1 monoclonal Ab (mAb). In some experiments, DCs and MΦ were also pulsed with different concentrations of OVA-conjugated Biomag beads.

For the experiments looking at the role of TLR stimulation, 1 μg purified LPS (obtained from Eicke Latz and Tom Thornley, UMass Medical School), 5 μg CpG 2395 (Coley Pharmaceutical Group, Ontario, Canada), or 5 μg polyI:C (Amersham, Piscataway, NJ) was added per 5 x 10^6 cells during peptide pulsing. The cells were washed once with complete media and twice with Hanks' Balanced Salt Solution (HBSS; Invitrogen/GIBCO, Carlsbad, CA) prior to immunization.

C. Phenotypic analysis of APCs

Prior to their use, DCs and MΦ were stained with different combinations of the following Ab: FITC-anti-CD80 (16-10A1), PE-anti-CD86 (PO3.1), PE-anti-IA^b (AF6-120.1), FITC-anti-H-$2K^b$ (AF6-88.5), PE-anti-IL-12 p40 (C15.6), and PE-anti-CD40 (HM40-3). The cells were also stained with 25D1 Ab to quantify the amount of SIINFEKL-K^b complexes. The cells were stained in the presence of 2.4G2 supernatant to block Fc receptors. Flow cytometry was done using a FACSCalibur (BD Biosciences, Mountain View, CA) and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

D. Cell lines

The fibroblast cell line L cell (DAP) transfected with full-length OVA and TfR-OVA fusion constructs were made by Lianjun Shen and have been described previously (Shen and Rock, 2004). Briefly, DAP cells were transfected with the indicated constructs

using FuGene6 (Roche Diagnostics, Indianapolis, IN) and cloned by limiting dilution. The C57BL/6 derived B16 tumor cells expressing Flt-3L (B16-Flt3L) were obtained from Dr. Ulrich von Andrian (CBR, Harvard Medical School, Boston, MA). These cells were originally made in the lab of Dr. Glen Dranoff by retroviral-mediated gene transfer into B16-F10 melanoma cells (Mach et al., 2000). The gene for murine Flt-3L was cloned into the pMFG vector. B16 melanoma cells stably expressing OVA (MO5) have also been previously described (Falo et al., 1995).

E. Analysis of T cell responses in vitro

Spleen and lymph node (LN) cells from the indicated TCR-Tg mice were depleted of red blood cells and re-suspended in complete media. They were then plated in 96-well tissue culture plates and mixed with WT or $CD40^{-/-}$ APCs that have been pulsed with the indicated Ag (peptide or OVA-beads). The cells were cultured in triplicates at an E:T ratio of 1:1. They were then incubated at 37°C in a 5% CO_2 incubator for 1-3 days. ^{3}H-Thymidine was added to the wells during the last 16-20 hours of culture, and T cell proliferation was measured by determining the amount of ^{3}H-Thymidine incorporation into DNA.

F. Adoptive transfer

Spleen and LN cells from P-14/$Thy1.1^{+}$, P-14/$CD45.1^{+}$, P-14/$Thy1.1^{+}$/$CD40L^{-/-}$, or OT-I/$Thy1.1^{+}$ mice that have been depleted of red blood cells were labeled with 1 μM carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) for 10-20 min at 37°C. After two washes with HBSS, 2 $x10^{6}$ total cells containing 30-50% TCR-Tg T cells were injected i.v. into the indicated hosts.

In experiments comparing WT and CD40L-deficient P-14 T cells, the proportion of TCR-Tg T cells was first determined by FACS so that identical numbers of TCR-Tg T cells could be injected into hosts.

In experiments analyzing the effect of precursor frequency on $CD8^+$ T cell responses, decreasing numbers of spleen and LN cells from OT-1 mice, starting from 1 $x10^6$ cells, were transferred into the indicated hosts.

G. Immunizations

One day after adoptive transfer, hosts were immunized i.v. with one of the following: a 13-mer peptide containing the LCMV gp33 epitope (KAVYNFATCGIFA; LCMV 13-mer), peptide-pulsed APCs, or OVA-transfected cells. The number of APCs and the amount of peptide injected into mice were ones that gave reproducibly strong but not maximal responses. Non-immunized mice or mice immunized with APCs that were not pulsed with Ag were used as negative control.

In some experiments 30-50 μg LPS, 100 μg CpG 2395, 50 μg polyI:C, or 25-100 μg agonistic anti-CD40 mAb (FGK45; Bioexpress, West Lebanon, NH) was injected i.p. per mouse at the time of peptide immunization. In some experiments hosts deficient in $CD4^+$ T cells and/or $NK1.1^+$ cells were used. $CD4^+$ T cells were depleted with anti-CD4 mAb (GK1.5; Taconic, Germantown, NY, Bioexpress or obtained from Dr. Dale Greiner, UMass Medical School), which was injected i.p. two consecutive days (150 μg/dose) prior to adoptive transfer. This consistently resulted in greater than 99% $CD4^+$ T cell depletion as verified by flow cytometry. $NK1.1^+$ cells were depleted with anti-NK1.1 Ab (obtained from Dr. Dale Greiner, UMass Medical School), which was injected i.p. one

day before adoptive transfer. This resulted in greater than 99% NK1.1$^+$ cell depletion. In some experiments, hosts that received the anti-CD40L Ab MR1 (obtained from Dr. Dale Greiner, UMass Medical School) were also used.

H. Analysis of TCR-Tg CD8$^+$ T cell responses

Except when indicated, spleens and LN were harvested on day 4 since the peak of the responses occurred at this time point. Single-cell suspensions were then stained with different combinations of the following Ab: PerCP-anti-CD8 (53-6.7; BD Pharmingen), APC-anti-Thy1.1 (HIS51), APC-anti-CD45.1 (A20; eBioscience), PE-anti-CD62L (MEL-14), PE-anti-CD44 (IM7), PE- or APC-anti-CD127 (A7R34), PE-anti-CD43 (1B11), PE-anti-CD25 (PC61.5). Samples were analyzed by flow cytometry. The Ab were purchased from either BD Pharmingen or eBioscience (San Diego, CA).

I. Intracellular cytokine staining

Spleen and LN cells were incubated with the indicated peptide in the presence of Brefeldin A (Golgi Plug; BD Pharmingen) and recombinant IL-2 for 5 hours at 37°C. The samples were stained with anti-CD8 and anti-Thy1.1 or anti-CD45.1 (A20) Ab, fixed and permeabilized using Cytofix/Cytoperm buffer (BD Pharmingen), and stained with anti-cytokine Ab diluted in Perm/Wash buffer (BD Pharmingen) according to manufacturer's instructions. Anti-IFN-γ (XMG1.2), anti-TNF-α (MP6-XT22), and anti-IL-2 (JES6-5H4) were purchased from BD Pharmingen or eBioscience. The samples were washed twice with Perm/Wash buffer and analyzed by flow cytometry.

J. In vivo CTL assay

The *in vivo* CTL assay was performed as described previously (Barber et al., 2003). Briefly, splenocyte targets were pulsed with relevant or irrelevant peptide and labeled with different concentrations of CFSE. The targets were then mixed at a 1:1 ratio and injected i.v. into immunized and unimmunized control mice. After 2-20 hrs spleens, LN, or blood samples were collected and analyzed by flow cytometry. The % target cell killing was calculated using the formula: 100 − {[(% rel. peptide-pulsed in immunized/% irrel. peptide-pulsed in immunized)/(% rel. peptide-peptide pulsed in control/% irrel. peptide-pulsed in control)] × 100}.

K. Assessment of endogenous $CD8^+$ T cell responses

In one set of experiments, WT, $CD40^{-/-}$, and $CD40L^{-/-}$ mice were immunized s.c. on one flank with 4-5 x 10^6 TfR-OVA cells. One week later, the mice were given peptide-pulsed target cells for an *in vivo* CTL assay.

In a second set of experiments, WT mice were immunized i.v. with 1 x 10^6 WT or $CD40^{-/-}$ DCs pulsed with OVA peptide. On day 7, some mice received targets for an *in vivo* CTL assay while some mice were sacrificed for *in vitro* re-stimulation of spleen cells. In some of the experiments, $CD4^+$ T cell-depleted hosts were used.

L. In vitro re-stimulation

Splenocytes from DC-immunized mice were depleted of red blood cells and plated at 5 x 10^6 cells per well in a 24-well plate and stimulated with 1 μg OVA peptide. After 4 to 6 days, OVA-specific $CD8^+$ T cell responses were evaluated by intracellular IFN–γ staining.

M. Tumor protection

WT, CD40$^{-/-}$, and CD40L$^{-/-}$ mice immunized 10-14 days previously with TfR-OVA cells were inoculated with 2 x 10^6 MO5 cells s.c. Tumor growth was monitored twice a week until the tumors reached a diameter of 2 cm, after which the mice were sacrificed.

N. Analysis of CD40L expression by CD8$^+$ T cells

Splenocytes from WT or CD40L-deficient P-14 mice were incubated with 1μg gp33 peptide or PMA/Ionomycin. Cells were collected every two hours starting from the beginning of culture and stained with PerCp-anti-CD8, FITC-anti-CD4 (RM4-5), and PE-anti-CD69 (H1.2F3) Ab. The cells were then washed and permeabilized with CytoFix/Cytoperm buffer, and then stained with biotinylated anti-CD40L (MR1) Ab followed by Steptavidin-APC. Samples were analyzed by flow cytometry.

O. Adoptive transfer and virus infection

Spleen and LN cells from P-14/CD45.1$^+$ mice were depleted of red blood cells and labeled with 1 μM CFSE (Molecular Probes) for 10-20 min at 37°C. After two washes with HBSS, 2 x10^6 total cells containing 30-50% TCR-Tg T cells were injected i.v. into WT or CD40$^{-/-}$ hosts. One day later, the mice were infected i.p. with 1 x 10^5 pfu of LCMV (Armstrong strain). P-14 responses were monitored in the blood at the indicated time points. Samples were depleted of red blood cells and then stained with different combinations of the following Ab: PerCP-anti-CD8, PE- or allophycocyanin-anti-CD45.1, Annexin V-PE (BD Pharmingen), APC-anti-CD127, PE-anti-CD44, and PE-anti-CD62. Samples were analyzed by flow cytometry.

P. Assessment of memory CD8$^+$ T cell maintenance

Spleens from WT and CD40$^{-/-}$ mice containing P-14 cells were collected 11-13 days after LCMV infection. Single cell suspensions were then depleted of red blood cells, labeled with CFSE, and injected i.v. into new, uninfected WT and CD40$^{-/-}$ hosts. The proportion of CD8$^+$/CD45.1$^+$ P-14 T cells from the donor spleens was determined by FACS prior to adoptive transfer to ensure that the hosts received similar number of P-14 T cells. Host mice were bled at the indicated time points and stained with anti-CD8 and anti-CD45.1 Ab to determine the number of P-14 cells remaining.

Q. Assessment of memory CD8$^+$ T cell function

WT and CD40$^{-/-}$ hosts containing memory P-14 cells were challenged i.p with 5 x 10^6 pfu of vaccinia virus expressing the LCMV glycoprotein (Vac-gp). Memory responses were evaluated by looking at the expansion and IFN-γ production of the P-14 cells on day 4 post-challenge.

WT mice containing memory P-14 cells were also challenged i.v. with WT or CD40$^{-/-}$ DCs pulsed with the gp33 peptide. Again memory P-14 expansion and IFN-γ production were analyzed on day 4 post-challenge.

R. DC immunization and assessment of endogenous CD8$^+$ T cell memory

WT mice were immunized i.v. with 1 x 10^6 WT or CD40$^{-/-}$ DCs pulsed with OVA peptide. In some experiments the mice were depleted of CD4$^+$ T cells with the GK1.5 Ab (Bioexpress) during immunization. After at least 6 weeks, the mice were challenged i.v. with 1 x 10^6 OVA peptide-pulsed WT or CD40$^{-/-}$ DCs. Memory responses were evaluated on day 4 post-challenge through *in vivo* CTL assay and intracellular IFN-γ staining.

S. Statistical analysis

Data for the primary $CD8^+$ T cell responses were analyzed for statistical significance with a two-tailed Student's *t* test using Microsoft Excel software. Differences in T cell responses were considered significant when a probability value of $p < 0.05$ was obtained. Data for the memory $CD8^+$ T cell responses were analyzed for statistical significance by performing an analysis of variance with a mixed model.

CHAPTER III.

CD40 SIGNALING IS IMPORTANT FOR INDUCTION OF MAXIMUM PRIMARY CD8$^+$ T CELL RESPONSES

Most of the previous studies showing a role for CD40 in APC activation and CD8$^+$ T cell responses *in vivo* relied on exogenous stimulation of the receptor with agonistic Ab (Bonifaz et al., 2002; Diehl et al., 1999; French et al., 1999; Lefrancois et al., 2000; van Mierlo et al., 2004). Two major limitations of these studies are the non-physiologic nature of Ab-mediated stimulation and the possibility of non-specific effects because of the numerous cell types that can express CD40. We therefore took the opposite approach and examined the priming of TCR-Tg CD8$^+$ T cells upon adoptive transfer into CD40-deficient animals. In this system, all APCs lack CD40 while the responding T cells express both CD40 and CD40L. In addition, we used peptide or transfected cells as Ag instead of viruses or bacteria; this is to avoid the potential complication of TLR stimulation, which might bypass a CD40 requirement.

RESULTS

A. Reduced P-14 T cell accumulation and effector function in CD40$^{-/-}$ hosts

We injected WT B6 and CD40$^{-/-}$ hosts with cells from the spleen and lymph nodes of P-14 mice, whose CD8$^+$ T cells express a transgenic TCR that recognize the LCMV gp33 peptide bound to H-2D^b (Fig. 1). In addition, we labeled the cells with CFSE, which is a dye that gets equally divided into daughter cells upon cell division and is therefore a

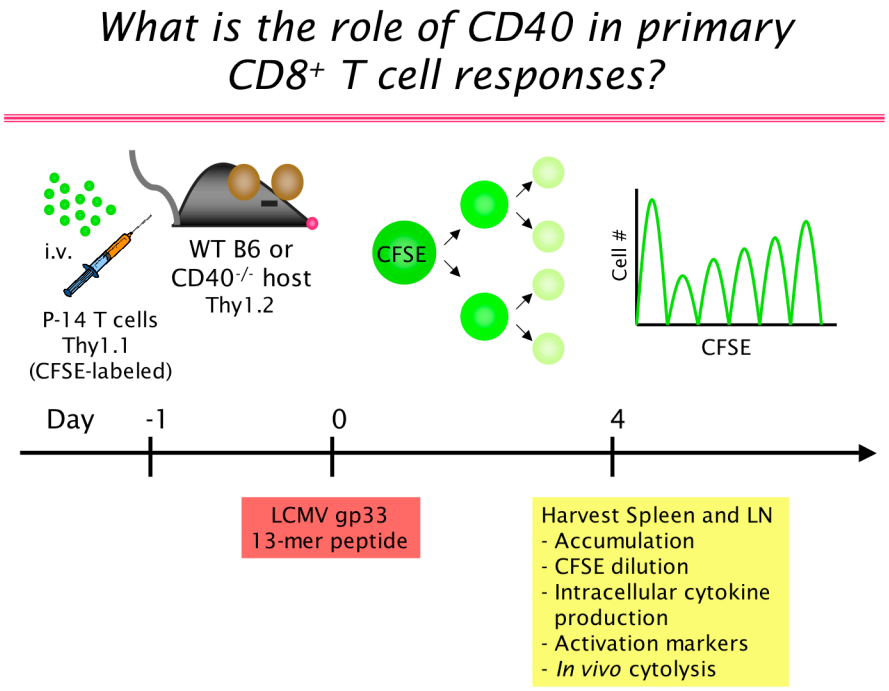


Figure 1. Experimental set-up for examining the role of CD40 expression on host APCs in primary CD8$^+$ T cell responses. *WT and CD40$^{-/-}$ hosts were adoptively transferred with CFSE-labeled spleen and lymph node cells from P-14 TCR-Tg mice and immunized with a 13-mer peptide containing the LCMV gp33 epitope. Spleens and lymph nodes were harvested on day 4 and examined for the above parameters. In this system all the host APCs are either expressing or not expressing CD40.*

useful tool for monitoring proliferation. One day later we immunized the hosts i.v. with a 13-mer peptide containing the minimal MHC class I epitope from LCMV gp33 (LCMV 13-mer). This Ag requires cross-presentation by host APCs in order to stimulate naïve T cells even without adjuvant (Ciupitu et al., 1998). On day 4 post-immunization, more than 95% of the P-14 T cells in both the WT and $CD40^{-/-}$ hosts had divided, as evidenced by the dilution of CFSE (Fig. 2B). The P-14 T cells in the WT hosts made up ~24% and ~15% of the total $CD8^+$ T cells in the spleen and LN, respectively (Fig. 2C). In contrast, the P-14 T cells in the $CD40^{-/-}$ hosts only comprised ~10% of the splenic and ~5% of LN $CD8^+$ T cells. When compared to the unimmunized control mice, there was 12-fold vs. 5-fold expansion of T cells in the spleens, and 7-fold vs. 3-fold expansion in the LN of WT and $CD40^{-/-}$ hosts, respectively.

We next examined whether the transgenic T cells became functional effectors by assaying for cytokine secretion. The percentage of P-14 T cells making IFN-γ in the spleen and LN of $CD40^{-/-}$ hosts is ~10-fold less than in the WT hosts (Fig. 2D). The same difference was observed for TNF-α and IL-2 production (data not shown).

B. Reduced numbers of P-14 T cells remaining in $CD40^{-/-}$ hosts at the end of the response

The effector $CD8^+$ T cell population undergoes extensive contraction upon resolution of the response. By day 12 post-immunization, the number of P-14 T cells had decreased significantly, making up 1.7% vs. 0.4% of $CD8^+$ T cells in the spleen and 0.5% vs. 0.1% of $CD8^+$ T cells in the LN of the WT and $CD40^{-/-}$ hosts, respectively (Fig. 3A). These data indicate that up to 98% of the effector cells at the peak of the response

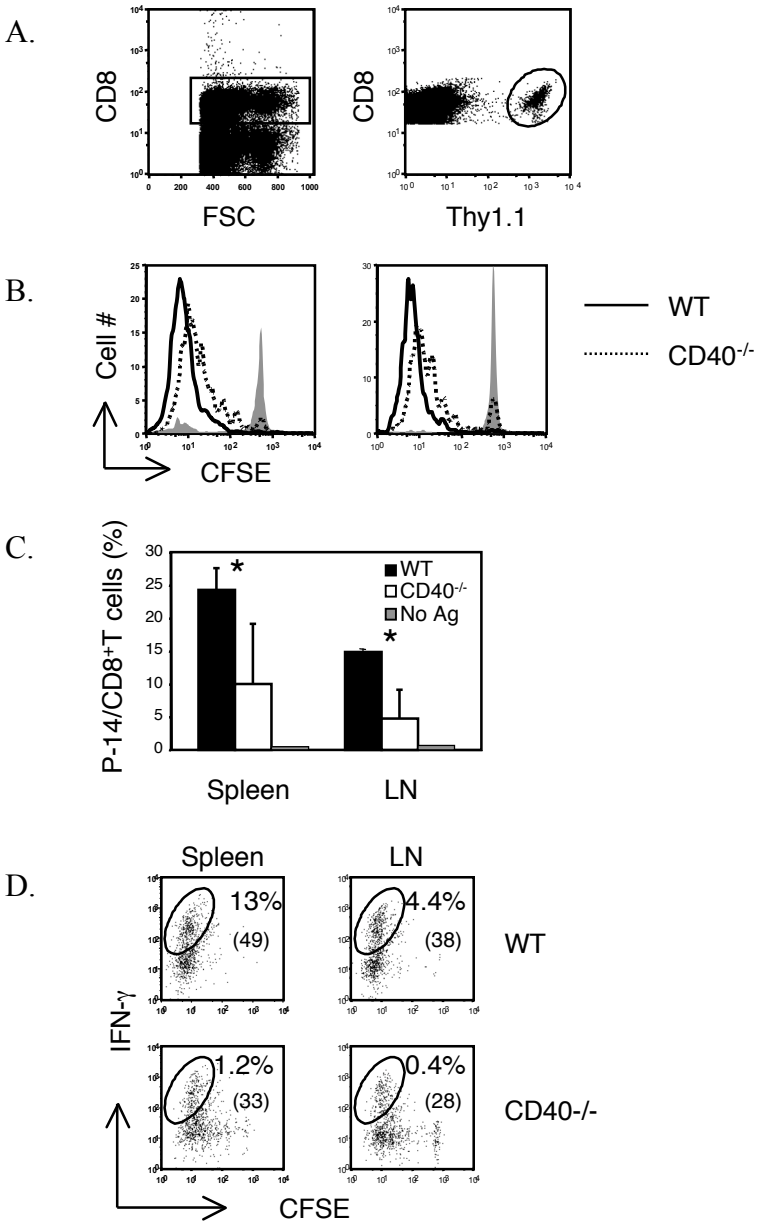


Figure 2. Reduced P-14 T cell response in CD40$^{-/-}$ hosts on day 4. *WT and CD40$^{-/-}$ hosts containing adoptively transferred CFSE-labeled Thy1.1^{+} P-14 T cells were immunized i.v. with 5 μg LCMV 13-mer peptide or left unimmunized. Four days later, spleens and lymph nodes were harvested, stained with anti-CD8 and anti-Thy1.1 Ab, and analyzed by*

*flow cytometry. (A) Gating strategy. (B) CFSE profiles of transferred T cells from representative mice. The shaded histogram represents P-14 T cells in an unimmunized mouse. (C) Overall accumulation of P-14 T cells was determined by calculating the percentage of Thy1.1$^+$ cells in the total CD8$^+$ T cell population. The data are presented as mean $^+$ SD. (D) Effector function was assayed by looking at IFN-γ production after a 5 hr incubation with gp33 peptide in the presence of Brefeldin A (GolgiPlug). Representative FACS plots gated on Thy1.1$^+$ P-14 T cells are shown. The numbers above indicate the percentage of IFN-γ-secreting P-14 T cells in the total CD8$^+$ T cell population while the numbers in parenthesis indicate the percentage of P-14 T cells secreting IFN-γ. In unimmunized mice the frequency of IFN-γ producing P-14 T cells was typically < 0.1%. The results shown are representative of three independent experiments with two to three mice per group. *, $p < 0.05$.*

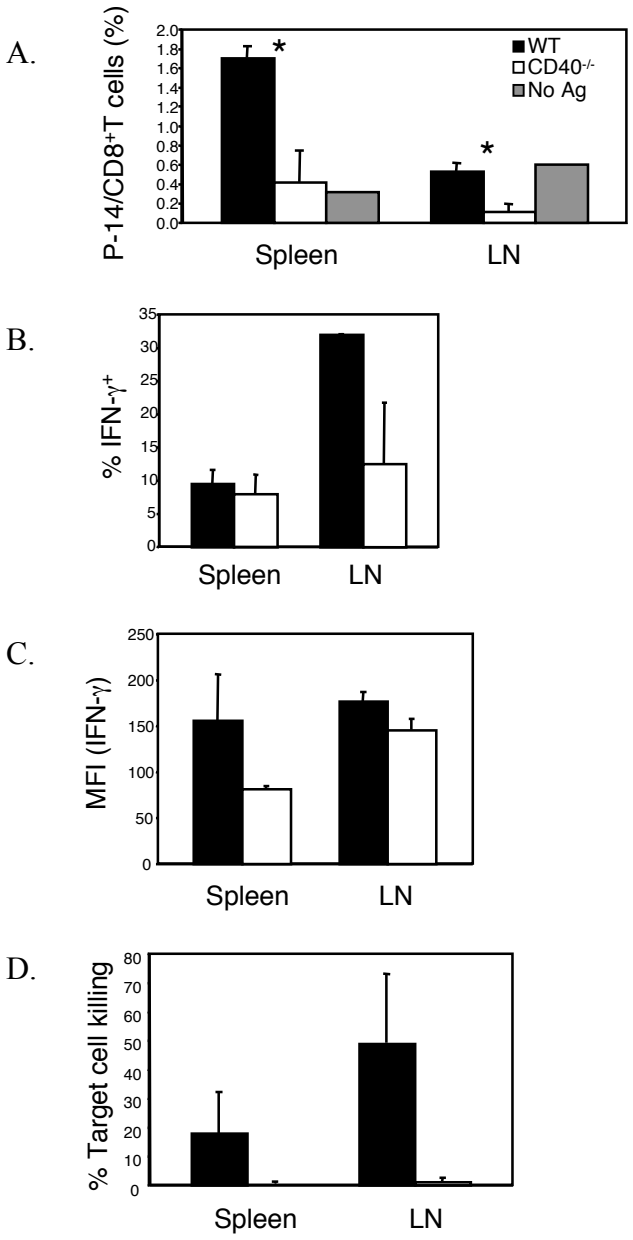


Figure 3. Reduced P-14 T cell numbers in CD40$^{-/-}$ hosts on day 12. *WT and CD40$^{-/-}$ hosts containing adoptively transferred CFSE-labeled Thy1.1^{+} P-14 T cells were immunized as in Fig. 1 and responses were analyzed in the spleen and lymph nodes on day 12. (A) Percentage of P-14 T cells in the total CD8^{+} T cell population. (B)*

*Percentage of IFN−γ^+ P-14 T cells. (C) MFI of IFN−γ staining. (D) In vivo CTL activity. Immunized mice were injected with a 1:1 mixture of gp33 peptide-pulsed and OVA peptide-pulsed splenocytes. The data are presented as the mean + SD of specific target killing. The results shown are representative of two independent experiments with two to three mice per group. *, $p < 0.05$.*

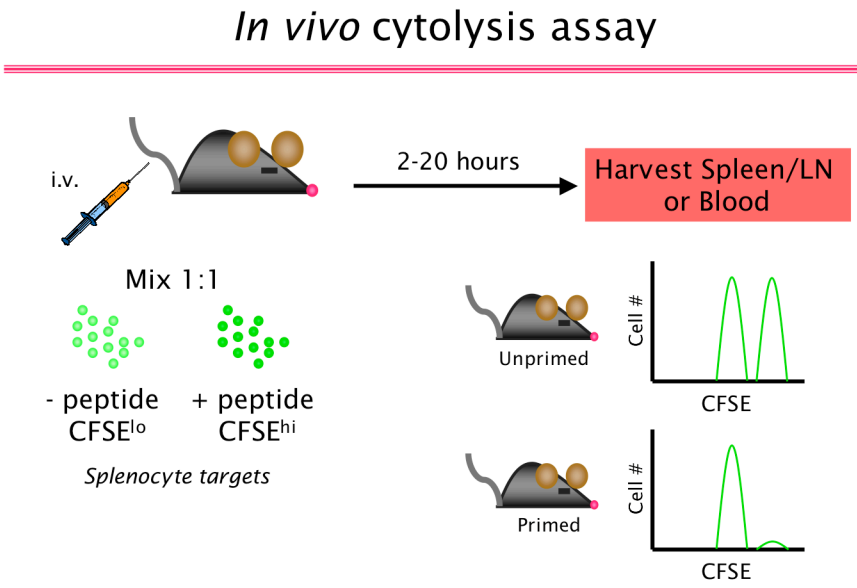


Figure 4. In vivo cytolysis assay. *Splenocyte targets are pulsed with relevant peptide while control targets are pulsed with irrelevant peptide or left unpulsed. The targets are then labeled with different concentrations of CFSE, mixed at a 1:1 ratio, and injected i.v. into experimental animals. In unprimed animals, there is no antigen-specific killing and the proportion of* $CFSE^{hi}$ *and* $CFSE^{lo}$ *cells is equal. In contrast, the* $CFSE^{hi}$ *cells will be reduced or disappear altogether in animals that have been primed.*

have died. Interestingly, because the P-14 T cells expanded to a greater extent in the WT hosts, the number of P-14 T cells that remained at the end of the response was also greater compared to the $CD40^{-/-}$ hosts. This implies that the greater the magnitude of the primary response, the greater the number of memory cells that can be possibly formed.

While the percentage of IFN–γ^+ P-14 T cells in the spleens of WT and $CD40^{-/-}$ hosts was equal, there were more IFN–γ producing P-14 T cells in the LN of the WT hosts (Fig. 3B). Moreover, the P-14 T cells in the WT hosts secreted higher levels of IFN–γ (Fig. 3C). Furthermore, when we examined *in vivo* CTL activity (Fig. 4), we found that the P-14 T cells in the spleens and LN of the WT hosts still exhibited significant target cell killing (Fig. 3D). In contrast, we did not observe target cell killing in either the spleen or LN of the $CD40^{-/-}$ hosts (Fig. 3D).

C. Reduced OT-I T cell accumulation and effector function in $CD40^{-/-}$ hosts

We also tested the response of adoptively transferred OT-I TCR-Tg T cells, which are specific for the OVA peptide SIINFEKL bound to H-$2K^b$, to make sure that the effects we observed were not confined to the P-14 TCR-Tg T cells. Instead of peptide, we immunized the hosts i.v. with a stable OVA-transfected cell line that also gets cross-presented by host DCs (Shen and Rock, 2004; Fig. 5). On day 4 of the response, almost all of the OT-I T cells in both the WT and $CD40^{-/-}$ hosts had divided more than 8 times (data not shown). However, the OT-I T cells accumulated to a lesser extent in the spleens and LN of $CD40^{-/-}$ compared to the WT hosts (Fig. 6A). The proportion, as well as the absolute number of IFN-γ-secreting cells, was also reduced by as much as 50% in the $CD40^{-/-}$ hosts (Fig. 6B and data not shown). Furthermore, the killing of SIINFEKL-

pulsed target cells was reduced in the $CD40^{-/-}$ hosts compared to the WT hosts (94% vs. 56%; Fig. 6C).

Upon s.c. immunization of the OVA-transfectants, the main difference we observed was that the OT-I response was greater in the draining LN compared to the spleen. There was still a similar reduction in the accumulation as well as IFN-γ production of OT-I cells in $CD40^{-/-}$ hosts (Fig. 7A and 7B). Surprisingly however, there was comparable *in vivo* CTL activity between the WT and $CD40^{-/-}$ hosts (Fig. 7C).

Altogether, these results demonstrate that although CD40 on APCs is not absolutely required to initiate naïve $CD8^{+}$ T cell priming, it is important in inducing T cells to undergo maximum expansion and differentiation into effectors.

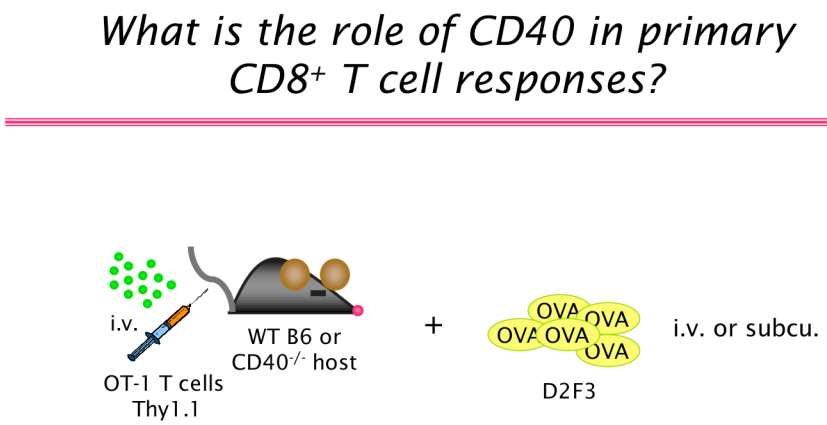


Figure 5. Experimental set-up to determine the role of CD40 on APCs in the induction of primary OT-I T cell responses. *WT and CD40$^{-/-}$ hosts were adoptively transferred with CFSE-labeled cells from the spleens and lymph nodes of OT-I mice and immunized with OVA-transfected cells either i.v. or s.c.. In this system all host APCs are either expressing or not expressing CD40.*

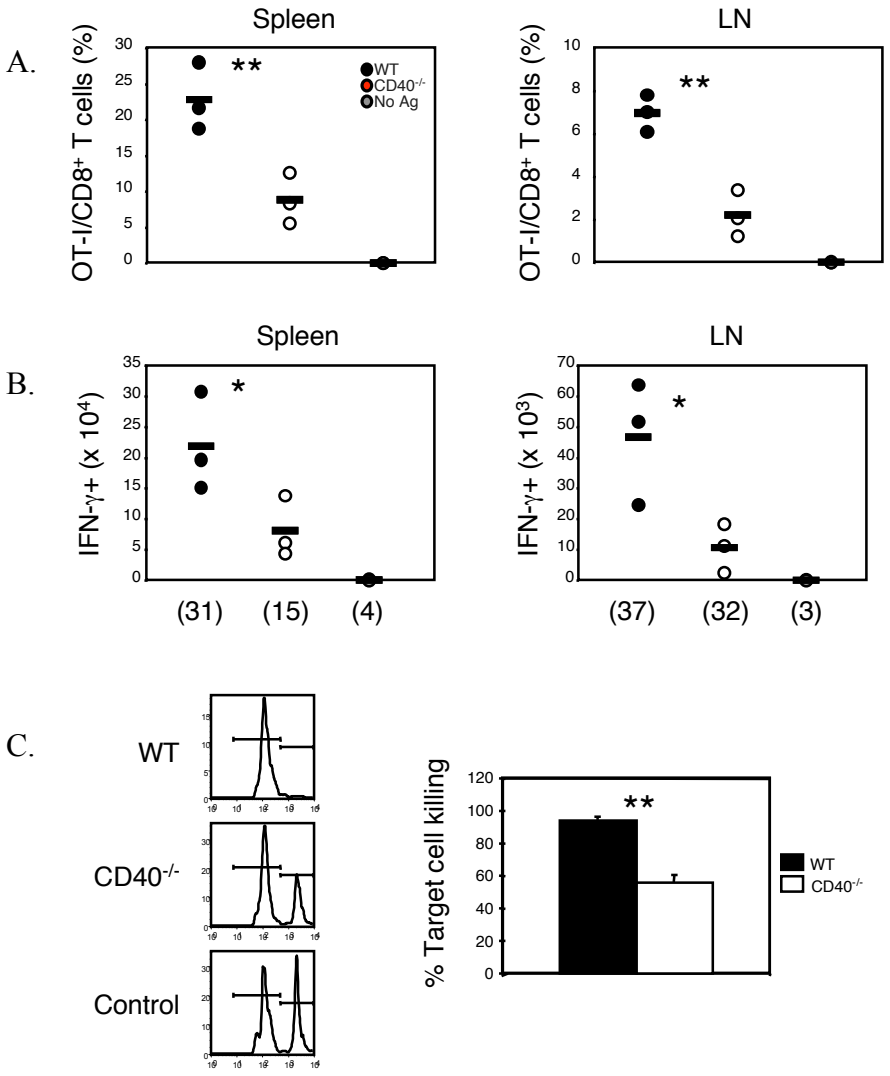


Figure 6. Reduced OT-I T cell response in CD40$^{-/-}$ hosts after i.v. immunization. *WT and CD40$^{-/-}$ hosts containing adoptively transferred CFSE-labeled Thy1.1^{+} OT- I T cells were immunized i.v. with 1 x 10^{6} OVA-transfected cells or left unimmunized. Spleens and lymph nodes were harvested on day 4 and analyzed as in Fig. 1. (A) Percentage of OT-1 T cells in the total CD8^{+} T cell population. (B) Spleen and lymph node cells were stimulated for 5 hours in vitro with OVA peptide. The absolute number of IFN-γ*

*producing OT-I T cells is shown. Each circle in (A) and (B) represents an individual mouse; the lines represent the means. The numbers in parenthesis below indicate the percentage of OT-I T cells secreting IFN-γ. (C) In vivo CTL activity in the blood. Immunized mice were injected with a 1:1 mixture of OVA peptide-pulsed and gp33 peptide-pulsed splenocytes. The data are presented as the mean + SD of specific target killing. Representative histograms are also shown. The results shown are representative of three independent experiments with three mice per group. *, $p < 0.05$; **, $p < 0.01$.*

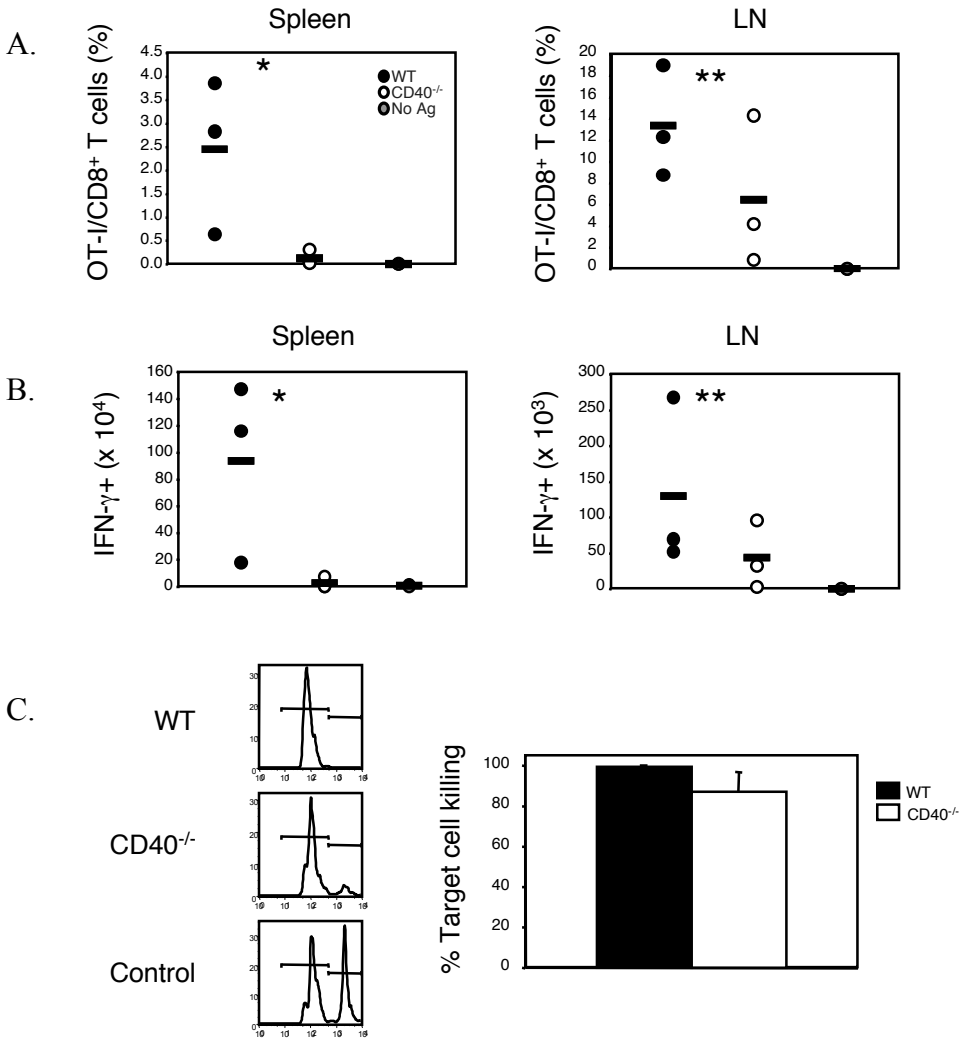


Figure 7. Reduced OT-I T cell response in CD40$^{-/-}$ hosts after s.c. immunization. *WT and CD40$^{-/-}$ hosts containing adoptively transferred CFSE-labeled Thy1.1^{+} OT- I T cells were immunized s.c. with 1 x 10^{6} OVA-transfected cells or left unimmunized. Spleens and lymph nodes were harvested on day 4 and analyzed as in Fig. 1. (A) Percentage of OT-1 T cells in the total CD8^{+} T cell population. (B) Absolute number of IFN-γ producing OT-I T cell. Each circle in (A) and (B) represents an individual mouse; the lines represent the*

*means. (C) In vivo CTL activity in the blood. The results shown are representative of three independent experiments with three mice per group. *, $p < 0.01$; **, $p < 0.05$.*

CHAPTER IV.

CD40 SIGNALING ON APCs, PARTICULARLY DCs, IS IMPORTANT FOR INDUCING MAXIMUM PRIMARY $CD8^+$ T CELL RESPONSES

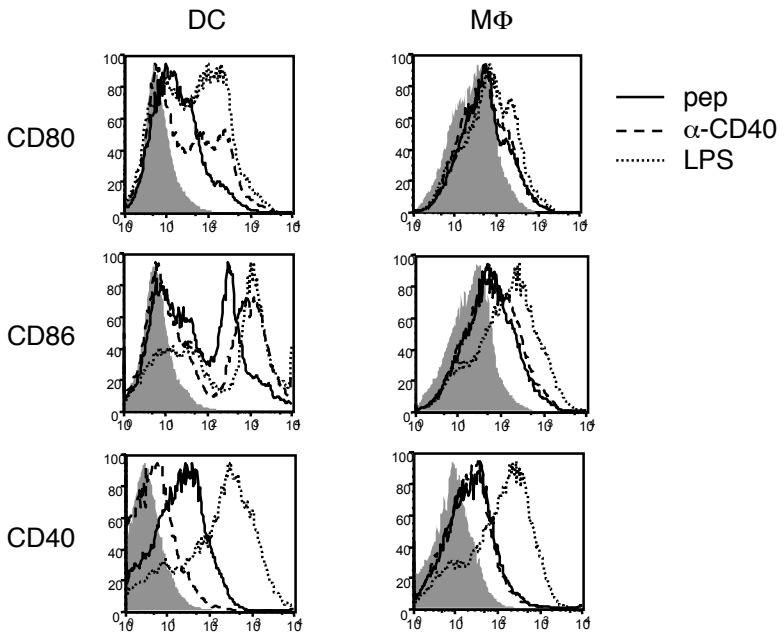
The preceding experiments examined T cell responses in which all APCs in the host are either expressing or not expressing CD40. DCs are thought to be the most potent APC for inducing naïve T cell responses (Banchereau and Steinman, 1998), however our lab has shown previously that MΦ are also capable of stimulating naïve $CD8^+$ T cells *in vivo* (Pozzi et al., 2005). Therefore we sought to investigate how CD40 signaling affects the ability of these APCs to prime naïve $CD8^+$ T cells.

RESULTS

A. Ability of CD40-stimulated APCs to activate naïve $CD8^+$ T cells in vitro

We first obtained bone marrow derived DCs and MΦ from WT (CD40-sufficient) mice and analyzed their phenotype upon stimulation with LPS, a classical APC activator, or anti-CD40 Ab. Both LPS and anti-CD40 Ab were able to induce up-regulation of the costimulatory molecules CD80 and CD86 on DCs (Fig. 8A). However, only LPS was able to induce CD80 and CD86 up-regulation on MΦ (Fig. 8A). LPS stimulation resulted in increased levels of CD40 on the surface of both DCs and MΦ (Fig. 8A). We then pulsed the APCs with OVA-conjugated beads and used them to stimulate OT-I T cells *in vitro*. DCs stimulated with LPS or anti-CD40 Ab induced much higher levels of T cell proliferation compared to DCs containing Ag alone (Fig. 8B). Addition of MR1 Ab,

A.



B.

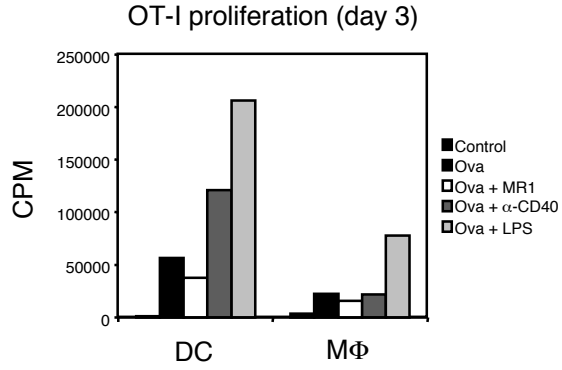


Figure 8. Phenotype of DCs and MΦ. *(A) Bone marrow derived DCs and MΦ from WT mice were incubated overnight with peptide +/- LPS or agonistic anti-CD40 Ab. The cells were then stained with Ab against CD80, CD86, and CD40 to analyze their phenotype. (B) BMDCs and MΦ were pulsed with 5 μg OVA-beads overnight and used to stimulate OT-I T cells in vitro. 1 μg LPS, 5 μg anti-CD40 Ab, or 5 μg MR1 Ab was added*

to some of the wells. T cell proliferation was measured on day 3 of culture. APCs pulsed with control beads were used as negative control. The results shown in (B) are representative of two independent experiments with each sample done in triplicate.

which blocks CD40L, to the DC/T cell cultures resulted in lower proliferation. On the other hand, MΦ in general induced lower T cell proliferation compared to DCs. However, while T cell proliferation increased upon stimulation of MΦ with LPS, there was no change in proliferation when the MΦ were stimulated with anti-CD40 Ab (Fig. 8B). Nevertheless, addition of MR1 Ab to the MΦ/T cell cultures also resulted in lower levels of proliferation compared to Ag alone (Fig. 8B). This suggests that the MΦ respond to CD40 stimulation but that the T cells are able to provide as much CD40L as needed. Thus, while the T cell response did not increase with anti-CD40 Ab, it was reduced when CD40L was blocked. We obtained similar results when the APCs were pulsed with either 5 μg or 1 μg of OVA-beads (data not shown). These results indicate that DCs are more responsive to CD40 stimulation compared to MΦ.

B. Ability of CD40-deficient APCs to activate naïve CD8$^+$ T cells in vitro

To determine whether different APCs have an absolute requirement for CD40 in order to induce naïve T cell responses, we next examined bone marrow derived DCs and MΦ from CD40$^{-/-}$ mice. Upon stimulation with LPS, WT and CD40$^{-/-}$ DCs had similar levels of CD80, CD86, and H-2K^b; the same was true for WT and CD40$^{-/-}$ MΦ (Fig. 9A). As expected, stimulation with anti-CD40 Ab resulted in up-regulation of CD80, CD86, and H-2K^b only in WT but not in CD40$^{-/-}$ DCs. In contrast to what we saw previously, stimulation with anti-CD40 Ab also resulted in up-regulation of CD80 and CD86 in WT MΦ but not in CD40$^{-/-}$ MΦ (Fig. 9A). WT and CD40$^{-/-}$ DCs pulsed with gp33 peptide induced the same level of P-14 proliferation *in vitro* (Fig. 9B). LPS stimulation increased the amount of T cell proliferation, and again, the WT and CD40$^{-/-}$ DCs induced the same

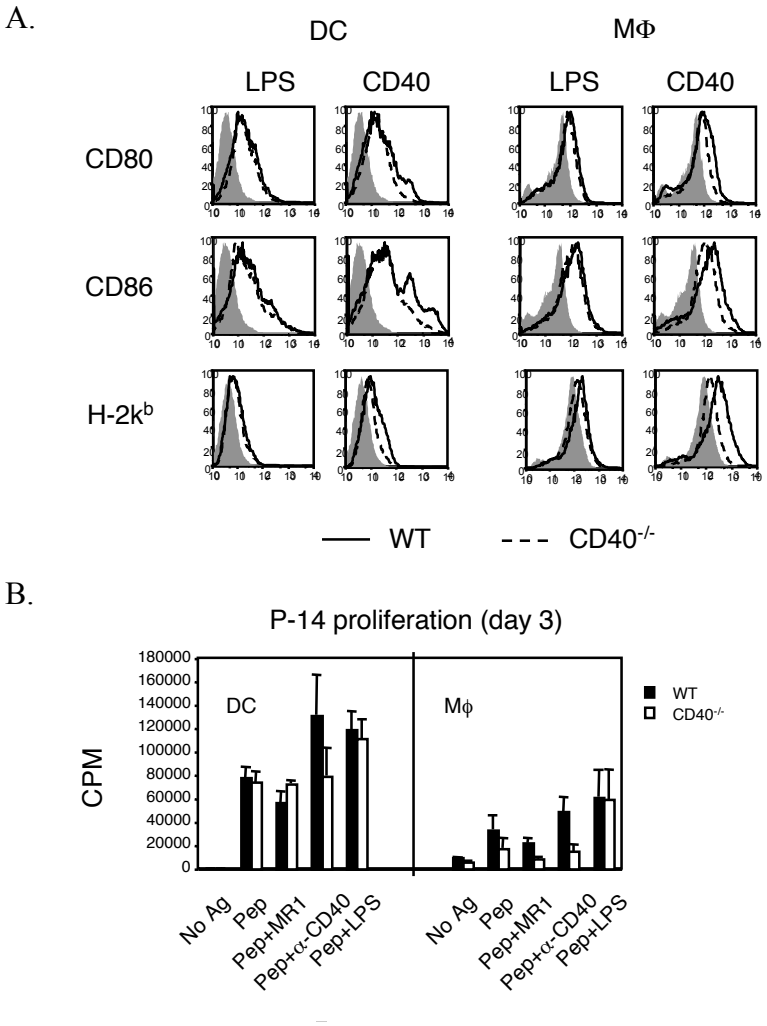


Figure 9. Phenotype of WT and CD40$^{-/-}$ APCs. *(A) Bone marrow derived DCs and MΦ from WT or CD40$^{-/-}$ mice were incubated for 2 hours with gp33 peptide alone or gp33 peptide + 1 μg LPS or 5 μg agonistic anti-CD40 Ab. The cells were then stained with Ab against CD80, CD86, and H-2K^b to analyze their phenotype. (B) BMDCs and MΦ from (A) were used to stimulate P-14 T cells in vitro. T cell proliferation was measured on day 3 of culture. 5 μg MR1 Ab was added to some of the wells and APCs that were not pulsed with peptide were used as negative control.*

responses (Fig. 9B). As expected, CD40 stimulation resulted in increased proliferation of the P-14 cells that were incubated with WT but not $CD40^{-/-}$ DCs (Fig. 9B). Addition of MR1 Ab resulted in a slightly lower T cell proliferation but only of those cells that were stimulated with WT DCs (Fig. 9B).

We observed the same general reduction in P-14 proliferation when they were incubated with MΦ. Nevertheless, the pattern was similar to that of the DCs in that WT and $CD40^{-/-}$ MΦ induced equal T cell proliferation (Fig. 9B). LPS stimulation resulted in increased proliferation of P-14 cells incubated with either WT or $CD40^{-/-}$ MΦ, while CD40 stimulation increased the proliferation of P-14 cells that were incubated with WT but not $CD40^{-/-}$ MΦ (Fig. 9B). Addition of MR1 Ab also resulted in slightly lower proliferation of T cells that were stimulated with WT but not $CD40^{-/-}$ MΦ (Fig. 9B). Altogether, these results indicate that activation of naïve $CD8^{+}$ T cells *in vitro* does not depend on CD40 signaling on APCs.

C. $CD40^{-/-}$ BM-derived APCs induce suboptimal T cell responses in vivo

We next analyzed how CD40-deficiency affects the ability of DCs and MΦ to prime naïve $CD8^{+}$ T cells *in vivo*. To do this, we immunized WT mice containing adoptively transferred P-14 T cells with DCs or MΦ from WT or $CD40^{-/-}$ mice pulsed with LCMV gp33 peptide. We used peptide as Ag in order to avoid possible complications that could be caused by a role of CD40 in antigen processing. In this situation the only cells that lack CD40 are the immunizing DCs or MΦ; at the time of immunization, the WT and CD40-deficient APCs had the same activation phenotype based on the expression of CD80, CD86, and MHC class II (data not shown). We used a

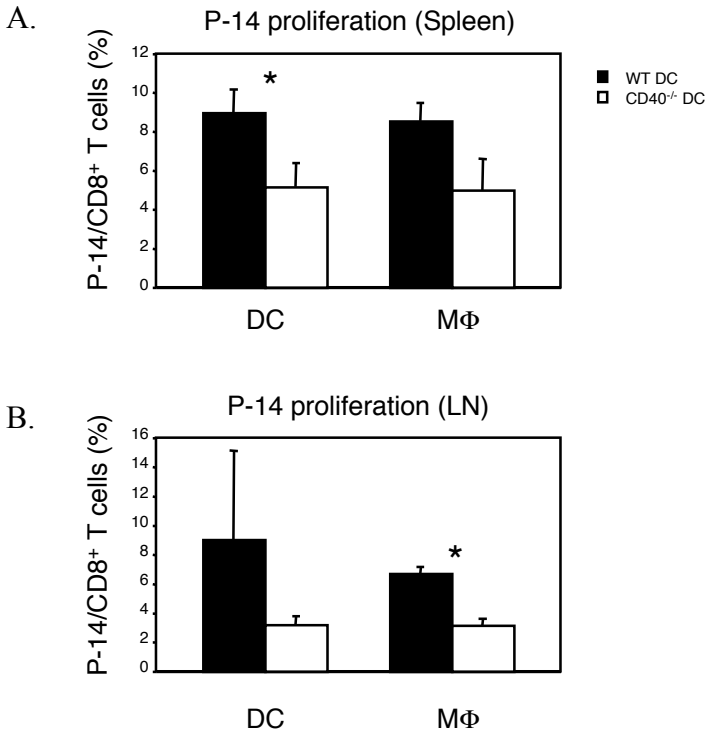


Figure 10. CD40-deficient APCs induce sub-optimal T cell responses in vivo. *WT mice containing adoptively transferred CFSE-labeled Thy1.1^{+} P-14 T cells were immunized i.v. with 1 x 10^{6} gp33 peptide-pulsed or unpulsed DC or MΦ from WT or CD40$^{-/-}$ mice. The percentage of P-14 T cells in the total CD8^{+} T cell population in the spleens (A) and LN (B) on day 4 of the response is shown. The results shown are representative of two independent experiments with two to three mice per group. *, $p < 0.05$.*

single dose of 1 x 10^6 APCs based on previous experiments that showed this number of cells stimulated reproducibly good responses. Nevertheless, compared to the previous immunizations with peptide or cell-associated Ag, immunization with peptide-pulsed DCs or MΦ generally resulted in lower $CD8^+$ T cell responses. Interestingly, $CD40^{-/-}$ DCs and MΦ induced less P-14 accumulation compared to their WT counterparts. The reduction in P-14 cell numbers was about two-fold in both the spleen and LN (Fig. 10A and 10B).

We then performed a time course study, and for this, as well as all subsequent experiments we decided to focus on DCs because they are considered as the most important APC for priming of naïve T cells *in vivo* (Fig. 11). WT DCs induced expansion of P-14 T cells in the spleen and LN as early as day 2 after immunization; peak P-14 expansion was observed at day 4 post-immunization (Fig. 12A). On the other hand, $CD40^{-/-}$ DCs also induced P-14 expansion by day 2 post-immunization. However, at all time points, the P-14 expansion induced by $CD40^{-/-}$ DCs was significantly weaker compared to that of the WT DCs (Fig. 12A). Analysis of CFSE profiles also revealed that $CD40^{-/-}$ DCs induced less P-14 proliferation compared to WT DCs across all time points (Fig. 12B and data not shown). We compared the phenotype of the activated, CFSE-negative P-14 T cells that were simulated by WT or $CD40^{-/-}$ DCs and found that both of them were $CD44^{hi}$, $CD69^{hi}$, $CD43^+$, $CD25^+$, $CD62L^{lo}$, and $CD127^{lo}$ (data not shown). In contrast to effector cells, the naïve, CFSE-positive P-14 T cells were $CD44^{lo}$, $CD69^{lo}$, $CD43^-$, $CD25^-$, and $CD62L^{hi}$ (data not shown). We next examined effector function and found that again, at all time points, $CD40^{-/-}$ DCs induced a lower percentage of IFN–γ

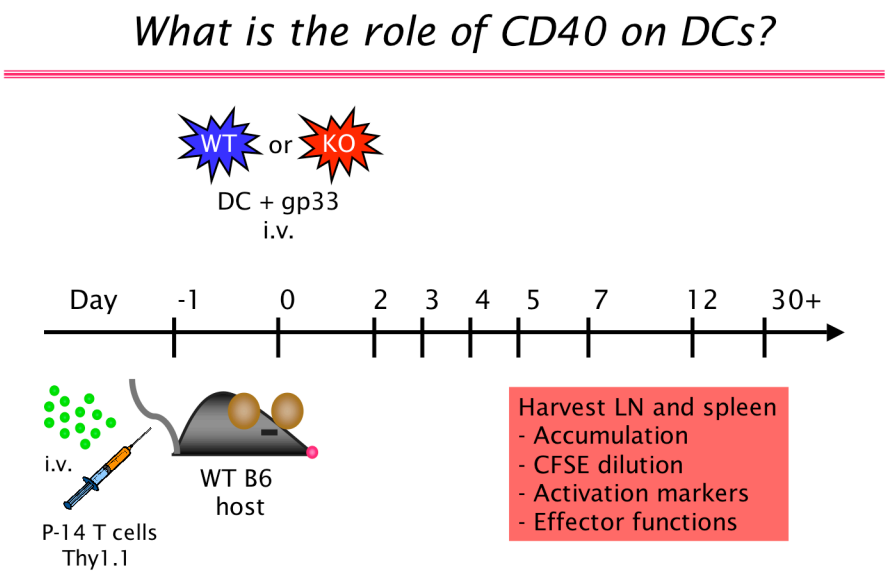


Figure 11. Experimental set-up to determine the role of CD40 expression on DCs in the priming of naïve CD8$^+$ T cell responses. *WT hosts adoptively transferred with CFSE-labeled P-14 T cells were immunized with DCs derived from the bone marrow or spleen of WT or CD40$^{-/-}$ (KO) mice that have been pulsed with gp33 peptide. Spleens and lymph nodes were harvested at different time points and analyzed for the above parameters. In this system CD40 deficiency is restricted to the immunizing DCs.*

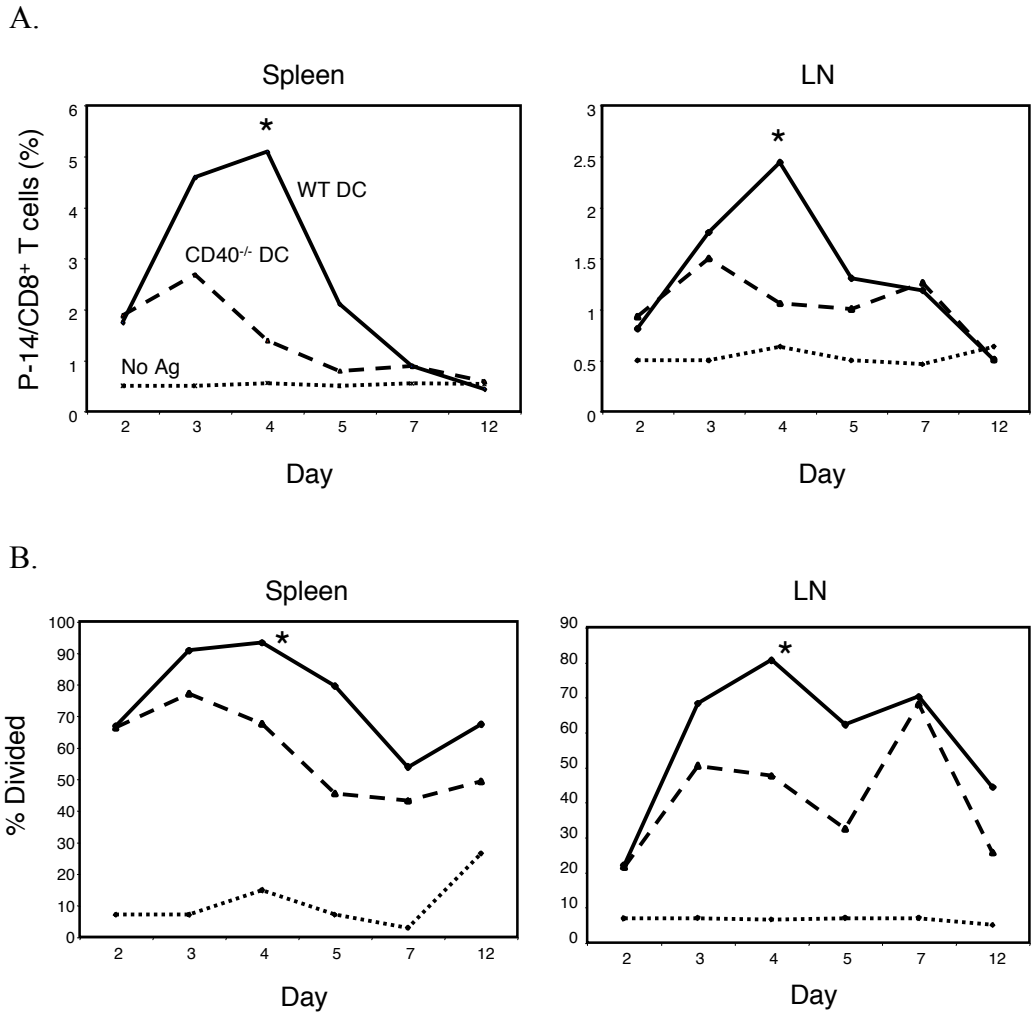


Figure 12. Time course of P-14 T cell responses in vivo. *WT mice containing adoptively transferred CFSE-labeled Thy1.1^{+} P-14 T cells were immunized i.v. with 1 x 10^{6} gp33 peptide-pulsed or unpulsed DC from WT or CD40$^{-/-}$ mice. (A) Mean percentage of P-14 T cells in the total CD8^{+} T cell population in the spleen and LN at different time points after immunization. (B) Percentage of P-14 T cells that have divided based on CFSE staining. The results shown are representative of two independent experiments with two to three mice per group. *, $p < 0.01$.*

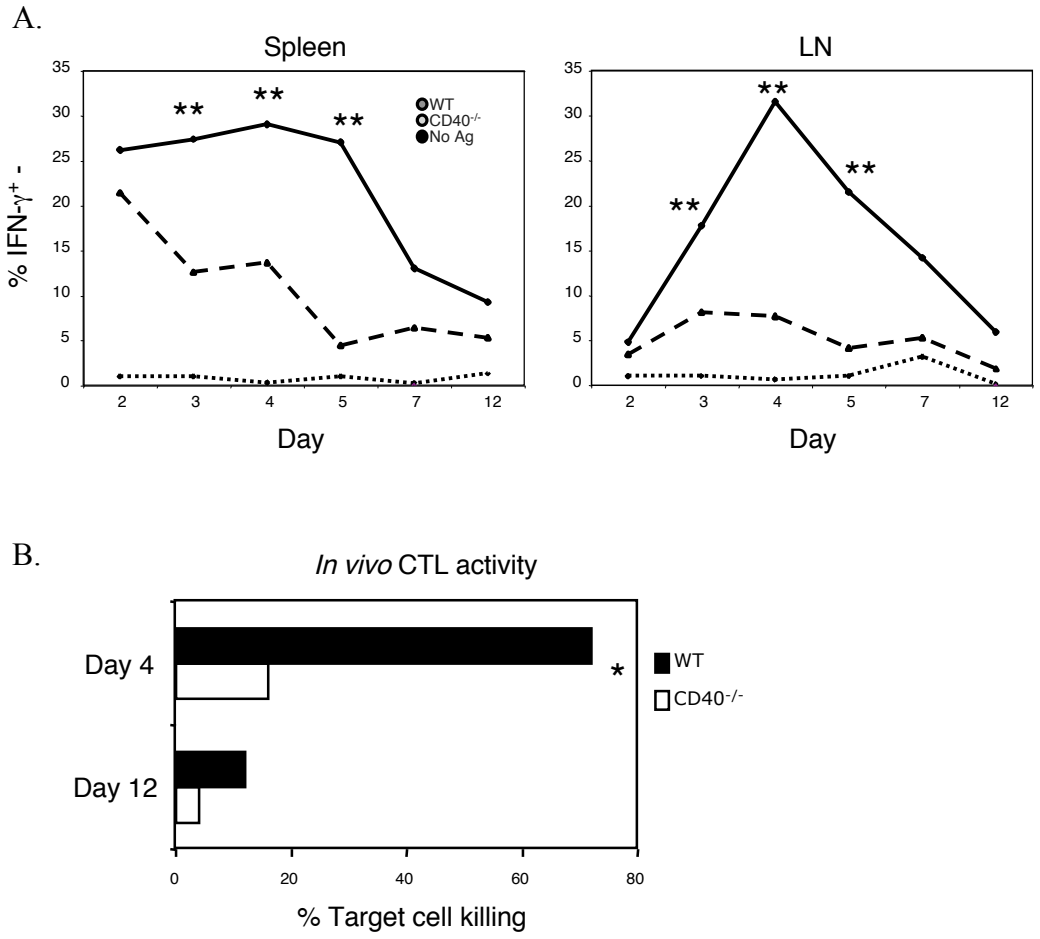


Figure 13. Time course of P-14 T effector function in vivo. *(A) Mean percentage of IFN-γ producing P-14 T cells in the total CD8$^+$ T cell population in the spleen and LN at different time points after immunization. (B) In vivo CTL activity against gp33 peptide-pulsed targets. The results shown are representative of two independent experiments with two to three mice per group. **, $p < 0.01$; *, $p < 0.05$.*

producing P-14 cells compared to WT DCs in both the spleen and LN (Fig. 13A). The reduced T cell response induced by the $CD40^{-/-}$ DCs led to a corresponding decrease in the ability of the immunized hosts to eliminate peptide-pulsed targets during the peak of the response (Fig. 13B). By day 12, however, there is very little CTL activity left regardless of the immunizing DC. This is most probably due to the low numbers of effector cells that remain at this point.

D. In vivo generated $CD40^{-/-}$ DCs induce sub-optimal T cell responses in vivo

Up to this point, we have only used APCs that were derived *in vitro* from bone marrow cells. It is possible that the environment in which APCs are generated could affect their function. Therefore, we also analyzed the ability of APCs, specifically DCs, which were generated *in vivo*, to induce naïve $CD8^+$ T cell responses. Because there is only a small population of DCs in the spleen and they are difficult to isolate in large numbers, we first injected WT and $CD40^{-/-}$ mice with B16 tumor cells that secrete Flt-3 ligand. Flt-3L has previously been shown to induce the differentiation and expansion of functionally mature DC subsets *in vivo* (Mach et al., 2000; Maraskovsky et al., 1996). In our case, injection of B16-Flt3L cells resulted in up to 50-fold expansion of $CD11c^+$ cells in the spleen (data not shown). We initially compared the *in vivo* priming ability of WT bone marrow derived DCs (BMDC) and Flt-3L induced splenic DCs (Flt-3L DCs) that were pulsed with different concentrations of peptide. We found that immunization with either BMDCs or Flt-3L DCs resulted in similar levels of P-14 expansion (Fig. 14).

We then obtained WT and $CD40^{-/-}$ Flt-3L DCs and used them to immunize hosts containing adoptively transferred P-14 T cells. The Flt-3L DCs from WT and $CD40^{-/-}$

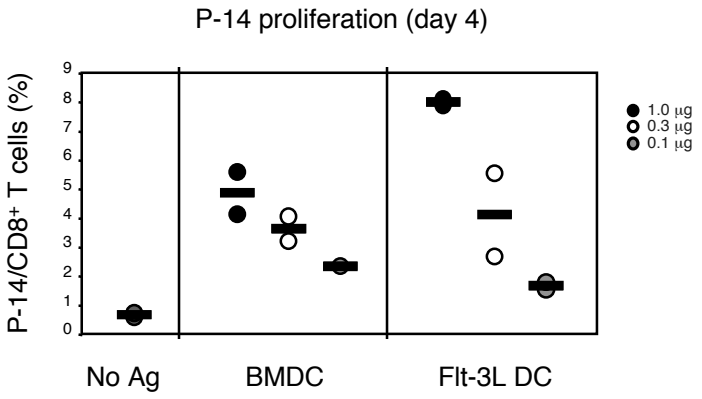


Figure 14. P-14 T cell responses upon immunization with BMDC or Flt-3L DCs. *WT mice containing adoptively transferred CFSE-labeled Thy1.1^{+} P-14 T cells were immunized i.v. with 1 x 10^{6} BMDC or Flt-3L DCs that have been pulsed for 2 hours with 1.0, 0.3, 03 0.1 μg gp33 peptide. The mean percentage of P-14 T cells in the total CD8^{+} T cell population in the spleen on day 4 of the response is shown. The results shown are representative of to independent experiments with two to three mice per group.*

mice contained similar proportions of the different splenic DC subsets (Fig. 15A). In addition, they also had the same activation phenotype (Fig. 15B). Similar to the *in vitro* derived BMDCs, $CD40^{-/-}$ splenic DCs induced much less P-14 T cell accumulation on day 4 of the response (Fig. 16A). Moreover, the proportion as well as the absolute number of IFN–γ^{+} P-14 cells was significantly reduced in mice immunized with $CD40^{-/-}$ Flt-3L DCs (Fig. 16B and 16C). Furthermore, the P-14 cells stimulated with $CD40^{-/-}$ Flt-3L DCs also made less IFN–γ compared to those that were stimulated with WT Flt-3L DCs (Fig. 16D).

All of the results using CD40-deficient APCs are concordant with the data we obtained using CD40-deficient hosts. Moreover, they directly demonstrate a key role for CD40 on APCs, particularly on DCs, in inducing maximal naïve $CD8^{+}$ T cell responses since they are the only cells lacking this receptor in these experiments.

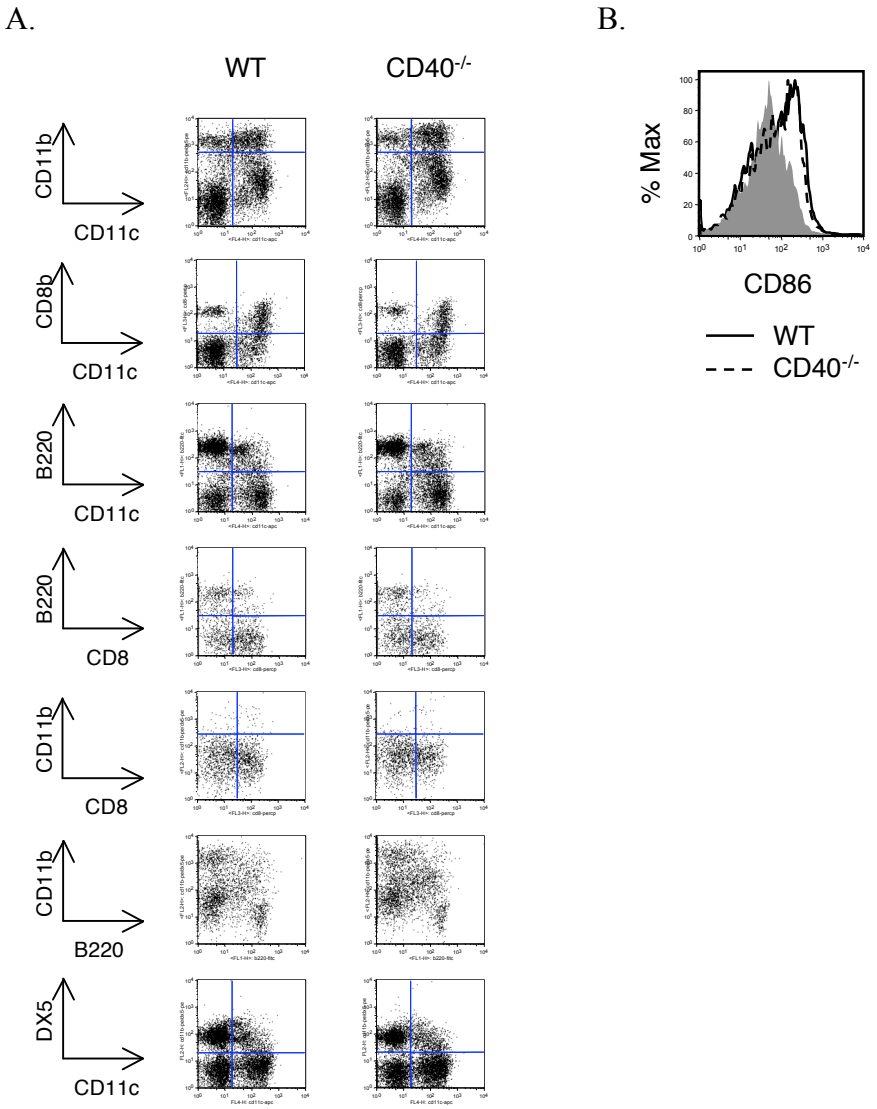


Figure 15. Phenotype of Flt-3L DCs from WT and CD40$^{-/-}$ mice. *Spleens were harvested from WT and CD40$^{-/-}$ mice injected with B16-Flt-3L tumor cells 12-14 days previously. Single-cell suspensions were stained with Ab against CD11c, CD11b, B220, CD8, and a pan-NK marker (A) or CD86 (B). Samples were analyzed by flow cytometry. The data shown in (A) are from either total splenocytes or gated on CD11c^{+} cells while the data in (B) is gated on CD11c^{+} cells.*

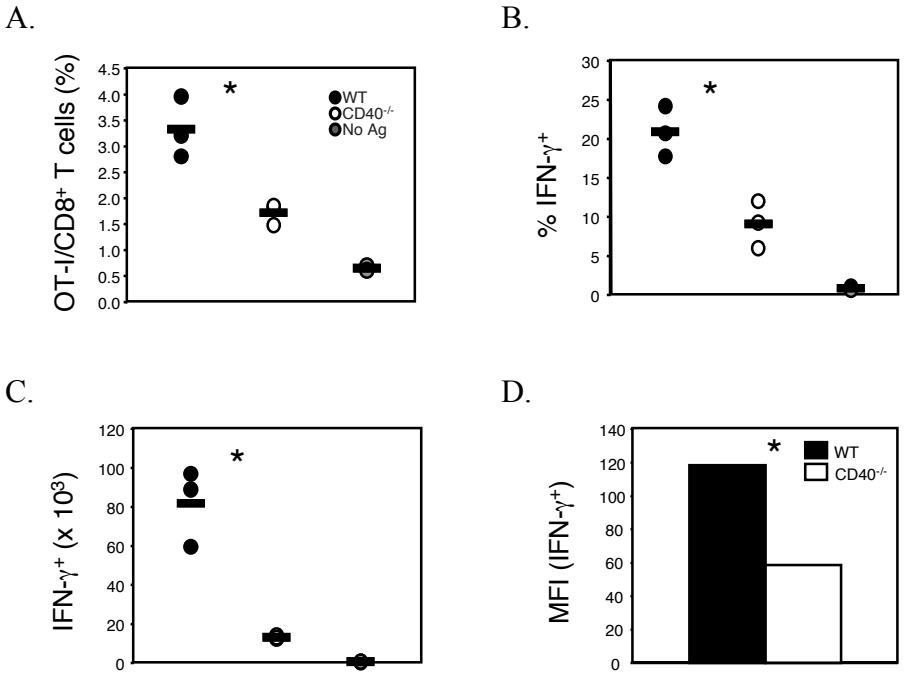


Figure 16. Flt-3L DCs from CD40$^{-/-}$ mice induce sub-optimal P-14 T cell responses in vivo. *WT mice containing adoptively transferred CFSE-labeled Thy1.1^{+} P-14 T cells were immunized i.v. with 1 x 10^{6} Flt-3L DCs from WT or CD40$^{-/-}$ mice that have been pulsed with gp33 peptide. Spleens were harvested on day 4 and analyzed as in Fig. 1. (A) Mean percentage of P-14 T cells in the total CD8^{+} T cell population in the spleen. (B) Percentage of IFN-γ^{+} P-14 T cells. (C) Absolute number of IFN-γ producing P-14 T cells. (D) MFI of IFN-γ staining. Each circle represents an individual mouse; the lines represent the means. The results shown are representative of three independent experiments with three mice per group. *, $p < 0.01$.*

CHAPTER V.

CD40-DEPENDENT $CD8^+$ T CELL RESPONSES IN THE ABSENCE OF $CD4^+$ T CELL HELP

$CD8^+$ T cell responses to non-inflammatory Ag such as peptides, soluble proteins, particulate Ag, and cell-associated Ag, including peptide-pulsed DCs are largely dependent on $CD4^+$ T helper cells (Behrens et al., 2004; Clarke, 2000). It is believed that CD40L on activated $CD4^+$ T cells is needed to stimulate CD40 on APCs and trigger licensing, and that this is the mechanism by which $CD4^+$ T cells provide help for $CD8^+$ T cell responses. In our experimental systems, it is possible that $CD4^+$ T cells might be generated to bovine serum proteins presented by cultured BMDCs or if the LCMV 13mer peptide contained a MHC class II epitope. Therefore we sought to investigate whether the data we have so far on the importance of CD40 signaling reflects the role of $CD4^+$ T cell help in generation of $CD8^+$ T cell responses.

RESULTS

A. Help-independent $CD8^+$ T cell responses to peptide or peptide-pulsed DCs

A previous study from our lab showed that the strength of antigenic stimulation determines whether a primary $CD8^+$ T cell response will be help-dependent or help-independent (Rock and Clark, 1996). Thus, immunization with a low concentration of a particulate Ag resulted in priming of CTLs in WT but not MHC $II^{-/-}$ mice while immunization with a higher concentration of Ag resulted in priming of CTLs in both WT

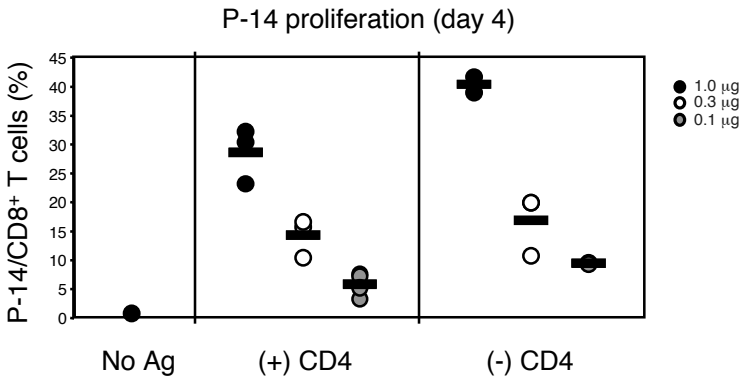


Figure 17. P-14 T cell responses in WT or CD4$^+$ T cell-deficient hosts. *CFSE-labeled Thy1.1$^+$ P-14 T cells were adoptively transferred into WT mice, which were depleted of CD4$^+$ T cells or left untreated. One day later the mice were immunized i.v. with 1.0, 0.3, or 0.1 µg LCMV 13-mer peptide. Spleens were harvested on day 4 and analyzed as in Fig. 1. The mean percentage of P-14 T cells in the total CD8$^+$ T cell population is shown. Each circle represents an individual mouse; the bars represent the means. The results shown are representative of two independent experiments with two to three mice per group.*

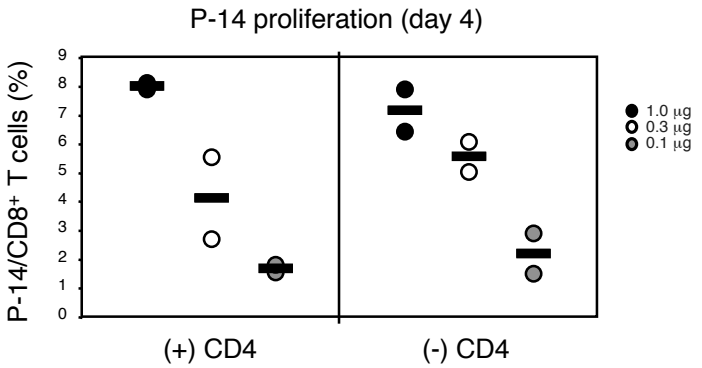


Figure 18. Helper-independent CD8$^+$ T cell responses to peptide-pulsed DCs. *CFSE-labeled Thy1.1$^+$ P-14 T cells were adoptively transferred into WT mice, which were depleted of CD4$^+$ T cells or left untreated. One day later the mice were immunized i.v. with 1 x 10^6 gp33 Flt-3L DCs that were pulsed with 1.0, 0.3, or 0.1 μg gp33 peptide or left unpulsed. Spleens were harvested on day 4 and analyzed as in Fig. 1. The mean percentage of P-14 T cells in the total CD8$^+$ T cell population is shown. Each circle represents an individual mouse; the bars represent the means. The results shown are representative of two independent experiments with two to three mice per group.*

and MHC $II^{-/-}$ mice. We asked if our experimental systems using peptide or peptide-pulsed DCs are subject to the same concentration-dependent requirement for $CD4^+$ T cell help. To answer this, we adoptively transferred WT and $CD4^+$ T cell-deficient mice with P-14 T cells and immunized them with different concentrations of LCMV 13-mer or DCs pulsed with different concentrations of gp33 peptide. We chose to do acute $CD4^+$ T cell depletion with GK1.5 Ab because this gives the most complete elimination of these cells, with the cells starting to disappear as early as one day after injection of the Ab. Surprisingly, we found that the P-14 T cell response to LCMV 13-mer is independent of $CD4^+$ T cell help. Regardless of the amount of peptide injected, similar levels of P-14 proliferation and expansion were observed in WT and $CD4^+$ T cell-deficient mice (Fig. 17). The P-14 T cell response to DCs pulsed with different concentrations of peptide is likewise similar in WT and $CD4^+$ T cell-deficient hosts (Fig. 18).

Altogether, these results indicate that the $CD8^+$ T cell response to peptide or peptide-pulsed DCs can occur independently of $CD4^+$ T cell help, in contrast to some earlier studies.

B. $CD40^{-/-}$ DC induce suboptimal responses in the absence of $CD4^+$ T cells

We next determined whether $CD4^+$ T cells are the sole source of CD40L that is needed to stimulate CD40 in our system. To do this, we immunized WT and $CD4^+$ T cell-deficient hosts containing P-14 T cells with WT or $CD40^{-/-}$ DCs pulsed with gp33 peptide (Fig. 19). Again, we found that WT DCs stimulated equivalent P-14 T cell responses with or without $CD4^+$ T cells (Fig. 20A and 20B). Depletion of $CD4^+$ T cells even resulted in a

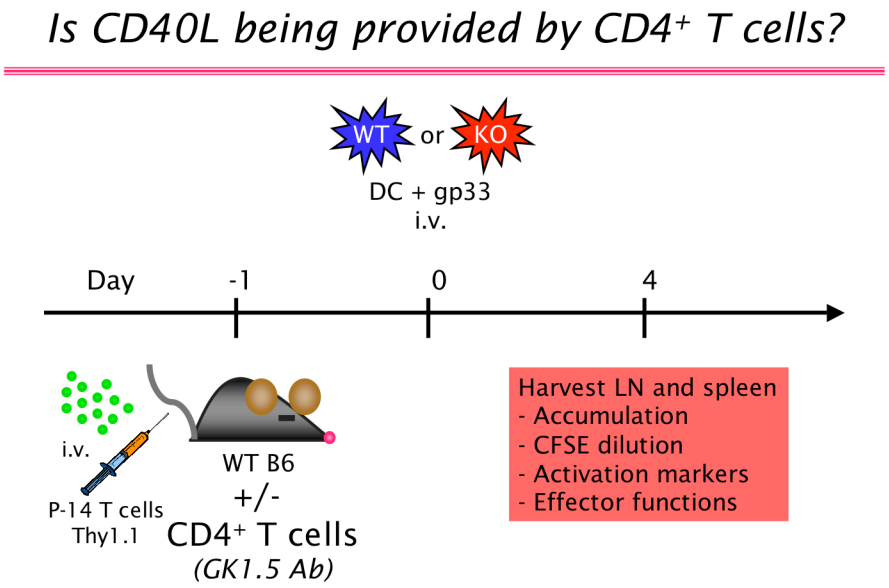


Figure 19. Experimental system to determine whether CD40L is being provided by CD4$^+$ T cells. *WT hosts were either depleted or not depleted of CD4$^+$ T cells using the GK1.5 Ab. They were then adoptively transferred with CFSE-labeled P-14 T cells and immunized with DCs from WT or CD40$^{-/-}$ (KO) mice that have been pulsed with gp33 peptide. Spleens and lymph nodes were harvested on day 4 and examined for the above parameters.*

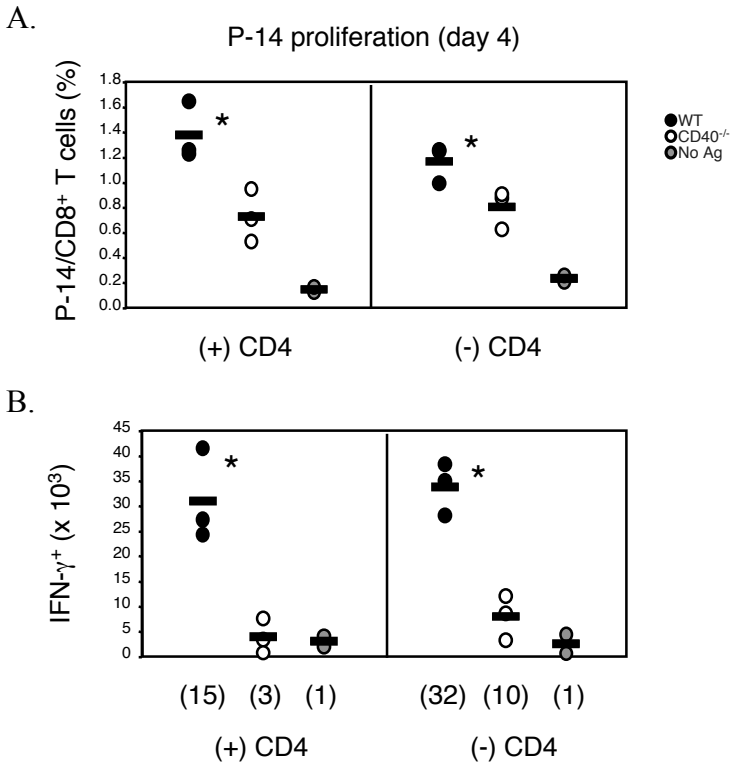


Figure 20. Helper-independent CD8$^+$ T cell responses induced by WT and CD40$^{-/-}$ DCs. *CFSE-labeled Thy1.1$^+$ P-14 T cells were adoptively transferred into WT mice, which were depleted of CD4$^+$ T cells or left untreated. One day later the mice were immunized i.v. with 1 x 10^6 gp33 peptide-pulsed or unpulsed DCs from WT or CD40$^{-/-}$ mice. Spleens were harvested on day 4 and analyzed as in Fig. 1. (A) Percentage of P-14 T cells in the total CD8$^+$ T cell population. (B) Absolute number of IFN-γ producing P-14 T cells. Each circle represents an individual mouse; the bars represent the means. The numbers in parenthesis below indicate the percentage of P-14 T cells secreting IFN-γ. The results shown are representative of three independent experiments with three mice per group. *, $p < 0.01$.*

slight increase in the absolute number of IFN–γ producing cells, perhaps suggesting a possible effect of depletion of the natural regulatory T cell (T_{REG}) population, which also express CD4. However, the difference we observed was not statistically significant. Interestingly, $CD40^{-/-}$ DCs still induced weaker P-14 T cell priming in the absence of $CD4^+$ T cells (Fig. 20A and 20B). This implies that $CD4^+$ T cell and CD40 activity are not always equivalent. Moreover, it suggests that $CD4^+$ T cells are not the only cells capable of activating CD40 on APCs during an immune response. To investigate this further, we did most of our subsequent experiments in $CD4^+$ T cell-depleted hosts.

C. OT-I T cell responses in $CD4^+$ T cell-deficient hosts

In order to make sure that the effects we observed were not confined to P-14 T cells, we again performed experiments using adoptively transferred OT-I cells and DCs pulsed with OVA peptide. Intriguingly, there was no difference in OT-I expansion upon immunization with WT or $CD40^{-/-}$ DCs (Fig. 21A). Nevertheless, the percentage of IFN–γ^+ OT-I cells was slightly lower in mice immunized with $CD40^{-/-}$ DCs (Fig. 21B). There is evidence that the precursor frequency of Ag-specific $CD8^+$ T cells affects whether their response will require $CD4^+$ T cell help (Mintern et al., 2002b). Therefore, one possible explanation for the above result is that the precursor frequency of the OT-I cells was high enough that their response became independent of $CD4^+$ T cells and even CD40. To test this, we titrated the number of OT-I T cells that we adoptively transferred into the $CD4^+$ T cell-deficient hosts. We then immunized the mice with WT or $CD40^{-/-}$ DCs pulsed with OVA peptide. This time, both the OT-I T cell expansion and IFN–γ production were lower in mice that were immunized with $CD40^{-/-}$ DCs (Fig. 22A and 22B). These data

indicate that CD40 signaling on APCs is important in the response of $CD8^+$ T cells with a different specificity. Furthermore, they suggest that CD40 signaling may not be as important when the frequency of Ag-specific $CD8^+$ T cells is high enough.

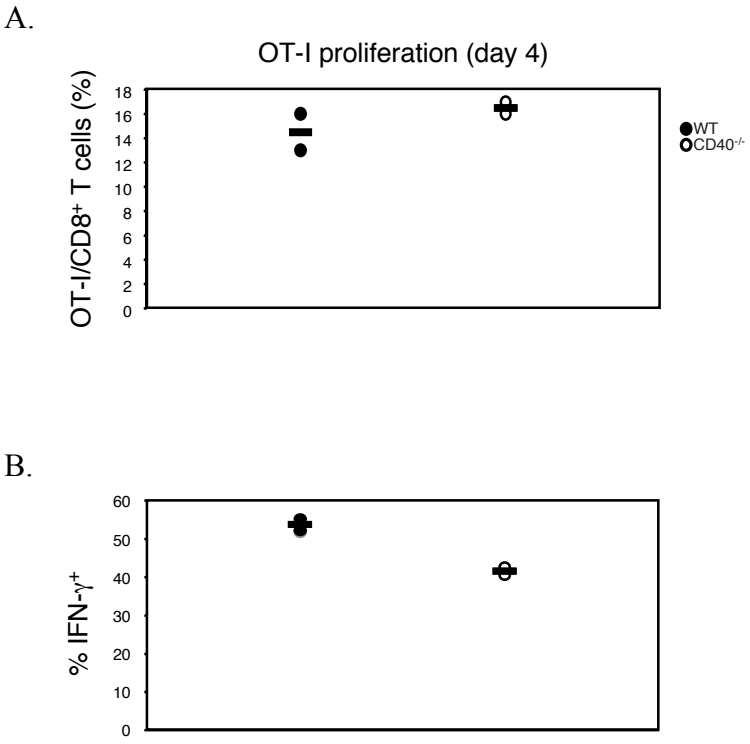
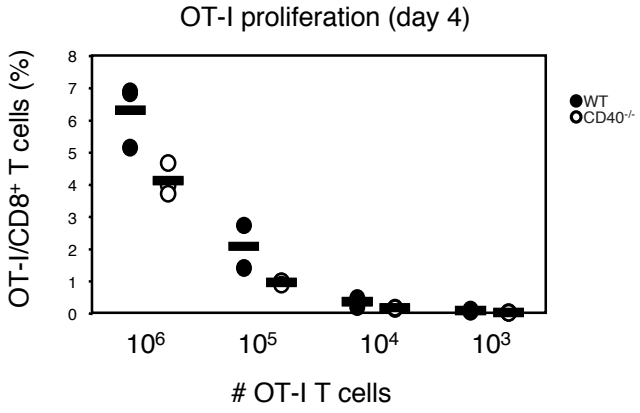


Figure 21. OT-I T cell responses induced by WT versus CD40$^{-/-}$ DCs. *2 x10^6 CFSE-labeled Thy1.1$^+$ OT-I T cells were adoptively transferred into WT mice, which were depleted of CD4$^+$ T cells. One day later the mice were immunized i.v. with 1 x 10^6 OVA peptide-pulsed or unpulsed DCs from WT or CD40$^{-/-}$ mice. Spleens were harvested on day 4 and analyzed as in Fig. 1. (A) Percentage of OT-I T cells in the total CD8$^+$ T cell population. (B) Percentage of IFN-γ producing OT-I T cells. The results shown are representative of two independent experiments. Each circle represents an individual mouse; the bars represent the means.*

A.



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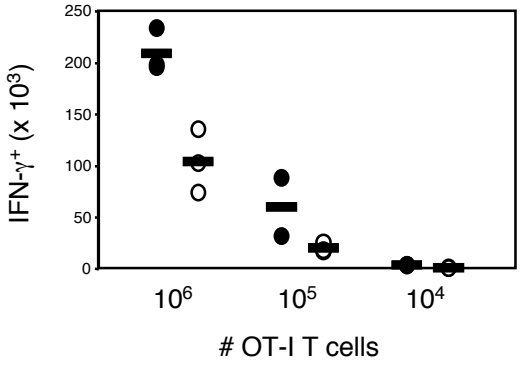


Figure 22. Reduced OT-I T cell responses induced by CD40$^{-/-}$ DCs. *Mice depleted of CD4^{+} T cells were adoptively transferred with 1 x10^{6}, 1 x10^{5}, 1 x 10^{4}, or 1 x 10^{3} CFSE-labeled Thy1.1^{+} OT-I T cells. One day later the mice were immunized i.v. with 1 x 10^{6} OVA peptide-pulsed or unpulsed DCs from WT or CD40$^{-/-}$ mice. Spleens were harvested on day 4 and analyzed as in Fig. 1. (A) Percentage of OT-I T cells in the total CD8^{+} T cell population. (B) Absolute number of IFN-γ producing OT-I T cells. Each circle represents an individual mouse; the bars represent the means.*

CHAPTER VI.

PIVOTAL ROLE OF CD40-CD40L INTERACTION IN ENDOGENOUS $CD8^+$ T CELL RESPONSES

Up until this point we have been analyzing the response of TCR-Tg $CD8^+$ T cells. These cells have a relatively high affinity for their cognate MHC-peptide and are present at high frequencies in the host mice. We next sought to extend our analysis of naïve $CD8^+$ T cell priming to a non-transgenic (non-Tg) system, in which host mice have normal T cell frequencies. In addition, the Ag-specific $CD8^+$ T cells in these mice are polyclonal and are therefore composed of cells with different affinities.

RESULTS

A. Reduced CTL activity in immunized $CD40^{-/-}$ and $CD40L^{-/-}$ mice

We immunized WT, $CD40^{-/-}$, and $CD40L^{-/-}$ mice with OVA-transfected cells and evaluated naïve $CD8^+$ T cell priming by performing an *in vivo* CTL assay one week later (Fig. 23). As expected, non-immunized WT, $CD40^{-/-}$, and $CD40L^{-/-}$ mice were unable to lyse OVA peptide-pulsed targets (Fig. 24A and data not shown). Immunized WT mice exhibited strong CTL responses, being able to lyse as much as 80% of targets (Fig. 24A and 24B). In contrast, both the immunized $CD40^{-/-}$ and $CD40L^{-/-}$ mice showed much weaker $CD8^+$ T cell priming, with target cell killing in these mice being reduced to ~20% (Fig. 24A and 24B). We then inoculated the mice with a melanoma cell line that stably

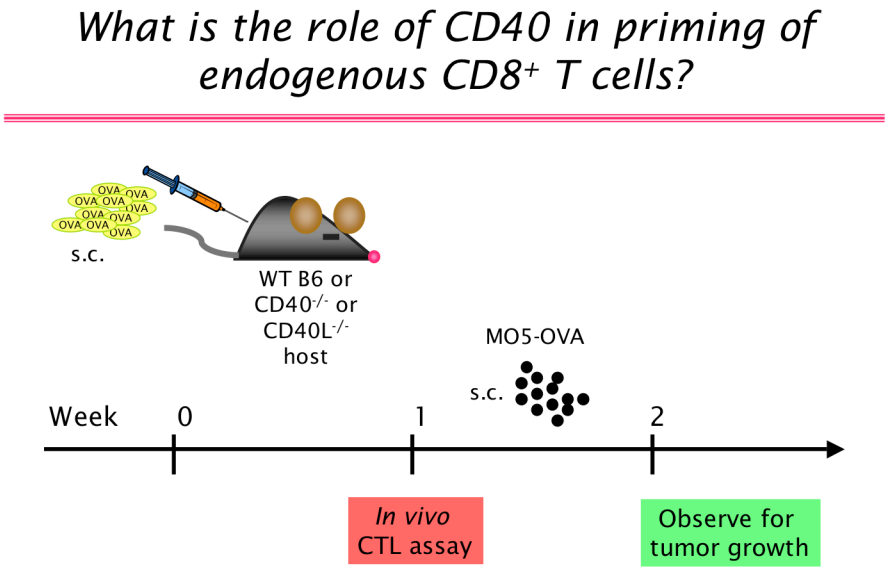
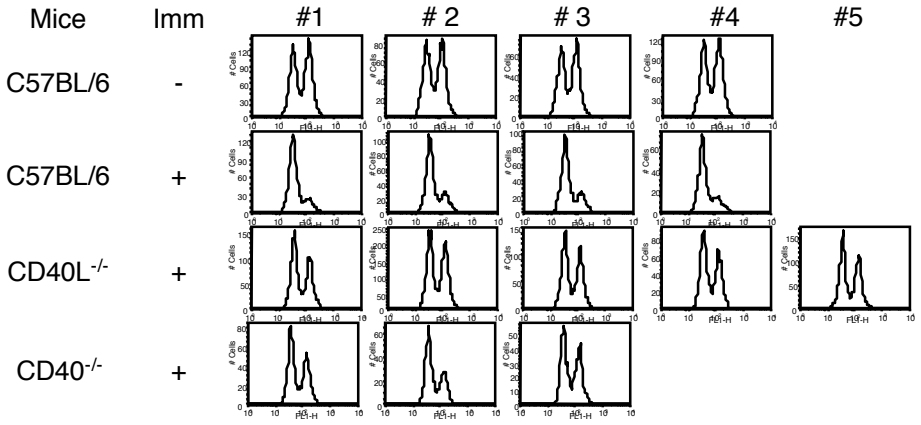
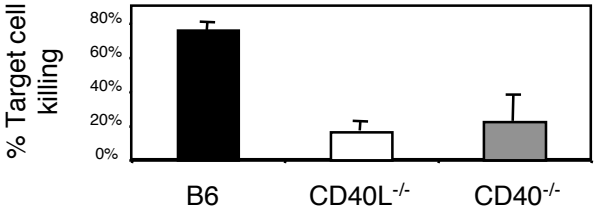


Figure 23. Experimental set-up to determine the role of CD40-CD40L interactions on the priming of endogenous CD8$^+$ T cells. *WT, CD40$^{-/-}$, and CD40L$^{-/-}$ mice were immunized s.c. with stable OVA-transfectants (D2F3). Primary responses were evaluated one week later using an in vivo CTL assay. Following this, the mice were inoculated with melanoma cells expressing OVA (MO5-OVA) and observed for tumor growth. In this system all host cells are either sufficient or deficient in CD40 or CD40L. Moreover, since there is no adoptive transfer, the responses are those of the endogenous CD8$^+$ T cells, which are not only polyclonal but also present at physiological frequencies.*

A.



B.



C.

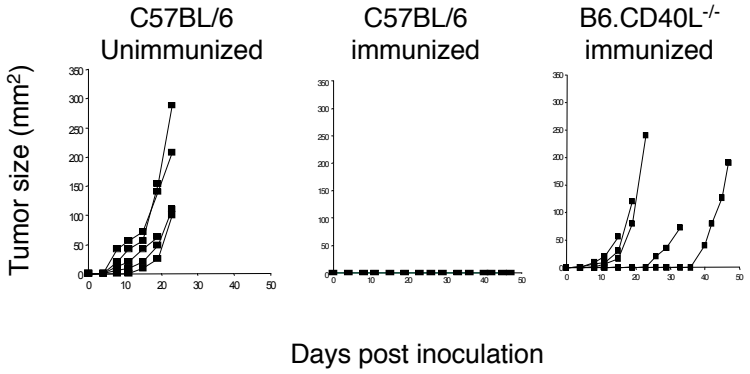


Figure 24. Endogenous CD8$^+$ T cell responses are compromised in the absence of CD40 or CD40L. *WT, CD40$^{-/-}$, and CD40L$^{-/-}$ mice were immunized s.c. with 5 x10^6 OVA-transfected cells or left unimmunized. One week later, OVA peptide-specific CTL*

responses were assessed using an in vivo CTL assay. (A) FACS data showing lysis of CFSE-hi target cells. (B) Specific target cell killing from the different mice in (A) are presented as mean + SD. (C) Indicated mice were immunized as in (A) and injected s.c 10-14 days later with 2 x10^6 MO5 cells. Tumor growth was monitored twice a week and mice were sacrificed when the tumors reached a diameter of 2 cm. The results shown are representative of 3 independent experiments with 3 to 5 mice per group. (Data provided by Lianjun Shen)

expresses OVA (MO5; Fig. 23). Whereas no tumor growth was observed in immunized WT mice, immunized $CD40^{-/-}$ and $CD40L^{-/-}$ mice eventually succumbed to tumors (Fig. 24C and data not shown). These results indicate that although naïve $CD8^+$ T cells can be primed in the absence of CD40-CD40L interaction, the lower magnitude of the response leads to responses are not protective.

B. $CD40^{-/-}$ DCs induce sub-optimal endogenous $CD8^+$ T cell responses

We also examined endogenous $CD8^+$ T cell responses in WT hosts upon immunization with WT or $CD40^{-/-}$ DCs pulsed with OVA peptide (Fig. 25). WT DCs induced a very strong CTL response, resulting in almost complete elimination of target cells (Fig. 26A and 26B). In contrast, $CD40^{-/-}$ DCs induced more modest CTL activity and this weaker response was observed whether or not $CD4^+$ T cells were present (Fig. 26A and 26B). We could not detect cytokine production by $CD8^+$ T cells directly *ex vivo* in either WT DC or $CD40^{-/-}$ DC immunized mice (data not shown). Upon *in vitro* re-stimulation, we found that only $CD8^+$ T cells from mice immunized with WT DCs were able to secrete IFN-γ (Fig. 26C). Interestingly, we observed a reduction in the number of IFN–γ producing cells in the absence of $CD4^+$ T cells. This appears to be a difference between the TCR-Tg versus the endogenous (polyclonal) T cell response and could be due to differences in T cell affinity and/or precursor frequency. However, it is important to note that in spite of the reduced number of endogenous IFN–γ producing $CD8^+$ T cells in CD4-depleted hosts, the response remains CD40-dependent.

Overall, the above results indicate that the CD40-CD40L interaction is required to generate a highly effective and protective primary response from an endogenous

polyclonal $CD8^+$ T cell population. This requirement is observed even in the absence of $CD4^+$ T cell help.

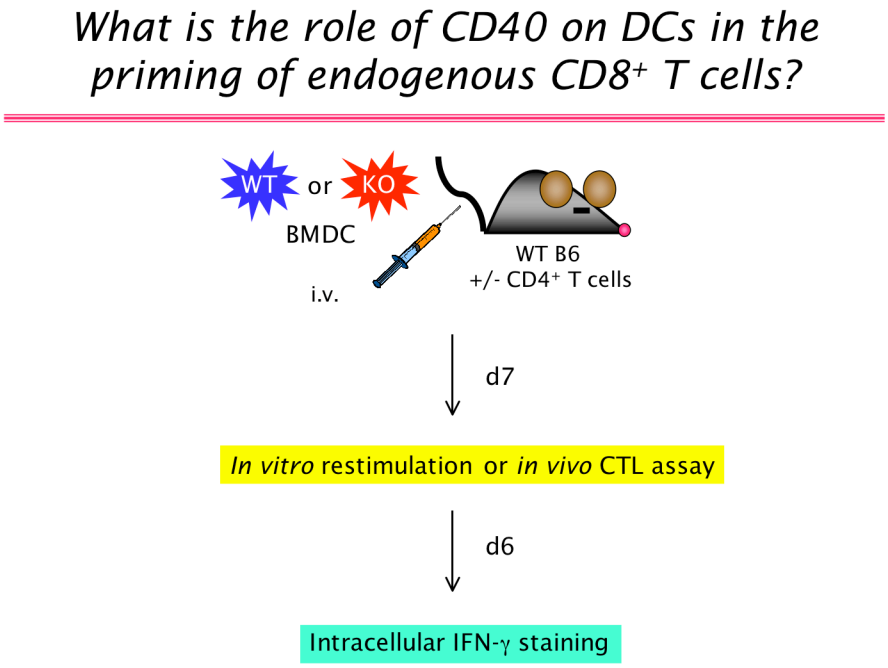


Figure 25. Experimental set-up to determine the role of CD40 on DCs in the priming of endogenous polyclonal CD8$^+$ T cell responses. *WT or CD4-depleted mice were immunized with BMDC from WT or CD40$^{-/-}$ (KO) mice that have been pulsed with OVA peptide. After one week, in vivo CTL assay was performed and spleen cells were restimulated in vitro with OVA peptide. Intracellular cytokine staining was performed after six days of culture. In this system CD40 deficiency is restricted only to the immunizing DC.*

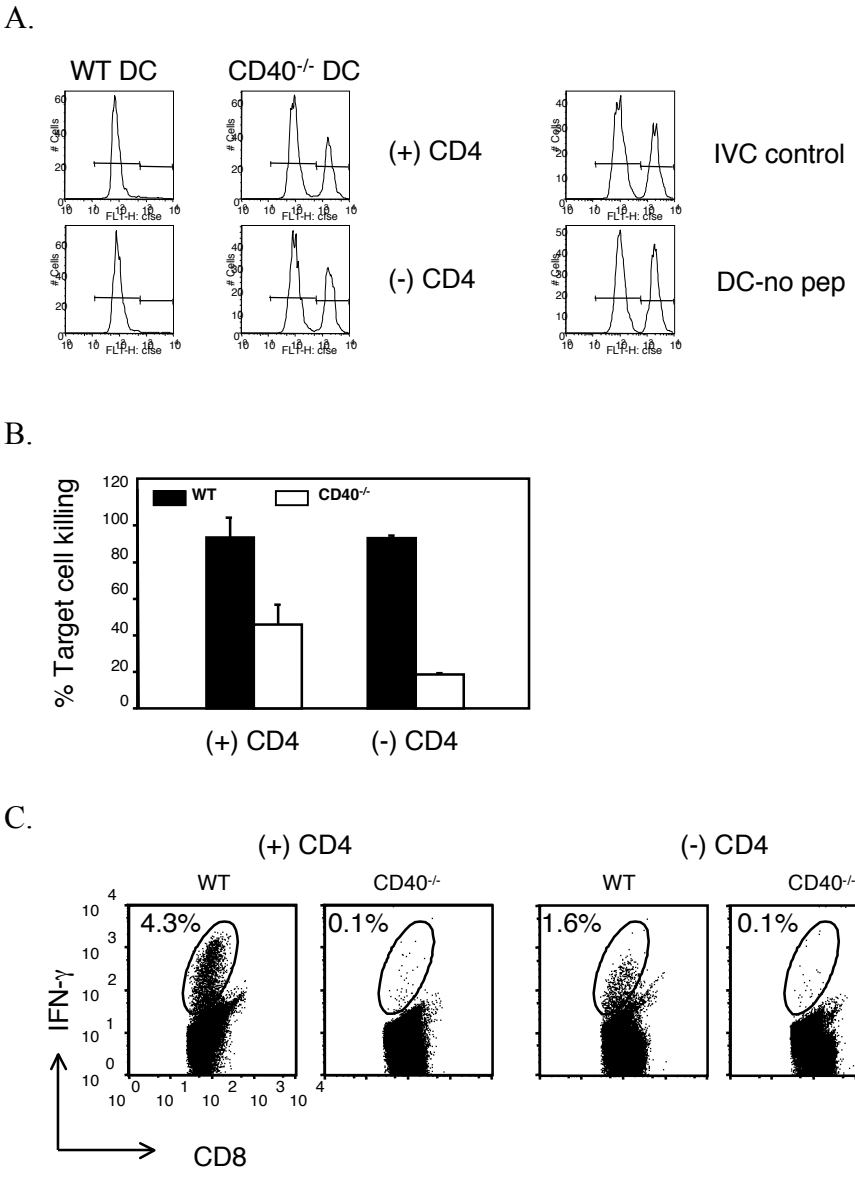


Figure 26. Endogenous CD8$^+$ T cell responses upon immunization with WT or CD40$^{-/-}$ DCs. *CD4-depleted or undepleted WT mice were immunized i.v. with 1 x 10^6 OVA peptide-pulsed or unpulsed DCs from the indicated mice. In vivo CTL activity against OVA peptide-pulsed targets was determined one week later. (A) Representative FACS data showing lysis of CFSE^{hi} target cells. (B) Specific target cell killing from the different*

mice in (A) are presented as mean + SD. (C) CD4-depleted or undepleted mice were immunized as in (A). On day 7, splenocytes were harvested and restimulated in vitro with 1 μg/mL OVA peptide. After six days, intracellular IFN-γ production was assessed following an additional 5-hour incubation with OVA peptide. Representative plots are shown, with the numbers indicating the percentage of $CD8^+$ T cells producing IFN-γ. The frequency of IFN-γ^+ cells in mice immunized with unpulsed DCs was ~ 0.5%. The results shown are representative of two independent experiments with three mice per group.

CHAPTER VII.

CD40L EXPRESSION BY $CD8^+$ T CELLS CONTRIBUTES TO MAXIMUM RESPONSES

$CD4^+$ T cells are considered to be the primary source of CD40L for stimulating CD40 on APCs. However, our observations that CD40 signaling is important in inducing maximum primary $CD8^+$ T cell responses even in the absence of $CD4^+$ T cells implies that CD40L is coming from a different source. Some of the cells that can express CD40L include $CD8^+$ T cells, B cells, NK cells, NKT cells, and platelets (Schonbeck and Libby, 2001). We sought to identify which among these candidates is responsible for providing CD40L.

RESULTS

A. $CD8^+$ T cells express CD40L

We hypothesized that in the absence of $CD4^+$ T cells, CD40L might be provided to DCs by the responding $CD8^+$ T cells themselves. To test this, we first bred the P-14 mice to the $CD40L^{-/-}$ background to obtain CD40L-deficient P-14 T cells. *In vitro*, the response of the CD40L-deficient P-14 T cells to peptide, peptide-pulsed DCs, and PMA/Ionomycin was comparable to that of WT P-14 T cells (Fig. 27).

We next confirmed that the WT P-14 T cells are capable of expressing CD40L. To do this, we stimulated the cells *in vitro* with either gp33 peptide or PMA/Ionomycin. By 4 hours after incubation, we could detect CD40L expression on the WT P-14 T cells,

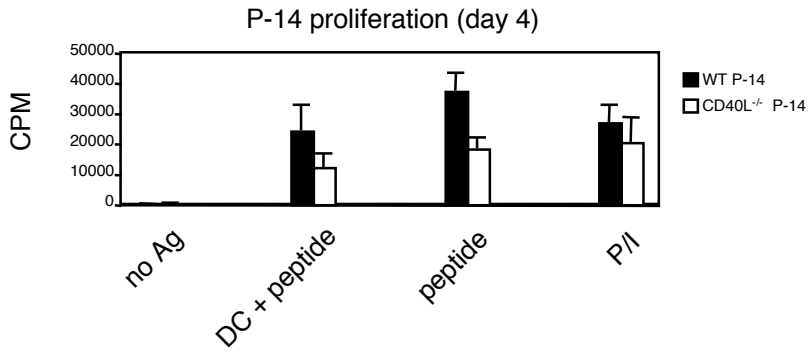


Figure 27. In vitro response by CD40L-deficient P-14 T cells. *Spleen and LN cells from WT or CD40L-deficient P-14 mice were incubated with DCs pulsed with 1 μg gp33 peptide, 1 μg gp33 peptide, or PMA and Ionomycin. T cell proliferation, measured by CPM, was determined after three days of culture.*

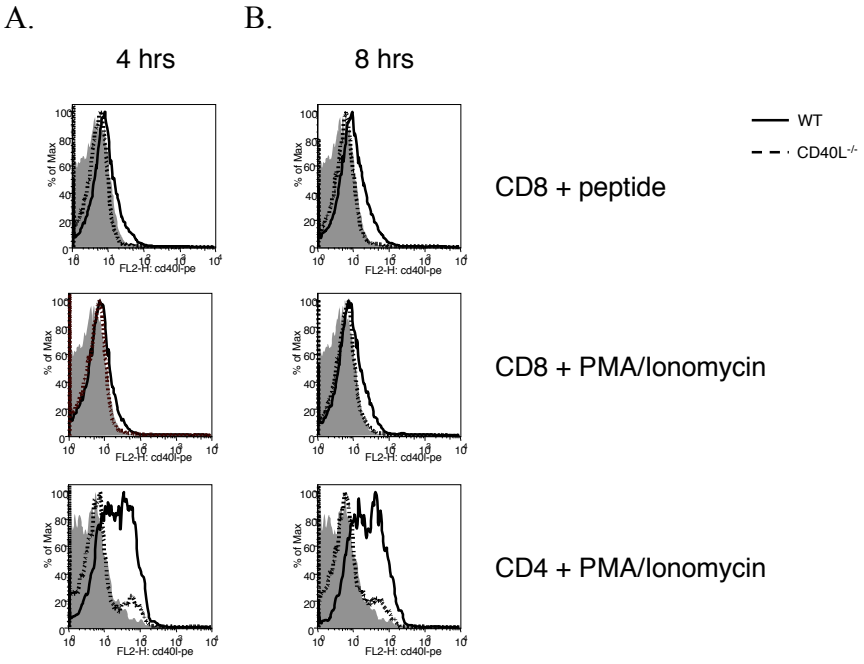


Figure 28. CD8$^+$ T cells express CD40L. *Spleen and LN cells from WT or CD40L-deficient P-14 mice were incubated with gp33 peptide or PMA and Ionomycin. At the indicated time points, the cells were stained for both surface and intracellular CD40L as well as CD8 and CD4. Samples were analyzed by flow cytometry. (A) CD40L expression at 4 hours. (B) CD40L expression at 8 hours. The shaded histogram represents staining with control Ab.*

albeit at much lower levels than in $CD4^+$ T cells incubated with PMA/Ionomycin (Fig. 28A). By 8 hrs, there was a slight increase in CD40L expression on the WT P-14 T cells, but the levels were still much lower compared to that of the $CD4^+$ T cells (Fig. 28B). We used CD40L-deficient P-14 T cells as a control and as expected, these cells did not express CD40L.

B. Reduced responses by CD40L-deficient $CD8^+$ T cells in vivo

We then analyzed the response of CD40L-deficient P-14 T cells in WT hosts after immunization with the LCMV 13-mer peptide. In this system all the host APCs express CD40 and all the host T cells express CD40L; only the adoptively transferred T cells are unable to express CD40L (Fig. 29). CD40L-deficient P-14 T cells proliferated about 2-fold less compared to WT P-14 T cells upon immunization with two different concentrations of peptide (Fig. 30A and 30B). The proportion and absolute number of CD40L-deficient P-14 T cells secreting IFN–γ was also significantly decreased (Fig. 30C and 30D). We also examined the response of CD40L-deficient P-14 T cells in WT versus $CD4^+$ T cell-deficient hosts. We found a similar 2-fold reduction in the CD40L-deficient P-14 T cell response in the presence or absence of $CD4^+$ T cells (Fig. 31A-31C). These results are reminiscent of the WT P-14 T cell response upon immunization with $CD40^{-/-}$ DCs.

C. CD40L-deficient $CD8^+$ T cells are not inherently defective

We next investigated whether the reduced P-14 T cell response was due to deficient activation of the CD40-positive host APCs and not to an inherent defect of the

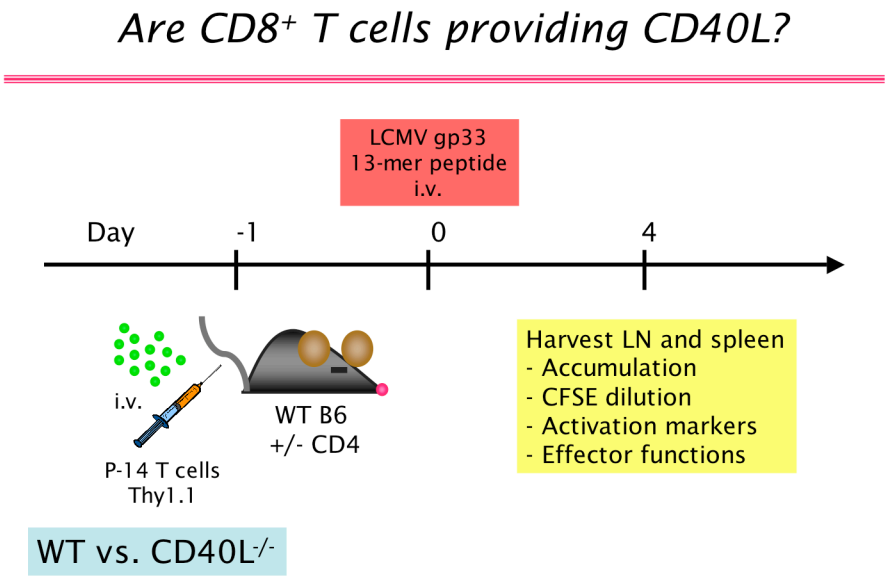


Figure 29. Experimental set-up to determine whether CD40L is being provided by antigen-specific CD8$^+$ T cells. *P-14 TCR-Tg mice were bred to CD40L$^{-/-}$ mice to obtain CD40L-deficient P-14 T cells. WT or CD4-depleted hosts were then adoptively transferred with either WT or CD40L-deficient P-14 T cells and immunized with LCMV 13-mer. Spleens and lymph nodes were analyzed on day 4. In this system all of the host cells are CD40 and CD40L sufficient; the only variable is CD40L expression on the transferred TCR-Tg CD8$^+$ T cells.*

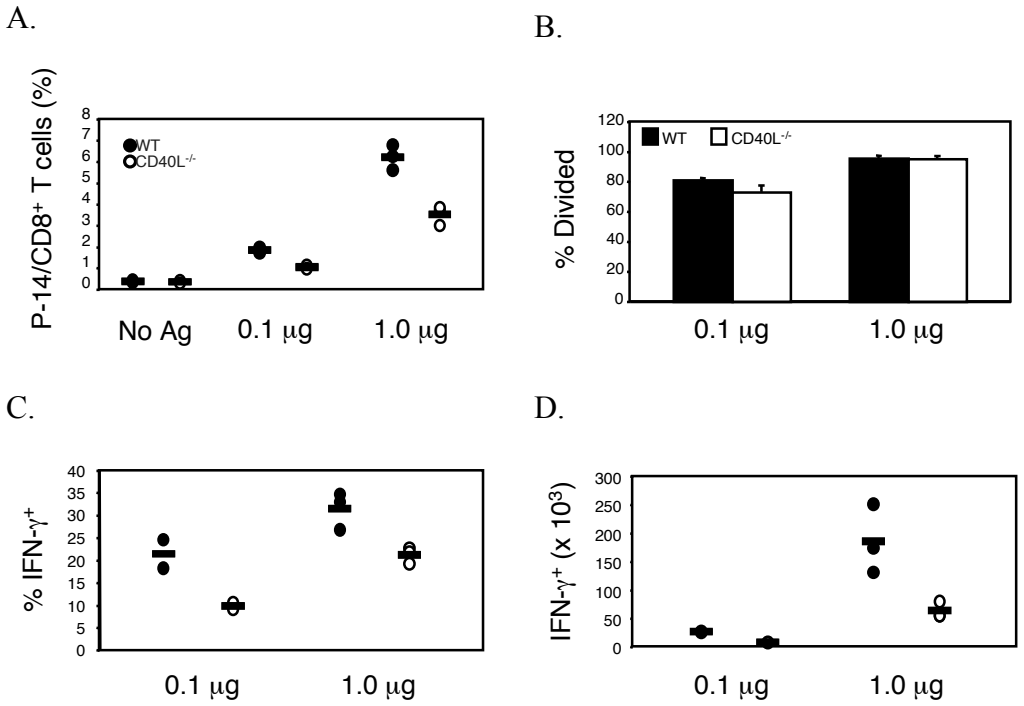


Figure 30. Reduced responses by CD40L-deficient CD8$^+$ T cells. *WT mice were adoptively transferred with CFSE-labeled Thy1.1$^+$ WT or CD40L-deficient P-14 T cells. One day later the mice were immunized i.v. with 0.1 μg or 1.0 μg LCMV 13-mer or left unimmunized. Spleens were harvested on day 4 and analyzed as in Fig. 1. (A) P-14 T cell expansion. (B) Percentage of P-14 T cells that have divided. (C) Percentage of IFN–γ producing P-14 T cells. (D) Absolute number of IFN–γ producing P-14 T cells. (A) and IFN-γ production (B) were analyzed as in Fig. 1. Each circle in (A), (C), and (D) represents an individual mouse; the bars represent the means.*

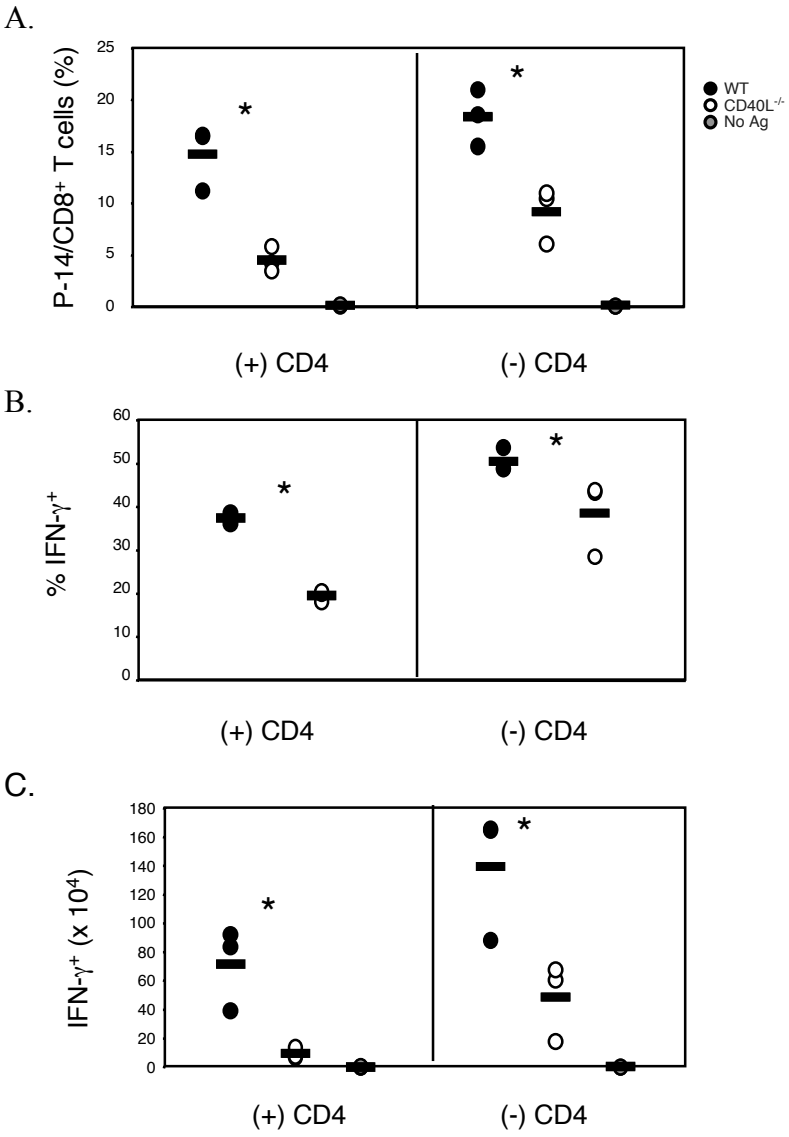


Figure 31. WT and CD40L-deficient P-14 T cell responses in WT and CD4+ T cell-deficient hosts. *CD4+ T cell-depleted or undepleted WT mice were adoptively transferred with CFSE-labeled Thy1.1+ WT or CD40L-deficient P-14 T cells. One day later the mice were immunized i.v. with 1 μg LCMV 13-mer or left unimmunized. Spleens were*

*harvested on day 4 and analyzed as in Fig. 1. (A) P-14 T cell expansion. (B) Percentage of IFN-γ producing P-14 T cells. (C) Absolute number of IFN–γ producing P-14 T cells. were analyzed as in Fig. 1. Each circle in (A) to (C) represents an individual mouse; the bars represent the means. The results shown are representative of three independent experiments with three mice per group. *, $p < 0.01$.*

CD40L-deficient P-14 T cells. To do this we injected the mice with agonistic anti-CD40 Ab at the time of immunization. This should result in activation of host APCs, thereby eliminating their need for CD40L. Indeed, treatment with agonistic anti-CD40 Ab increased the CD40L-deficient P-14 T cell response to WT levels (Fig. 32A-32C). This increased response was not observed when $CD40^{-/-}$ mice were used as hosts (Fig. 33). This rules out the possibility that the increased responses were caused by the antibody directly activating the transferred T cells, which can express CD40.

D. WT $CD8^+$ T cells can provide help to CD40L-deficient $CD8^+$ T cells

To further investigate whether the reduced response by CD40L-deficient P-14 T cells is not due to an intrinsic defect, we performed adoptive transfers using a 1:1 mixture of WT and CD40L-deficient P-14 T cells (Fig. 34). This resulted in equal expansion and IFN-γ production of both T cell populations (Fig. 35A-35C). Injection of the anti-CD40L blocking Ab MR1 inhibited the ability of the WT P-14 T cells to rescue the response of the CD40L-deficient P-14 T cells (Fig. 35D). Therefore, the CD40L-deficient T cells were fully functional when the WT $CD8^+$ T cells provided the CD40L signal, presumably to the CD40-expressing APCs.

E. Other CD40L-expressing cells do not contribute to the $CD8^+$ T cell response

Our experiments thus far do not address whether other CD40L-expressing cells from the host might be providing "help" to the adoptively transferred T cells. To address this issue, we used $CD40L^{-/-}$ mice, which are completely unable to express CD40L, as hosts. The magnitude of T cell expansion was very similar in WT and $CD40L^{-/-}$ hosts,

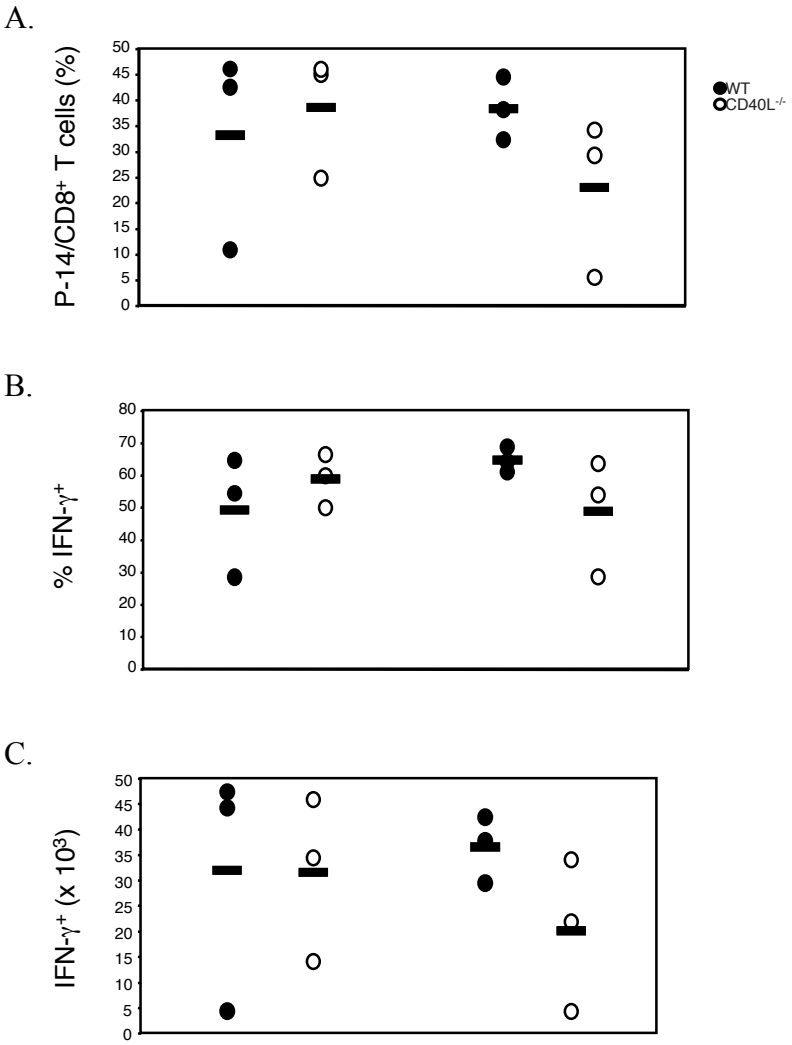


Figure 32. Agonistic anti-CD40 Ab boosts WT and CD40L-deficient P-14 T cell responses. *CD4-depleted or undepleted WT mice containing WT or CD40L-deficient P-14 T cells that have been labeled with CFSE were immunized i.v. with 1μg LCMV 13-mer and injected i.p. with 50 μg of the agonistic anti-CD40 Ab FGK45. Spleens were harvested on day 4 and analyzed as in Fig. 1. (A) Percentage of P-14 T cells in the total $CD8^+$ T cell population. (B) Percentage of IFN-γ producing P-14 T cells. (C) Absolute*

number of IFN−γ producing P-14 T cells. Each circle in (A) to (C) represents an individual mouse; the bars represent the means. The results shown are representative of two independent experiments.

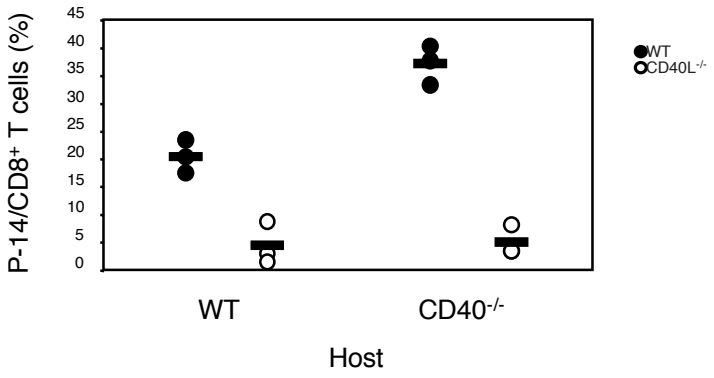


Figure 33. Agonistic anti-CD40 Ab does not directly activate P-14 T cells. *WT or CD40$^{-/-}$ mice were adoptively transferred with CFSE-labeled Thy1.1^{+} WT or CD40L-deficient P-14 T cells. One day later the mice were immunized i.v. with 1 μg LCMV 13-mer together with 50 μg of the agonistic anti-CD40 Ab FGK45 injected i.p.. Spleens were harvested on day 4 and the percentage of P-14 T cells in the total CD8^{+} T cell population was determined. Each circle represents an individual mouse; the bars represent the means.*

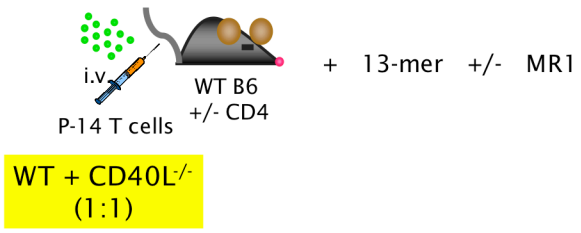


Figure 34. Experimental set-up to determine whether CD8$^+$ T cells can provide CD40L for CD40 stimulation. *WT or CD4-depleted mice were adoptively transferred with WT or CD40L-deficient P-14 T cells mixed at a 1:1 ratio. The mice were then immunized with LCMV 13-mer in the presence or absence of the anti-CD40L blocking Ab MR1. This system tests whether CD40L-deficient CD8$^+$ T cells can respond normally in the presence of other CD40L-expressing CD8$^+$ T cells that can activate CD40 on APCs.*

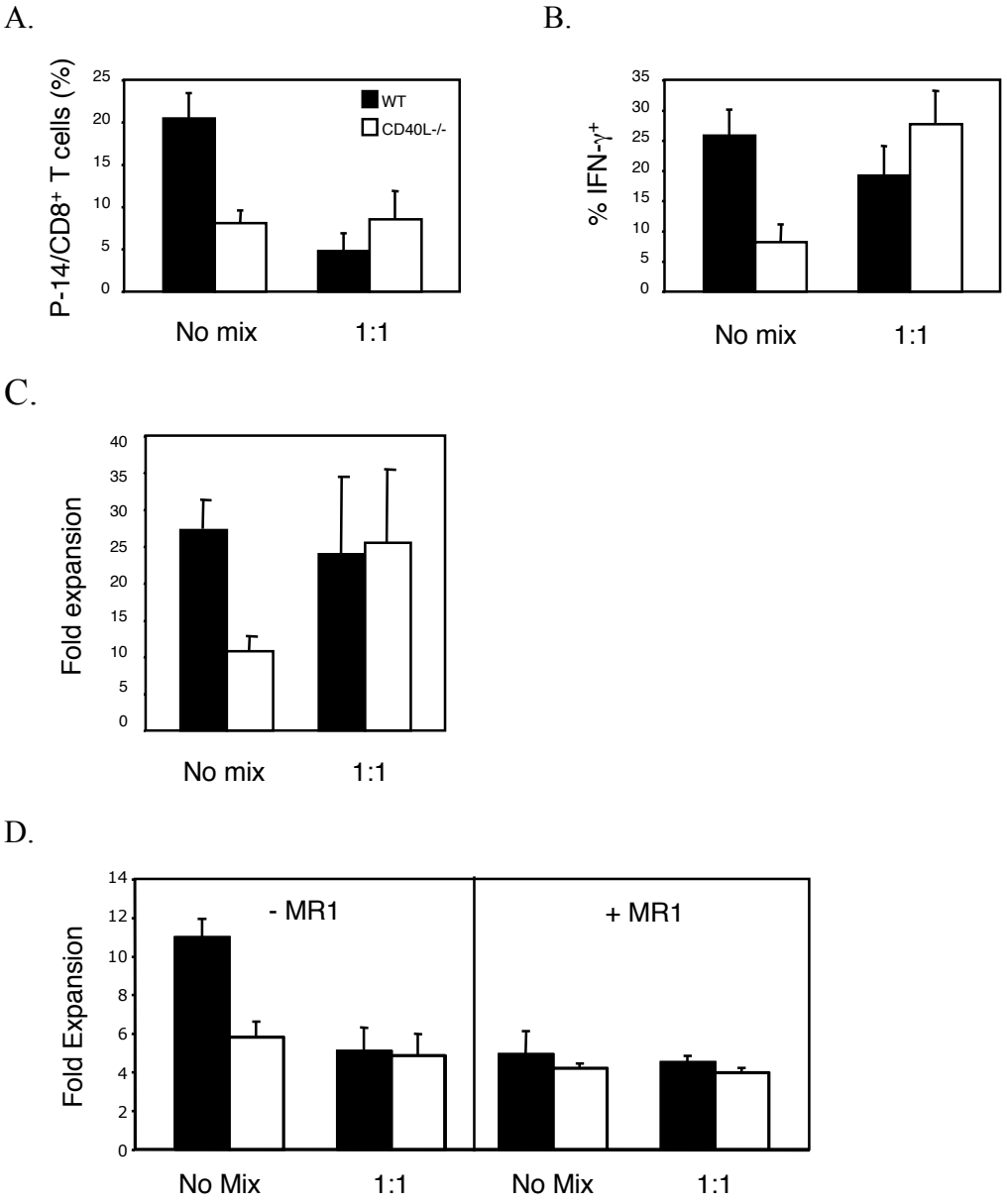


Figure 35. WT P-14 T cells can rescue the response of CD40L-deficient P-14 T cells. *CD4-depleted WT hosts were divided into two groups and adoptively transferred with either CD45.1$^+$ WT or Thy1.1$^+$ CD40L-deficient P-14 T cells alone or a 1:1 mixture of WT and CD40L-deficient P-14 T cells. One day later the mice were immunized i.v. with 1*

μg LCMV 13-mer in the presence (D) or absence (A-C) of MR1 Ab and the spleens were harvested on day 4. (A) Percentage of P-14 T cells in the total $CD8^+$ T cell population. (B) Percentage of IFN−γ producing cells. (C) and (D) Fold expansion was calculated by dividing the percentage of WT or $CD40L^{-/-}$ P-14 T cells in the spleens of immunized mice by the percentage in unimmunized mice. The data are presented as mean + SD. The results shown are representative of two independent experiments with three mice per group.

but more importantly, the CD40L-deficient P-14 T cells still exhibited ~2-fold reduction in accumulation compared to the WT P-14 T cells (Fig. 36A and 36B). In preliminary experiments, we also obtained similar results using $RAG^{-/-}$ hosts, which lack both endogenous $CD4^+$ and $CD8^+$ T cells (data not shown).

Additionally, we found that upon depletion of $NK1.1^+$ cells, the proliferation of CD40L-deficient P-14 T cells was still reduced compared to that of WT P-14 T cells (Fig. 37). Furthermore, in hosts that have been depleted of $NK1.1^+$ cells, the proliferation of WT P-14 T cells stimulated with $CD40^{-/-}$ DCs was also still reduced compared to those that were stimulated with WT DCs (Fig. 38). Depletion of platelets did not affect the ability of mice immunized with OVA-transfected cells to mount a primary CTL response (data not shown)

All of these results show that there is no inherent defect in the ability of the CD40L-deficient $CD8^+$ T cells to be primed and that their reduced response can be attributed to their inability to activate APCs. These data also provide functional evidence that CD40L expression by the responding $CD8^+$ T cells contributes to the generation of a maximal primary response in the presence or absence of $CD4^+$ T cells.

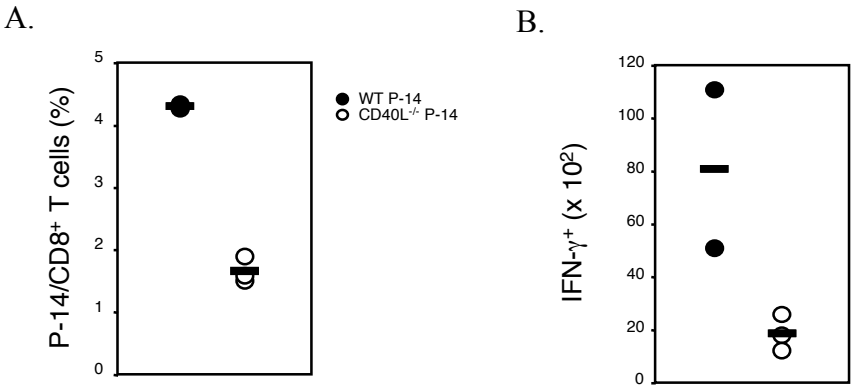


Figure 36. WT and CD40L-deficient P-14 T cell responses in CD40L$^{-/-}$ hosts. *CFSE labeled WT or CD40L-deficient P-14 T cells were adoptively transferred into CD40L$^{-/-}$ hosts, which were immunized one day later with 1 μg LCMV 13-mer peptide. Spleens were harvested four days later and analyzed as in Fig. 1. (A) Percentage of P-14 T cells in the total CD8^{+} T cell population. (B) Absolute number of IFN–γ producing P-14 T cells. The results are representative of two experiments with two to three mice per group.*

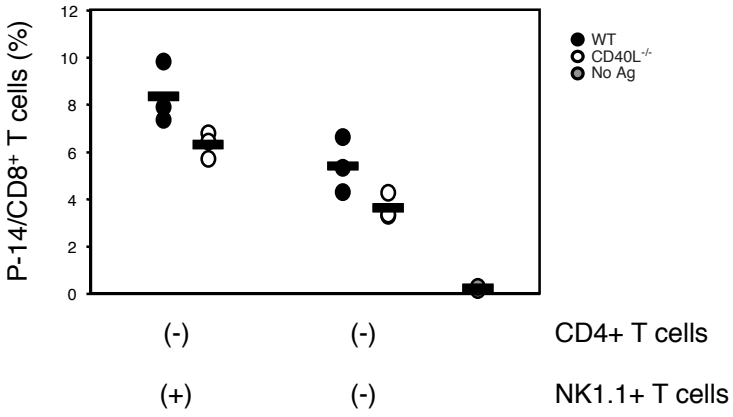


Figure 37. Response of WT and CD40L-deficient P-14 T cells in hosts depleted of NK1.1$^+$ cells. *WT mice were injected with GK1.5 Ab to deplete CD4$^+$ T cells or with a combination of GK1.5 and anti-NK1.1 Ab to deplete CD4$^+$ T cells as well as NK and NKT cells. They were then adoptively transferred with CFSE-labeled WT or CD40L-deficient P-14 T cells and immunized the next day with 1 μg LCMV 13-mer peptide. Spleens were harvested on day 4 and the percentage of P-14 T cells out of the total CD8$^+$ T cell population was determined. The results shown are representative of two independent experiments with two to three mice per group.*

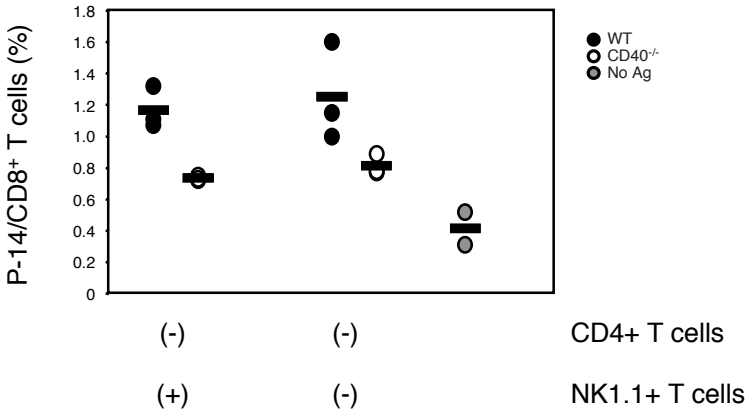


Figure 38. P-14 T cell responses induced by WT versus CD40$^{-/-}$ DCs in hosts depleted of NK1.1^{+} cells. *WT mice were injected with GK1.5 Ab to deplete CD4^{+} T cells or with a combination of GK1.5 and anti-NK1.1 Ab to deplete CD4^{+} T cells as well as NK and NKT cells. They were then adoptively transferred with CFSE-labeled P-14 T cells and immunized the next day with 1 x 10^{6} DCs from WT or CD40$^{-/-}$ mice pulsed with gp33 peptide. Spleens were harvested on day 4 and the percentage of P-14 T cells out of the total CD8^{+} T cell population was determined. The results shown are representative of two independent experiments with two to three mice per group.*

CHAPTER VIII.

TLR STIMULATION DOES NOT COMPENSATE FOR CD40 OR CD40L DEFICIENCY

Toll-like receptors (TLRs) comprise a subset of innate receptors that recognize pathogen associated molecular patterns (PAMPS) (Takeda and Akira, 2005). There are 11 TLRs in mice and 10 in humans, each with a different specificity. They are expressed on various cell types, but more importantly, they are expressed on APCs. It is thought that TLRs serve as the bridge between the innate and adaptive arms of the immune response (Akira et al., 2001). This is because TLR stimulation activates APCs and enhances their ability to induce T cell activation and differentiation (Pasare and Medzhitov, 2005; Reis e Sousa, 2004b). TLR stimulation induces similar effects as that of CD40 stimulation, e.g., activation of NF-κB, up-regulation of costimulatory molecules, production of cytokines, and promotion of cross-priming (Janeway and Medzhitov, 2002; Pasare and Medzhitov, 2005; Reis e Sousa, 2004b). Hence, we determined whether TLR stimulation could replace CD40 function in naïve $CD8^+$ T cell priming.

RESULTS

A. Reduced P-14 T cell responses in $CD40^{-/-}$ hosts even with TLR stimulation

We immunized WT and $CD40^{-/-}$ hosts containing adoptively transferred P-14 T cells with LCMV 13-mer alone or LCMV 13-mer plus the representative TLR agonists

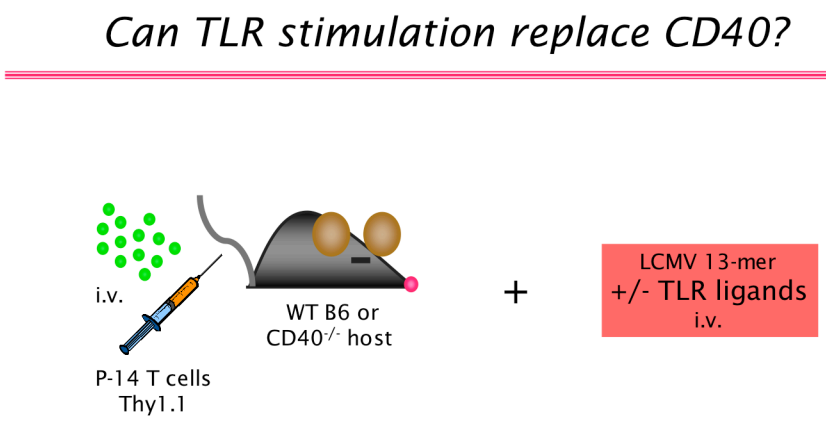


Figure 39. Experimental set-up to determine whether TLR stimulation can substitute for CD40 activation. *WT or CD40$^{-/-}$ hosts were adoptively transferred with CFSE-labeled P-14 T cells and immunized with LCMV 13-mer alone or with representative TLR ligands LPS, CpG, or polyI:C. In this system, all host APCs are either expressing or not expressing CD40. Furthermore, because the TLR ligands are injected into the mice, they could stimulate TLRs on cells other than APCs.*

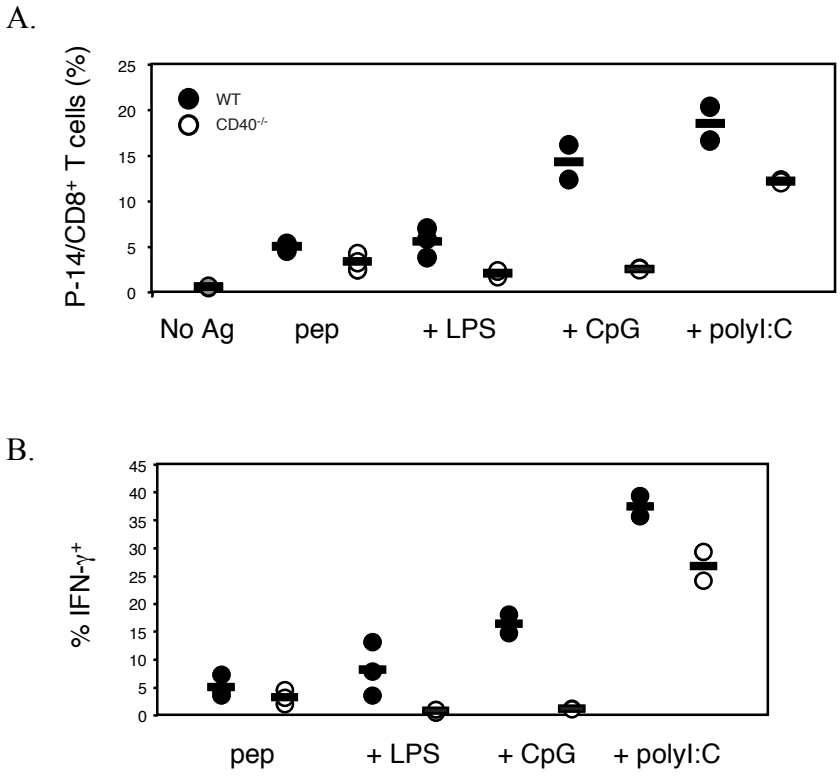


Figure 40. Reduced CD8$^+$ T cell responses in CD40$^{-/-}$ mice immunized with peptide in the presence of TLR agonists. *WT and CD40$^{-/-}$ mice were adoptively transferred with CFSE-labeled P-14 T cells. One day later the mice were immunized i.v. with 1 μg LCMV 13-mer peptide alone or together with the indicated TLR agonist injected i.p. Spleens were harvested on day 4 and analyzed as in Fig. 1. (A) Percentage of P-14 T cells in the total CD8$^+$ T cell population. (B) Absolute number of IFN-γ producing P-14 T cells in the spleen. Each circle represents an individual mouse; the bars represent the means. The results shown are representative of three independent experiments with two to three mice per group.*

LPS (TLR4), CpG (TLR9), or polyI:C (TLR3) (Fig. 39). As we have seen previously, P-14 T cell responses were reduced in the absence of CD40 signaling (Fig. 40A and 40B). In the presence of LPS, CpG, or polyI:C, in WT hosts there was a significant increase in P-14 T cell expansion as well as IFN–γ production (Fig. 40A and 40B). However, the P-14 T cell responses in the $CD40^{-/-}$ hosts were still reduced compared to the WT hosts in the presence of LPS or CpG (Fig. 40A and 40B). Interestingly, immunization in the presence of polyI:C induced a marked increase in the number of P-14 T cells in $CD40^{-/-}$ hosts. Nonetheless, when compared to the P-14 T cells in WT hosts, their number was still reduced approximately two-fold. These results indicate that in the absence of CD40 signaling, TLR stimulation is not sufficient to induce maximal primary $CD8^{+}$ T cell responses.

B. Phenotype of TLR-stimulated DCs

In the above experiment, all the APCs in the host are either CD40-sufficient or CD40-deficient. Since we showed that DCs are the major APCs that mediate CD40 signaling, we next examined whether TLR stimulation can substitute for the effect of CD40 stimulation on DCs. First, we analyzed the phenotype of WT and $CD40^{-/-}$ DCs that were incubated with peptide in the presence or absence of LPS, CpG, or polyI:C. There was no difference in activation status between WT and $CD40^{-/-}$ DCs incubated with peptide alone, similar to what we have shown before. Addition of TLR agonists resulted in up-regulation of CD80, CD86, $I\text{-}A^{b}$, $H\text{-}2K^{b}$, and IL-12, but again the WT and $CD0^{-/-}$ DCs had similar levels of these molecules (Fig. 41 and Fig. 42). We also quantified the number of peptide-MHC complex on DCs pulsed with SIINFEKL peptide using the

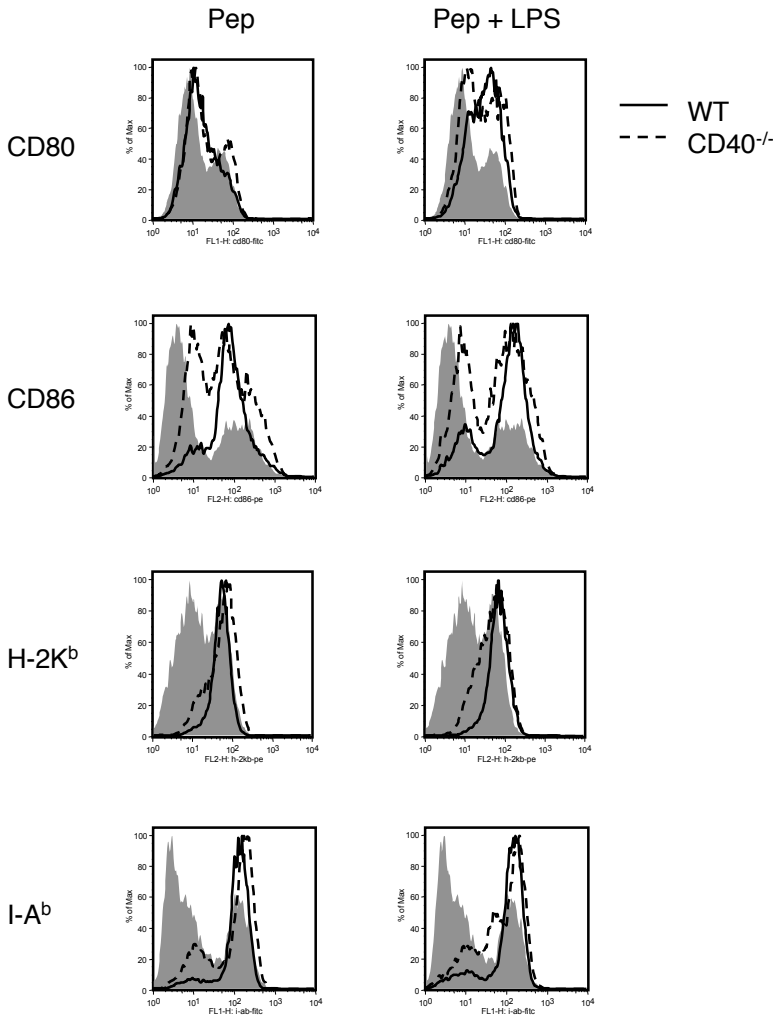


Figure 41. Phenotype of WT and CD40$^{-/-}$ DCs stimulated with TLR agonists. *WT and CD40$^{-/-}$ DCs were incubated overnight with peptide and the TLR agonists LPS, CpG, and polyI:C and stained with Ab against CD80, CD86, MHC class I (H-2K^b), and MHC class II (I-A^b). Representative FACS profiles are shown for DCs stimulated with peptide alone or peptide + LPS. Staining of DCs that were not pulsed with antigen is indicated by the solid histograms.*

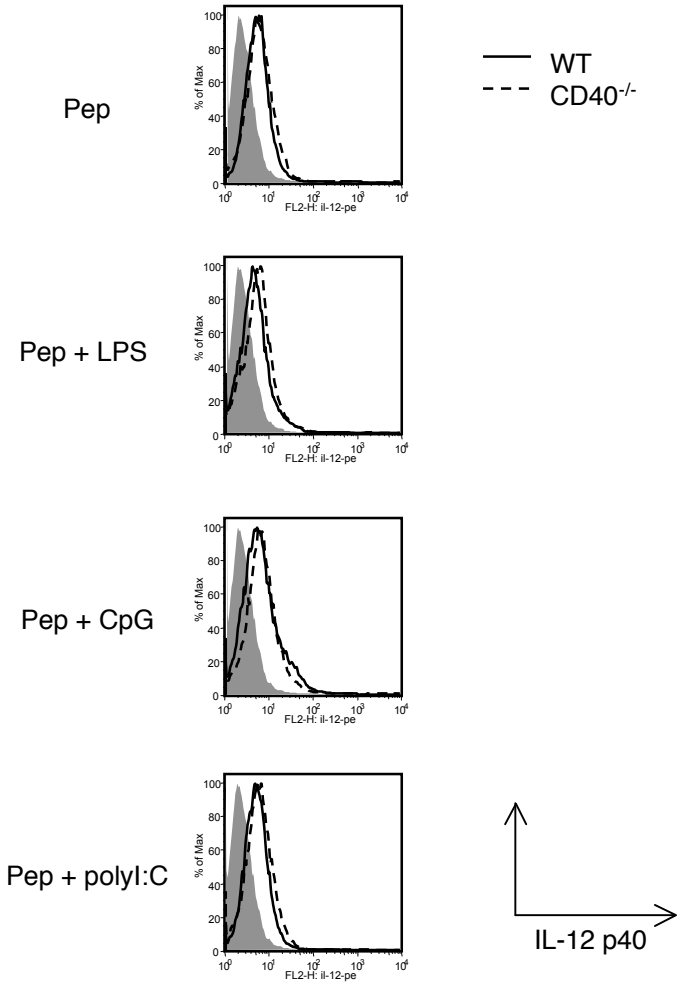


Figure 42. IL-12 production in WT and CD40$^{-/-}$ DCs stimulated with TLR agonists. *WT and CD40$^{-/-}$ DCs were incubated overnight with peptide and the TLR agonists LPS, CpG, and polyI:C and stained with Ab against the IL-12 p40 subunit. Representative FACS profiles are shown. Staining of DCs that were not pulsed with antigen is indicated by the solid histograms.*

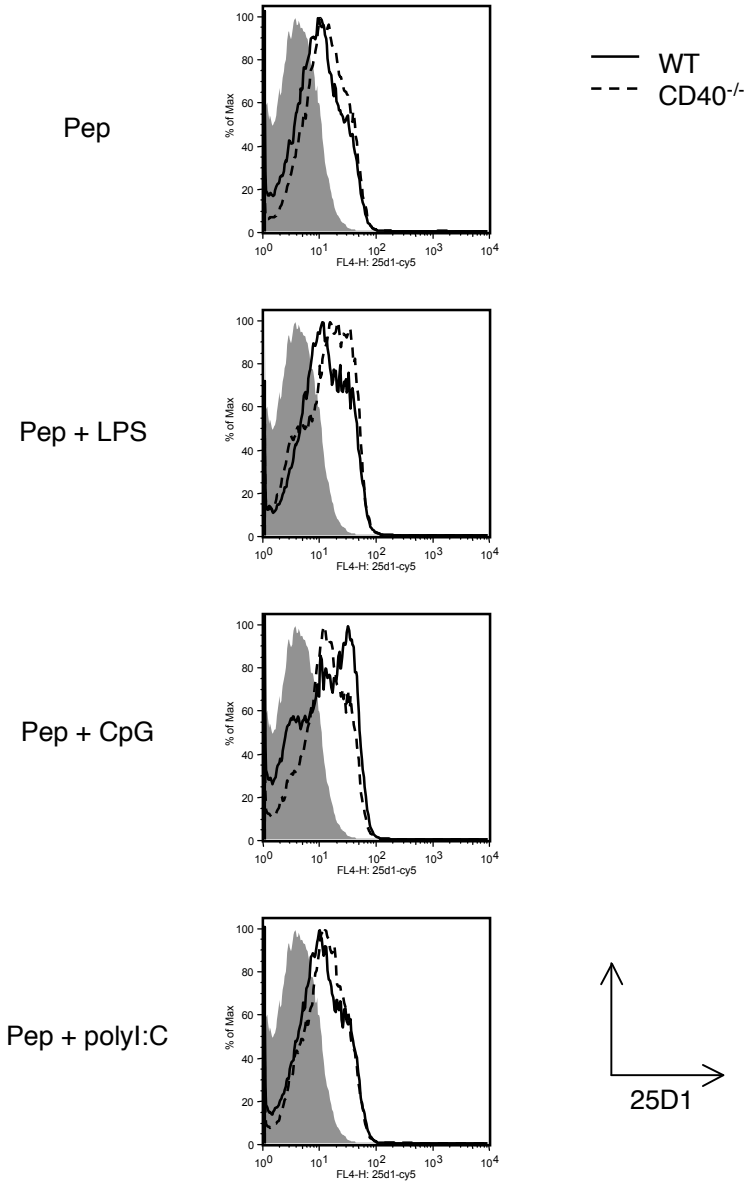


Figure 43. MHC-peptide levels in WT and CD40$^{-/-}$ DCs stimulated with TLR agonists. *WT and CD40$^{-/-}$ DCs were incubated overnight with the OVA peptide SIINFEKL and the TLR agonists LPS, CpG, and polyI:C. The cells were then stained with the 25D1 Ab, which recognizes SIINFEKL-K^{b} complexes. Representative FACS profiles are shown. Staining of DCs that were not pulsed with antigen is indicated by the solid histograms.*

25D1 Ab and found no difference between the WT and $CD40^{-/-}$ DCs (Fig. 43). These results show that there are no overt phenotypic differences in the markers examined between WT and $CD40^{-/-}$ DCs even with TLR stimulation.

C. Reduced responses induced by TLR-stimulated $CD40^{-/-}$ DCs

We then examined P-14 T cell responses in CD4-depleted WT hosts upon immunization with WT or $CD40^{-/-}$ DCs that were pulsed with gp33 peptide and incubated *in vitro* with the above TLR agonists (Fig 44). Consistent with our previous results, $CD40^{-/-}$ DCs pulsed with peptide alone stimulated less P-14 T cell proliferation compared to WT DCs (Fig. 45A). As expected, activation of DCs with any one of the TLR ligands augmented T cell expansion. However, whereas TLR-activated WT DCs induced a tremendous increase in T cell numbers (3 to 4-fold greater compared to peptide alone), TLR-activated $CD40^{-/-}$ DCs only induced a more modest increase (2-fold greater compared to peptide alone). More strikingly, $CD40^{-/-}$ DCs induced consistently lower P-14 T cell expansion and IFN-γ production compared to WT DCs (~50% less) despite TLR stimulation (Fig. 45A-45C). In other words, in the presence of TLR ligands CD40-deficient DCs were still inferior to CD40-sufficient DCs in stimulating naïve $CD8^{+}$ T cells.

D. Reduced responses of CD40L-deficient P-14 T cells even with TLR stimulation

In similar experiments, we analyzed the response of WT and CD40L-deficient P-14 T cells in CD4-depleted WT hosts immunized with different concentrations or LCMV 13-mer peptide with or without LPS as a representative TLR ligand (Fig. 46). In this situation all the host APCs express CD40 and CD40L-deficiency is restricted to the

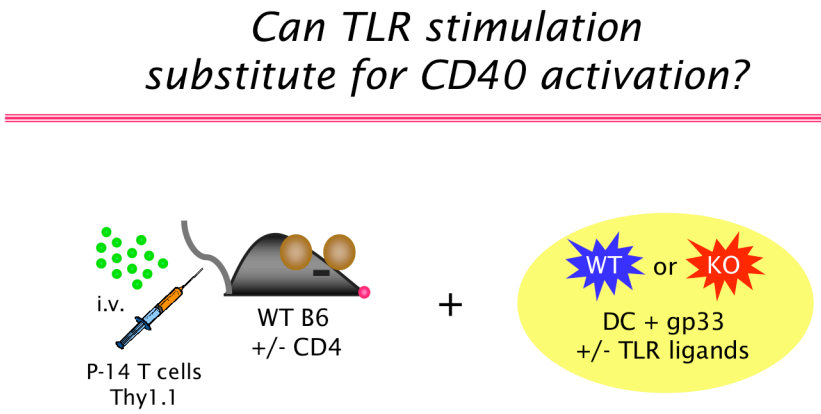


Figure 44. Experimental set-up to determine whether TLR stimulation can replace CD40 stimulation on the ability of DCs to prime naïve CD8$^+$ T cell responses. *WT and CD40$^{-/-}$ hosts were adoptively transferred CFSE-labeled P-14 T cells. They were then immunized with DCs from WT or CD40$^{-/-}$ mice that have been pulsed with gp33 peptide and incubated with or without the representative TLR ligands LPS, CpG, and polyI:C. In this system, CD40 deficiency as well as TLR stimulation is restricted only to the immunizing DCs.*

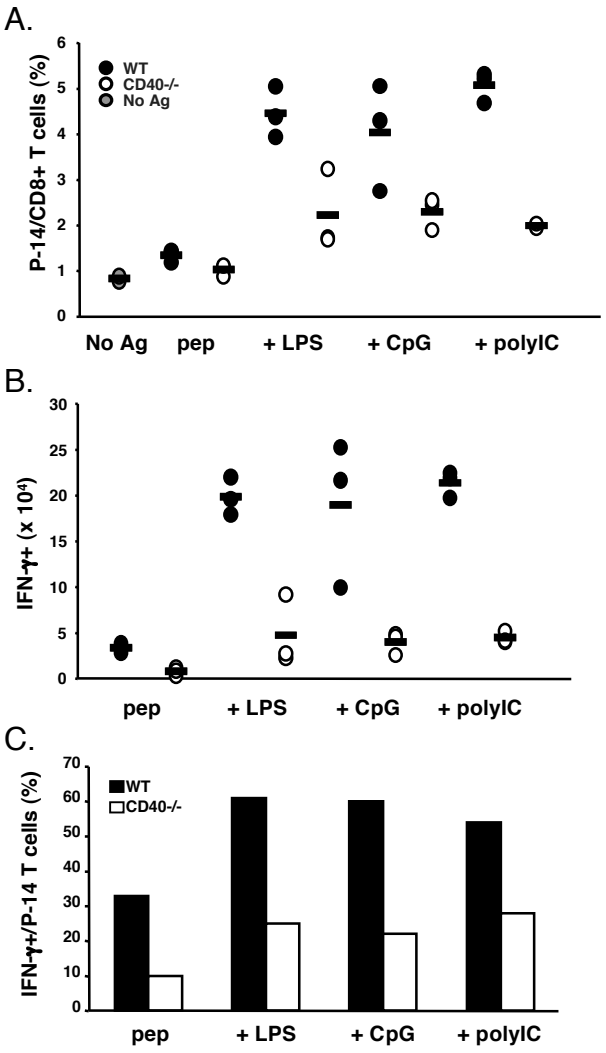


Figure 45. CD40$^{-/-}$ DC stimulated with TLR agonists induce reduced CD8^{+} T cell responses. *WT mice were depleted of CD4^{+} T cells and adoptively transferred with CFSE-labeled Thy1.1^{+} P-14 T cells. One day later the mice were immunized i.v. with 1 x 10^{6} gp33 peptide-pulsed or unpulsed DCs from the indicated mice that were stimulated with representative TLR agonists in vitro or left untreated. Spleens were harvested on day 4 and analyzed as in Fig. 1. (A) Percentage of P-14 T cells in the total CD8^{+} T cell*

population. (B) Absolute number of IFN-γ producing P-14 T cells in the spleen. (C) Percentage of IFN–γ producing P-14 T cells. Each circle in (A) and (B) represents an individual mouse; the bars represent the means. Mice immunized with unpulsed DC had a background of 2.5 x 10^3 IFN-γ^+ cells. The results shown are representative of three independent experiments with two to three mice per group.

adoptively transferred P-14 T cells. There was about a 2-fold reduction in proliferation of CD40L-deficient P-14 T cells compared to WT P-14 T cells upon immunization with either 0.1 μg or 1.0 μg of LCMV 13-mer peptide (Fig. 47A). Co-injection of LPS increased the response of both WT and CD40L-deficient P-14 T cells. Immunization with 0.1 μg peptide in the presence of LPS induced responses similar to that induced by 1 μg peptide alone. However, there was still a difference in expansion as well as the number of IFN–γ producing cells between WT and CD40L-deficient P-14 T cells (Fig. 47A and 47B).

We also observed that the CD40L-deficient P-14 T cells accumulated to a lesser extent upon co-injection of CpG and polyI:C with peptide (Fig. 48A). The absolute number of IFN-γ producing cells was also reduced (Fig. 48B). In these experiments, the reduced response of CD40L-deficient P-14 T cells again paralleled that of WT P-14 stimulated with $CD40^{-/-}$ DCs. Taken together, these results establish that TLR stimulation cannot completely compensate for CD40 or CD40L deficiency. Therefore, CD40-CD40L signaling has a unique function in inducing maximal primary $CD8^+$ T cell responses.

E. TLR stimulation fails to provide complete costimulatory repertoire to $CD8^+$ T cells

Our results indicate that the ability of DCs to prime naïve $CD8^+$ T cells is different when they receive TLR stimulation alone or when their stimulation includes CD40 ligation. We analyzed P-14 responses upon immunization with TLR-stimulated WT or $CD40^{-/-}$ DCs in the presence or absence of anti-CD40L Ab (MR1) (Fig. 49). As we have previously shown, although TLR stimulation improved the responses induced by $CD40^{-/-}$ DCs, the levels were still lower compared to those induced by WT DCs. When

mice immunized with WT DCs were treated with MR1, the responses were reduced and became equivalent to those induced by $CD40^{-/-}$ DCs (Fig. 50).

We also examined the responses of WT and CD40L-deficient P-14 cells in WT and $CD40^{-/-}$ hosts immunized with LCMV 13-mer and LPS. As expected, in WT hosts, the proliferation and IFN–γ production of CD40L-deficient P-14 cells was impaired compared to WT P-14 cells. In contrast, in $CD40^{-/-}$ hosts, the response of both the WT and CD40L-deficient P-14 was low and similar to that of CD40L-deficient P-14 cells in WT hosts (data not shown).

Taken together, these data further evidence that DCs are unable to provide a complete costimulatory repertoire to naïve $CD8^{+}$ T cells in the absence of CD40 signaling, even when there is TLR stimulation.

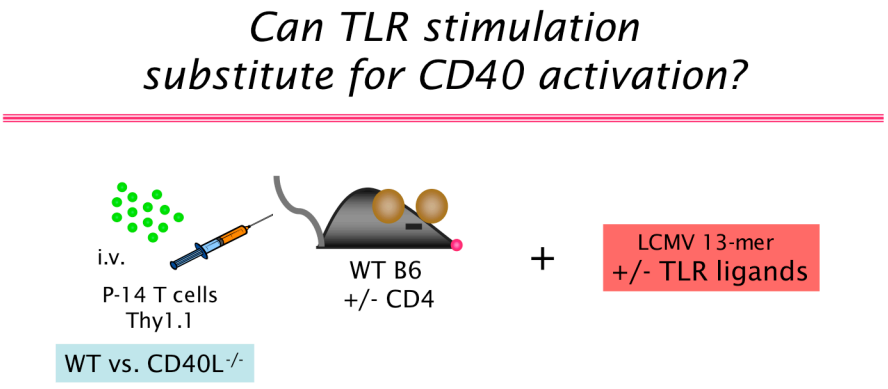
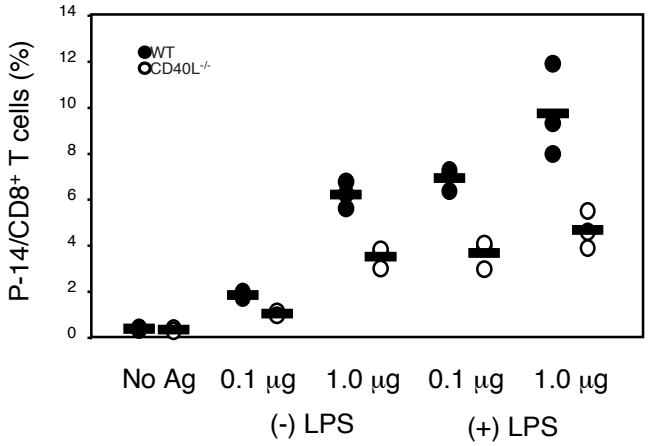


Figure 46. Experimental set-up to determine whether TLR stimulation can compensate for the absence of CD40L on CD8$^+$ T cells. *WT and CD4-depleted mice were adoptively transferred with WT or CD40L-deficient P-14 T cells that have been labeled with CFSE. They were then immunized with LCMV 13-mer alone or with the representative TLR ligands LPS, CpG, or polyI:C. In this system all the host cells express CD40 and CD40L; CD40L-deficiency is restricted only to the transferred TCR-Tg CD8$^+$ T cells.*

A.



B.

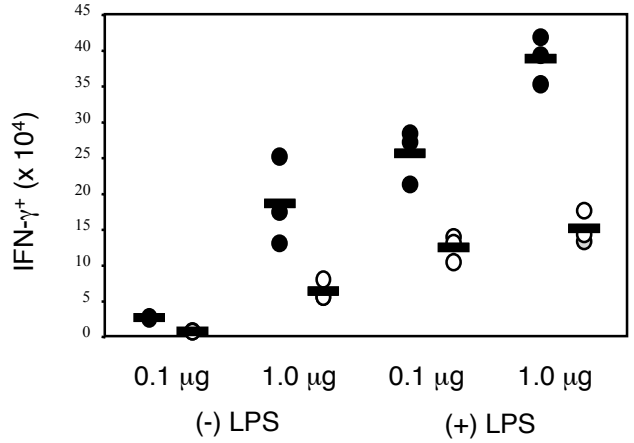


Figure 47. Reduced response by CD40L-deficient P-14 T cells in vivo even upon LPS stimulation. *WT mice were adoptively transferred with CFSE-labeled WT or CD40L-deficient Thy1.1$^+$ P-14 T cells. One day later the mice were immunized i.v. with 0.1 μg or 1.0 μg LCMV 13-mer alone, or together with LPS, which was injected i.p.. Spleens were harvested on day 4 and analyzed for (A) Percentage of P-14 T cells in the total CD8$^+$ T cell population and (B) Absolute number of IFN-γ producing P-14 T cells. Each circle represents an individual mouse; the bars represent the means. The results shown are representative of two independent experiments with two to three mice per group.*

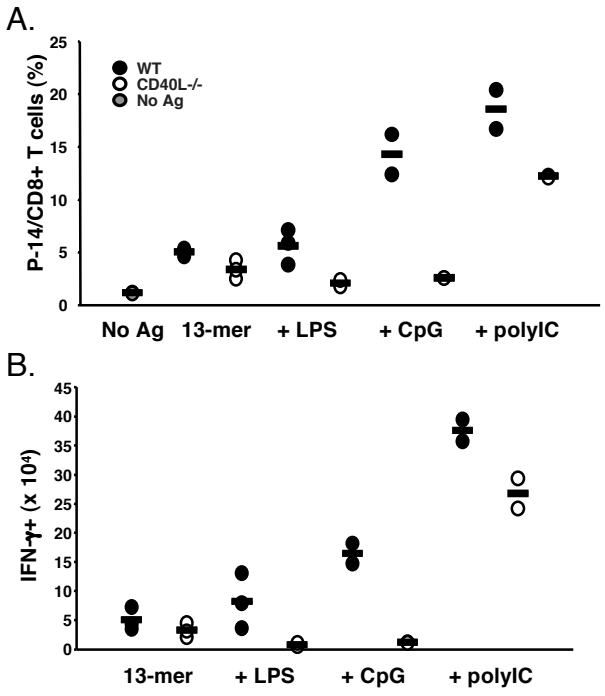


Figure 48. TLR agonists fail to completely restore impaired responses by CD40L-deficient P-14 T cells. *CD4-depleted WT mice were adoptively transferred with WT or CD40L-deficient Thy1.1$^+$ P-14 T cells. One day later the mice were immunized i.v. with 1 μg LCMV 13-mer alone, with LCMV 13-mer plus representative TLR agonists, or left unimmunized. Spleens were harvested on day 4 and analyzed for (A) Percentage of P-14 T cells in the total CD8$^+$ T cell population and (B) Absolute number of IFN-γ producing P-14 T cells. Each circle represents an individual mouse; the bars represent the means. Unimmunized mice had no IFN-γ$^+$ cells. The results shown are representative of two independent experiments with two to three mice per group.*

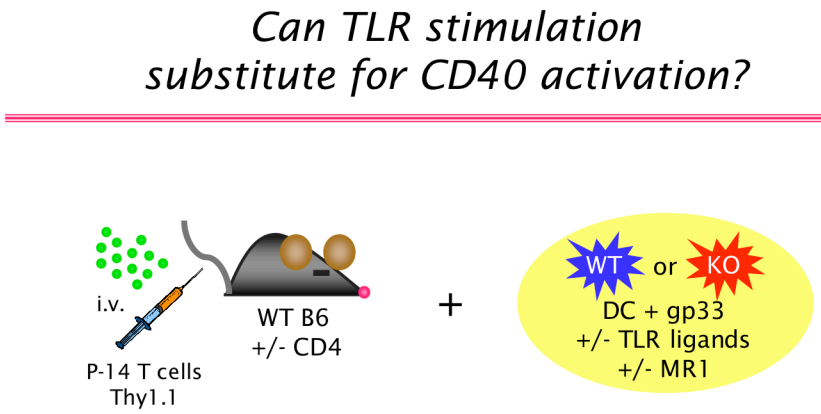


Figure 49. Experimental set-up to determine whether CD40L stimulation is providing unique signals to DCs to induce naïve CD8$^+$ T cell priming. *WT mice were adoptively transferred with CFSE-labled P-14 T cells. They were then immunized with WT or CD40$^{-/-}$ (KO) DC that have been pulsed with gp33 peptide alone or with TLR ligands. In addition, immunization was performed in the presence or absence of MR1 Ab in order to block CD40L. If stimulation through CD40L is responsible for inducing a complete costimulatory repertoire, then the responses induced by WT DC + MR1 and CD40$^{-/-}$ DC should both be reduced compared to the responses induced by WT DC.*

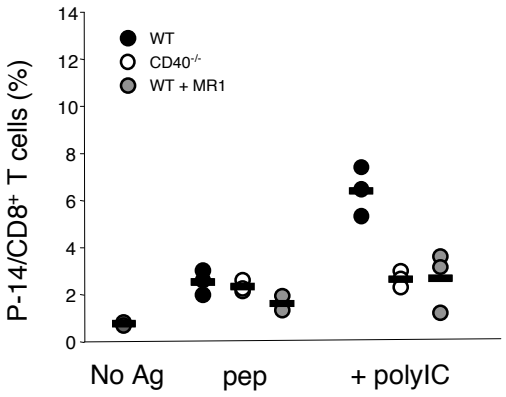


Figure 50. MR1 Ab reduces P-14 T cell proliferation induced by WT DCs. *WT mice were adoptively transferred with CFSE-labeled P-14 T cells. One day later they were immunized with One day later they were immunized i.v. with 1 x 10^6 gp33 peptide-pulsed or unpulsed DCs from the indicated mice that were stimulated with representative TLR agonists in vitro or left untreated. Additionally, immunization was done in the presence or absence of MR1 Ab, which was injected i.p.. Spleens were harvested on day 4 and the percentage of P-14 T cells in the total $CD8^+$ T cell population was determined. A representative result is shown with DCs treated with polyI:C as TLR agonist.*

CHAPTER IX.

CD40 SIGNALING AND CD8$^+$ T CELL MEMORY

Following exposure to Ag, naïve CD8$^+$ T cells undergo a proliferation and differentiation pathway that culminates in the establishment of memory. However, the signals that are required for memory CD8$^+$ T cell development and function are still not completely defined. There is evidence that Type I IFNs and/or IL-12 provide a third signal to naïve T cells in order to induce maximum proliferation and complete differentiation into memory cells (Curtsinger et al., 2005; Kolumam et al., 2005; Mescher et al., 2006; Valenzuela et al., 2002). In addition, the common γ-chain cytokines IL-7 and IL-15 have been shown to be critically involved in the homeostasis of memory cells (Fry and Mackall, 2005; Prlic et al., 2002) . More recently, CD4$^+$ T cells have been found to be essential in the programming as well as maintenance of memory CD8$^+$ T cells even when the primary response is independent of CD4$^+$ T cell help (Janssen et al., 2003; Masopust et al., 2004; Shedlock and Shen, 2003; Sun and Bevan, 2003; Sun et al., 2004; Williams et al., 2006a). The exact nature of the key programming and maintenance signals provided by CD4$^+$ T cells is however, still unknown.

In the preceding chapters we showed that CD40-CD40L interactions contribute to the induction of maximal primary CD8$^+$ T cell responses independently of CD4$^+$ T cell help. In this chapter we ask what role CD40 signaling plays in the generation, maintenance and function of memory CD8$^+$ T cells.

RESULTS

A. Decreased numbers of memory cells in CD40$^{-/-}$ hosts

Similar to what has been reported for CD4^{+}T cells, CD40 signals may also be involved not only in the programming/generation of memory CD8^{+} T cells but also in their maintenance and/or function. In order to have a better understanding of what role CD40 plays in each of these processes, we again used an adoptive transfer system. We injected WT and CD40$^{-/-}$ mice with CFSE-labeled P-14 T cells expressing the CD45.1 congenic marker and infected them one day later with LCMV (Armstrong strain) in order to stimulate strong primary responses. We then monitored the P-14 response by staining for CD8^{+}/CD45.1^{+} cells in the blood of the infected animals at different time points (Fig. 51). The P-14 cells proliferated robustly in both WT and CD40$^{-/-}$ hosts, with the peak of expansion occurring at days 5-6 post-infection. However, in a majority of the experiments (5 of 7), even though the P-14 cells expanded considerably in the CD40$^{-/-}$ hosts, there was still a greater number of P-14 cells in the WT hosts at the peak of the response (Fig. 52A). Nevertheless, there was no difference in the percentage of IFN–γ producing cells, indicating that they were able to differentiate into functional effectors (Fig. 52B). When we looked at the number of P-14 cells remaining, we found that the P-14 cells in the CD40$^{-/-}$ underwent a much greater contraction compared to those in the WT hosts. While ~50% of the peak numbers of P-14 still remained in the WT hosts two weeks post-infection, only ~30% remained in the CD40$^{-/-}$ hosts. By 4 weeks post-infection, the P-14 numbers have gone down to 34% and 16% of the respective peak responses (Fig. 52C).

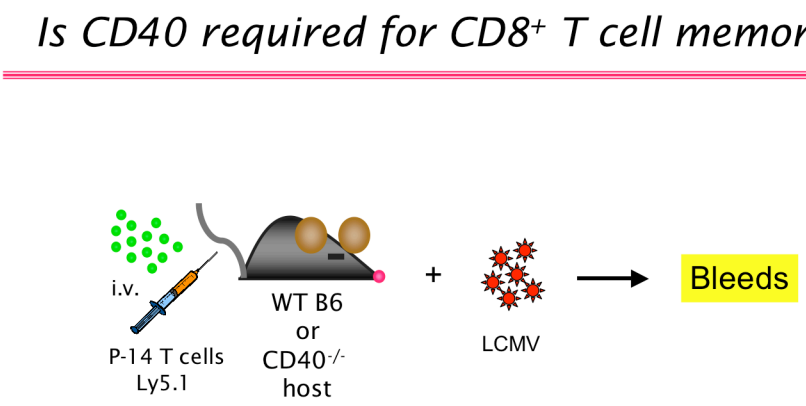


Figure 51. Experimental set-up to determine whether CD40 is involved in CD8$^+$ T cell memory. *WT and CD40$^{-/-}$ mice were adoptively transferred with CFSE-labeled P-14 T cells and then infected one day later with LCMV-Armstrong. P-14 T cell responses were monitored in the blood at different time points. In this system all host APCs are either sufficient or deficient in CD40 while the transferred TCR-Tg CD8$^+$ T cells are also CD40-sufficient. LCMV infection was used to stimulate a very robust CD8$^+$ T cell response; thus, making it more feasible to track the cells into the memory phase.*

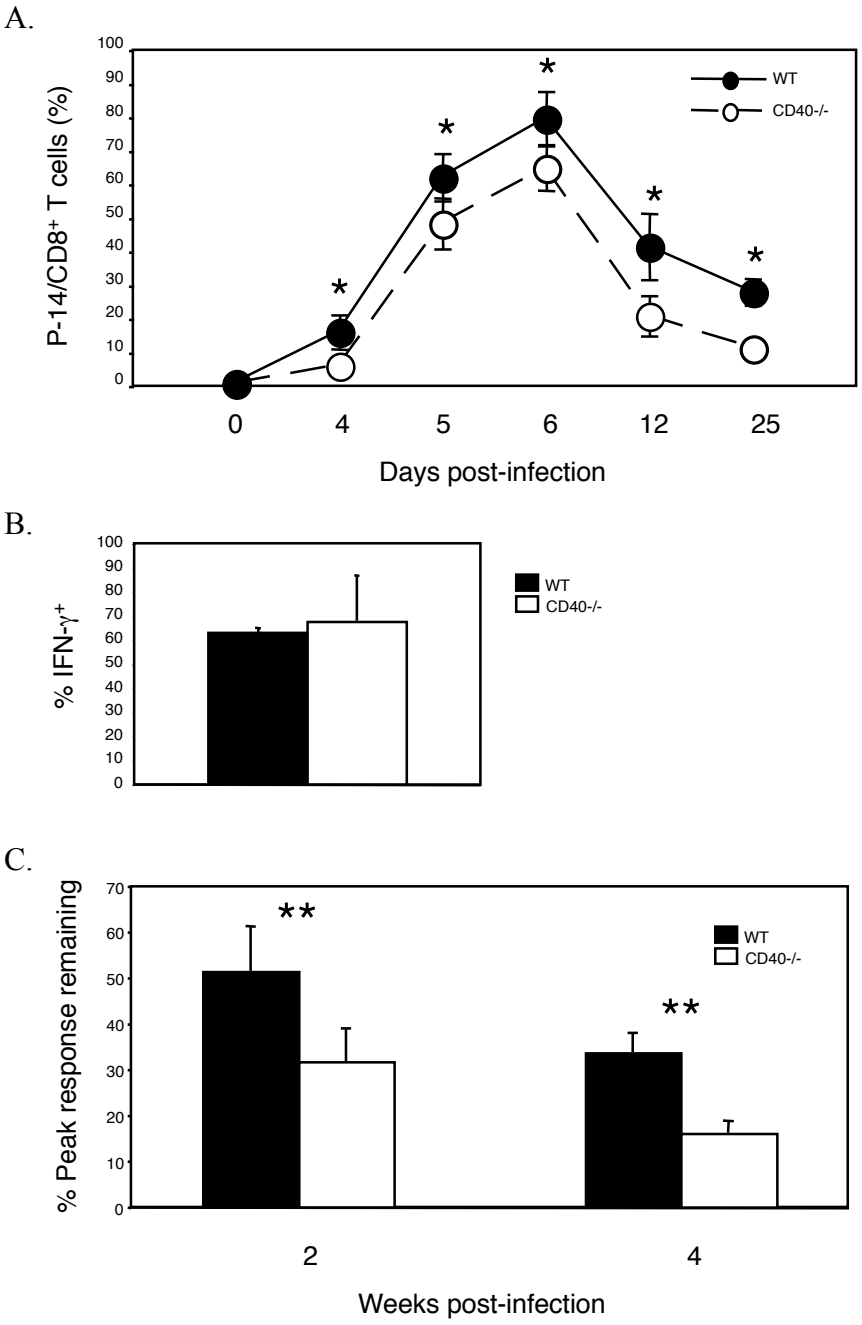


Figure 52. Reduced P-14 T cell accumulation in CD40$^{-/-}$ hosts upon LCMV infection. *WT and CD40$^{-/-}$ mice were adoptively transferred with CFSE-labeled CD45.1^{+} P-14 T cells. One day later the mice were infected with 1 x 10^{5} LCMV Armstrong strain i.p. The*

*mice were bled at the indicated time points, and the samples were stained with Ab against CD8 and CD45.1. (A) Percentage of P-14 T cells in the total $CD8^+$ T cells in the blood. (B) Blood samples were depleted of red blood cells and incubated for 5 hours with gp33 peptide in the presence of Brefeldin A and IL-2. The cells were stained with Ab against CD8 and CD45.1, and intracellular cytokine staining was performed as in Fig. 1. The percentage of IFN–γ producing P-14 T cells is shown. (C) Percentage of the peak P-14 T cell response remaining at the indicated time points. The results shown are representative of five different experiments with 5-10 mice per group. *, $p < 0.005$; **, $p < 0.0001$.*

In some experiments (2 of 7), the initial P14 T cell response was similar in magnitude between wild type and CD40-deficient hosts. The basis for this variation between experiments is not clear but presumably reflects some difference in the strength of the initial stimulation. The P-14 T cells made up as much as 70% of the $CD8^+$ T cells in the blood, and in addition, more than 60% of the P-14 cells are producing IFN-γ (Fig. 53A and data not shown). However, this parity was not maintained and at later time points the number of P-14 cells remaining in the $CD40^{-/-}$ hosts was approximately two-fold less compared to the WT hosts (Fig. 53A). Examination of earlier time points revealed that whereas the P-14 population in the WT hosts was still at peak levels one week after infection, the P-14 population in the $CD40^{-/-}$ hosts had already started to decline (Fig. 53B). Not only was the rate of P-14 contraction faster in the $CD40^{-/-}$ hosts, the magnitude of contraction was also greater.

The P-14 cells that remained in the WT and $CD40^{-/-}$ hosts had the same phenotype – they were CFSE-negative, CD44-hi, and contained both CD62L-hi and CD62L-lo cells (data not shown). Moreover, they were able to readily secrete IFN-γ directly ex vivo (Fig. 54). However, while 40% of the P-14 cells in the WT hosts were able to secrete IFN–γ, only 23% of the P-14 cells in the $CD40^{-/-}$ were able to do so. Overall, these results indicate that CD40 signaling is not necessary to generate memory $CD8^+$ T cells. However, in the absence of CD40 there is a roughly two-fold reduction in the number of functional memory cells. This effect seems to be independent of the role of CD40 in inducing maximal T cell expansion during a primary response.

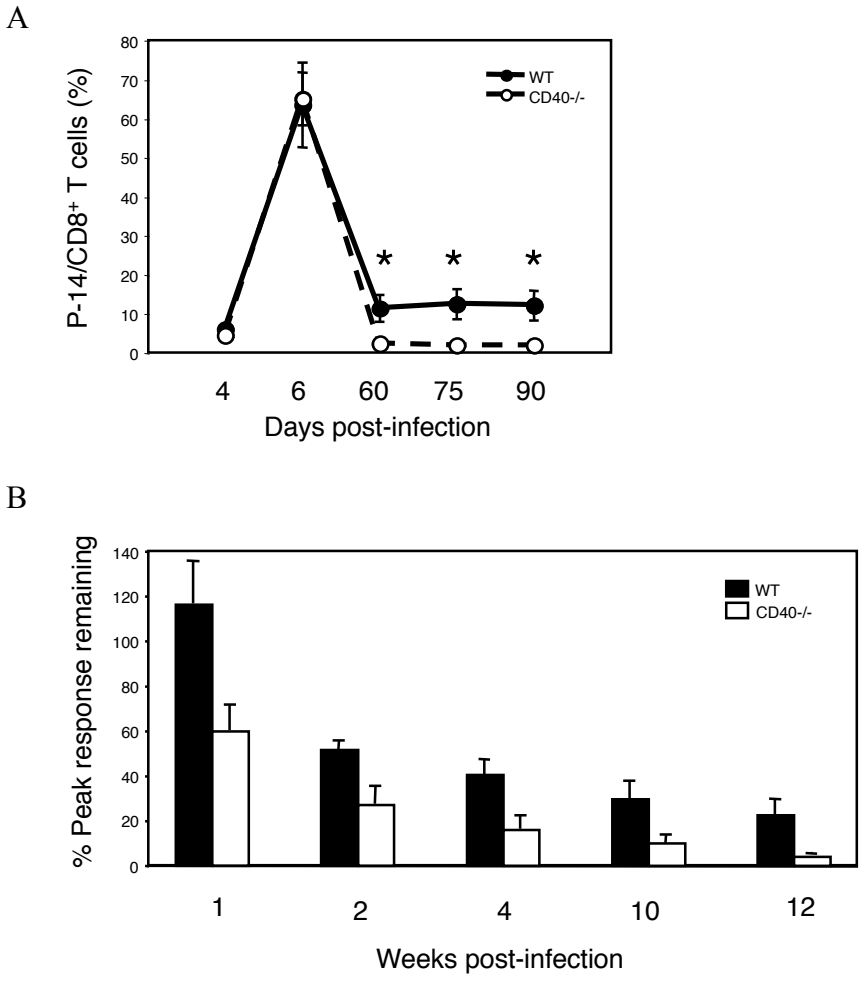


Figure 53. Equal P-14 T cell responses in WT and CD40$^{-/-}$ hosts upon LCMV infection. *WT and CD40$^{-/-}$ mice were adoptively transferred with CFSE-labeled CD45.1^{+} P-14 T cells and infected with 1 x 10^{5} LCMV Armstrong strain i.p. one day later. The mice were bled at the indicated time points and analyzed as in Fig. 38. (A) Percentage of P-14 T cells in the total CD8^{+} T cells in the blood. (B) Percentage of the peak P-14 T cell response remaining at the indicated time points. The results shown are representative of two different experiments with 5-10 mice per group. *, $p < 0.001$.*

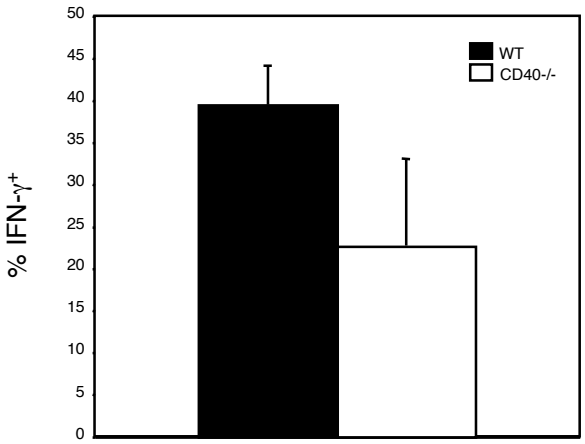


Figure 54. Functional memory CD8$^+$ T cells are generated in CD40$^{-/-}$ hosts. *WT and CD40$^{-/-}$ hosts were adoptively transferred with CFSE-labeled CD45.1$^+$ P-14 T cells and infected with 1 x 10^5 LCMV Armstrong strain i.p. one day later. Blood was collected 80 days after infection and stimulated in vitro with gp33 peptide for 5 hours. The percentage of IFN–γ producing P-14 T is shown. *, p = 0.02.*

B. Reduced memory CD8$^+$ T cells numbers in CD40$^{-/-}$ hosts is not due to differences in apoptosis

We hypothesized that the reduced numbers of memory CD8$^+$ T cell numbers in CD40$^{-/-}$ hosts could be due to differences in apoptotic cell death. To test this, we performed Annexin V staining. Annexin V is a Ca2$^+$-dependent protein that binds to phosphotidylserine (PS), a phospholipid that gets translocated from the inner to the outer leaflet of the plasma membrane starting at the earliest stages of apoptosis (Boersma et al., 2005). On day 4 post-infection, the majority of P-14 cells in both the WT and CD40$^{-/-}$ hosts were Annexin V$^+$. However, there was a small, but statistically significant increase in the percentage as well as MFI of Annexin V$^+$ cells in the CD40$^{-/-}$ hosts (Fig. 55A). At the same time, we examined the host CD8$^+$ T cells and found that they exhibited very little Annexin V binding (Fig. 56). By day 6 post-infection, the percentage as well as the MFI of Annexin V$^+$ P-14 cells were similar and had started to go down (Fig. 55A and 55B). By day 12 post-infection, the percentage as well as MFI of Annexin V$^+$ P-14 cells had returned to similar levels as that of the host CD8$^+$ T cells (Fig. 55 and 56). At a later time point however, there was a small, but statistically significant difference in the MFI of Annexin V$^+$ cells in the WT compared to CD40$^{-/-}$ hosts (Fig. 55B and data not shown). Because the proportion of P-14 cells that bind Annexin V as well as the intensity of Annexin V binding was the same at all but one of the time points examined, the difference in memory CD8$^+$ T cell numbers between WT and CD40$^{-/-}$ hosts is most likely not due to an increased rate of apoptosis. However, we cannot exclude the possibility that

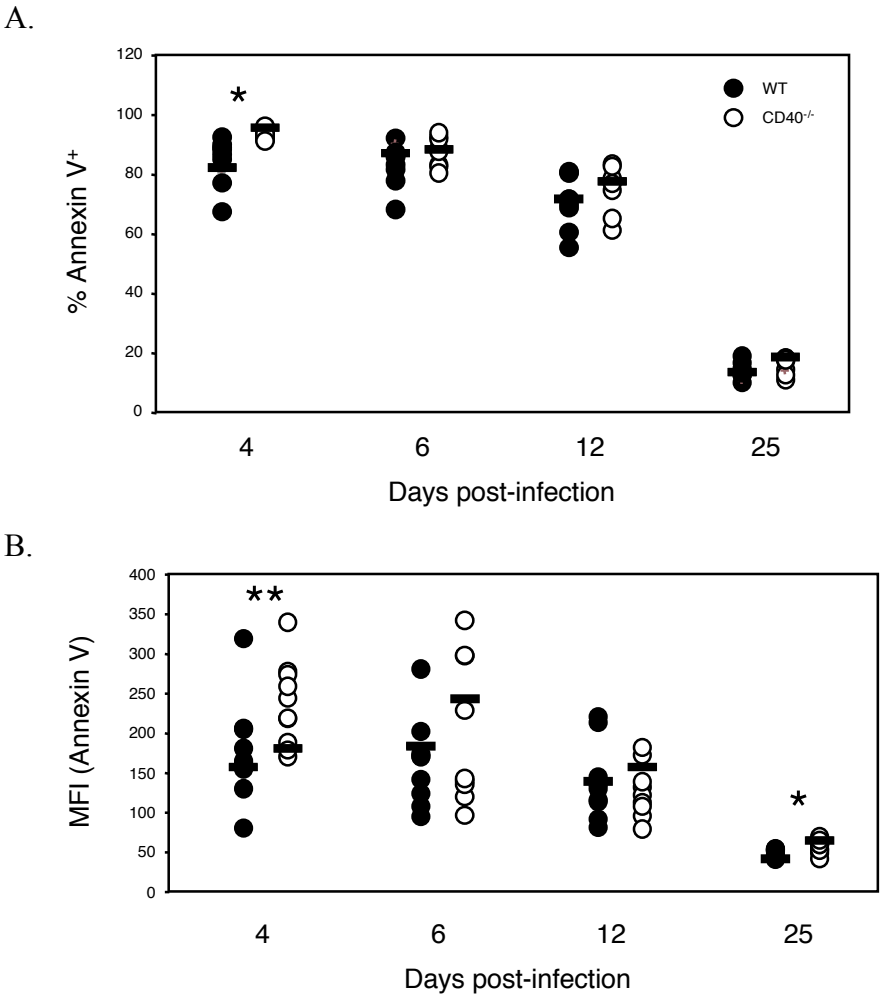


Figure 55. Similar levels of P-14 T cell apoptosis in WT and CD40$^{-/-}$ hosts infected with LCMV. *WT and CD40$^{-/-}$ mice were adoptively transferred with CFSE-labeled CD45.1^{+} P-14 T cells and infected with 1 x 10^{5} LCMV Armstrong strain i.p. one day later. The mice were bled at the indicated time points and stained with Ab against CD8 and CD45.1 as well as Annexin V-PE. (A) Percentage of Annexin V^{+} P-14 T cells. (B) MFI of Annexin V staining. Each circle represents an individual mouse; the bars represent the means.*

*The results shown are representative of three independent experiments with five to ten mice per group. *, $p < 0.01$; **, $p < 0.05$.*

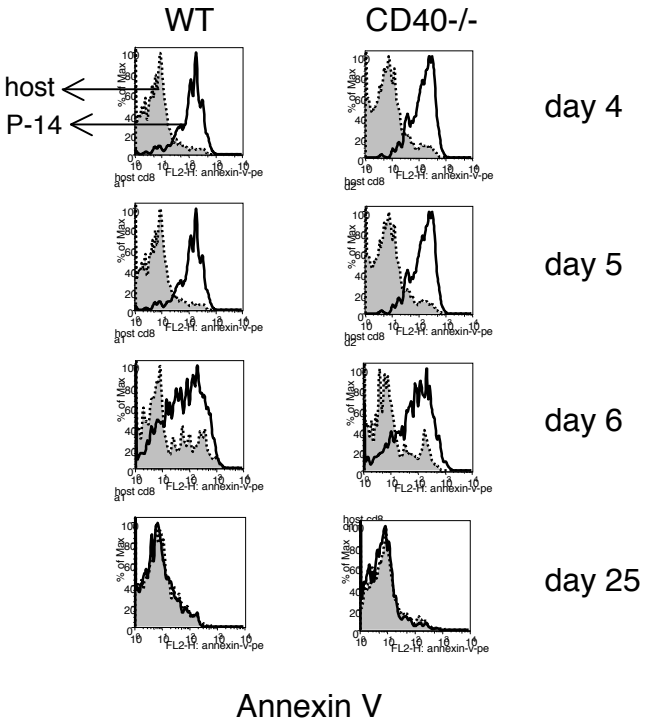


Figure 56. Comparison of Annexin V staining in P-14 vs. host CD8$^+$ T cells. *Representative histogram overlays of Annexin V staining in P-14 vs. host CD8$^+$ T cells at different time points after LCMV infection of WT and CD40$^{-/-}$ mice.*

differences in the rate of apoptosis that are too small to reliably measure could still contribute over time to the differences we observed.

C. Reduced memory CD8$^+$ T cells numbers in CD40$^{-/-}$ hosts correlates with IL-7R expression

The cytokine IL-7 is important in the homeostasis of naïve and memory CD8$^+$ T cells (Fry and Mackall, 2005; Prlic et al., 2002). IL-7 receptor (IL-7R) expression is high in naïve CD8$^+$ T cells, is down-regulated in effector cells, and goes back up again in memory cells. There have been recent reports that IL-7R expression serves as a marker for identifying effector cells that will develop into memory cells (Bachmann et al., 2005; Huster et al., 2004; Kaech et al., 2003). We therefore decided to examine IL-7R expression by the P-14 cells. During the peak of the response at day 6, the percentage of IL-7R$^+$ P-14 cells was similarly low in the WT and CD40$^{-/-}$ hosts (25% vs. 29%; Fig. 57). This finding is consistent with the down regulation of IL-7R expression by effector cells. By day 12 however, while the percentage of IL-7R$^+$ P-14 cells in the WT hosts had started to increase, the percentage of IL-7R$^+$ P-14 cells in the CD40$^{-/-}$ hosts remained low (44% vs. 27%; Fig. 57). Furthermore, whereas ~70% of the P-14 cells in the WT hosts had reacquired IL-7R expression by day 25, only ~33% of the P-14 cells in the CD40$^{-/-}$ hosts had done so (Fig. 57). Interestingly, this two-fold difference in IL-7R expression correlates with the roughly two-fold difference in memory CD8$^+$ T cell numbers between WT and CD40$^{-/-}$ hosts.

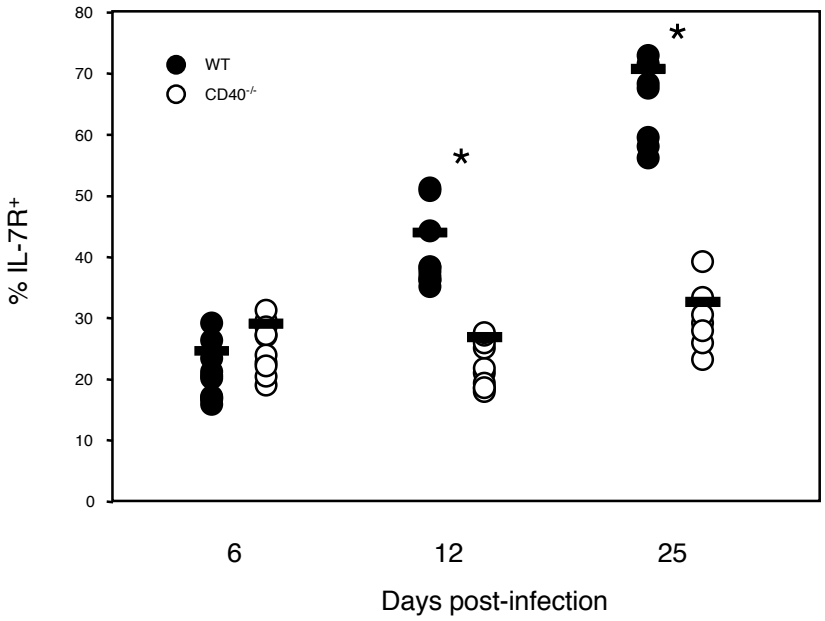


Figure 57. Reduced numbers of IL-7R$^+$ P-14 T cells in CD40$^{-/-}$ hosts. *WT and CD40$^{-/-}$ mice were adoptively transferred with CFSE-labeled CD45.1$^+$ P-14 T cells and infected with 1 x 10^5 LCMV Armstrong strain i.p. one day later. The mice were bled at the indicated time points and stained with Ab against CD8, CD45.1, and IL-7R. The percentage of IL-7R$^+$ P-14 T cells is shown. Each circle represents an individual mouse; the bars represent the means. The results shown are representative of three independent experiments with five to ten mice per group. *, $p < 0.00001$.*

D. CD40 is important in memory CD8$^+$ T cell differentiation and survival

We next investigated whether CD40 signaling is still important once the naïve CD8$^+$ T cells have already been activated and are undergoing differentiation into memory cells. To do this, we transferred P-14 cells from LCMV-infected mice into new, uninfected WT and CD40$^{-/-}$ hosts (Fig. 58). We harvested spleens 11-13 days post-infection to ensure that the virus has been cleared. We also normalized the number of splenocytes that we injected such that they contained equal numbers of P-14 cells.

We observed a gradual cell loss upon transfer of P-14 effector cells that have been primed in WT hosts into new WT hosts (Fig. 59A). This is expected since the majority of effector cells undergo apoptosis and only a small population becomes memory cells. By 5 weeks post-transfer, the P-14 population had stopped contracting and the number of cells remained stable. In contrast, there was a greater cell loss when we transferred the P-14 effector cells that have been primed in CD40$^{-/-}$ hosts into new WT hosts (Fig. 59A). By one week post-transfer, there was an approximately three-fold difference in the number of P-14 cells that initially came from the WT compared to the CD40$^{-/-}$ hosts. At later time points, there was a further decrease in the number of P-14 cells that came from CD40$^{-/-}$ hosts. Therefore, during the primary response CD40 in the host environment appears to be necessary to program CD8 T cells for optimal differentiation into long-lived memory cells.

Interestingly, when P-14 effector cells that were activated in WT mice were transferred into CD40-deficient hosts, there was also a marked reduction in their cell number (Fig. 59B). This indicates that after T cells have been activated and gone through

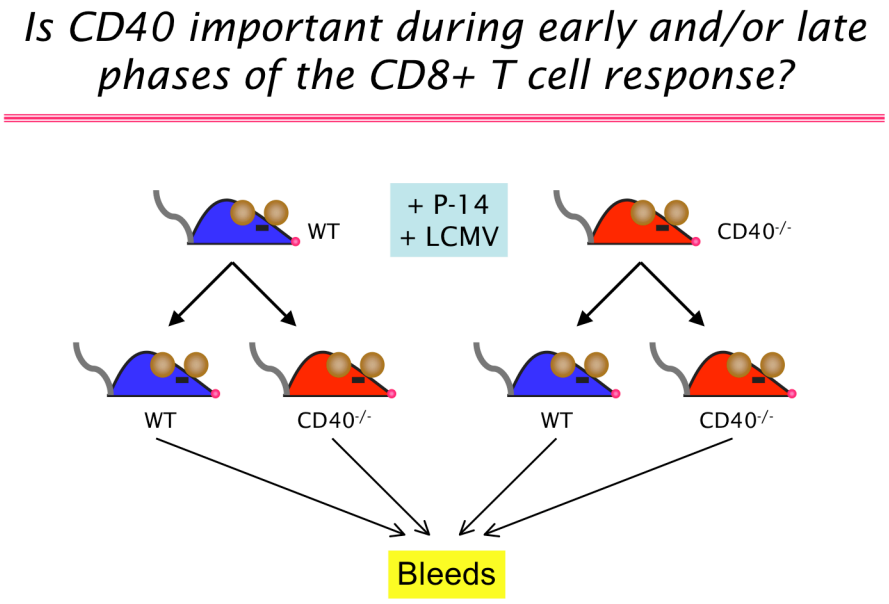


Figure 58. Experimental set-up to determine whether CD40 signals during and/or after priming are important for CD8$^+$ T cell memory. *Splenocytes were harvested from WT and CD40$^{-/-}$ mice containing adoptively transferred P-14 T cells 11-13 days post-infection with LCMV. The cells were then labeled with CFSE and transferred into new, uninfected WT or CD40$^{-/-}$ hosts. Bleeds were collected at different time points to monitor the survival of the transferred cells.*

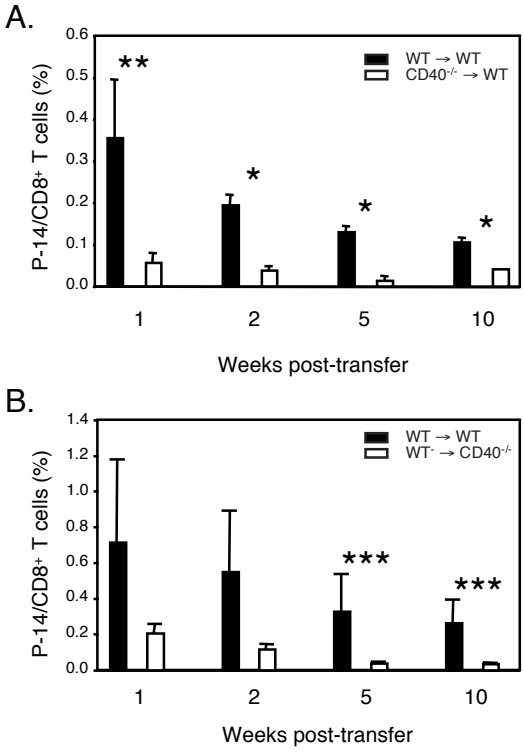


Figure 59. CD40 signals during and after priming affect CD8$^+$ T cell survival. *WT and CD40$^{-/-}$ mice were adoptively transferred with CFSE-labeled CD45.1$^+$ P-14 T cells and infected with 1 x 10^5 LCMV Armstrong strain i.p. one day later. Spleens were harvested 11-13 days after infection, depleted of red blood cells, and labeled with CFSE. They were then transferred into new uninfected WT or CD40$^{-/-}$ hosts. At the indicated time points the mice were bled and the survival of transferred P-14 T cells was determined. The data is presented as the percentage of P-14 T cells out of the total CD8$^+$ T cells in the blood. The results shown are representative of three independent experiments with three to five mice per group. ***, $p < 0.05$; **, $p < 0.01$; *, $p < 0.001$.*

their rounds of proliferation, there continues to be a role for CD40 signaling in the continued development and/or maintenance of CD8 memory cells.

E. CD40 signals are not required during a memory response

We then sought to address whether CD40 signaling plays a role in the recall response of memory $CD8^+$ T cells. To do this, we challenged the WT hosts that initially received effector P-14 T cells from either WT or $CD40^{-/-}$ mice with a vaccinia virus construct that expresses LCMV glycoprotein (Vac-gp). We performed the challenge 10 weeks post-transfer, when the effector cells had already differentiated into memory cells (Fig. 60). At this time point, the number of memory P-14 cells that were initially primed in WT donors was four times more than that of the memory P-14 cells that were initially primed in $CD40^{-/-}$ donors. We monitored memory responses by looking at the expansion of $CD45.1^+$ P-14 cells in the blood. Four days after challenge, the P-14 cells from WT donors comprised ~15% of the $CD8^+$ T cells and ~3% of the total cells in the blood of new WT hosts (Fig. 61A and 61B). In contrast, the P-14 cells from the $CD40^{-/-}$ donors comprised only ~4% of the $CD8^+$ T cells and ~1% of the total cells in the new WT hosts (Fig. 61A and 61B). Nonetheless, although there was a difference in cell numbers, the fold-expansion was similar as well as the percentage of IFN–γ producing cells (Fig. 61A-61C). Overall, these results indicate that CD40 signaling is not required during memory $CD8^+$ T cell responses.

We next performed experiments wherein we directly challenged the LCMV-immune WT and $CD40^{-/-}$ mice, which contain memory P-14 cells, with Vac-gp. Four days after challenge, the P-14 cells made up ~30% and ~5% of the $CD8^+$ T cells in the

blood of WT and CD40$^{-/-}$ hosts, respectively (Fig. 62A). Taking into account the differences in initial memory cell frequencies, these numbers represent a ~2-fold vs. ~1.3-fold expansion of P-14 cells in the respective hosts (Fig. 62A). When the P-14 cell numbers were expressed as the percent out of the total cells, the expansion became ~6-fold vs. ~2-fold in the WT and CD40$^{-/-}$ hosts, respectively (Fig. 62B). Although memory cell proliferation was not as robust as we expected, the P-14 cell numbers in the CD40$^{-/-}$ hosts were consistently much lower than in the WT hosts. On the other hand, analysis of memory effector function revealed that the percentage of IFN–γ^+ P-14 cells was the same in both hosts (Fig. 62C). Taken together, these results show that CD40 signaling is not absolutely required during a memory CD8$^+$ T cell response. However, they suggest that CD40 signals influence memory CD8$^+$ T cell programming or development. This is because the memory P-14 T cells that were initially activated and maintained in a CD40-deficient environment had reduced responses.

Finally, we challenged WT mice that were infected at least 3 months previously with LCMV, and which contained memory P-14 cells, with WT or CD40$^{-/-}$ DCs that have been pulsed with gp33 peptide (Fig. 63). We detected an increase in memory P-14 cell numbers four days after challenge with peptide-pulsed DCs. However, WT and CD40$^{-/-}$ DCs induced similar levels of memory P-14 expansion (Fig. 64A). When we looked at memory effector function, we found that the percentage of IFN–γ producing P-14 cells was the same regardless of whether WT or CD40$^{-/-}$ DCs were used for challenge (Fig. 64B). These results provide further evidence that CD40 signaling on DCs is not required during memory CD8$^+$ T cell responses.

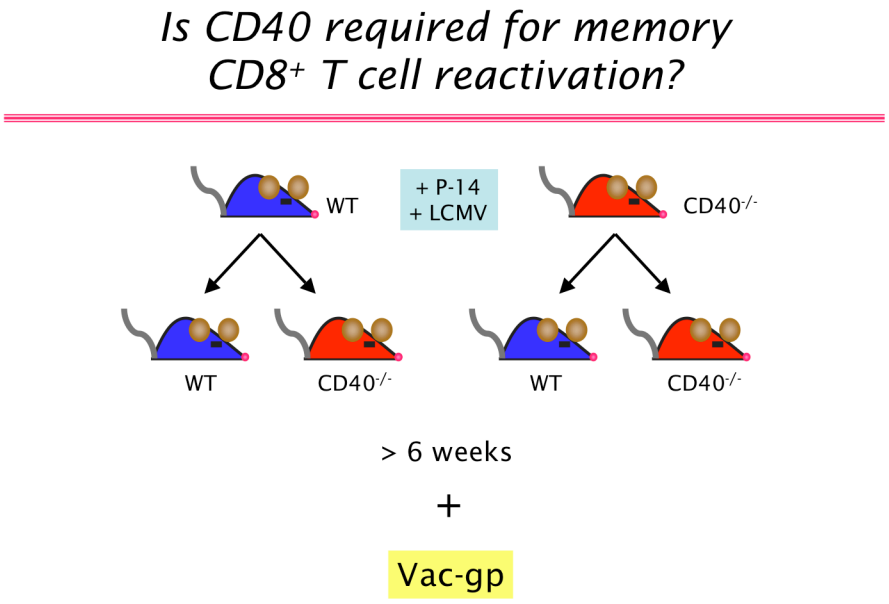


Figure 60. Experimental set-up to determine whether CD40 is required during reactivation of memory CD8$^+$ T cells. *WT and CD40$^{-/-}$ hosts containing P-14 T cells that have been previously activated in either WT or CD40$^{-/-}$ hosts were challenged with recombinant vaccinia virus expressing LCMV glycoprotein (Vac-gp). Challenge was performed at least six weeks post transfer to ensure that the cells have differentiated into memory.*

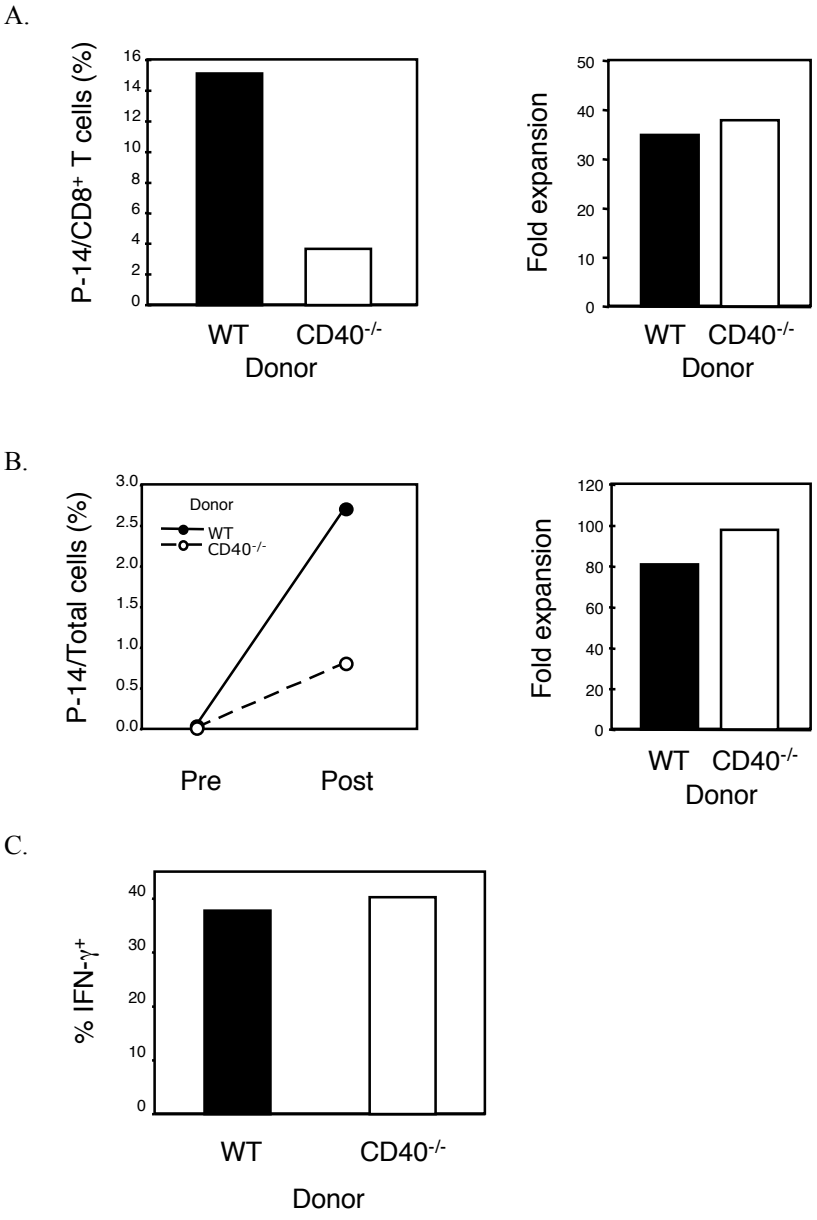


Figure 61. P-14 T cells primed in CD40$^{-/-}$ hosts are able to respond upon challenge. *Mice in Fig. 38 were challenged with 1 x 10^6 pfu of recombinant vaccinia virus expressing the LCMV glycoprotein (Vac-gp). Four days later, blood was collected and analyzed as in Fig. 38. (A) Percentage of P-14 T cells out of the total CD8$^+$ T cells in the*

blood. (B) Percentage of P-14 T cells out of the total cells in the blood before and after challenge with Vac-gp. The numbers in parenthesis in (A) and (B) indicate the fold expansion of the P-14 T cells. (C). Percentage of IFN−γ producing P-14 T cells upon challenge. The results shown are representative of three independent experiments with two to three mice per group.

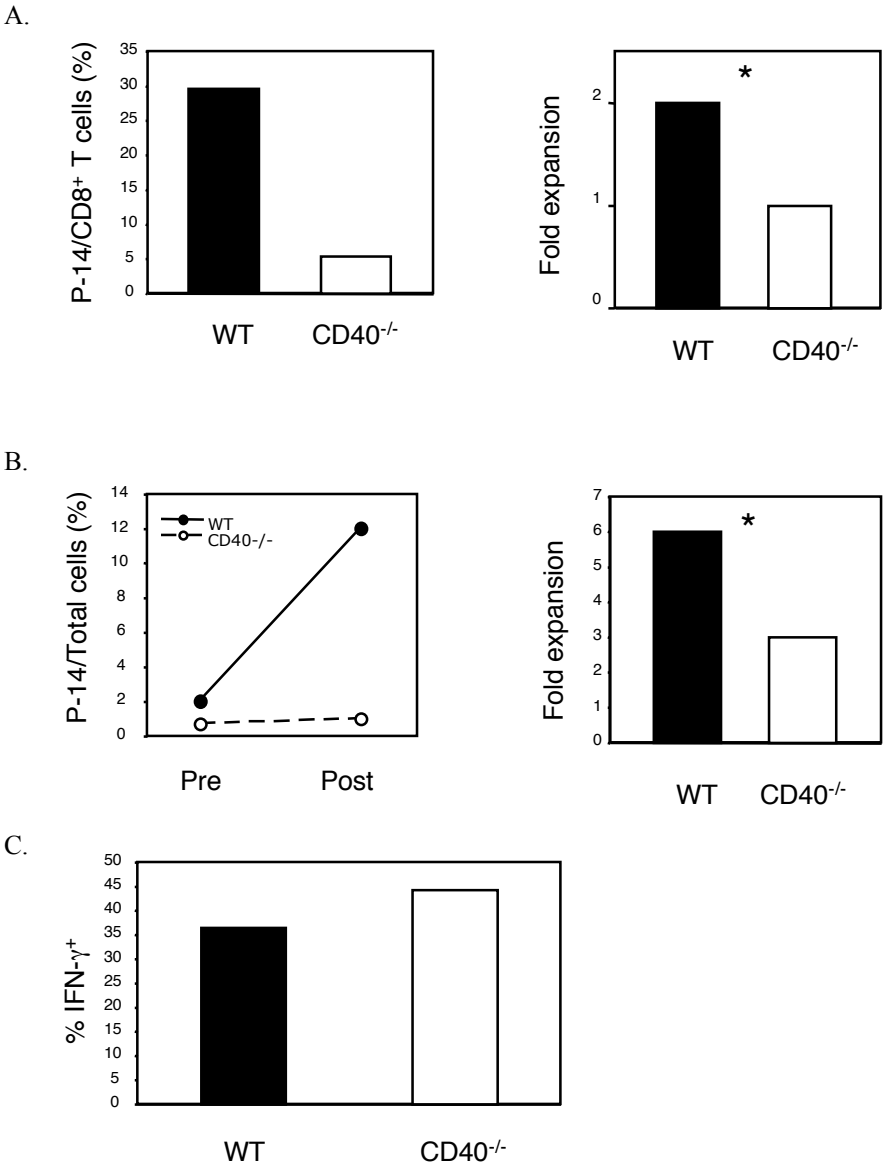


Figure 62. Impaired CD8$^+$ T cell memory in LCMV-infected CD40$^{-/-}$ hosts. *WT and CD40$^{-/-}$ hosts containing adoptively transferred CFSE-labeled P-14 T cells were infected with LCMV. After at lest six weeks, the mice were challenged with 1 x 10^6 pfu of Vac-gp. Blood was collected four days later and analyzed as in Fig. 38. (A) Percentage of P-14 T cells out of the total CD8$^+$ T cells in the blood. (B) Percentage of P-14 T cells out of the*

*total cells in the blood before and after challenge with Vac-gp. The numbers in parenthesis in (A) and (B) indicate the fold expansion of the P-14 T cells. (C). Percentage of IFN−γ producing P-14 T cells upon challenge. The results shown are representative of three independent experiments with two to five mice per group. *, $p < 0.01$.*

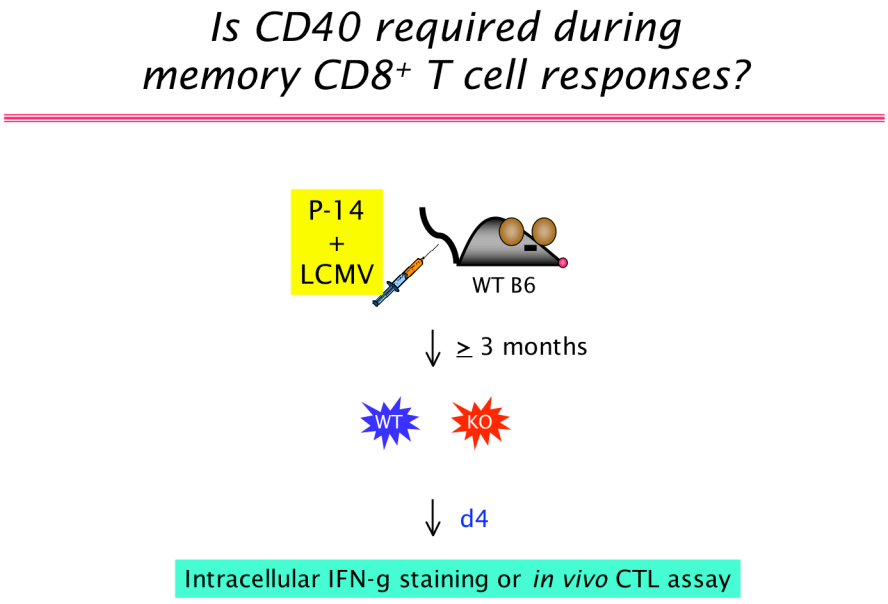


Figure 63. Experimental set-up to determine whether CD40 is required during memory CD8$^+$ T cell reactivation. *WT mice containing adoptively transferred P-14 T cells were infected with LCMV and allowed to rest for more than three months. The mice were then challenged with WT or CD40$^{-/-}$ DC that have been pulsed with gp33 peptide. In this system CD40 deficiency is restricted only to the challenge DCs.*

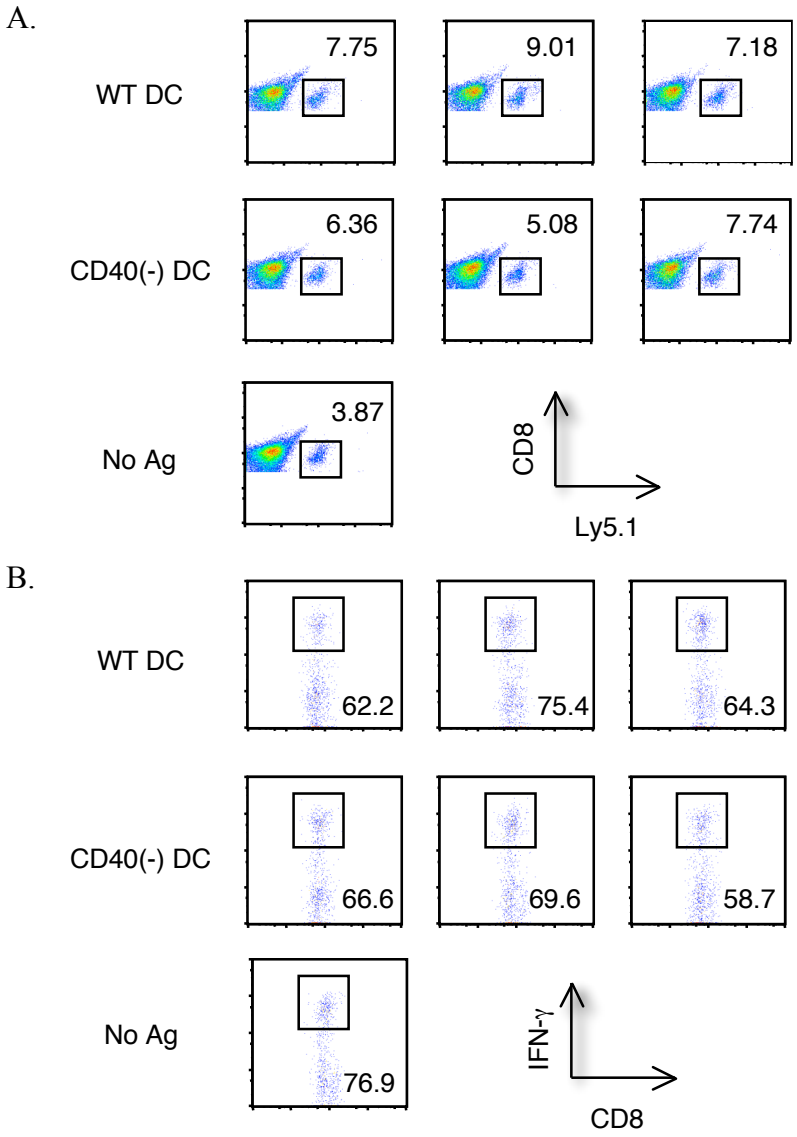


Figure 64. CD40 is not required during recall responses of memory CD8$^+$ T cells. *WT mice containing adoptively transferred P-14 T cells were immunized with LCMV. After three months the mice were challenged with 1 x 106 WT or CD40$^{-/-}$ BMDCs pulsed with gp33 peptide. Spleens were harvested after four days and analyzed as in Fig. 1. (A)*

Percentage of P-14 T cells in the total $CD8^+$ T cell population in the spleen. (B) Percentage of IFN−γ producing P-14 T cells.

CHAPTER X.

THE ROLE OF CD40 SIGNALING ON DCs IN CD8$^+$ T CELL MEMORY

We showed that CD40 signaling on APCs, specifically on DCs, is involved in maximizing primary CD8$^+$ T cell responses. Because our results also point to a role for CD40 signaling on APCs in the generation and maintenance, but not the function of memory CD8$^+$ T cells, we examined the importance of CD40 signaling on DCs in the above processes. We focused our experiments this time on endogenous polyclonal memory CD8$^+$ T cell responses.

RESULTS

A. Priming with CD40$^{-/-}$ DCs leads to a weaker memory response

In order to determine whether CD40 signaling on DCs plays a role in CD8$^+$ T cell memory, we first asked whether mice that were immunized with CD40$^{-/-}$ DCs can mount a recall response (Fig. 65). We injected WT mice i.v. with WT or CD40$^{-/-}$ DCs pulsed with OVA peptide and examined *in vivo* CTL responses one week later to make sure that priming occurred (data not shown). We then waited at least six weeks after immunization and challenged the mice i.v. with OVA peptide-pulsed WT DCs. Endogenous memory responses were evaluated by looking at *in vivo* CTL activity against OVA peptide-pulsed targets as well as IFN-γ production. Mice immunized with WT DCs showed strong memory CTL responses, exhibiting as much as 90% target cell killing by day 4 post-challenge (Fig. 66A). In contrast, mice immunized with CD40$^{-/-}$ DCs mounted

Are memory CD8$^+$ T cells formed in mice immunized with CD40$^{-/-}$ DCs?

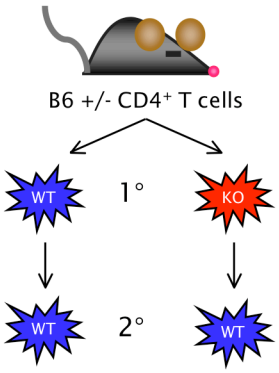


Figure 65. Experimental set-up to determine whether immunization with CD40$^{-/-}$ DCs can induce the development of endogenous CD8$^+$ T cell memory. *WT or CD4-depleted mice were immunized with peptide-pulsed DCs from WT or CD40$^{-/-}$ (KO) mice. They were then challenged with WT DCs that have been pulsed with the same peptide. In this system CD40 deficiency is restricted only to the immunizing DC.*

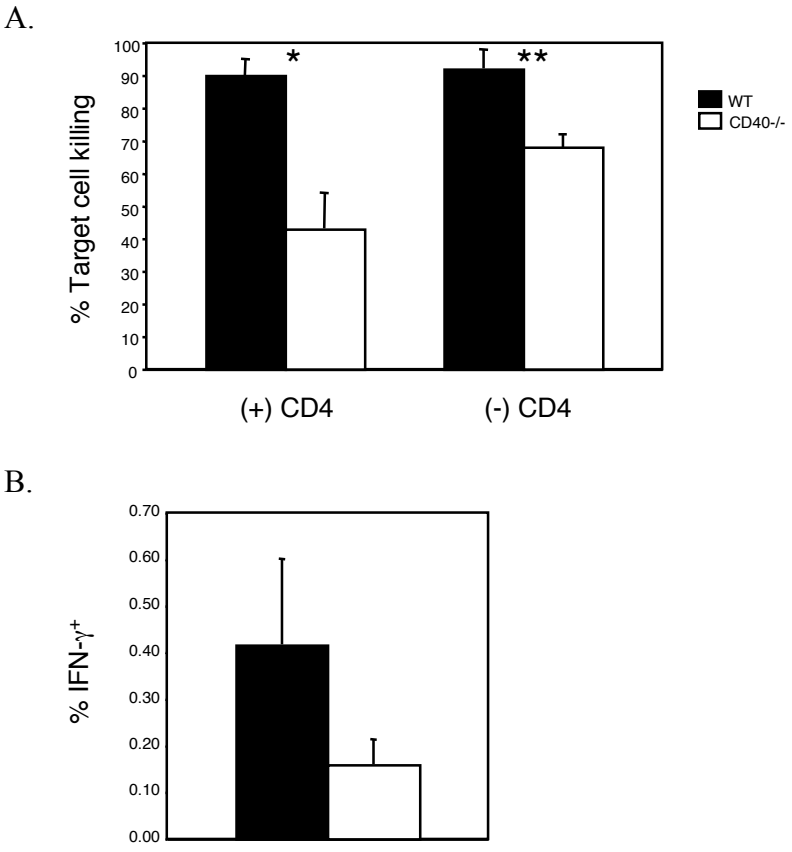


Figure 66. CD8$^+$ T cell memory induced upon immunization with WT or CD40$^{-/-}$ peptide-pulsed DCs. *WT or CD4$^+$ T cell-depleted mice were immunized i.v. with 1 x 10^6 and CD40$^{-/-}$ DCs were pulsed with OVA peptide. After at least six weeks, the mice were challenged with OVA peptide-pulsed WT DCs injected i.v. (A) On day 4 post-challenge mice were injected with target cells for an in vivo CTL assay. The percentage of target cell killing is shown. (B) Spleens were harvested four days after challenge and stimulated in vitro with OVA peptide in the presence of Brefeldin A and IL-2. The percentage of IFN−γ producing cells in the total CD8$^+$ T cell population is shown. The results shown*

*are representative of three independent experiments with two to three mice per group. *, p = 0.003; **, p = 0.01.*

significantly weaker memory CTL responses, with only ~43% target cell killing (Fig. 66A). In control mice that were left unimmunized, CTL activity was not observed until day 7 post-challenge (data not shown). Analysis of cytokine production by spleen cells directly ex vivo also revealed that mice immunized with $CD40^{-/-}$ DCs have less IFN-γ producing cells upon challenge compared to mice immunized with WT DCs (Fig. 66B). We obtained similar data when we performed the challenge 3 months after immunization (data not shown). These results imply that naïve $CD8^+$ T cells primed in the absence of CD40 can develop into memory cells; therefore, CD40 stimulation of APCs during priming is not absolutely required to induce a memory $CD8^+$ T cell response. However, there is an approximately two-fold reduction in memory responses in mice immunized with $CD40^{-/-}$ DCs. This parallels what we have observed for primary responses and is likely a direct consequence of a reduced number of memory cells arising from a reduced number of effector cells.

B. Memory $CD8^+$ T cell responses in the absence of $CD4^+$ T cell help

Several groups have reported that the presence of $CD4^+$ T helper cells during $CD8^+$ T cell priming is essential in the development of functional $CD8^+$ T cell memory while depletion of $CD4^+$ cells during challenge had no effect on memory responses (Janssen et al., 2003; Shedlock and Shen, 2003; Sun and Bevan, 2003). These findings were obtained using immunization with tumor cells or infection with vaccinia virus or *Listeria monocytogenes* to induce primary responses. We examined whether the $CD8^+$ T cell memory induced by DC immunization had a similar dependence on $CD4^+$ T cell help. Moreover, because help is thought to primarily occur through activation of CD40,

we also determined whether the reduction in memory responses that we observed in $CD40^{-/-}$ DC immunized mice was due to the involvement of $CD4^+$ T cells. We immunized host mice that were acutely depleted of $CD4^+$ T cells with OVA peptide-pulsed WT and $CD40^{-/-}$ DCs and challenged them six weeks later with WT DCs. Treatment of host mice with the anti-CD4 Ab GK1.5 for two consecutive days prior to immunization resulted in loss of $CD4^+$ T cells lasting up to two weeks. Surprisingly, we detected memory $CD8^+$ T cell responses even when we depleted the host mice of $CD4^+$ T cells during priming. In fact, there was no difference in target cell killing between CD4-sufficient and CD4-deficient mice (Fig. 66A). More interestingly, in mice that were depleted of $CD4^+$ T cells, the memory CTL activity was still weaker in the mice that were immunized with $CD40^{-/-}$ DCs compared to the mice that were immunized with WT DCs (Fig. 66A). This result is reminiscent of the data we obtained for primary responses. It suggests that the effect of CD40 on memory $CD8^+$ T cell responses can likewise be independent of $CD4^+$ T cell help.

C. Challenge with $CD40^{-/-}$ DCs results in weaker memory responses

Finally, we asked whether CD40 signaling on DCs is involved in the recall response of memory $CD8^+$ T cells. To answer this, we challenged mice immunized with either WT or $CD40^{-/-}$ DCs with the indicated DC pulsed with the same peptide (Fig. 67). As we have shown in Fig. 66A, mice immunized with $CD40^{-/-}$ DCs exhibited reduced *in vivo* CTL activity compared to mice immunized with WT DCs upon challenge with WT DCs (Fig. 68A). In contrast, we observed equally strong memory CTL responses when mice that were immunized with WT DCs were challenged with either WT or $CD40^{-/-}$

DCs (Fig. 68A). However, mice that were both immunized and challenged with $CD40^{-/-}$ DCs showed much lower memory CTL activity (Fig. 68A).

Interestingly, depletion of $CD4^{+}$ T cells at the time of immunization resulted in reduced memory CTL activity even in mice immunized with WT DCs and challenged with $CD40^{-/-}$ DCs (Fig. 68B). CD4-depleted mice that were both immunized and challenged with $CD40^{-/-}$ DCs had the most striking reduction in memory CTL responses (Fig. 68B). Altogether, these results indicate yet again that CD40 signals during priming affect the programming of memory $CD8^{+}$ T cells. And although CD40 signaling is not necessary to activate memory $CD8^{+}$ T cells, it plays a role in maximizing memory responses. The requirement for CD40 signals during memory is most evident in situations wherein primary responses are weak to begin with, consequently leading to memory T cells with reduced frequency and/or function.

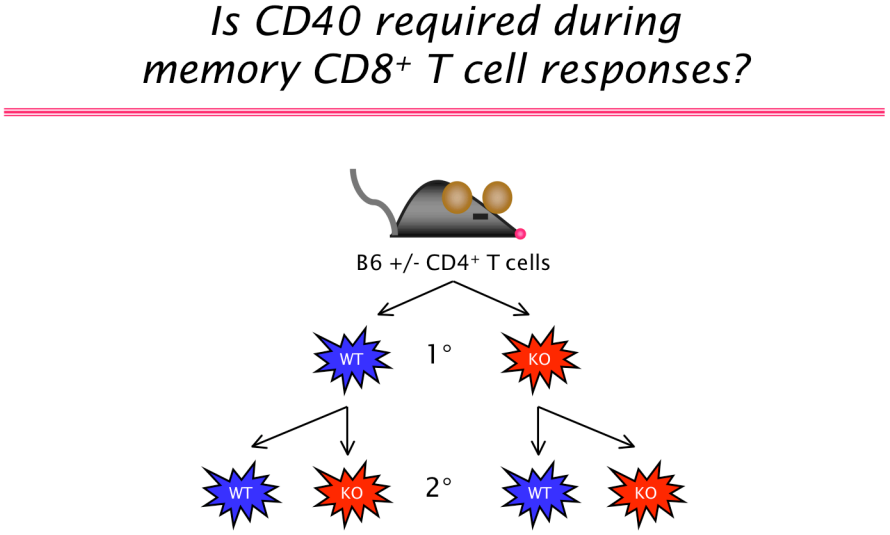


Figure 67. Experimental set-up to determine whether CD40 is required during reactivation of endogenous memory CD8$^+$ T cells. *WT or CD4-depleted mice were immunized with peptide pulsed DCs from WT or CD40$^{-/-}$ (KO) mice. They were then challenged with either WT or CD40$^{-/-}$ DC that have been pulsed with the same peptide. In this system CD40 deficiency is restricted only to the immunizing and/or challenge DCs.*

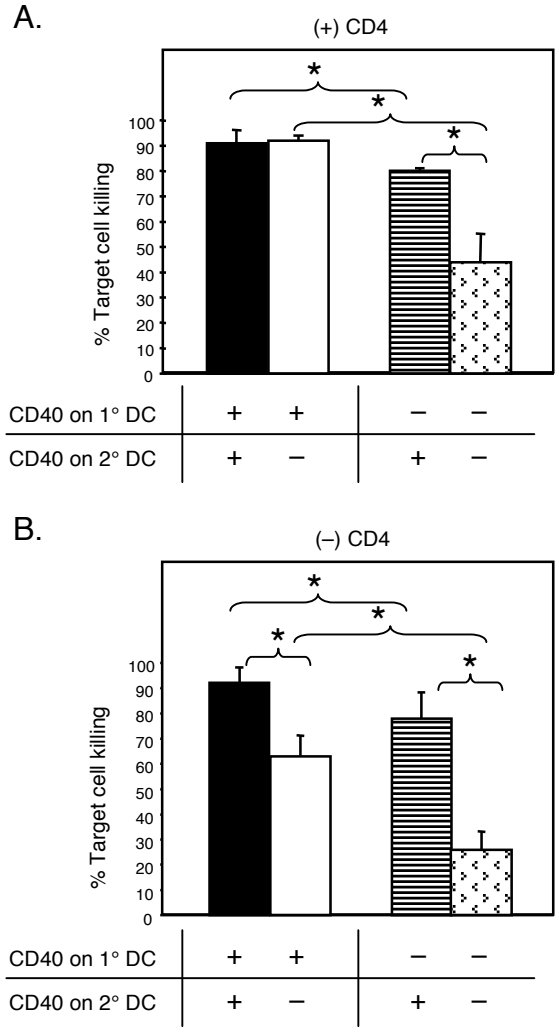


Figure 68. Role of CD40 and CD4$^+$ T cell help in CD8$^+$ T cell memory. *WT or CD4$^+$ T cell-depleted mice were immunized i.v. with 1 x 10^6 and CD40$^{-/-}$ DCs were pulsed with OVA peptide. After at least six weeks, the mice were challenged with OVA peptide-pulsed WT or CD40$^{-/-}$ DCs injected i.v. In vivo CTL assay was performed on day 4 post-challenge and the percentage of target cell killing in WT (A) and CD4$^+$ T cell-depleted (B) mice is shown. The results shown are representative of three independent experiments with two to three mice per group. *, $p < 0.05$.*

CHAPTER XI.

DISCUSSION

$CD8^+$ T cells are critical in protective immunity against various pathogens and tumors. Therefore it is important to understand the signals that are necessary for induction of a highly effective $CD8^+$ T cell response. It is well established that CD40 stimulation augments T cell proliferation and effector function, mostly through its effect on APC activation. However, there are many other stimuli, including TLR agonists and some inflammatory cytokines, which can activate APCs and enable them to efficiently prime T cells. The experiments we performed were designed to address whether there is in fact, a requirement for CD40 in primary as well as memory $CD8^+$ T cell responses.

A. Role of CD40-CD40L interaction in priming of naïve $CD8^+$ T cells

We first asked what the consequence of CD40 deficiency is on the magnitude and quality of primary $CD8^+$ T cell responses. We found that there is no absolute requirement for CD40 in order to generate naive $CD8^+$ T cell responses. However, the proliferation, cytokine production, and cytolytic activity of adoptively transferred TCR-Tg $CD8^+$ T cells were consistently reduced by as much as 50% when they were primed in $CD40^{-/-}$ hosts. One caveat of our adoptive transfer experiments is that we were using total spleen and lymph node cells instead of purified $CD8^+$ T cells. However, based on our experiments as well as those of other people in the lab, there seems to be no difference in the homing of the non-purified cells because we can detect them in the lymphoid tissues

of both unimmunized and immunized mice. Moreover, in preliminary experiments, we also observed similar responses whether or not the transferred TCR-Tg $CD8^+$ T cells were purified (data not shown).

Another caveat of using an adoptive transfer system is that the frequency of the transferred cells is much higher than what is normally found. Several studies have shown that having high frequencies of Ag-specific T cells can skew their response. In fact, we showed that the response of OT-I T cells is CD40-independent at lower frequencies; however, there is a threshold above which their response becomes CD40-independent. In order to get around this caveat, we examined endogenous (non-Tg) $CD8^+$ T cell responses and found that they were also compromised in the absence of CD40 or CD40L. These results, combined with previous studies showing augmented T cell responses upon CD40 stimulation by exogenous anti-CD40 Ab, point to an important role for CD40 signaling in maximizing primary $CD8^+$ T cell responses.

This finding is in contrast with several earlier studies, which showed that CD40-CD40L interactions are dispensable for primary $CD8^+$ T cell responses. However, these studies were done using $CD40L^{-/-}$ mice that have been infected with viruses (LCMV, VSV, or Pichinde) or bacteria (Listeria) (Andreasen et al., 2000; Borrow et al., 1996; Shedlock et al., 2003; Thomsen et al., 1998; Whitmire et al., 1999; Whitmire et al., 1996). These pathogens are able to directly activate APCs, induce inflammation and are extraordinarily potent immunogens (potentially overriding CD40's contribution to responses). The main difference in our study is that we used peptide and peptide-pulsed

APCs as antigens, which are weaker immunogens because of their non-inflammatory (or less-inflammatory) nature.

Interestingly, there is a more stringent requirement for CD40 in the priming of naïve $CD4^+$ T cells, whether the antigens are inflammatory or non-inflammatory. For example, Ag-specific $CD4^+$ T cells from $CD40L^{-/-}$ mice fail to expand and differentiate into cytokine-producing cells and are also unable to induce autoimmunity in an EAE model (Grewal et al., 1996; Grewal et al., 1995). On the other hand, $CD40L^{-/-}$ mice infected with LCMV have a ten-fold reduction in virus-specific $CD4^+$ T cells (Whitmire et al., 1999). In addition, $CD40^{-/-}$ and $CD40L^{-/-}$ mice have impaired resistance to the intracellular parasite *Leishmania*, which relies on a T_H1 response (Soong et al., 1996). Furthermore, no $CD4^+$ T cell priming is observed in $CD40^{-/-}$ and $CD40L^{-/-}$ immunized with peptide in complete Freund's adjuvant (CFA) or peptide-pulsed DCs (MacLeod et al., 2006).

We have not determined the exact mechanism(s) for the lower numbers of $CD8^+$ T cells that responded in the $CD40^{-/-}$ hosts. CD40 may affect the number of T cells that are initially recruited to proliferate, the number of divisions a cell undergoes, and/or the survival of activated cells. At the peak of the response, the majority of the cells have already divided more than eight times, as evidenced by the loss of CFSE. This suggests that the lower overall accumulation of $CD8^+$ T cells in the absence of CD40 may be due to a failure of these cells to survive. However, it is possible that the cells stop dividing earlier than their wild type counterparts (i.e. sometime after 8 divisions) and we have not been able to detect consistent differences in the percent of mutant cells undergoing

apoptosis. In future studies it will also be useful to perform a cell cycle progression analysis.

CD40 is expressed not only by professional APC but also by hematopoietic precursors, epithelial cells, endothelial cells, and even activated T cells (Quezada et al., 2004; Schonbeck and Libby, 2001; van Kooten and Banchereau, 2000). Hence, we next asked what cells are important for CD40 function. This issue has not been resolved in the many previous studies that have explored CD40 function using agonistic anti-CD40 Ab, which bind to all CD40-expressing cells. Our adoptive transfer experiments map the key role of CD40 to APCs in the host. Moreover, we demonstrate that when DCs are the only APC lacking CD40, the reduction in T cell responses is similar to that observed when all host APCs were CD40-deficient. Therefore, CD40 is working at least in part on DCs. In an earlier study, it was found that only tumor-loaded $CD40^{+/+}$ but not $CD40^{-/-}$ DCs can restore protective anti-tumor responses in $CD40^{-/-}$ mice (Mackey et al., 1998b). This, together with our findings, further supports the notion that DCs are the major initiators of naïve T cell activation and that the outcome of a T cell response can be shaped by the activation status of DCs. Interestingly, while $CD40^{-/-}$ DCs induced reduced $CD8^{+}$ T cell responses *in vivo*, they were as efficient as WT DCs in stimulating T cell proliferation *in vitro*. This indicates that the costimulatory requirements for naïve $CD8^{+}$ T cell activation are different *in vitro* and *in vivo*. In another study, it was shown that the *in vivo* priming of naive $CD4^{+}$ T cells by the dendritic cell line JawsII is dependent on CD40, but *in vitro* priming was not (Haase et al., 2004).

Nevertheless, it is possible that CD40 also influences the function of other APCs. In fact, we showed that although MΦ are not as responsive as DCs to CD40 stimulation *in vitro*, CD40-deficient MΦ also induced reduced $CD8^+$ T cell responses *in vivo*. Recent reports show that activation of B cells through CD40 converts them into efficient stimulators of both $CD8^+$ and $CD4^+$ T cells (Ritchie et al., 2004; Rodriguez-Pinto and Moreno, 2005). It remains to be tested whether or not CD40-deficient B cells show a reduced capacity to stimulate $CD8^+$ T cells in our system.

It has been proposed that CD40 also plays a role on the responding $CD8^+$ T cells themselves. In this alternative model of CD40 function, it is thought that binding of CD40L-expressing $CD4^+$ T cells to CD40-expressing $CD8^+$ T cells directly stimulates their expansion (Bourgeois et al., 2002). This is unlikely in our system because our adoptive transfer experiments show that CD40 expression is critical on host cells. Moreover, agonistic anti-CD40 Ab augmented the response of the adoptively transferred T cells, which come from a CD40-sufficient background, only in WT but not $CD40^{-/-}$ hosts. Therefore, our findings do not support the alternative model of direct CD40 activation on $CD8^+$ T cells. Rather, they provide further evidence for the importance of CD40 in the APC licensing model.

Another important question was what cell was the source of CD40L that was needed to stimulate APCs *in vivo*. It has generally been thought that $CD4^+$ T helper cells are the principal source of CD40L in CD40-dependent responses. However, our finding that the absence of helper $CD4^+$ T cells did not affect the CD40-dependence of the $CD8^+$ T cell responses indicated that some other cells provided CD40L for APC activation.

$CD8^+$ T cells can express CD40L but there are few studies showing any functional significance for this expression (Cronin et al., 1995; Hermann et al., 1995; Roy et al., 1993; Sad et al., 1997; Whitmire et al., 1999). Nevertheless, since they are able to directly interact with APCs presenting cognate Ag, we reasoned that they might be able to provide their own help. The experiments in which we use CD40L-deficient $CD8^+$ TCR-Tg T cells provided the first direct test of this hypothesis. We found that CD40L is not absolutely required by the transferred T cells in order to proliferate. However, the mutant cells exhibited the same defective responses that were observed when WT T cells were stimulated with CD40-deficient APCs in the presence or absence of $CD4^+$ T cells. It is possible that the defective response of CD40L-deficient $CD8^+$ T cells is due to a role for CD40L in transducing signals to the T cell. However, the WT P-14 T cells were able to rescue the P-14/$CD40L^{-/-}$ T cell response. This demonstrates that CD40L is not involved in signaling to the $CD8^+$ T cells because if it was, then the WT T cells should not have been able to rescue the response of the CD40L-deficient T cells. This result also formally shows that $CD8^+$ T cells can provide help in "trans". We conclude that antigen-specific $CD8^+$ T cells can directly activate APCs through CD40L and thereby provide their own help in the absence of $CD4^+$ T cells. Nevertheless, it is also possible that the WT P-14 T cells could simply have provided IL-2 to promote the proliferation of the CD40L-deficient P-14 T cells. This can be tested by looking at whether the addition of exogenous IL-2 can also rescue the response of the CD40L-deficient $CD8^+$ T cells.

Our data are in contrast with those from another study, which showed that the ability of large numbers of $CD8^+$ T cells to overcome the requirement for help is not

mediated via CD40L (Mintern et al., 2002b). Nevertheless, they are consistent with and extend the findings of earlier studies that suggested that CD40L could be delivered by cells other than $CD4^+$ T cells. In the first one, CD40L blockade in conjunction with soluble antigen administration inhibited the accumulation of Ag-specific $CD8^+$ T cells in mucosal tissues (Lefrancois et al., 1999). Since similar results were obtained using $CD40L^{-/-}$ hosts, the authors concluded that CD40L expressed by the responding $CD8^+$ T cells was responsible for binding CD40 on the host APCs. In the second study, the $CD8^+$ T cell response to VSV was found to depend on CD40L but was unaffected by acute $CD4^+$ T cell depletion or MHC class II deficiency (Andreasen et al., 2000). The authors took this as indirect evidence of $CD8^+$ T cell "self-help" through CD40L. The third study made use of GK1.5-transgenic mice, in which peripheral $CD4^+$ T cells were permanently depleted. These mice mounted CTL responses to allogeneic P815 cells that were inhibited upon CD40L blockade (Zhan et al., 2000). The authors interpreted this as an indication that direct CD40-CD40L interaction between APCs and $CD8^+$ T cells provides an accessory signal for CTL induction.

It has been shown in other systems that $CD4^+$ T cells provide help through CD40L (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998). On the other hand, we show that $CD8^+$ T cells can also provide "self-help" through CD40L. Our results do not suggest that $CD4^+$ T cells play absolutely no role in $CD8^+$ T cell responses. Rather, it is likely that CD40L expressed by both $CD4^+$ and $CD8^+$ T cells can activate APCs and contribute to the amplification of a normal immune response. It is remarkable that we observed $CD8^+$ T cell responses in the absence of help in light of the general

requirement for $CD4^+$ T cells in $CD8^+$ T cell responses against non-inflammatory antigens. The precursor frequency and affinity of responding $CD8^+$ T cells have been shown to affect helper dependence (Franco et al., 2000; Mintern et al., 2002b; Wang et al., 2001). However, titrating the number of adoptively transferred T cells still resulted in detectable CD40-dependent responses in the presence or absence of $CD4^+$ T cells. Moreover, we were able to detect endogenous primary CTL responses; aside from having a low frequency, the responders in this case also consisted of a spectrum of affinities. In the APC licensing model, $CD4^+$ T cell help is usually equated with CD40 stimulation. The fact that we observed CD40-dependent responses despite the absence of helper $CD4^+$ T cells indicates that CD40 and $CD4^+$ T cell function are not always equivalent.

While it is known that APCs must be activated in order to stimulate naïve T cell responses, it has been unclear whether all activating stimuli are similarly effective in this process, particularly for responses *in vivo*. We therefore asked how CD40 stimulation compares to microbial (TLR) stimulation for licensing APCs to prime naïve $CD8^+$ T cells. Some anti-bacterial and anti-viral $CD8^+$ T cell responses develop in the absence of $CD4^+$ T cell help or CD40-CD40L interactions (Andreasen et al., 2000; Clarke, 2000; Hamilton et al., 2001; Ruedl et al., 1999; Shedlock et al., 2003; Whitmire et al., 1999). This implies that CD40 and TLR signaling may play similar roles in APC activation. However, one study has found that only CD40-stimulated, but not LPS-stimulated DCs can induce naïve polyclonal $CD8^+$ T cell activation *in vitro* (Kelleher and Beverley, 2001). Moreover, HIGM patients, who have no functional CD40L, are more susceptible to opportunistic infections (DiSanto et al., 1993; Etzioni and Ochs, 2004). These

observations raise the possibility that CD40-CD40L interactions are still necessary even when TLR stimulation is present. Indeed, it has been shown that HIGM3 patients, who have mutations in the CD40 gene, have functionally defective DCs even after LPS or cytokine stimulation (Fontana et al., 2003).

By incubating WT and $CD40^{-/-}$ DCs with TLR ligands prior to immunization, we were able to directly show that the two signals have non-redundant effects on the stimulatory property of DCs. Although TLR ligands were able to amplify $CD8^+$ T cell responses in the absence of CD40 signaling, these responses never reached the levels that were induced when CD40 signaling was present. In other words, maximum $CD8^+$ T cell proliferation could only be achieved when both CD40 and TLR are stimulated. This result is in accord with studies showing that microbial signals are required for CD40-induced cytokine production by DCs, and that CD40 triggering can amplify TLR-induced cytokine production (Edwards et al., 2002; Schulz et al., 2000). It is also consistent with a previous report, which showed that the TLR and CD40 pathways synergize to amplify $CD8^+$ T cell responses (Ahonen et al., 2004). However, in those studies CD40 stimulation was achieved through a pharmacologic agent (agonistic anti-CD40 Ab). Again, it is possible that antibody stimulation of other cell types expressing CD40 could have contributed to the increased T cell responses. Our studies demonstrate the importance of CD40, specifically on APCs. More importantly, we show that in the presence or absence of PAMPs, and even without exogenous stimulation**,** CD40 naturally participates in the priming of naïve $CD8^+$ T cell responses. While it has been shown that TLR engagement could convert $CD8^+$ T cell autoreactivity into overt autoimmune disease in one system

(Lang et al., 2005), in another TLR ligation broke peripheral cross-tolerance to self-antigens and induced autoimmunity only when $CD4^+$ T cell help was present (Hamilton-Williams et al., 2005). Since help is presumably needed to stimulate CD40 on APCs, this latter study, together with our findings, suggests that CD40 plays a fundamental role in the induction of $CD8^+$ T cell responses.

In vitro stimulation with either LPS, CpG, or polyI:C was able to significantly augment the ability of WT DCs to prime naïve $CD8^+$ T cell responses while only having little effect on $CD40^{-/-}$ DCs (Fig. 45). Interestingly however, when these TLR agonists were injected directly into host mice, only CpG and polyI:C were able to induce a marked increase in $CD8^+$ T cell expansion in the WT hosts while only polyI:C induced increased CD8+ T cell responses in $CD40^{-/-}$ hosts (Fig. 40). There are at least three explanations for this discrepancy. First, it could be due to the single dose of TLR agonists used in the experiments. Ideally, a dose response for each of the TLR agonists needs to be performed in order to ensure that the inability of TLR agonists to replace CD40 is not just simply because the strength of stimulation is too low. Second, it could be that when the TLR agonists are directly injected into the hosts, they are stimulating cells other than APCs, whereas in the *in vitro* stimulation system, they are only stimulating the immunizing DCs. In vivo, not only are TLRs expressed in non-APCs, but even within different DC subsets, they are differentially expressed. Third, it could be due to differences in the downstream signaling pathways that the respective TLRs activate. LPS (TLR4) and CpG (TLR9) both activate a MyD88-dependent pathway. However, LPS additionally activates

a MyD88-independent pathway involving the adaptors TIRAP and TRAM. Meanwhile, polyI:C activates a TRIF-dependent pathway that is independent of MyD88.

How can the difference in the ability of CD40-stimulated and TLR-stimulated DCs to induce $CD8^+$ T cell responses be explained? CD40 stimulation of DCs has been shown to be important for production of IL-12, which promotes $CD8^+$ T cell expansion and differentiation (Cella et al., 1996; Valenzuela et al., 2002). However, we found that WT and CD40-deficient DCs made similar levels of IL-12 upon incubation with either WT or CD40L-deficient $CD8^+$ T cells (data not shown). In connection with this, there is evidence that the ability of CD40-stimulated Langerhans cells to prime $CD8^+$ T cells is independent of IL-12 (Gorbachev and Fairchild, 2004). Moreover, we found that with or without TLR ligation, there was no difference in MHC-peptide levels as well as co-stimulatory molecule expression (CD80 and CD86) and IL-12 production between WT and $CD40^{-/-}$ DCs. Therefore, the different responses induced by CD40- and TLR-stimulated DCs are not due to differences in conventional "co-stimulatory repertoire" and the underlying molecular mechanism(s) remains to be determined. This is especially important in light of recent efforts to distinguish between phenotypically and functionally mature DCs (Reis e Sousa, 2006). CD40-matured DCs have been reported to be more phenotypically stable compared to TLR-matured DCs (Nakamura et al., 2004). In addition, it has also been shown that CD40 induces higher levels of CD70 (CD27L) on DCs compared to TLRs and this correlates with increased immunogenicity even in the absence of helper $CD4^+$ T cells (Bullock and Yagita, 2005; Taraban et al., 2004). Furthermore, CD40 stimulation has been shown to increase the lifespan and antigen-

presenting capacity of DCs and there is even some evidence that CD40-deficient DC have an impaired survival *in vivo* (Miga et al., 2001). If the CD40-deficient DCs indeed have a survival defect, then the reduced T cell responses could be due to the limited period that antigen is getting presented. DCs are able to secrete IL-2, which is crucial for T cell proliferation (Feau et al., 2005). It has been reported that CD40-deficient DCs produce less IL-2 upon culture with T_{REG} cells (Guiducci et al., 2005). It is possible that CD40 is also involved in IL-2 production by DCs upon encounter with Ag-specific $CD8^+$ T cells. Whether any of these previously reported mechanisms and/or other ones account for the effects we observed will require further studies.

B. Role of CD40 in memory $CD8^+$ T cell responses

Several things need to occur in order to have a highly effective T cell memory. First, memory T cells that have a much higher frequency and avidity have to be generated from a polyclonal pool of antigen-specific naïve T cells. Next, these memory T cells have to persist for a long time in the host. Finally, these memory T cells have to rapidly and robustly respond by proliferating and exerting effector function upon re-encounter with antigen. It is evident that the signals that T cells receive during activation have a huge impact in their subsequent differentiation not only into effector cells but also into memory cells. Therefore, we asked what is the ultimate fate of $CD8^+$ T cells that were primed in the absence of CD40. Specifically, we examined whether CD40 signaling plays a role in the generation, maintenance, and function of memory $CD8^+$ T cells.

We initially used an infection model using LCMV in order to have an extremely potent primary stimulation. We also used an adoptive transfer system so we could easily track the responding cells at different stages of the response. Strikingly, we found that regardless of how strong the primary response is, CD40 deficiency resulted in lower frequencies of functional memory cells. Moreover, it seems that CD40 mostly affected the survival of effectors cells that contain memory cell precursors, although there was also a small effect on their subsequent maintenance.

Several studies have shown that CD40 is dispensable for the development of functional $CD8^+$ T cell memory to the bacteria *Listeria monocytogenes* (Montfort et al., 2004; Shedlock et al., 2003). However, there are also a number of reports showing that CD40 is important for anti-viral $CD8^+$ T cell memory. $CD40L^{-/-}$ mice mount strong primary CTL responses to viruses such as LCMV, Pichinde, and VSV (Andreasen et al., 2000; Borrow et al., 1996; Borrow et al., 1998; Thomsen et al., 1998; Whitmire et al., 1999; Whitmire et al., 1996). The primary activation, clonal expansion, and differentiation of virus-specific endogenous $CD8^+$ T cells were normal in CD40L-deficient mice (Thomsen et al., 1998). However, there was a rapid impairment of effector activity resulting in an inability to permanently control virus replication (Andreasen et al., 2000; Thomsen et al., 1998). It was eventually shown that the weaker $CD8^+$ T cell memory in $CD40L^{-/-}$ mice is caused by the generation of lower numbers of memory cells and not due to a problem in memory $CD8^+$ T cell maintenance (Borrow et al., 1998). The results we obtained using $CD40^{-/-}$ mice directly complement the findings from the above studies. We showed that in the absence of CD40, adoptively transferred $CD8^+$ T cells

proliferated robustly upon LCMV infection. Despite this, there was a marked reduction in the number of effector cells soon after the peak of the response. Therefore, the absence of either CD40 or CD40L leads to the production of fewer memory $CD8^+$ T cells; we conclude that CD40-CD40L interaction plays an important role in memory $CD8^+$ T cell development.

LCMV induces extremely robust $CD8^+$ T cell responses in part because of its ability to directly activate DCs as well as stimulate TLRs. In the absence of MyD88, there is a defect in both the innate and adaptive immune response to LCMV (Zhou et al., 2005). Particularly, cytokine production by virus-specific $CD8^+$ T cells is impaired and this results in viral persistence. In the absence of CD40, although there is a reduction in the number of primary effector cells, these cells nonetheless exhibit no defect in cytokine production. Therefore, it seems that even for acute viral infections, CD40 and TLR stimulation play unique roles.

There is data showing that CD40 expression on $CD8^+$ T cells, and not APCs, is important in generation of memory $CD8^+$ T cells (Bourgeois et al., 2002). In this previous study, it was found that CD40-deficient TCR-Tg $CD8^+$ T cells fail to proliferate rapidly and secrete high levels of cytokines upon *in vitro* re-stimulation with Ag. However, it is unlikely that the defect in $CD8^+$ T cell memory in our study could be attributed to the lack of CD40 on $CD8^+$ T cells. This is because the adoptively transferred T cells come from a WT, CD40-sufficient background and CD40 deficiency was confined only to the host cells. Hence, our results indicate that CD40 signaling on $CD8^+$ T cells is of little or no significance to the development of memory. The role of CD40 is most probably on

APCs, similar to what we have shown for primary responses. Two other studies have shown that there is no requirement for direct CD40 activation on $CD8^+$ T cells in order to generate memory. In the first study, it was found that optimal $CD8^+$ T cell responses to influenza are dependent on CD40 expression on hematopoietic cells but not T cells (Lee et al., 2003). Specifically, the proliferation as well as differentiation of endogenous, polyclonal $CD40^{+/+}$ and $CD40^{-/-}$ $CD8^+$ T cells was the same as long as they were primed in mice containing $CD40^{+/+}$ APCs. In the second study, it was likewise found that CD40 expression on $CD8^+$ T cells was not essential to mount a primary or secondary response against an acute viral (LCMV) or bacterial (*Listeria*) infection (Sun and Bevan, 2004). Bone marrow chimeric mice containing $CD40^{+/+}$ and $CD40^{-/-}$ $CD8^+$ T cells were made, and no difference in expansion or trafficking into non-lymphoid tissues was observed between the two different cells.

We showed that CD40L expression on antigen-specific $CD8^+$ T cells contributes to the generation of maximal primary responses. However, it remains unclear whether CD40L expression on $CD8^+$ T cells is also important for memory. In one of the studies above wherein $CD40L^{-/-}$ mice were infected with LCMV, it was determined that CD40L expression by the $CD8^+$ T cells is not essential and that the defective memory was mostly due to the absence of CD40L on $CD4^+$ T cells (Borrow et al., 1998). This conclusion was derived using bone marrow chimeric mice containing WT and $CD40L^{-/-}$ T cells, which, upon infection with LCMV, had similar frequencies of memory T cells of both types. But we have shown that WT $CD8^+$ T cells can rescue the *in vivo* expansion defect of $CD40L^{-/-}$ $CD8^+$ T cells. In order to definitively examine the role of CD40L expression on $CD8^+$ T

cells in memory, experiments wherein CD40L deficiency is restricted only to $CD8^+$ T cells need to be done. For example, the ability of WT versus CD40L-deficient $CD8^+$ T cells to differentiate into memory cells can be compared in WT or $CD40L^{-/-}$ hosts.

How important are CD40-CD40L interactions in other phases of the immune response? Again, in our LCMV infection system, the adoptively transferred T cells underwent a much greater contraction in the $CD40^{-/-}$ hosts compared to WT hosts. Further, transfer into new CD40-deficient hosts also resulted in a more pronounced loss of effector $CD8^+$ T cells compared to transfer into new CD40-sufficient hosts. Nevertheless, the population of cells that remained in the $CD40^{-/-}$ hosts that were either directly infected or used as recipients was eventually maintained at stable levels and they exhibited functional characteristics of memory cells. Therefore, CD40 signaling plays a role in the survival of memory $CD8^+$ T cell precursors during the antigen-independent contraction phase of the immune response. However, once they are formed, the memory $CD8^+$ T cells are not critically dependent on CD40 signaling for survival and maintenance of function.

$CD4^+$ T cells have been implicated in the programming as well as maintenance of memory $CD8^+$ T cells (Janssen et al., 2003; Masopust et al., 2004; Shedlock and Shen, 2003; Sun and Bevan, 2003; Sun et al., 2004; Williams et al., 2006a). Indeed, while $CD4^+$ T cell-deficient mice can mount normal primary CTL responses upon LCMV infection, the generation as well as maintenance of memory CTL activity is impaired in these mice (Andreasen et al., 2000). It is thought that the effect of $CD4^+$ T cell help occurs mostly through CD40 stimulation. However, we unequivocally showed that for $CD8^+$ T cell

priming, CD40 and $CD4^+$ T cell help do not always have equivalent function. In our LCMV infection system, it is still not clear whether the role of CD40 in $CD8^+$ T cell memory can likewise be independent of $CD4^+$ T cells. This is because depletion of $CD4^+$ T cells from the WT or $CD40^{-/-}$ hosts would also lead to defective memory and complicate the interpretation of the results.

Immunization of $CD4^+$ T cell-depleted mice with a helper-dependent antigen, adenovirus-transformed mouse embryonic cells (5E1 MECs), has been shown to result in failure of "un-helped" $CD8^+$ T cells to undergo secondary expansion upon re-activation *in vitro* or *in vivo* (Janssen et al., 2003). Even infection of $CD4^+$ T cell-depleted mice with LCMV, a helper-independent antigen, also resulted in defective secondary expansion of virus-specific $CD8^+$ T cells (Janssen et al., 2003). Two other studies showed that $CD4^+$ T cell-depleted mice acutely infected with either recombinant vaccinia virus or recombinant Listeria had impaired recall responses upon challenge. In all of these studies, it was found that the $CD4^+$ T cell help was only required during priming and it was important for imprinting or programming of memory $CD8^+$ T cells (Shedlock and Shen, 2003; Sun and Bevan, 2003). However, it was not clear whether help was being mediated by direct or indirect CD40-CD40L interactions between $CD4^+$ T cells, $CD8^+$ T cells, and APCs.

Our data reveal that immunization with peptide-pulsed DCs, a non-inflammatory antigen, can induce the development of functional memory $CD8^+$ T cells. Remarkably, this can occur even in the absence of $CD4^+$ T cell help during priming but more importantly, it depends on CD40. Therefore, even for the development of $CD8^+$ T cell

memory, the function of $CD4^+$ T cell help and CD40 can be independent of each other. Furthermore, because in our experiments CD40-deficiency was restricted only to the immunizing DCs, this suggests that CD40-CD40L interactions between DCs and the responding $CD8^+$ T cells are also involved in memory generation. Notably, the assay we used for evaluating memory only relied on functional activity of memory cells whereas in the other studies, the ability of the memory cells to undergo secondary expansion was also examined. It is important to quantify the number of responding memory cells in order to determine whether CD40-CD40L interactions influence not just the quantity but also the quality of the memory $CD8^+$ T cells that are generated.

The signals that are needed to initiate memory $CD8^+$ T cell responses are not clearly understood. It is thought that memory $CD8^+$ T cells are generally less dependent on co-stimulatory signals and could thus be efficiently activated by cells other than APCs (Bugeon and Dallman, 2000; Gause et al., 1997). However, there is evidence that DCs are important for driving maximal memory $CD8^+$ T cell responses to viral or bacterial infections (Zammit et al., 2005). In addition, the maturation state of DCs has also been shown to affect the activation of influenza-specific memory $CD8^+$ T cells (Larsson et al., 2000). Since CD40 plays a unique role in stimulating DC maturation, we examined whether it is required for memory $CD8^+$ T cell activation. We found that the proliferation, cytokine-production, and cytotoxic activity of memory $CD8^+$ T cells does not depend on CD40 signaling either on all APCs or only on DCs. However, we found that recall responses were reduced when CD40 is absent during both the priming and re-activation of $CD8^+$ T cells. Further, the reduction in memory CTL activity is more severe when

$CD4^+$ T cells were depleted during priming. There are at least three explanations, which are not necessarily mutually exclusive, that could account for these results. First, since we and others have shown that $CD8^+$ T cell responses can occur independently of CD40 or $CD4^+$ T cell help when the frequency of responders is high enough, it is possible that the requirement for CD40 or $CD4^+$ T cells during memory is a direct consequence of the lower numbers of memory cells that are formed in the absence of CD40. Depletion of $CD4^+$ T cells during priming could have resulted in a more limiting CD40L stimulation, thereby further reducing the number of memory cells that are formed. Second, it is possible that the $CD4^+$ T cells are delivering some other signal that could not provided by CD40 stimulation. Finally, it is also possible that the memory cells generated in the presence of CD40 are in fact qualitatively different from the ones that were primed in the absence of CD40 in that the former do not need CD40 stimulation again whereas the latter do need it. It could be that the CD40-dependent APC to T cell signal needs to have been received, but it can be either during priming or at challenge. This implies that the fate of antigen-specific $CD8^+$ T cells primed in the absence of CD40 is not fixed or irreversible. If this is true, it would be reminiscent of the ability of exogenous IL-2 to rescue the proliferative and functional defects of "un-helped" memory $CD8^+$ T cells (Janssen et al., 2003).

What is the mechanism of CD40 function in $CD8^+$ T cell memory? The most prominent effect of CD40 is on the frequency of memory $CD8^+$ T cells that are generated. At any time, the number of T cells is determined by a balance between proliferation, survival, apoptosis, and homeostatic turnover. However, we did not find a difference in

the percentage of apoptotic P-14 T cells between WT and $CD40^{-/-}$ hosts. What we did find was a correlation between CD40 expression in the host and IL-7R expression in the P-14 T cells. IL-7, a γ-chain cytokine, has been implicated in the survival of both naïve and memory $CD8^+$ T cells, owing to its ability to induce the expression of the anti-apoptotic molecules Bcl-2 and Bcl-x_L in IL-7R expressing cells (Bachmann et al., 2005; Fry and Mackall, 2005; Huster et al., 2004; Kaech et al., 2003; Prlic et al., 2002; Schluns et al., 2000). It is not clear at this point how CD40 affects the ability of the responding $CD8^+$ T cells to reacquire IL-7R expression. Two other members of the γ-chain cytokine family play an important role in $CD8^+$ T cell memory. IL-15 has been shown to be critical in the generation as well as homeostasis of memory $CD8^+$ T cells (Prlic et al., 2002). Meanwhile, IL-2 has been found to be involved in programming memory $CD8^+$ T cells to undergo secondary expansion as well as in re-activation of memory $CD8^+$ T cells (Blachere et al., 2006; Williams et al., 2006b). Two other cytokines, IL-12 and Type I IFNs, have been shown to provide a third signal to naïve $CD8^+$ T cells for activation and memory differentiation (Curtsinger et al., 2005; Mescher et al., 2006; Valenzuela et al., 2002). All of the above cytokines can be produced by DCs, but it is worth noting that memory $CD8^+$ T cells can be maintained in the absence of continuous interaction with APCs (Murali-Krishna et al., 1999). We have not yet been able to compare the cytokine levels in WT versus $CD40^{-/-}$ mice or DCs.

How do $CD8^+$ T cells compare to $CD4^+$ T cells in terms of their requirement for CD40 in order to generate and maintain memory? Because CD40 is strictly required for the activation of naïve $CD4^+$ T cells, no memory cells are formed in the absence of

CD40, particularly on DCs (Hochweller and Anderton, 2004). Nevertheless, memory $CD4^+$ T cells do persist in a CD40-deficient environment and they rapidly proliferate upon secondary encounter with antigen (MacLeod et al., 2006). Interestingly however, these cells are incapable of producing effector cytokines such as IFN–γ. On the other hand, we show that memory $CD8^+$ T cells can be formed in the absence of CD40, whether on all APCs or only on DCs. However, CD40 affected the number or memory cells that are generated. The maintenance as well as reactivation of memory $CD8^+$ T cells is largely independent of CD40.

It has been shown that T cell priming in conjunction with CD40L blockade can induce deletion of both $CD8^+$ and $CD4^+$ T cells and subsequently, tolerance (Iwakoshi et al., 2000). In addition, it has also been found that systemic administration of antigen-loaded $CD40^{-/-}$ DCs failed to sustain the activation and led to deletional tolerance of $CD4^+$ T cells (Hochweller and Anderton, 2004). Why then did we not elicit tolerance and instead only observed memory $CD8^+$ T cell responses? The answer may lie in one or more of the following. First, is the nature as well as dose of antigens that were used in our study versus the co-stimulatory blockade studies. Second, is the presence of other stimuli that can overcome the effect of CD40L. Indeed, virus infection and TLR stimulation have both been shown to prevent induction of tolerance by co-stimulatory blockade (Thornley et al., 2006; Turgeon et al., 2000). Third, CD40L blockade affects both $CD4^+$ and $CD8^+$ T cells, and as we and others have shown, $CD4^+$ T cells are very important in generating and maintaining $CD8^+$ T cell memory. Fourth, it is possible that administration of anti-CD40L blocking Ab acted in ways other than by preventing productive APC-T cell

interactions. For example, the Ab could have caused direct T cell lysis through ADCC or complement activation. In addition, the Ab could have also blocked binding of CD40L to a different receptor. Lastly, there is evidence of CD40L signal transduction by CD40L despite the absence of known signaling motifs (van Essen et al., 1995). Therefore, it is possible that the anti-CD40L Ab also blocked this signaling function.

There are a number of issues that remain to be addressed regarding the role of CD40 in $CD8^+$ T cell memory. So far, we have only studied memory responses in the blood. It will be important to examine memory responses in other organs to determine how CD40 affects central and effector memory T cells, which are differentially localized. Also, it is imperative to examine the ability of memory $CD8^+$ T cells that are primed in the absence of CD40 to effectively clear virus or bacteria from a recall challenge. Finally, it would be interesting to compare the role of CD40 stimulation to TLR stimulation in memory $CD8^+$ T cell production and persistence.

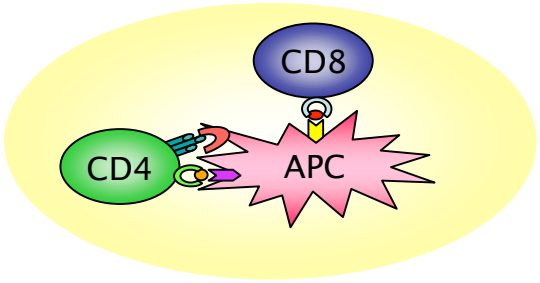
CHAPTER XII.

CONCLUSION

We demonstrate that priming of naïve $CD8^+$ T cells in the absence of CD40 results in reduced T cell expansion as well as development of effector function. Our findings also reveal a unique role for CD40 signaling on APC activation that cannot be fully replaced by TLR stimulation. Importantly, our data support a new model of $CD8^+$ T cell-mediated APC "licensing", in which CD40L expressed by Ag-specific $CD8^+$ T cells interacts with CD40 on APCs, leading to maximal $CD8^+$ T cell responses that can be primed in the absence of $CD4^+$ T cell help (Fig. 49).

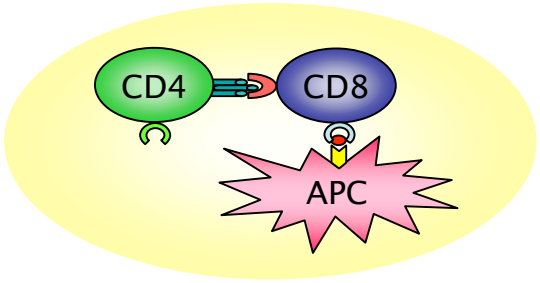
A major consequence of reduced primary responses in the absence of CD40 is the generation of fewer memory $CD8^+$ T cells. However, once formed, these memory cells are not critically dependent on CD40 for survival or maintenance of function (Fig. 50). Therefore, stimulation of CD40 is an essential consideration in the development of vaccines that can induce large numbers of highly effective and long-lived memory cells.

I.



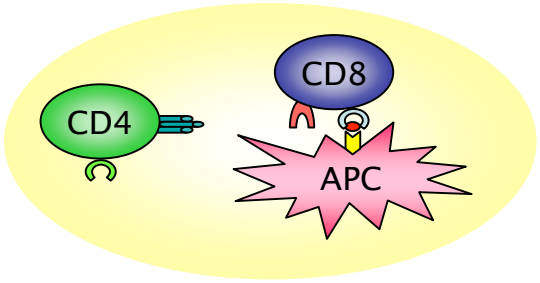
APC licensing

II.



Direct effect on CD8 T cells

III.



Direct CD40-CD40L interaction
between APC and CD8 T cells

Figure 49. Model for how CD40-CD40L interactions influence the generation of CD8$^+$ T cell responses. *(A) CD4$^+$ T cell mediated licensing of APCs. CD40L expressed by*

activated $CD4^+$ T cells binds to CD40 on APCs and stimulates APC activation. (B) Direct $CD4^+$-$CD8^+$ T cell interactions. CD40L expressed by activated $CD4^+$ T cells binds to CD40 expressed on $CD8^+$ T cells and directly stimulates differentiation. (C) $CD8^+$ T cell mediated APC licensing. CD40L expressed by antigen-specific $CD8^+$ T cells binds to CD40 on APCs and stimulates activation. CD40-CD40L interactions between $CD8^+$ T cells and APCs is important for the generation of maximal primary $CD8^+$ T cell responses.

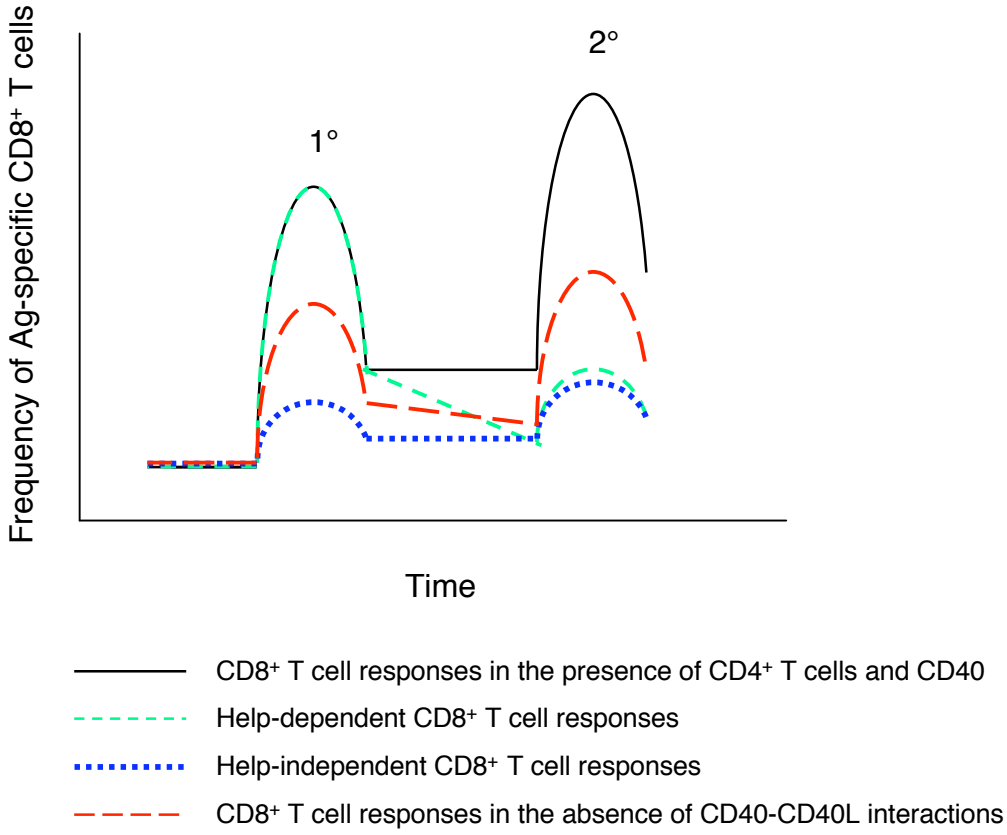


Figure 50. Role of CD40-CD40L interactions in naïve and memory CD8⁺ T cell responses. *Encounter with APCs presenting cognate antigen and appropriate costimulatory signals stimulates naïve CD8⁺ T cells to undergo exponential expansion. This is followed by a contraction phase in which the vast majority of effector cells die by apoptosis, leaving a population of long-lived memory cells that have a higher frequency and affinity to antigen. Depending on the antigen, primary CD8⁺ T cell responses can be CD4⁺ T cell help-dependent or independent. However, it has been found that the presence of CD4⁺ T cells during priming is critical for programming memory CD8⁺ T cells to undergo secondary expansion. Moreover, CD4⁺ T cells have also been shown to*

be important for maintenance of functional $CD8^+$ T cell memory. $CD4^+$ T cell help is mediated primarily through CD40-CD40L interactions. In the absence of CD40 or CD40L, primary $CD8^+$ T cell expansion is reduced and fewer memory $CD8^+$ T cells are generated. Nevertheless, memory $CD8^+$ T cells induced in the absence of CD40 are functional and are capable of undergoing secondary expansion upon reencounter with Ag. The differential requirement for CD40 and $CD4^+$ T cell help during different phases of $CD8^+$ T cell responses suggests that they provide non-redundant signals to $CD8^+$ T cells.

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