THE CRITICAL ROLE OF $CD4^+$ TH CELLS IN $CD8^+$ CTL RESPONSES AND ANTI-TUMOR IMMUNITY

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By

Channakeshava Sokke Umeshappa

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Department of Pathology and Laboratory Medicine

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ABSTRACT

The goal of this body of research was to elucidate the mechanism by which $CD4^+$ T cells provide help for $CD8^+$ cytotoxic T lymphocyte (CTL) responses in different immunization types. The establishment of diseases, such as chronic infections and cancers, is attributed to severe loss of or dysfunctions of $CD4^+$ T cells. Even in acute infections, $CD4^+$ T cell deficiency leads to poor memory responses. While the role of $CD4^+$ T cells is being increasingly appreciated in these diseases, the timing and nature of $CD4^+$ T help and associated molecular mechanisms are not completely understood. Growing evidence suggests that, depending on the type of infections or immunizations, the requirements of $CD4^+$ T cells can vary for optimal $CD8^+$ CTL responses. In order to understand the modulatory effects of $CD4^+$ T cells for optimal $CD8^+$ CTL responses, two distinct immunization types were chosen. These include: 1) non-inflammatory dendritic cell (DC) immunization, which fails to provide inflammatory/danger signals; and 2) inflammatory adenovirus (AdV) immunization, which provides profound inflammatory/danger signals. This allowed us to study $CD4^+$ T cell's participation under different inflammatory conditions.

The studies described in Chapters 2 and 3 of this thesis were performed to further understand the concept of how $CD4^+$ T cells mediate optimal $CD8^+$ CTL responses. This has been called the "new dynamic model of $CD4^+$ T helper – antigen (Ag)-presenting cells (Th-APCs)," proposed in 2005 by our laboratory. The study described in Chapter 2 shows that Th-APCs participate not only in augmenting CTL-mediated immune responses, perhaps during early phase, but also in regulating cellular immunity, perhaps during a later phase. Through enhanced IL-2, CD80 and CD40L singnaling, and weaker peptideMHC I (pMHC) signaling, Th-APCs stimulated naïve $CD8^+$ T cells to differentiate into effector CTLs, capable of developing into, central memory CTLs. Th-APC-stimulated $CD4^+$ T cells behaved like Th cells in function, augmenting the overall magnitude of CTL responses. In contrast, Th-APCs were able to kill DCs and other Th-APCs, predominantly through perforin-mediated pathway. The experiments described in Chapter 3 revealed a novel co-operative role of cognate Th-CTL interactions, contrary to previously known immune-regulatory mechanisms among Th-Th or CTL-CTL interactions. In our experiments, Th cells, via CD40L, IL-2, and acquired pMHC-I signaling, enhanced CTL survival and transition into functional memory CTLs. Moreover, RT-PCR, flow cytometry and western blot analysis demonstrate that increased survival of Th cell-helped CTLs is matched with enhanced Akt1/NF-κB activation, down-regulation of FasL and TRAIL, and

altered expression profiles with up-regulation of prosurvival (Bcl-2) and down-regulation of proapoptotic (NFATc1, Bcl-10, Casp-3, Casp-4, Casp-7) genes/ molecules. Finally, helped CTLs were also able to induce protection against highly metastasizing tumor challenge, explaining why memory CTLs generated under cognate Th1's help show survival and recall advantages.

The studies in Chapter 4 showed how the precursor frequency (PF) of $CD8^+$ T cells impacts $CD4^+$ T helper requirements for functional CTL responses. At endogenous PF, $CD4^+$ T helper signals were necessary for both primary and memory CTL responses. At increased PF, $CD4^+$ T help, and its CD40L but not IL-2 signal became dispensable for primary CTL responses. In contrast, memory CTL responses required $CD4^+$ T cell signals, largely in the form of IL-2 and CD40L. Thus, these results could impact the development of novel immunotherapy against cancers, since their efficacy would be determined in part by $CD4^+$ T help and $CD8^+$ T cell PF.

Finally, the study showed the importance of $CD4^+$ T cells for multiple phases of AdV transgene product-specific CTL responses. These include: a) cognate $CD4^+$ T cells enhanced CTL responses via IL-2 and CD40L signaling during primary, maintenance and memory phases; b) polyclonal $CD4^+$ T environment enhanced the survival of AdV-specific CTL survival, partially explaining protracted CTL contraction phase; and c) during the recall phase, the $CD4^+$ T environment, particularly memory $CD4^+$ T cells, considerably enhanced not only helped, but also unhelped, memory CTL expansion. Thus, these results suggest the participation of both cognate and polyclonal $CD4^+$ T cells for multiple phases of AdV-specific CTLs.

Taken together, the current work delineated the critical roles of $CD4^+$ T cells in different stages of CTL responses and in the development of anti-tumor immunity. The results presented here will significantly advance our current understanding of immunity to cancers, autoimmunity and chronic infections, since pathogenesis of these diseases is largely determined by $CD4^+$ T helper functions. As most immunization procedures use the principle that is based on functions of memory cells, the knowledge gained from this work will also have a major impact on designing vaccines against intractable diseases, including cancers and chronic infections. Moreover, in advanced tumors, vaccines developed using this knowledge may act synergistically with other cancer treatments such as irradiation, chemotherapy and microsurgery, minimizing their side effects and prolonging the lives of patients.

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DEDICATED TO

My family members for their selfless love and continuous encouragement

And

All the animals which sacrificed their valuable lives for the welfare of mankind

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ABBREVIATIONS

Abs	Antibodies
AdV	Adenovirus
Ag(s)	Antigen(s)
AICD	Activation-induced cell death
APC(s)	Antigen presenting cell(s)
APM	Antigen-presenting machinery
BTLA	B and T lymphocyte attenuator
CCR	C-C chemokine receptor
CD	Cluster of differentiation
cDC(s)	Conventional DC(s)
CFSE	Carboxyfluorescein succinimidyl ester
CMI	Cell-mediated immunity
CTLA	Cytotoxic T-lymphoctye antigen
CTLs	Cytotoxic T lymphocytes
DC(s)	Dendritic cell(s)
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte macrophage- colony-stimulating factor
HMI	Humoral-mediated immunity
ICOS	Inducible costimulators
Ig	Immunoglobulin
IL	Interleukin
IFN(s)	Interferon(s)
iNOS	Inducible Nitric oxide synthase
IRF	Interferon regulator factor
iTreg	Inducible T reglatory
i.v.	Intravenous
KO	Knockout

LDC(s)	Langerhans DC(s)
LN(s)	Lymph node(s)
LT	Lymphotoxin
LFA-1	Lymphocyte function-associated Ag 1
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
NK	Natural killer
nTreg	Natural T regulatory
OVA	Ovalbumin
OT	OVA-specific TCR-transgenic
PAMP(s)	Pathogen associated molecular pattern(s)
PD	Programmed death
PE	Phycoerythrin
pMHC	Peptide MHC
pDC(s)	Plasmatoid-derived DC(s)
PF	Precursor frequency
TNF	Tumor necrotic factor
Th	T helper
TLR	Toll-like receptor
Tip DC(s)	TNF-α iNOS-producing DC(s)
Tfh	T follicular helper
Th	T helper
T_{CM}	Central memory CTLs
T_{EM}	Effector memory CTLs
TCR	T cell receptor
Treg	T regulatory
TGF	Transforming growth factor
STAT	Signal transducer and activator of transcription

CHAPTER 1

1. Introduction, literature review, hypothesis and objectives

1.1 General overview

In this 'manuscript style' of thesis, the entire project work is presented in Chapters 2 to 5, each of which has its own hypothesis and objectives leading to achieve a common goal of the thesis. In the beginning, an introduction and a general literature review pertaining to the subject of the thesis leading to hypothesis and objectives of the thesis in its entirety are given. Subsequently, each chapter containing relevant literature review, introduction, materials and methods, results and discussion is presented. The style of all these chapters has been uniformly maintained irrespective of journals considered for publication. At the end, a general discussion of the thesis in its entirety is given.

1.2 Introduction to aspects of the immune system relevant to this thesis

Following entry of foreign substances, usually infectious pathogens, our body responds in a collective and coordinated way to eliminate the foreign substance. This defensive response is called the 'immune response/immunity.' Our immune system is made up of various immune cells and their secretary products. Based on immune cells' origin, location and functions, immune system is broadly divided into three parts (1). It includes primary lymphoid organs (fetal liver, thymus and adult bone marrow). These are sites of lymphocyte maturation where pre-B and pre-T lymphocytes mature into native B and T lymphocytes, respectively, in the absence of foreign antigen (Ag). Immune system also includes secondary lymphoid organs [lymph nodes (LNs), spleen, tonsils and mucosal-associated lymphoid tissues]. These organs harbor B and T lymphocytes and are strategically located in anatomically distinct areas, where Ag-presenting cells (APCs) presenting peptides of invading microbes accumulate and activate rare Ag-specific lymphocytes to initiate adaptive immunity and development of long-lived protective immunity. Immune system further includes tertiary lymphoid organs (eg. inducible bronchus-associated lymphoid tissues). These are ectopic collections of lymphoid cells that generate in non-lymphoid

organs when chronic inflammation occurs as a result of autoimmunity, microbial infection, and graft rejection (2).

1.3 Classification of Immunity

Based on Ag specificity and the nature of different immune responses, our immunity is broadly classified into innate and adaptive immunity.

1.3.1 Innate Immunity

Innate immunity is a potent non-specific protection that prevents or limits infections caused by most pathogens. It provides the immediate line of defense and is targeted towards structures that are frequent in related groups of pathogens. Essentially, this immunity responds similarly to repeated pathogen invasions. The main components of innate immune system are (3): 1) physical and chemical barriers (skin, mucosal epithelia and antimicrobial chemicals); 2) cells [phagocytes, mainly neutrophils and Ag-presenting cells [(APCs) – B cells, dendritic cells (DCs) and macrophages], and natural killer (NK) cells]; and 3) blood proteins (complements and inflammatory mediators, such as cytokines).

1.3.1.1 APCs: DCs

The specialized components of innate immunity that play a critical role in the initiation and development of adaptive immunity is called ‘APCs’. Among various APCs, DCs are regarded as professional APCs since they play a crucial role in induction and control of adaptive immune responses. DCs are distinct from other APCs in that they sometimes possess dendritic morphology, show elevated expression of major histocompatibility complex (MHC) I and II molecules as well as co-stimulatory molecules [cluster of differentiation (CD)40, CD80, CD86 and CD54], exhibit motility, and most importantly, convert from Ag-capturing status to a T cell sensitizing status (called ‘maturation’) (4). After 40 years since their discovery, it is now confirmed that DCs possess characteristic T cell sensitizing properties and control many aspects of immunity, forming a bridge between the innate and adaptive immune responses.

DCs form a physical link between skin/mucosae (periphery) and secondary lymphoid systems where they capture harmful pathogens in the periphery and induce the immune response

in the secondary lymphoid organs by activating T cells. Upon phagocytosing pathogens, DCs secrete important cytokines, principally type I interferons (IFNs) and tumor necrotic factor (TNF)-α. They also produce nitric oxide, and mediate antiviral defense mechanisms and NK cell activation (5). To achieve these functions, DCs undergo a definitive maturation process where they (in immature status) capture invaded pathogens, rearrange cytoskeleton structures to down-regulate their phagocytic activity, process and present Ags to T cells (6). The functions of DCs can vary depending on time, location and type of pathogens involved. For instance, in bacterial and parasitic infections, DCs are biased towards inducing the T helper (Th) 2 response and humoral immunity (7). In contrast, in intracellular pathogen infections, they mainly induce Th1 and/or Th17 responses and cellular immunity (7). In addition to acting as a physiological adjuvant in initiating innate and adaptive immunity, DCs also play a pivotal role in inducing central and peripheral tolerance (8, 9).

1.3.1.1.1 DC subsets

DC subsets have different propensities to reside in particular tissues. Their variable phenotypes endow them with distinct capabilities to ensure optimal tissue-specific immunity although some functions can overlap. The different DC subsets are classified based on their phenotype. Their specialized functions are described below.

A. Conventional DCs (cDCs): These are DCs present in the secondary lympoid organs (also called as 'resident DCs'). Based on the expression of CD4 and CD8α markers, cDCs are divided into three subsets (10); $CD11c^{+}CD8\alpha^{+}$ DCs, $CD11c^{+}CD8\alpha^{-}CD4^{+}$ DCs and $CD11c^{+}CD8\alpha^{-}CD4^{-}$ DCs. All cDCs are derived from a common precursor, reside in the spleen in an immature state, and perform screening of blood-born pathogens (11). The composition of these subsets varies with the type of lymphoid organs. For instance, LNs do not contain $CD4^{+}$DCs but largely possess $CD4^{-}CD8^{-}$ DCs (12). cDCs in LNs capture Ag either from migratory DCs, or from lymphatic conduits.

a) $CD8^{+}$ DCs

These DCs bear the $CD8\alpha^{+,}$ $CD205^{+,}$ $CD24^{+}$ and $CD11b^{-}$ antigenic markers. They have been extensively studied owing to their crucial functions in the immune system. During steady

state when body is not challenged by foreign entities (such as pathogens), these cells play a crucial role in establishing peripheral tolerance to self Ags (9). Unlike other resident DCs, $CD8^+$ DCs typically present in T cell areas of the spleen and LN (13), where they get maturation (licensing) signals from $CD4^+$ T cells during intracellular pathogen infections and cross-present Ags to $CD8^+$ T cells (14). Although most DC subsets cross-present exogenous Ag on MHC I molecules, $CD8^+$ DCs possess exceptional ability, suggesting their key roles in viral diseases (14-16). They express high levels of toll-like receptor (TLR)-3 but not TLR7, suggesting they are specialized for protection against dsRNAs viruses (17). Following infection, $CD8^+$ DCs show up-regulation of MHC Ags and co-stimulatory molecules, and secrete interleukin (IL)-12p70, which in turn induces $CD4^+$ Th1, and $CD8^+$ primary and memory cytotoxic T lymphocyte (CTL) generation (18). These cDCs are also chiefly responsible for clearing apoptotic bodies from the blood (19).

b) $CD8^-$ DCs

These DCs are characterized by their absence of CD8α and CD24 and the presence of the 33D1 and CD11b markers. They are the most abundant cDCs in the spleen (~80% of DC population) but are only present in low levels in LNs. Unlike $CD8^+$ cDCs, which reside in T cell areas, $CD8^-$ cDCs reside in the marginal zones of the LN and spleen. $CD8^-$ cDC activity is dependent on the presence of the C-C chemokine receptor (CCR6) and macrophage inflammatory protein (MIP)-3α. They are specialized for Ag-presentation to $CD4^+$ T cells (20). In contrast to $CD8^+$ cDCs, $CD8^-$ cDCs express principally TLR7 but not TLR3. Following LPS or other TLR agonists, $CD8^-$ cDCs secrete large amounts of the inflammatory chemokine RANTES, MIP-1αβ and MIP-1β and migrate to T cell areas (18).

B Plasmatoid-derived DCs (pDCs)

These DCs, in immature status, have a plasmacytoid shape, and contain round eccentric nucleus. They also have a distinct gene expression profile compared to cDCs (12). pDCs are phenotypically Ly6C/Gr-1^+, $B220^+$, $CD11c^{low/int}$, $120G8^+$, $MHCI^{low}$, $MHCII^-$ and $CD4^+$. They express low levels of co-stimulatory molecules (21-23). In mice, pDCs are present in the blood,

thymus, bone marrow, liver and lymphoid organs. pDCs play an important role in pathogen surveillance, including against bacterial, viral and certain TLR agonists.

C Migratory DCs

These include dermis-derived DCs, epidermis-derived Langerhans DCs (LDCs), and $CD103^+$ DCs. The $CD103^+$ DCs usually reside in the periphery (skin and mucosal layers). LDCs possess longer processes, contain characteristic Birbeck granules (made of langerin), and express the surface marker Ag, $CD207^+$ (langerin). Langerin is a type II transmembrane cell surface receptor that known to helps in non-classical Ag-processing pathway (23). LDCs reside in the epidermis of skin and stratified squamous epithelia in the pharynx, upper esophagus, vagina and external cervix. They have the potential to convert into DCs following Ag encounter (24). Dermal-derived $Langerin^+$ DCs are $CD103^+$ and play a crucial role in cross-presentation of self and viral Ags (12). Dermal classic DCs are $CD11b^+$ $langerin^-$ and reside in the dermis of skin.

D Resident DCs

These DCs are $CD11c^{hi}CD45RA^{lo}MHC\text{-}II^{int}$, immature steady-state cDCs, and are further divided into $CD8\alpha^+$ DCs and $CD8\alpha^-$ DCs. $CD8\alpha^-$ DCs make up the majority of the $CD4^+$ DC population (20). A detailed description of these DC subsets has been described above.

E Circulating DCs

These DCs act as circulating sentinels against blood-borne pathogens. These include some pre-DCs, such as pDCs and $Ly6C^{hi}$ monocytes, $CD11c^+B220^+$ pDCs, and $CD11c^+MHCII^-$ pre-DCs or $Ly6C^{lo}$ monocytes. These DCs are regarded as poor presenters of Ags to T cells although some researchers reported otherwise (20). Whether $DX5^+$ NK cells, which are $CD11c^+$, also possess DC characteristics is not known. Monocytes are chief components of the circulating DCs and possess the capacity to enter from the blood stream to organs to initiate innate immune responses. Limited studies have also shown that they can differentiate into $CD11c^+MHCII^+$ DCs in the presence of granulocyte macrophage- colony-stimulating factor (GM-CSF), with or without IL-4 during inflammation (20). However, under steady state, they form important sources of DCs in skin (Langerhans cells) and mucosa (lamina propria of the gastrointestinal,

respiratory, and urogenital tracts) (25). During inflammation, monocytes also provide defense against pathogens by linking both adaptive and innate immune responses (25). They activate cognate $CD4^+$ T cells and cross-prime $CD8^+$ T cells during viral, bacterial, and parasitic infections.

F Interstitial DCs

These comprise of the dermal DCs of skin and DCs of the mucosae. Interstitial DCs are similar to LCs with some differences. Upon encountering foreign Ags, these DCs migrate to draining lymphoid organs and show as $CD11c^{int}$MHC-II^{hi} $DEC\text{-}205^{int}$ DCs (26). They play important roles in some cutaneous viral infections and in immune-regulation.

G Inflammatory TNF-α inducible nitric oxide synthase (iNOS)-producing DCs (Tip DCs)

Under the influence of GM-CSF and CCR2, these DCs develop and produce high levels of TNF-α and iNOS. Unlike splenic cDCs, which are $CD11c^{hi}CD11b^{low}MAC\text{-}3^-$, Tip DCs are $CD11c^{int}CD11b^{hi}MAC\text{-}3^+$ under steady state. Studies have also shown that $CD11c^-MHCII^-$ $Ly6C^{hi}$ monocytes can develop into $CD11c^{int}MHCII^+Mac3^+$ DCs (27). Tip DCs play a crucial role in variety of infectious diseases, including bacterial, viral and parasitic diseases and they have also been implicated in the autoimmune disorders.

H FL-derived DCs

Under the influence of Flt3 in cultures, these DCs develop and are analogous to steady-state $CD8^-$, $CD8^+$, and pDCs (20).

I Veiled cells

These DCs are named after their sheet-like processes and reside in afferent lymphatic vessels. Veiled cells are thought to form peptide MHC (pMHC) complexes by capturing Ag from matured DCs that are undergoing apoptosis, thereby playing an important role in amplifying immune responses.

1.3.1.1.2 DC vaccines in cancer therapy

All currently available cancer therapies, including irradiation and chemotherapy, are nonspecific in action and generally kill healthy tissues in addition to tumors. Furthermore, these therapies themselves have the potential to induce tumors. Therefore, immunotherapy appears to be very promising approach in the treatment of cancers. Studies have shown that people with increased Ag-experienced T cell infiltration at the tumor site show an improved clinical outcome (28-30). As DCs are extremely potent APCs and robustly activate T cells, they can potentially be used as cellular adjuvant in the treatment of cancers. In support of this, DCs loaded with tumor Ags have proven to induce T cell-mediated tumor destruction (31, 32). These findings have led to clinical trials to understand the protective roles of Ag-loaded DCs as a therapeutic vaccine in patients with cancer (33). Although DC vaccination in cancer therapy is a promising approach, several factors could potentially influence such vaccination outcomes. These include (34): 1) Ag factors - identifying immunogenic non-mutating tumor Ag; 2) DC factors - introducing Ag into MHC I and II processing pathways, methods of purifying and maturation of the DCs, and route of administration; and 3) T cell factors – precursor frequency (PF). Although optimizing these parameters in DC-based vaccination methods is cumbersome, the results of many clinical trials with solid tumors, such as melanoma and prostate cancer, imply that DC vaccination may eventually prove efficacious and could be applicable to the treatment of other tumors (34).

1.3.2 Adaptive immunity

Following pathogenic invasion, a non-specific innate immune response immediately sets in followed by a specific immune response that targets Ags specific for a pathogen. The magnitude and protective abilities of specific immunity augment with repeated infections of particular pathogen. This definitive immune response is called adaptive immunity. The hallmarks of adaptive immunity are discriminating specificity for distinct molecules of a pathogen, enormous diversity (can recognize any foreign Ags exist on the earth), and immunological memory, the capacity to remember and respond more quickly to repeated invasions of pathogens. The main components of adaptive immunity are (3): 1) cellular and chemical barriers [epithelial

lymphocytes and antibodies (Abs)]; 2) blood proteins (Abs); and 3) cells (lymphocytes). Depending on the types of immune cells involved and their functions, adaptive immunity is broadly divided into 1) humoral-mediated immunity (HMI); and 2) cell-mediated immunity (CMI).

1.3.2.1 Humoral Immunity or HMI

HMI is mediated by B lymphocytes, which secrete specialized molecules called antibodies (Abs). Naïve B cells undergo activation upon encountering foreign Ag under the influence of a type of T cell subset, called T follicular helper cells (Tfh), and differentiate into Ab-producing plasma cells and memory B cells. Abs principally act against extracellular pathogens, such as bacteria, and some parasites, and their toxins (35). Abs form effector arms of humoral immunity and provide defense in many ways, depending on the type of Abs involved (36): 1) Neutralization – Abs help in neutralization of bacterial toxins, viruses and bacteria, preventing the cellular entry; 2) Opsonization – Abs also coat the pathogen and aid the phagocytosis by APCs; and 3) Ab-dependent cell-mediated cytotoxicity – Abs also activate complement pathway by coating pathogens. This allows complement receptor binding, attraction of phagocytic cells, and even the lysis of the target cells.

1.3.2.2 Cellular Immunity or CMI

CMI is principally mediated by T lymphocytes, such as $CD4^+$ Th1 and $CD8^+$ CTLs. Once naive T cells develop in thymus, they migrate and re-circulate between blood and peripheral lymphoid tissues. T cells are mainly involved in defending our body against intracellular pathogens, such as viruses and some bacteria (37, 38). To engage in adaptive immunity, T cells have to recognize Ags in the form of short peptide present on MHC of APCs. Ag-recognition leads to activation and differentiation of naïve T cells into effector T cells. For instance, naïve $CD8^+$ T cells, upon recognition of pathogen peptides presented by MHC I, differentiate into CTLs. Naïve $CD4^+$ T cells, upon recognition of pathogen peptides presented by MHC II complexes, differentiate into different effector Th cells, including Th1, Th2, Th17, Tfh and various regulatory T (Treg)-cell subsets. In typical activation of T cells, 3 signals are very essential: 1) signal 1 – comprises antigenic signals derived from the interaction of a specific

pMHC with the T cell receptor; 2) signal 2 – are co-stimulatory signals that support the survival and expansion of T cells; and 3) signal 3 – are cytokine signals that mediate/direct T cell differentiation into the different subsets of effector T cells.

Th cells confer protection indirectly by activating macrophages to kill phagocytosed microbes, B cells to induce Ab production, and DCs or $CD8^+$ T cells to mediate cellular immunity (39). On the other hand, CTLs form final effector arms of CMI and confer protection by directly destroying virally-infected and cancerous cells that present antigenic peptides of pathogens (called target cells) (37). Naïve T cells follow definite kinetics in response to Ag recognition, and undergo activation and clonal expansion called primary response/priming. During priming, long-lived memory T cells that retain imprints of invaded pathogen generate and swiftly respond to subsequent invasion of similar pathogen, the process called secondary/memory/recall responses. Although both $CD4^+$ and $CD8^+$ T cells follow definite kinetics, the magnitude and timing of each phase as well as its regulatory factors can vary depending on the type of pathogen/immunogen involved.

1.4 Phases of $CD8^+$ CTL responses

$CD8^+$ T cells, which are regarded as guided missiles of the immune system, provide protection against numerous cancers and intracellular pathogens, such as viruses and some bacteria. Hence, the induction of an effective $CD8^+$ CTL response is one of the most important steps in any vaccination strategy. In general, their response to an acute infection is divided into 4 phases: (i) 'effector phase' – naïve, Ag-specific $CD8^+$ T cells interact with APCs expressing foreign Ags, undergo activation and differentiation into effector CTLs; (ii) 'contraction phase' - more than 90% of effector CTLs undergo activation-induced cell death, leaving behind only small proportion of cells as long-lived memory CTLs; (iii) 'maintenance phase' - memory CTLs maintained for very long period of time, perhaps life-long in some cases; and (iv) 'recall phase' - memory CTLs undergo rapid proliferation and differentiation into effector CTLs during subsequent exposure to similar Ags (40). $CD4^+$ T cells are also known to behave in a similar manner but differentiate into different T helper subsets, including Th1, Th2, Th17 and Tfh, depending on the microenvironment they receive (41). The factors that control $CD4^+$ and $CD8^+$ T cell fates include nature of Ag, duration of exposure to Ag and surrounding microenvironment in terms of signaling by cytokines and costimulation. Although both types of T cells follow self

program of differentiation, the factors regulating the differentiation, kinetics and efficiency of $CD8^+$ T cells differ significantly from that of $CD4^+$ T cells (37). When mounting immunity, various factors affect the differentiation process and control the quality and quantity of $CD4^+$ and $CD8^+$ T effector and memory cells (42-44). Among various regulatory factors, Th1 cells appear to play central role in orchestrating one or several phases of CTL responses in many disease conditions.

1.4.1 Primary or expansion phase

Upon pathogen entry, DCs ingest pathogen or pathogen-infected cells, and present pathogen-derived peptides on their MHC I (pMHC I). Once naïve, cognate $CD8^+$ T cells recognize pMHC I (signal 1), they undergo activation and expansion (priming). As mentioned earlier, this process is facilitated by other signals such as stimulations from co-stimulatory molecules (signal 2) and cytokines (signal 3) (40). In signal 2, OX40L, 41BBL, and CD80/CD86 co-stimulatory molecules on DCs respectively binds to OX40, 41BB and CD28 on $CD8^+$ T cell surface, which results in increased survival, cytokine production (IL-2) and differentiation into effector and memory CTLs (45). Depending on the type of disease involved, primary CTL response also requires help from $CD4^+$ T cells, which will be discussed later in detail. In signal 3, cytokines, mainly IL-12 and type I IFNs, further drive $CD8^+$ T cell clonal expansion (46, 47). During primary response, generation of millions of copies of effector CTLs occurs due to repeated divisions of activated $CD8^+$ T cells. Also, some researchers believed that the programming of effector and memory responses occurs largely during first 2-3 days of infection (48-51) although no consensus has been achieved yet.

Differentiated $CD8^+$ CTLs leave lymphoid tissues and find the target cells that display pMHC complexes for killing. During differentiation, $CD8^+$ T cells acquire effector functions, include cytokine-secreting (IFN-γ, TNF-α and lymphotoxin (LT)-α) and target cell killing (by developing membrane-bound cytoplasmic granules containing perforin and granzyme-B) (40, 52, 53). $CD8^+$ CTLs form an immunological synapse with the target cells to aid signaling and to directly release effector molecules. Upon recognizing pMHC I complexes on target cells, $CD8^+$ CTLs release perforin, which facilitates the delivery of granzymes. Granzymes are pro-proteases that undergo activation in cytoplasm and induce apoptosis in the target cell. CTLs also express membrane-bound effector molecule Fas ligand that binds to Fas (CD95) on Fas-bearing target

cells and initiates apoptosis. IFN-γ secreted by CTLs inhibit viral replication, increase expression of MHC I complexes and Ag presentation in the infected cells, and activate and recruit macrophages to sites of infection, or even remove virus from target cells without killing them. TNF-α and LT-α can synergize with IFN-γ and induce macrophage activation or apoptosis in target cells upon interacting with tumor necrotic factor receptor-1.

1.4.2 Effector/transitiona/contraction phase

Once invaded pathogen gets cleared from the body, which typically takes 1-2 weeks, about 90-95% of effector CTL pool undergoes death by a process called apoptosis. At least two types of apoptosis have been described in CTL contraction: 1) activation-induced cell death (AICD), also called Ag-driven apoptosis, is triggered by Fas-FasL interactions; and 2) activated T cell autonomous cell death, also called growth factor withdrawal-induced apoptosis (54). It was proposed that inflammation during effector phase determines extent of proliferation and directly influences the contraction rate of effector CTLs (55, 56). The availability of growth factors (cytokines) during peak of effector phase have also been implicated for contraction of CTLs (57). For instance, IL-15 is known to reduce the contraction of effector CTLs by inducing expression of antiapoptotic molecules (58). In contrast, the recent study has suggested that contraction of effector CTLs is predetermined before the onset of contraction and is postulated to be due to epigenetic mechanisms and asymmetric mitotic divisions (56). Interestingly, during contraction phase, about 5-10% of effector CTL pool escapes AICD and develops into functional memory CTLs, the size of which depends on the extent of effector CTLs that survive contraction (58). Although memory development occurs in this phase, whether regulatory factors, particularly of $CD4^+$ T helper signals, have any influence on the size and quality of memory T cell generation is not completely understood.

1.4.3 Memory maintenance phase

Immunological memory, a hallmark of acquired immunity, is defined as transformed and heightened immune response of a vertebrate host to subsequent encounter of same Ags. The realistic use of this dynamic process was noted in the earliest periods by Greek historian, Thucydides during the outbreaks of Plague in Athens. He pointed that person recovered from the

disease often immune to the same disease again and thus he can assist the diseased person without contracting infection (59). Although the development of long-lived memory CTLs programmed reported to occur during first few days of effector phase (48), the growing literatures suggest that memory CTL generation occur in the late stage of immune response, and is believed to form from a minute fraction of effector cells that have undergone some degree of proliferation and differentiation (42). Despite extensive studies, the mechanism by which the lineage of memory CTLs forms is not completely understood. Various models have been put forward to explain how memory generation occurs. These include: 1) Linear Differentiation Model, which states "memory cells are descended directly from the effector cells" (60); 2) Modified Linear Differentiation Model, which states that "a brief exposure to antigenic stimulation favors the development of central memory CTLs (T_{CM}) cells, whereas prolonged exposure to antigenic stimulation lead to development of effector memory CTLs (T_{EM})" (41); and 3) Decreasing Potential Model of Memory $CD8^+$ T cell Development, which states that "effector T cells which receive strong and prolonged Ag-induced signals die by apoptosis, whereas those effector cells which receive moderate and short duration of antigenic stimulation develop into T_{EM} and T_{CM}, respectively (61)."

Various factors, notably signals from $CD4^+$ T cells, are known to influence both quality and quantity of memory T cells generation. These factors are discussed in detail specific to each chapter. During the production of T memory cells, various surface markers are either expressed or downregulated to fulfill their functions effectively. These markers are often used to characterize and distinguish T memory cells from naïve and effector cells. These markers are discussed in detail later wherever appropriate. Once memory CTLs are generated, they maintained for very long period under the influence of survival signals (62). Even the size of memory pool remains fairly constant over extended duration of time. Naïve $CD8^+$ T cells occupy distinct location in the secondary lymphoid organs. However, their memory counter parts destined to home different non-lymphoid tissues apart from secondary lymphoid organs in order to fulfill their functions (63). Such a distinct homing property of memory cells is an outcome of interaction of various molecular tools of the immune system, including adhesive molecules, chemokines and cytokines. These events are necessary to bring about effective immunosurveillence of various re-entries of foreign Ags. Expression of various adhesion molecules and chemokine receptors by T memory cells reflects their residence and migrating

properties (63). Recent studies have suggested that signals from various cytokines (IL-15 and IL-7), co-stimulatory molecules, and $CD4^+$ T cells play important role in memory CTL maintenance and homeostasis [reviewed in (42)].

1.4.4 Recall or secondary phase

During memory maintenance phase, if our body encounters invasion of similar pathogen, memory CTLs respond quickly and robustly compared to their naïve $CD8^+$ T cell counterparts, and eliminate pathogens efficiently. It has been shown that memory CTLs possess special characters, perhaps epigenetically, enabling them to respond more rapidly and vigorously (64). Recall response is contributed by $CD8^+$ memory T cells. Protection has shown to be contributed by both resting type of memory cells, T_{CM}, and a more activated type of memory cells, T_{EM} (65, 66). Although both types exhibit some common functions, they have shown to differ in cytolytic and overall cytokine-secreting abilities (67-69). Overall, T_{CM} are characterized by rapid proliferative capacity in lymphoid organs whereas T_{EM} are characterized by immediate effector functions in the peripheral tissues. Depending on the model system used, investigators have reported that $CD4^+$ T cell signals during priming and memory maintenance can influence the magnitude and quality of recall responses (49, 62, 70-72). As recall responses represent hallmarks of any vaccination regimen, there is a great need to understand mechanisms and regulatory factors that influence $CD8^+$ T memory recall responses. In this context, the role of $CD4^+$ T cells during recall responses as such is scantily available and needs detailed study. Such an understanding would aid better design of vaccines for treating and preventing infectious diseases.

1.5 $CD4^+$ T cells and their subsets

$CD4^+$ T cells are one of the most versatile immune cell types and exhibit multi-faceted roles in regulating diverse immune responses. Generally, both naïve $CD4^+$ and $CD8^+$ T cells live for few to several months. During this period, unless they encounter foreign Ags, they inexorably migrate through circulation and lymphoid organs; do extensive sampling of self-pMHC, exit secondary lymphoid organs, and return to circulation (73, 74). Both $CD4^+$ T cells and lymphoid organs work in concert to provide immunity to hosts against dangerous invading pathogens. To

fulfill this task, both $CD4^+$ and $CD8^+$ T cells constantly search for foreign Ags in lymphoid organs through a mechanism called 'immunosurveillance', where they screen DCs in T cell areas for foreign pMHC complexes (75). If a pathogen breaks innate immunity, then DCs ingest the pathogen, and undergo maturation via both pathogen- (TLR, NOD and C-type lectin) and host-derived (IFN-γ, IL-1, TNF-α and CD40L) signals (76). If a productive T cell receptor (TCR)-pMHC interaction occurs between DCs and cognate $CD4^+$ T cells via immunological synapse, activation and proliferation of $CD4^+$ T cells begins. Immunological synapse comprised of central T cell signaling molecules (TCR, CD28) encircled by adhesion molecules (LFA-1), and cytokine and chemokine receptors (77). Once the pathogen is cleared from the system, a variety of host signals, particularly Tregs, control ongoing immune reactions to prevent host tissue damage.

Unlike $CD8^+$ T cells, $CD4^+$ T cells, being very sensitive to environmental cues, have potential to develop into many subsets, each of which shows distinct phenotype and gene expression, and performs different tasks. Depending on the type of pathogens, DCs take blueprint of pathogens via combinatorial ligation of pattern recognition receptors and generate distinct cytokine microenvironment, which in turn decides the fate of $CD4^+$ T cell choices, ensuring immunity specific for different classes of pathogens with diverse life cycles and evasion strategies (75, 77). In addition, it is believed that formation of immunological synapse, which might determine strength of TCR and co-stimulatory signals, also plays an important role in $CD4^+$ T cell lineage commitments. $CD4^+$ T cell subsets can be distinguished based on the cytokine secretion and the surface marker expression [reviewed in (78)]. In contrast to 4 distinct subsets, Th1, Th2, Th17 and inducible T regulatory (iTreg) cells, which derive from naïve $CD4^+$ T cells that are selected from thymus in Ag-dependent manner, some $CD4^+$ T cell subsets, such as NKT and natural T regulatory (nTreg) cells, do not derive from naïve $CD4^+$ T cells but rather selected directly from thymus as distinct lineages in an Ag-independent manner without the aid of cytokines (78). Unlike $CD8^+$ T cells, most of the $CD4^+$ T cell functions are indirect. $CD4^+$ T cells play crucial roles in orchestration and/or recruitment of other immune including B cells, $CD8^+$ T cells, macrophages, mast cells, neutrophils, eosinophils and basophils and nonimmune cells, (79). A summary of these $CD4^+$ T cell subsets, their phenotype and functions is given below:

1.5.1 Th1 cells

Upon encountering cognate Ags in the presence of IL-12 and IFN-γ, naïve $CD4^+$ T cells express signal transducer and activator of transcription (STAT) 1, STAT4, and T-bet transcriptional factors (80) and differentiate into Th1 (81, 82). T-bet in combination with STAT4 brings about its effects by inducing IFN-γ production and inhibiting GATA3 ie Th2 differentiation factor. Other factors, interferon regulator factor (IRF)1, Ikaros and Runx3, are also required for fine-tuning Th1 differentiation (83). Th1 cells are characterized by the secretion of IFN-γ and LT as signature cytokines (78). In addition, Th1 cells also secrete IL-2 and TNF-α and also express cytokine receptors, IL-12Rβ2 and IL-18Rα, activation markers, CD25, CD69 and CD40L, and chemokine receptors, CXCR3 and CCR5 (84-86).

Th1 cells activate microbicidal activity of macrophages, NK cells and $CD8^+$ T cells by secreting IFN-γ, LTα, and IL-2 (87). Th1 cells are chief mediators of inflammation and play a crucial role in systemic immunity against various intracellular pathogens. Both IL-2 and CD40L costimulation provided by Th1 cells are very critical in the generation of primary and memory CTL responses (50, 51, 88-91). Absence of T-bet transcription factor in $CD4^+$ T cells leads to diminished IFN-γ and Th1 differentiation, resulting in the reduced protection against *Leishmania* infections (92). T-bet-expressing $CD4^+$ T cells also get reduced in asthmatic patients, suggesting the importance of Th1 cells in asthma (93, 94). Recently, another transcription factor, Eomesodermin (Eomes), has also been shown to involve in Th1 differentiation, and that the absence of this along with T-bet results in poor protection against lymphocytic choriomeningitis virus infection (83, 95).

1.5.1.1 Th1-derived co-stimulatory and cytokine signals in CTL memory development

Th1 cells modulate CTL responses in many ways (96). Among many signals provided by Th1 cells, two signals, such as CD40L and IL-2, have been frequently studied and implicated in the development of memory CTLs although they are reported to be required for primary responses in some situations. There are two schools of thought to describe how CD40L signaling from Th1 is targeted for memory CTL development. First, during DC interaction, $CD4^+$ T cells get activated and license the DCs through CD40-CD40L costimulation such that licensed DCs stimulate $CD8^+$ CTLs activation, and differentiation into effector and memory CTLs (97-99);

Second, this concept was challenged by the finding that activated $CD8^+$ T cells transiently expressing CD40 can directly receive CD40L signaling from Th1 (51, 88), Recently, several reports also suggested that $CD4^+$ T cells induce memory CTL development by providing IL-2 signaling (50). Subsequently, various results suggested that both IL-2 and IL-15 signals determine the generation of T_{CM} versus T_{EM} with IL-2 signals promoting T_{EM} cells (96, 100-104). Recently, it has been shown that Th1 cells license DCs to secrete IL-12, which in turn act on $CD8^+$ T cells to express IL-2Rα for efficient use of IL-2 secreted by Th1 (90).

1.5.2 Th2 cells

Th2 cells require IL-4 and to some extent IL-2 for their differentiation, and typically secrete IL-4, IL-5, IL-13 and IL-25 as signature cytokines. Evidence of these cells secreting IL-2, TNF-α and IL-9 has also been suggested. These cytokines are responsible for up-regulation of STAT5, STAT6, and a master transcriptional regulator, GATA3, which in turn influence Th2 differentiation and functions via induction of IRF4 (83, 95). Absence of this factor leads to resistance to autoimmune encephalomyelitis. In addition, c-Maf factor is also shown to induce IL-4 secretion from Th2 cells (83). Th2 cells also express surface markers, IL-4Rα, IL-2Rα (CD25), IL-33Rα, and chemokine receptors, CCR3, CCR4, CCR8, and CRTh2 (84). Th2 cells are critical to provide defense in mucosal and epithelial surfaces against extracellular pathogens, including bacterial and helminth infections.

1.5.3 Th17 cells

Th17 cells are induced under the influence of IL-6 and transforming growth factor (TGF)-β, and can be maintained well in the presence of IL-23 and IL-21 (83). Under these cytokine milieus, and in the presence of cognate Ag, naïve $CD4^+$ T cells upregulate STAT3, and master regulatory factors, RORγt and RORα, and differentiate into Th17 cells (105). STAT3 together with RORγt expressions in Th17 cells also down-regulates Foxp3 induction and Tregs differentiation. Another factor IRF1 is also required for fine-tuning Th17 differentiation (83). Under the influence of these molecules, they also produce IL-17A, IL-17F and IL-22 as signature cytokines. In addition, Th17 cells also secrete IL-21, IL-6, and TNF-α and express cytokine receptors, IL-23R, IL-1R1, and IL-18Rα, and chemokine receptors, CCR6 and CCR4 (84). Th17

cells mainly regulate inflammation and provide antitumor immunity, and in concert with neutrophils, provide defense against extracellular bacterial and fungal infections (106, 107). Over expression of Th17 cells have been implicated in the pathology of autoimmune disorders, particularly experimental autoimmune encephalomyelitis, collagen induced arthritis, inflammatory bowel disease and multiple sclerosis, and even in some conditions contribute to cancerous status (106, 107).

1.5.4 Tfh cells

Although it is controversial, it was thought that IL-6 or IL-21 cytokines together with TCR-mediate induce Bcl-6 expression, which in turn determine the differentiation of Tfh cells (83). Bcl-6 expression also mediates Tfh-related molecules, including CXCR5, PD-1, IL-6R, and IL-21R, and suppresses Th1, Th2 and Th17 differentiation (83). Tfh cells are identified as p-selectin glycoprotein ligand-1^- and $CXCR5^+$, and can secrete IL-4 or IFN-γ depending on the priming conditions (108, 109). For instance, in *Schistosoma mansoni* infections, Tfh cells secrete IL-4 to mediate their functions (83). They remain in LNs and spleen, and are responsible for mediating Ab responses by activating B cells and inducing germinal centers formation (108, 110). In addition, they are also responsible for somatic hypermutation and Ab class-switching. Th17 cells mediate these functions in germinal centers via CD40 signaling and secretion of Th1- and Th2-like cytokines. How far these cells are distinct from Th1 or Th2 lineages is not yet clear.

1.5.5 Treg cells

Growing experience in regulatory immune response studies where Treg cells were involved suggest that different Treg populations may control the functions of different $CD4^+$ T cell subsets. Treg cells express moderate levels of GATA3 although its function is not clear. $CD4^+CD25^+FoxP3^+$ Treg cells are induced by $CD103^+$ DCs in respiratory and gastrointestinal tracts following exposure to commensal microorganisms, food proteins, and possibly pathogens, and this is required to maintain balance between tolerance and immunity (111).

1.5.5a iTreg cells

These cells derive from naïve $CD4^+$ T cells under the control of distinct transcription factors parallel to Th1, Th2 and Th17 lineages (112, 113). For example, IRF4 induces differentiation of Tregs that possess the ability to control Th1 functions. Similarly, STAT3 expression in Tregs controls Th17 cell's functions. In all these situations, TGF-β and IL-2 together with TCR stimulation are critically required for iTregs differentiation (114). These cytokines mediate STAT5 activation required for Tregs development (115). These Tregs produce TGF-β as their signature molecule, and express CD25, IL-2Rα, CTLA-4, glucocorticoid-induced TNFR related protein, Folr4 and CD103, which can be used to distinguish naïve conventional nTregs (84). Although these cells can develop from naïve $CD4^+$ T cells, their *in vivo* relevance has not been demonstrated yet in humans unlike in mice (116).

1.5.5b nTreg cells

In contrast to iTregs, nTregs do not derive from naïve $CD4^+$ T cells but rather selected from thymus as distinct lineages (117). nTreg cells naturally express a master transcription factor, Foxp3. This factor together with TGF-β, regulate nTregs differentiation and its maintenance of suppressive activity. Mutations in the Foxp3 lead to various autoimmune disorders, such as immunodeficiency, polyendocrinopathy, and enteropathy, X-linked syndrome, in humans. These patients often develop insulin-dependent diabetes, increased serum immunoglobulin (Ig)E, eczema and psoriasis. Furthermore, patients also develop hypothyroidism, anemia, thrombocytopenia, and neutropenia (83).

1.6 Costimulation in cellular immunity

T cells are stimulated through recognition of Ag-peptide bound MHC I (in $CD8^+$ T cells) or MHC II (in $CD4^+$ T cells) molecules on APCs, such as macrophages and DCs. However, as discussed earlier, this TCR complex engagement is alone not sufficient for productive T cell activation (118). In addition to antigenic stimulation, costimuation-mediated signal 2 and cytokine-mediated signal 3 are very important for T cell activation. Signal 2 is mediated by one of the many co-stimulatory receptors and transmits independent signals or facilitates the signal cascade triggered by the engagement of TCR complex.

1.5.1 CD28

CD28 signal is major costimulus for both $CD4^+$ and $CD8^+$ T cells and is required for many functions in T cells. Although many co-stimulatory receptors expressed on the resting T cells are able to provide signal 2, CD28 appears to be very much required for activation of both $CD4^+$ and $CD8^+$ T cells (119). CD28 is present on all resting T cells and binds to CD80 (B7-1) and CD86 (B7-2) expressed on mature professional APCs (45). Uniquely, this binding is known to induce enhanced secretion of IL-2 from T cells. This results in the effective T cell response due to activation of c-Jun kinase and P13K pathways. These pathways in turn enhance T cell activation as well as T cell survival by inhibiting apoptosis and up-regulating anti-apoptotic molecules via AKT activation (120-122). CD28 signals also up-regulate CD40L expression on T cells, which leads CD40 signaling on APCs and on APCs lead to up-regulation of B7-1 and B7-2 ligands,

1.5.2 Inducible costimulators (ICOS)

Recently, studies have also shown that ICOS, another member of CD28 family, also engages in T cell activation. In contrast to CD28 that express on all resting T cells, ICOS express mostly on active T cells, suggesting sequential involvement of these costimulators in productive T cell activation (123). Since ICOS ligand express relatively on many cell types (nonlymphoid cell types, including epithelial and endothelial cells) in addition to APCs (B cells, DCs and macrophages), it is postulated that insufficiently activated T cells can undergo further activation in tissues that express ICOS ligand (124). ICOS signals are known to stimulate mainly P13K pathway and required for regulating ability of T cells to provide help to B cells for both class-switching and germinal centers formation (125). The level of ICOS expression has been shown to associate with the type of cytokines produced, with $ICOS^{hi}$ T cells linked to IL-10 production, $ICOS^{medium}$ T cells linked to IL-4, IL-5 and IL-13 production, whereas $ICOS^{low}$ T cells are linked to IL-2, IL-6 and IFN-γ production (5). ICOS is also known to suppress Tregs *in vivo* (126).

1.5.3 TNF/TNFR super family costimulators

In addition to the above co-stimulatory molecules, additional accessory molecules also provide essential signals required for full activation of T cells. These molecules are TNF/TNFR

super family members, such as CD27, 4-1BB (CD137), OX-40 (CD134) and CD30 receptors on T cells and their ligands CD27L, 4-1BBL, OX-40L and CD30L, respectively, on APCs. CD27 acts as costimuatory molecule to induce T and B cell responses. It is present excessively on NK cells, on induced B cells, and on $CD4^+$ and $CD8^+$ T cells. It is known to control the size of primary and memory T cell responses, particularly in respiratory viral infections by preventing the death of activated T cells (5, 127). CD27 ligand, CD70, expresses in DCs only transiently during interaction with T cells. Persistent CD27-CD70 interactions in chronic infections lead to excessive secretion of TNF-α and IFN-γ, which results in the loss of splenic architecture, increased tissue pathology and increased T cell exhaustion and reduced virus-specific neutralizing Ab responses leading to lethal immunodeficiency (127). These undesired effects can be reverted by blocking this signal. OX40 signaling is required for maintaining T cell numbers. In addition, OX40 signal facilitates TNF secretion in $CD4^+$ and $CD8^+$ T cells, and cytotoxic functions in $CD8^+$ T cells. This signaling has also been implicated in the IL-12-mediated Th1 autoimmunity. OX40 is known to express transiently on T cells. 4-1BB expresses on activated $CD4^+$ and $CD8^+$ T cells, activated NK cells, and DCs, whereas its ligand expresses on resting B cells and activated APCs. 4-1BB costimulation enhances CTL generation and immunity against tumors and viruses. Interestingly, 4-1BB costimulation inhibits autoimmunity, suggesting its effects can vary with the stages of T cell responses. CD40L (CD154), expresses on activated $CD4^+$ T cells, some $CD8^+$ T cells, eosinophils, basophils, and NK cells. Its ligand, CD40, expresses on B cells, activated macrophages, DCs, thymic epithelium, and endothelial cells (5). Signaling from CD40L is crucial for effective T and B cell responses. Principally, this signaling is mediated through activated $CD4^+$ T cells. CD40L-expressing $CD4^+$ T cells binds to CD40 on APCs, which on B cells result in B cell expansion, Ig production, and isotype class switching, and on DCs result in maturation of DCs, production of cytokines and chemokines, and prolonged survival, thereby increasing overall magnitude and quality of CMI responses (5, 128).

1.5.4 Inhibitory co-stimulatory molecules

Cytotoxic T-Lymphocyte Ag (CTLA)-4, programmed death (PD)-1 and B and T lymphocyte attenuator (BTLA) are inhibitory receptors that bind to the negative costimulator B7 family molecules.

CTLA-4 (CD152) expressed on activated and memory $CD4^+$ and $CD8^+$ T cells (similar to ICOS) (129). Upon T cell activation, CTLA-4 traffics to the sites of TCR engagement and has high affinity for B7 molecules than CD28 and thus suppresses the activation of T cell by out competing CD28 for B7 binding, which in turn lead to aborting of T cell response (129). Mechanistically, CTLA-4 is known to inhibit Akt directly in T cells, transmit suppressive signals to DCs via B7 and suppress natural Tregs to maintain tolerance to self-Ags (5).

PD-1 (CD279), in contrast to CD28 and CTLA-4, inducibly express in wide range of cells, including $CD4^+$ and $CD8^+$ T cells, NKT cells, B cells, and monocytes upon activation (5). PD-1 transmits signals only when engaged along with TCR ligation. APC ligands for PD-1 receptor are B7-H1 (PD-L1) and B7-DC (PD-L2). PD-L1 expression is induced by IFN-γ. Both PD-L1 and PD-L2 synergistically inhibit T cell activation. Tumor cells often use PD-L1 to evade immune recognition. It has been shown that PD-1 also expresses on $CD8^+$ T cells during chronic infections, such as LCMV and HIV-1, and blocking PD-1/PD-L1 pathway has shown to increase T cell proliferation and effector functions. Similar to CTLA-4, PD-1 has shown to inhibit Akt phosphorylation, suggesting both CTLA-4 and PD-1 may regulate T cell activation (130).

B and T (especially Th1 cells) cells express BTLA (5). Upon binding to its ligand, herpes virus entry mediator, BTLA negatively regulates T cell responses (5).

1.7 Immunity in vaccination

Vaccination, by markedly decreasing mortality from infections, forms one of the most important contributions to public health in the last century. Vaccines mediate their effects by inducing adaptive immunity and immune memory (53). Pre-existing Abs in the blood and tissues act as the first line of defense and readily attack the invading pathogen. Abs maintain their levels for long period, presumably due to the presence of Ab-secreting long-lived plasma cells. Memory B cells act as a source of Abs during pathogen invasion. Similarly, T cell memory is contributed by T_{EM} cells, which reside in infected peripheral tissues and provide an immediate line of defense by killing pathogen-infected target cells, and T_{CM} cells, which reside mainly in LNs and undergo activation and differentiation into effector T cells upon encountering pathogens (67, 131, 132). T cell memory is contributed by both $CD4^+$ and $CD8^+$ memory T cells (132).

Since the discovery of small pox vaccination by Edward Jenner, our understanding of immunity as a result of vaccination has tremendously increased, particularly in the last two

decades with the advent of modern immunological technologies. The development of vaccination began with two empirical approaches (reviewed in (53)): 1) killed or inactivated, in which the infective ability of pathogens is destroyed by killing, yet retaining immunogenesity; and 2) live or attenuated vaccines, in which the infective ability of pathogen is reduced by modifying the organism, yet they retain a natural behavior of an original pathogen. Killed vaccines act mainly by inducing humoral immunity. They are often targeted to bacteria and viral infections. In contrast, attenuated vaccines act as a larger and more sustained dose of Ags (by pathogen replication), and thus can able to induce both Ab and T cell memories. Although attenuated vaccines are potent inducers of T memory, they are contraindicated in certain infections, such as those which induce immunosuppression or undergo genetic recombination. Though smallpox was eradicated without having much knowledge of cellular and humoral (both primary and secondary) immune responses, currently we are still struggling to control various chronic diseases and cancers even with much immunological background. Still we have not been completely able to understand the basic reasons for success or failure of our vaccination regimens. Hence, immunologists began to develop novel approaches. One such effective approach is the use of microbial vectors in vaccines preparation (133). In this approach, attenuated recombinant viral vectors, such as adenovirus (AdV), fowl pox, canary pox and modified vaccinia Ankara virus that infect mammalian hosts but are unable to replicate, are used to insert genes encoding proteins of pathogen. This approach acts as a powerful vaccination strategy since it is endowed with several benefits, including its ability to induce potent humoral and cellular immunity (133, 134). These approaches appear to be invaluable especially in the treatment of chronic diseases, such as HIV, malaria, tuberculosis and hepatitis.

1.8.1 AdV vectors

In the last two decades, the transfer of genetic information that encodes desired proteins in target cells using viruses and viral vectors has gained more interest for the prevention or treatment of infectious diseases, autoimmune disorders, allergies, and cancers (135). Although non-vector systems, such as naked plasmid DNA, DNA complexed with cationic lipids, and particles comprising DNA condensed with cationic polymers, are available, viral vectors have been commonly used due to their potent ability to transfer genes to broad range of host cells (136). In cancer immunotherapy, several of these vectors have been used to stimulate patient's

immune system to destroy tumors. Although several replication-deficient viral vectors derived from retroviruses, AdV, adeno-associated virus, semliki forest virus and herpes simplex virus families also have been used in gene expression studies. AdV vectors have been most commonly used in the treatment of cancers and chronic diseases due to their several inherent features (133, 137).

AdVs are non-enveloped viruses and contain linear double-stranded DNA genome. AdV-based vectors are considered to be the widely-used vectors in clinical studies worldwide, representing major part of all the trials (138). AdVs have several advantages, enabling them the choice of vector for many preventive and therapeutic applications (136). One of the best traits in AdVs is their ability to transiently transfect the therapeutic genes into both dividing and non-dividing cancer cells (136). Another advantages of using AdVs in vaccine preparation is our in-depth understanding of lifecycle and immunological effects of adenoviral vector delivery (139). Currently, almost all serotypes that infect humans naturally have been identified and characterized. AdVs immunological consequences in various doses and routes have also been extensively studied. This knowledge is particularly relevant especially when excessive immune response in host that needs to be controlled. For instance, in situations where controlled immunity is desired, one needs to reduce innate immunity (by reducing dose, avoiding coexisting inflammation, etc.) and adaptive immunity (delivery during neonatal immune systems, avoiding APC transduction, blocking co-stimulatory molecules, delivery to organs such as liver, eye, mucosa and muscle) (138). Characterization of various aspects of AdVs and their genetic modification have also now allowed us to modify them for use in a safer and efficacious way (140).

1.7.1.1 Immunity to adenoviral vaccination

AdV vectors have attracted considerable attention as a platform for eliciting productive immunity against intractable diseases, including chronic infectious and cancerous diseases and are at the forefront of vaccine development against such diseases. One of the most notable characteristics of AdV vectors is their capacity to induce exceptionally high and sustained levels of transgene product-specific $CD8^+$ CTL responses (133, 134). They are also regarded as exceptionally strong inducers of innate as well as humoral immune responses (133). Transgene product-specific Abs are mainly of IgG2a isotype although IgG1 are also known to be induced,

suggesting AdV induce a mixed Th1/Th2 response with Th1-type predominance. AdVs have shown to induce very quick and persistent immune responses in both adults and neonatal mice (133). This potent immunogenicity of AdV vectors appears to be due to their capacity to activate innate immune cells, particularly APCs (141). Compared to other vaccine candidates, such as poxvirus vectors, DNA vaccines, and alphavirus vectors, AdV vectors perform superiorly in terms of inducing transgene-specific Abs and $CD8^+$ T cell responses (142, 143). Hence, AdV are being currently considered in treating various infectious diseases, including AIDS, hepatitis, Rabies, SARS and Ebola. AdV vectors do not induce potent transgene product-specific $CD4^+$ T cell responses (133). However, AdV vectors appear to require $CD4^+$ T help for optimal $CD8^+$ T cell responses (144). Although the usage of AdV vectors largely depends on their ability to induce $CD8^+$ CTL responses, our knowledge on how $CD4^+$ T cells regulate different phases of CTL responses is incomplete. This knowledge is very critical since AdV vectors are currently being used in diseases such as AIDS, hepatitis and other chronic diseases, and cancers, which often induce $CD4^+$ T cell dysfunctions in the body.

1.9 Models to explain mechanism of $CD4^+$ T cell help for $CD8^+$ CTL responses

For the development of effective CMI responses, multiple, at times rare immune cell types must communicate within organized lymphoid tissues. Despite our technological advancement in the immunology field, the mode by which $CD4^+$ T cells provide help for $CD8^+$ CTL responses has been debated frequently. Our perception on where $CD4^+$ T cells participate in the CTL responses has been changing gradually. Various models have been proposed to explain the development of $CD8^+$ CTLs [reviewed in (145, 146)] **(Fig. 1)**.

1.9.1 Traditional model of three-cell interactions

Initial studies in allograft rejection and *in vitro* studies of allogeneic mixed lymphocyte reactions suggested that $CD4^+$ T cell "help" is vital for the clonal expansion of naive $CD8^+$ T cells (147-149). Based on these observations and recent studies, researchers thought that both $CD4^+$ and $CD8^+$ T cells have to recognize peptide Ag on the same APC simultaneously for optimal delivery of $CD4^+$ T cell help (98, 150, 151). Justifications in formulating this model include: a) $CD4^+$ T cells were a chief source of IL-2 and its help often thought to occur in

paracrine manner (152); and b) neutralization of IL-2 or blockade of the IL-2 receptor in culture severely limited CTL proliferation (153-155). Hence, according to this model, $CD4^+$ and $CD8^+$ T cells recognize antigenic peptide on the same APC so that IL-2 produced by $CD4^+$ T cells can act on neighboring IL-2-receptor-expressing $CD8^+$ T cells (150, 156). Two main limits of this model include:

a) this model cannot explain how two rare Ag-specific cells, $CD4^+$ and $CD8^+$ T cells manage to interact on the same Ag-bearing APC. Such an event has extremely low probability; and

b) CTL responses can also be elicited without $CD4^+$ T cells in various models, suggesting the need for $CD4^+$ T help is conditional rather than absolute.

1.9.2 Model of sequential two-cell interactions by APC by Polly Matzinger

The above notion of $CD4^+$ T and $CD8^+$ T cell cooperation raised many questions when immunologists came to realize that mature DCs and activated $CD8^+$ T cells can also transiently secrete IL-2, questioning earlier notions that $CD4^+$ T cells are unique producers of IL-2 that mediates $CD4^+$ T cell help (157-159). Furthermore, $CD4^+$ T cells were not essential in the control of acute viral infections, challenging the notion that Th1 help is absolutely required for CTL clonal expansion (160). These results led to the belief that Th1 help is only required for noninfectious Ags that lack molecular pathogen-derived stimuli [pathogen-associated molecular patterns (PAMPs)] for the innate immune cells, whereas it is dispensable for acute microbial infections, whose PAMPs acts as potent stimuli for APCs (161, 162). Thus, this model states that, in the absence of PAMPs [eg. TLRs, such as dsRNA (TLR3), lipopolysaccharide (LPS) (TLR4), and CpG-containing oligonucleotides (TLR9) and type 1 IFNs], $CD4^+$ T cells, during activation, recognize APC and deliver all signals required for APC maturation (163). It was shown that such activation occur through CD40L and CD40 interactions in immunity involving noninfectious Ags (97-99). The licensed APCs (typically DCs) can then directly stimulate $CD8^+$ T cells. (97-99). Subsequently, it was also reported that, even in acute infections, memory/recall responses require $CD4^+$ T helper signals (51, 62, 72, 164). Furthermore, $CD4^+$ T cell requirement was also shown to be required for the clearance of persistent pathogens in chronic infections (165-167).

This model is supported by the fact that Ag-specific CTL responses can be elicited by activating APCs *in vitro/in vivo* by agonistic anti-CD40 Ab, which mimic CD40L signaling (97, 99). Limitations to this model include:

a) very small numbers of Ag-bearing APCs must first be conditioned by a rare and naïve, Ag-specific $CD4^+$ T cells, and then have to find and activate equally rare and naïve, Ag-specific CTLs;

b) the model does not explain how T cell's IL-2 precisely targeted to Ag-specific CTLs *in vivo*; and

c) the experiments used to propose this model may not represent the physiological phenomenon.

1.9.3 New dynamic model of sequential two-cell interactions by $CD4^+$ Th-APC by Jim Xiang

Trogocytosis (Greek: trogo; *gnaw*) is a process through which certain lymphocytes interacting with APCs pinch off surface molecules from these cells and express them on their own surface. Currently, the transfer of cell surface proteins via trogocytosis among different types of cells, and its biological function are becoming the subject of study in immunology (168-170). Trogocytosis is a wide-spread phenomenon and many immune cells utilize this mechanism for cellular communication. Recent studies have shown that $CD4^+$ T cell subsets are particularly involved in acquiring APC's Ag-presenting machinery (APM), attracting the attention of many immunologists (88, 146, 169, 171-174). Other correlative studies included Ag-presenting functioning of human γδ T cells expressing MHC-II and co-stimulatory molecules, which stimulated naive αβ T cell proliferation and differentiation (175), Ag-specific peptide pulsed Th lymphocytes which can cross-prime memory CTL responses, and human $CD4^+$ T cells bearing viral epitopes which can elicit functional virus-specific memory $CD8^+$ T cell responses (176). Consistent with these concepts, Xiang *et al*. showed the *in vivo* relevance of Ag-presenting nature of $CD4^+$ T cells. This model states that $CD4^+$ T cells while interacting with APCs acquire APM and behave like APCs called "Th Ag-presenting cell" (Th-APC) (146). During APC and $CD4^+$ T cells interaction, APCs directly transfer pMHC II as well as bystander pMHC I and co-stimulatory molecules (CD54 and CD80) to expanding populations of IL-2-producing Th1 cells. Th-APCs with acquired pMHC I and co-stimulatory molecules can then directly stimulate naïve cognate $CD8^+$ T cells for proliferation and differentiation into effector CTLs. Th-APCs are efficient in induction of an effective antitumor immunity by triggering $CD8^+$ T cell proliferaftion

and differentiation into CTLs through their acquired molecules, pMHC I complexes and CD80, and via their inherent ability to secrete IL-2 cytokines (146). These Th-APCs are also known to promote CTLs infiltration and survival via IL-2 secretion and targeting effect of pMHC I complexes and regression of established tumors *in vivo* (164). It was found that Th-APCs can even stimulate memory CTL proliferation during recall responses via acquired pMHC I complexes and CD80 molecules (177-179). Furthermore, it was shown that Th-APC's stimulatory power is independent of endogenous $CD4^+$ T cells. They also have the ability to break tolerance and induce autoimmunity. Although these observations provided the evidence of $CD4^+$ T cell's role in CMI responses via direct Ag presentation, many issues associated with this model were not understood. These include: 1) whether the acquired pMHC II on $CD4^+$ Th-APC are also functional; 2) which type of T cells these $CD4^+$ Th-APC-stimulated $CD4^+$ and $CD8^+$ T cells belong to; and 3) what is the molecular mechanism responsible for Th-APC's stimulatory effect on *in vivo* stimulation of $CD8^+$ CTL responses and antitumor immunity.

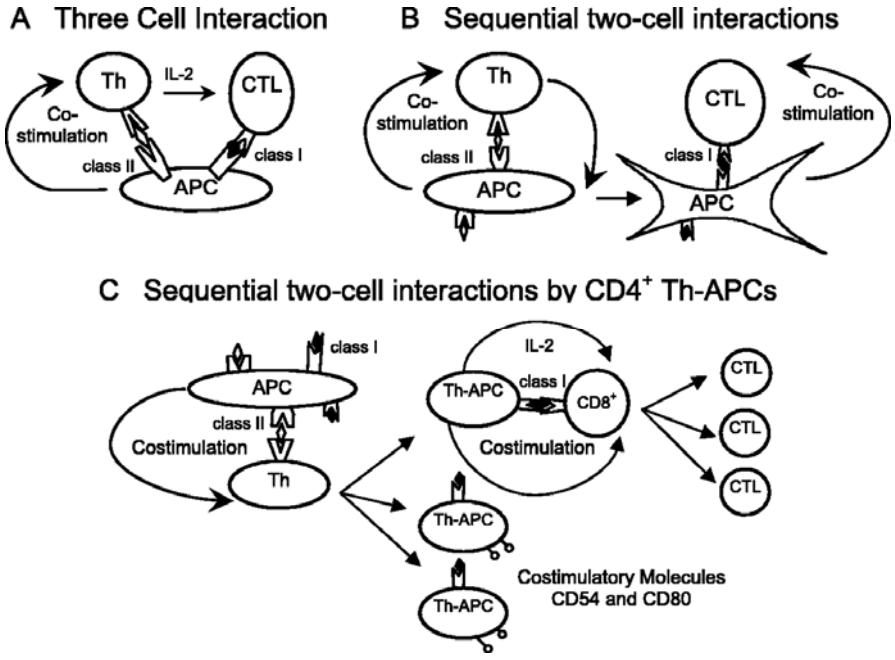

Figure. 1. Models to explain development of CTL responses via $CD4^+$ T cell help (146): a) Model of three-cell interaction; b) Model of sequential two-cell interactions; and c) New dynamic model of two-cell interactions by $CD4^+$ Th-APC.

1.10 Rationale, hypothesis and objectives

Among the various factors that regulate $CD8^+$ T cell responses, $CD4^+$ T cells play a predominant role in modulating one or several phases of CTL responses. Studying the requirements of $CD4^+$ T cell help is intriguing since this requirement varies with type of disease or immunization. Generally, in infectious conditions where inflammatory signals play a significant role, it was found that $CD4^+$ T cells have little role in most phases of CTLs with the exception of memory phase (62, 180). On the contrary, in immunity associated with minor histocompatibility Ags, such as non-inflammatory signals derived from cell-associated foreign Ags, transplantation Ags and tumor Ags, it was shown that $CD4^+$ T cells play a crucial role in multiple phases of CTL responses (49, 51, 88, 98, 99, 180). These results suggest that the requirements of $CD4^+$ T helper cells for effective CTL responses vary with the type of infections or immunizations. Furthermore, researchers have also shown that, in addition to inflammatory signals, the PF of $CD8^+$ T cells also impact the requirements of $CD4^+$ T-helper signals for CTL responses (181, 182). Although at higher PF, $CD4^+$ T cell help is not required (181, 182), how higher PF impacts $CD4^+$ T-helper requirements for memory CTL responses is not completely known. Hence, keeping in view the above knowledge gap, the main focus of the current work is to understand the requirements of $CD4^+$ T cell help and its molecular mechanisms for effective $CD8^+$ T cell responses and anti-tumor immunity. As inflammatory signals are known to alter $CD4^+$ T cell requirements, two immunization methods were selected to investigate the requirements of $CD4^+$ T cell help for CTL responses: 1) the non-inflammatory DC immunization; and 2) the inflammatory AdV immunization. To investigate the function of CTLs in these immunization methods under $CD4^+$ T-helper influence, the mouse melanoma tumor challenge model was used. A detailed rationale and hypothesis specific for each part of the project has been further discussed in the introductory part of each chapter.

In the first part of the project, the study was designed to further characterize the "new dynamic model of sequential two-cell interactions by $CD4^+$ Th-APC," giving particular attention to Th-APC's stimulatory effect on primary and memory CTL responses. Here, the main objectives were to investigate:

1. whether $CD4^+$ Th-APC can acquire co-stimulatory molecules and the pMHC complexes by *in vivo* DC_{OVA} stimulation;

2. whether the acquired pMHC II on these $CD4^+$ Th-APC are functional;
3. which type of subsets these $CD4^+$ Th-APC-stimulated $CD4^+$ and $CD8^+$ T cells belonged to; and
4. the molecular mechanisms responsible for Th-APC's stimulatory effect on *in vivo* stimulation of $CD8^+$ CTL responses and anti-tumor immunity.

In the second part of the project, the study was designed to understand the interaction between Th-APCs and differentiated effector CTLs. Here, the main objectives were to investigate:

1. Th1 cells' signaling during primary and transitional period for memory CTL development;
2. the nature of $CD4^+$ T-helper requirement for memory CTL development;
3. the molecular mechanisms of Th1's help during transitional period for memory CTL development; and
4. genes or molecules that regulate effector CTL's death or survival under the influence of Th1's signals.

In the third part of the project, the study was designed to understand the impact of altered PF on the requirement of $CD4^+$ T-helper signals for primary and memory CTLs responses. Here, the main objectives were to understand:

1. $CD4^+$ T-helper signals for primary and memory CTL responses at endogenous PF;
2. $CD4^+$T-helper signals for primary CTL responses at higher PF;
3. the impact of altered PF on the therapeutic efficacy of DCova immunization; and
4. $CD4^+$T-helper signals for memory CTL responses at higher PF.

In the final part of the project, the study was designed to understand the roles of $CD4^+$ T cells on different phases of AdV transgene product-specific CTL responses. Here, the main objectives were to investigate:

1. the nature and mechanisms of $CD4^+$ T help for primary CTL responses;
2. the nature of $CD4^+$ T help for AdV-specific effector and memory CTLs maintenance;
3. the role of $CD4^+$ T help and its molecular mechanism for functional memory CTL responses; and
4. the role of $CD4^+$ T help for functional recall responses.

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CHAPTER 2

CD4$^+$ TH-APC WITH ACQUIRED PEPTIDE/MHC CLASS I AND II COMPLEXES STIMULATE TYPE 1 HELPER CD4$^+$ AND CENTRAL MEMORY CD8$^+$ T CELL RESPONSES

Channakeshava Sokke Umeshappa*, Hui Huang*, Yufeng Xie*, Yangdou Wei†, Sean J. Mulligan‡, Yulin Deng§, and Jim Xiang*,§

*Research Unit, Research Division, Saskatchewan Cancer Agency and Department of Oncology, Department of Immunology, †Department of Biology, and ‡Department of Physiology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada; and §School of Life Sciences and Technology, Beijing Institute of Technology, Beijing, China

Correspondence: Dr. Jim Xiang, Saskatoon Cancer Center, 20 Campus Drive, Saskatoon, Saskatchewan S7N 4H4, Canada, Tel: 306 6552917, Fax: 306 6552635, Email: jim.xiang@saskcancer.ca

Key Words: T helper cell, pMHC I complex, central memory CTL, IL-2, CD40L, CD80

Abbreviations used in this paper: DCOVA, OVA-pulsed dendritic cell; CMCTL, central memory CTL; EMCTL, effector memory CTL; ECD, energy-coupled dye; pMHC, peptide/MHC; CMA, concanamycin A; KO, knockout; Tm, memory T; Tr, regulatory T.

Note:

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2.1 Brief introduction to Chapter 2

The main goal of the present study is to understand and expand our knowledge on how and when $CD4^+$ T cells provide help to $CD8^+$ CTLs for optimal protective responses under different immunization conditions. This chapter is mainly focused on understanding how $CD4^+$ T cells and their associated signals during priming phase modulate $CD8^+$ CTL effector, survival and memory responses. A non-inflammatory DCova immunization model was used in this study. Furthermore, this study characterized the "new dynamic model of $CD8^+$ CTL responses via DC-stimulated $CD4^+$ T helper – Ag-presenting cells (Th-APCs)," proposed recently by our laboratory.

2.2 Abstract

T cell-T cell Ag presentation is increasingly attracting attention. We previously showed that the *in vitro* OVA-pulsed dendritic cell (DC_{OVA})-activated $CD4^+$ Th cells acquired OVA peptide/MHC (pMHC) class I and co-stimulatory molecules such as CD54 and CD80 from DC_{OVA} and acted as $CD4^+$ Th-APC capable of stimulating OVA-specific $CD8^+$ CTL responses. In this study, we further applied the OVA-specific TCR-transgenic OT I and OT II mice with deficiency of various cytokines or co-stimulatory molecule genes useful for studying the molecular mechanisms underlying in Th-APC's stimulatory effect. We demonstrated that DC_{OVA}-stimulated OT II $CD4^+$ Th-APC also acquired co-stimulatory molecules such as CD40, OX40L, and 4-1BBL and the functional pMHC II complexes by DC_{OVA} activation. $CD4^+$ Th-APC with acquired pMHC II and I were capable of stimulating $CD4^+$ Th1 and central memory $CD8^+44^+CD62L^{high}IL\text{-}7R^+$ T cell responses leading to antitumor immunity against OVA-expressing mouse B16 melanoma. Their stimulatory effect on $CD8^+$ CTL responses and antitumor immunity is mediated by IL-2 secretion, CD40L, and CD80 signaling and is specifically targeted to $CD8^+$ T cells *in vivo* via acquired pMHC I. In addition, $CD4^+$ Th-APC expressing OVA-specific TCR, FasL, and perforin were able to kill DC_{OVA} and neighboring Th-APC expressing endogenous and acquired pMHC II. Taken together, we show that $CD4^+$ Th-APC can modulate immune responses by stimulating $CD4^+$ Th1 and central memory $CD8^+$ T cell responses and eliminating DC_{OVA} and neighboring Th-APC. Therefore, our findings may have great impacts in not only the antitumor immunity, but also the regulatory T cell-dependent immune tolerance *in vivo*.

2.3 Introduction

The intercellular transfer of cell surface proteins and the biological function of transferred proteins on recipient cells have increasingly attracted great attention for study (1). T cell-to-T cell (T-T) antigen presentation, dependent upon $CD4^+$ T cells acquiring major histocampatibility complex (MHC) II and CD80 molecules from antigen-presenting cells (APC), has also been frequently reported. However, the role such $CD4^+$ T cells may play have been as yet ill defined, and the results of the relevant studies were disparate, in part because multiple experimental systems have been used. It has been reported that $CD4^+$ T cells induced T cell apoptosis and anergization of $CD4^+$ T cell lines (2, 3) and the transfer of $CD4^+$ T cell line resulted in immunosuppression in the context of autoimmune responses (4). In these studies, helper T (Th) cells were derived from rather uncharacterized Con A-stimulated allogenic T cells or T cell lines. Brandes et al demonstrated that human γδ T cells expressing MHC II and co-stimulatory molecules can stimulate naive αβ T cell proliferation and differentiation (5). Kennedy et al reported that the active Th lymphocytes when pulsed with Ag peptides can cross-prime memory cytotoxic T lymphocytes (CTL) responses (6). Adamopoulou et al showed that human $CD4^+$ T cells displaying viral epitopes elicited a functional virus-specific memory $CD8^+$ T cell response (7).

Recently, we have developed a new concept of "sequential two-cell interactions by $CD4^+$ Th-antigen presenting cells," a new dynamic model of $CD8^+$ T effector cell responses via $CD4^+$ Th cells (8). We demonstrated that the ovalbumin (OVA)-pulsed dendritic cell (DC_{OVA})-activated $CD4^+$ Th cells were able to acquire from DC_{OVA} the immunological synapse-comprised peptide/MHC (pMHC) II complexes and co-stimulatory molecules (CD54 and CD80) as well as the bystander pMHC I since both pMHC I and II as well as co-stimulatory molecules localize in the same immunological synapse formed between a DC_{OVA} and a $CD4^+$ T cell (9). These $CD4^+$ Th cells with acquired pMHC I and co-stimulatory molecules by DC_{OVA} stimulation termed $CD4^+$ Th-APC were able to stimulate naïve OT I $CD8^+$ T cell proliferation and differentiation into CTL, and induce effective OVA-specific anti-tumor immunity (8). However, some important issues related to this new concept of $CD4^+$ Th-APC remain unclear. These include (i) whether $CD4^+$ Th-APC can also acquire the other co-stimulatory molecules and the pMHC complexes by *in vivo* DC_{OVA} stimulation, (ii) whether the acquired pMHC II on these $CD4^+$ Th-

APC are functional, (iii) which type of CTLs these $CD4^+$ Th-APC-stimulated $CD4^+$ and $CD8^+$ T cells are belonged to, and (iv) what is the molecular mechanism responsible for Th-APC's stimulatory effect on *in vivo* stimulation of $CD8^+$ CTL responses and antitumor immunity.

In this study, we applied the OVA-specific T cell receptor (TCR) transgenic OT I and OT II mice with or without deficiency of various cytokines or co-stimulatory molecules. This model system provides a monoclonal source of OVA-specific $CD4^+$ and $CD8^+$ T cells, which is useful for studying cellular interactions between OVA-specific $CD4^+$ and $CD8^+$ T cells at the molecular level to address the above questions. We found that both *in vitro* and *in vivo* DC_{OVA}-stimulated OT II $CD4^+$ Th cells acquired pMHC I and II complexes. $CD4^+$ Th-APC with acquired pMHC II and I were capable of stimulating $CD4^+$ Th1 cell and $CD8^+44^+CD62L^{high}IL\text{-}7R^+$ central memory CTL responses, and antitumor immunity against OVA-expressing mouse B16 melanoma. Their stimulatory effect on $CD8^+$ CTL responses and antitumor immunity is mediated by IL-2 secretion, CD40L and CD80 costimulation, and specifically targeted to $CD8^+$ T cells *in vivo* via acquired pMHC I complexes.

2.4 Materials and methods

2.4.1 Tumor cells, reagents and animals

The mouse B16 melanoma BL6-10 and thymoma EL-4 tumor cell lines were maintained in DMEM containing 10% FCS, whereas the OVA-transfected BL6-10 ($BL6\text{-}10_{OVA}$) and EL-4 (EG7) tumor cell lines were maintained in DMEM containing 10% FCS and G418 (0.5 mg/ml) (8). The mouse B cell hybridoma cell line LB27 expressing both $H\text{-}2K^b$ and Ia^b was obtained from American Type Culture Collection. The mouse T cell hybridoma cell lines RF3370 and MF72.2D9 expressing TCR specific for K^b-OVAI and Ia^b-OVAII peptide complexes, respectively, were obtained from Dr. K. Rock (University of Massachusetts Medical Center, Worcester, MA) (10). The biotin-labeled and fluorescent dye (FITC, PE, and energy-coupled dye (ECD)-labeled Abs specific for CD3 (145-2C11), CD4 (GK1.5), CD11c (HL3), CD25 (7D4), CD40 (3/23), CD40L (TRAP1), CD44 (C26), CD45.1 (A20), 4-1BBL (TKS-1), OX40L (RM134L), CD62L (MEL-14), CD69 (H1.2F3), CD80 (16-10A), IL-7R (4G3), CD40L (MR1), FasL (NOK-1), IL-4 (11B11) and IFN-γ (XMG1.2) Abs from BD Pharmingen and perforin (CB5.4) from Alexis Biochemicals were obtained. The recombinant GM-CSF, IL-2, IL-4, IL-7, and IL-12 as well as the anti-IL-4 Ab were obtained from R&D Systems. The anti-$H\text{-}2K^b$/OVAI

(pMHC I) Ab was obtained from Dr. J. Germain (National Institutes of Health, Bethesda, MD) (11). The PE-H-2K^b/OVAI tetramer and FITC-anti-CD8 Ab were obtained from Beckman Coulter. The OVAI ($OVA_{257–264}$, SIINFEKL), OVAII ($OVA_{323–339}$, ISQAVHAAHAEINEAGR) (12), and irrelevant Mut1 (FEQNTAQP) (13) peptides for H-2K^b were synthesized by Multiple Peptide Systems. Concanamycin A (CMA) and emetin were obtained from Sigma-Aldrich Canada. C57BL/6 (B6, CD45.2$^+$), C57BL/6/B6.1 (B6.1, CD45.1$^+$), $OVA_{257–264}$, and $OVA_{323–339}$-specific TCR-transgenic OT I and OT II mice and IL-2, IFN-γ, TNF-α, CD28, CD40, CD40L, OX40L, and CD80 gene knockout (KO) mice on a C57BL/6 background were obtained from The Jackson Laboratory. 4-1BBL gene KO mice on a C57BL/6 background were obtained from Amgen. Homozygous OT II/H-2K$^{b-/-}$, OT II/CD40$^{-/-}$, OT II/CD40L$^{-/-}$, OT II/4-1BBL$^{-/-}$, OT II/OX40L$^{-/-}$, OT II/CD80$^{-/-}$, OT II/IL-2$^{-/-}$, OT II/TNF-α$^{-/-}$, and OT II/IFN-γ$^{-/-}$ mice were generated by backcrossing the designated gene KO mice onto the OT II background for two generations. Homozygous OT II/B6.1 and OT I/B6.1 mice were generated by backcrossing B6.1 mice onto the OT II and OT I backgrounds, respectively. The homozygosity was confirmed by PCR according to The Jackson Laboratory's protocols or flow cytometry. All mice were treated according to animal care committee guidelines of the University of Saskatchewan.

2.4.2 Preparation of DC

Bone marrow-derived DC generated using GM-CSF (20 ng/ml) and IL-4 (20 ng/ml) and pulsed overnight at 37°C with 0.3 mg/ml OVA (Sigma-Aldrich) (8) were referred to as DC_{OVA}. These DC displayed cell surface H-2K^b, Ia^b, CD40, CD54, CD80, and pMHC I (14), indicating that they are mature DC. OVA-pulsed DC generated from H-2K^b, CD40, 4-BBL, OX40L, and CD80 gene KO mice were referred to as (K$^{b-/-}$)DC_{OVA}, (CD40$^{-/-}$)DC_{OVA}, (4-1BBL$^{-/-}$)DC_{OVA}, (OX40L$^{-/-}$)DC_{OVA}, and (CD80$^{-/-}$)DC_{OVA}, respectively.

2.4.3 Preparation of naive CD4$^+$ and CD8$^+$ T cells and active CD4$^+$ T cells

The naive CD4$^+$ and CD8$^+$ T cells were prepared from splenocytes of OT II mice, OT II mice with various gene KO, and OT I mice were enriched by passage through nylon columns (C&A Scientific) and purified by negative selection using anti-mouse CD8 (Ly2) and CD4 (L3T4) paramagnetic beads (Dynal). To generate the *in vitro* DC_{OVA}-activated CD4$^+$ T cells,

naive $CD4^+$ T cells (4×10^6 cells/ml, 100 μl/well) from OT II mice were stimulated for 3 days with irradiated (4000 rad) DC_{OVA} (1×10^6 cells/ml, 100 μl/well) in the presence of IL-2 (10 U/ml), IL-12 (5 ng/ml), and anti-IL-4 Ab (10 μg/ml) (8). These *in vitro* DC_{OVA}-activated $CD4^+$ T cells were then isolated by Ficoll-Paque (Sigma-Aldrich Canada) density gradient centrifugation and further purified by positive selection using CD4 microbeads (Miltenyi Biotec). The $CD4^+$ T cells derived from OT II mice with respective IL-2, IFN-γ, TNF-α, CD40L, CD40, 4-1BBL, OX40L, and CD80 gene KO were referred to as $CD4^+$ $(IL\text{-}2^{-/-})$T, $(IFN\text{-}\gamma^{-/-})$T, $(CD40L^{-/-})$T, $(CD40^{-/-})$T, $(4\text{-}1BBL^{-/-})$T, $(OX40L^{-/-})$T, and $(CD80^{-/-})$T cells, respectively. The *in vitro* DC_{OVA}-activated $CD4^+$ T, $(IL\text{-}2^{-/-})$T, $(IFN\text{-}\gamma^{-/-})$T, $(TNF\text{-}\alpha^{-/-})$T, and $(CD40L^{-/-})$T cells were referred to as $CD4^+$ Th-, $(IL\text{-}2^{-/-})$Th-, $(IFN\text{-}\gamma^{-/-})$Th-, $(TNF\text{-}\alpha^{-/-})$Th-, and $(CD40L^{-/-})$Th-APC, respectively. The *in vitro* $(CD40^{-/-})DC_{OVA}$-, $(4\text{-}1BBL^{-/-})DC_{OVA}$-, and $(OX40L^{-/-})DC_{OVA}$-activated $CD4^+$ T cells were referred to as $CD4^+$ $(CD40^{-/-})$Th-, $(4\text{-}1BBL^{-/-})$Th-, and $(OX40L^{-/-})$Th-APC, respectively. The *in vitro* $(K^{b-/-})DC_{OVA}$-activated OT II/$K^{b-/-}$ $CD4^+$ T cells without acquired pMHC I complexes were referred to as $(K^{b-/-})$Th-APC, whereas the *in vitro* $(CD80^{-/-})DC_{OVA}$-activated OT II/$CD80^{-/-}$ $CD4^+$ T cells were referred to as $(CD80^{-/-})$Th-APC. The phenotypes and cytokine profiles of the above $CD4^+$ Th-APC with various gene KO were similar to $CD4^+$ Th cells except for the specifically designed gene deficiency (data not shown). To prepare *in vivo* DC_{OVA}-activated $CD4^+$ Th-APC, the OT II/$K^{b-/-}$ mice were s.c. immunized with irradiated (4000 rad) DC_{OVA} or $(K^{b-/-})DC_{OVA}$ (1×10^6 cells/mouse). Three days later, $CD4^+$ T cells were purified from immunized mouse draining lymph nodes by positive selection using CD4 microbeads (Miltenyi Biotec) and referred to as $CD4^+$ Th-APCvivo and $(K^{b-/-})$Th-APCvivo, respectively. Con A-stimulated OT II $CD4^+$ T (Con A OT II) cells were generated by incubating OT II mouse splenocytes with Con A (1 μg/ml) and IL-2 (10 U/ml) for 3 days. The Con A-stimulated $CD4^+$ T cells were then purified on density gradients and then using CD4 microbeads (Miltenyi Biotec). The cytokine secretion in the supernatants of the above $CD4^+$ Th-APC was assessed using cytokine ELISA kits (R & D Systems). The phenotypes and cytokine profiles of Con A-stimulated $CD4^+$ T cells were similar to *in vitro* DC_{OVA}-stimulated $CD4^+$ Th cells (data not shown).

2.4.4 Phenotypic analysis of $CD4^+$ Th-APC

The BM-derived OVA-pulsed DC_{OVA} and the purified DC_{OVA}-activated $CD4^+$ Th-APC or Th-APC with various gene KO were stained with a panel of Abs or with PE-anti-CD4, FITC-anti-CD11c and ECD-CD3 Abs and then analyzed by flow cytometry (14). For intracellular cytokines, cells were restimulated with OVAII-pulsed irradiated (4000rad) LB27, permeabilised using Cytofix/CytoPerm Plus reagent (BD Pharmingen) and stained for detecting the expression of IL-4, IFN-γ or perforin (14, 35). In another set of experiments, $CD4^+$ Th-APC and Th-APC($K^{b-/-}$) cells derived from DC_{OVA} and ($K^{b-/-}$)DC_{OVA} activation were stained with biotin-anti-pMHC I Ab and FITC-avidin and then analyzed by confocal fluorescence microscopy.

2.4.5 Ag presentation

RF3370 and MF72.2D9 hybridoma cells (0.5×10^5 cells/well) were cultured with irradiated (4000 rad) DC_{OVA}, $CD4^+$ Th-APC, Th-APCvivo, and Con A OT II cells (1×10^5 cells/well) for 24 h. The supernatants were then harvested for measurement of IL-2 secretion using an ELISA kit (R & D Systems).

2.4.6 T cell proliferation assays

In the *in vitro* T cell proliferation assays, irradiated (4000 rad) stimulators, DC_{OVA} (0.1×10^5 cells/well), $CD4^+$ Th-APC, Th-APCvivo, and Con A OT II cells (each 0.4×10^5 cells/well), and their 2-fold dilutions, were cultured with a constant number of responders, the naive OT II $CD4^+$ or OT I $CD8^+$ T cells (0.5×10^5 cells/well). After 48 h, [^{3}H] thymidine incorporation was determined by liquid scintillation counting. In another set of experiments, the purified naive OT I $CD8^+$ or OT II $CD4^+$ T cells (0.5×10^5 cells/well) were first labeled with CFSE (final concentration, 0.3 μM) and then cocultured with irradiated (4000 rad) stimulators, DC_{OVA} ($0. 1 \times 10^5$ cells/well) and $CD4^+$ Th-APC or Con A OT II cells (each 0.4×10^5 cells/well). Three days after culture, the cultured cells were purified by Ficoll-Paque density gradient centrifugation before determining the number of CFSE-labeled T cell divisions by flow cytometry.

In the *in vivo* T cell proliferation assays, C57BL/6 mice (six mice per group) were i.v. injected with CFSE-labeled naive OT II $CD4^+$ and OT I $CD8^+$ T cells (4×10^6 cells each mouse). One day after the injection, the mice were i.v. immunized with the irradiated (4000 rad) DC_{OVA}

(0.5×10^6 cells/mouse) and Th-APC or Con A OT II T cells (3×10^6 cells/mouse). Three days after immunization, the mouse splenocytes were analyzed for determination of the number of CFSE-labeled T cell divisions by flow cytometry. To assess whether $CD4^+$ Th-APC with acquired pMHC I and II can stimulate $CD4^+$ and $CD8^+$ T cell responses, we performed another set of *in vivo* T cell proliferation assays. In the *in vivo* $CD4^+$ T cell proliferation assay, C57BL/6/6.1 ($CD45.1^+$) mice (six mice per group) were i.v. immunized with irradiated (4000 rad) DC_{OVA} (0.5×10^6 cells/mouse) and $CD4^+$ Th-APC or Th-APCvivo (3×10^6 cells/mouse). The tail blood samples derived from mice 4 days after immunization were incubated with PE-CD45.1, FITC-anti-CD4, and ECD-anti-CD44 Abs (Beckman Coulter) and analyzed by flow cytometry. In the *in vivo* the $CD8^+$ T cell proliferation assay, C57BL/6 mice (six mice per group) were i.v. immunized with irradiated (4000 rad) DC_{OVA} (0.5×10^6 cells/mouse) and $CD4^+$ Th-APC and Th-APCvivo or $CD4^+$ Th-APC with different gene KO (3×10^6 cells/mouse). The tail blood samples derived from these immunized mice 6 days after immunization were stained with PE-H-$2K^b$/OVAI tetramer and FITC-anti-CD8 Ab (Beckman Coulter) and analyzed by flow cytometry (14).

2.4.7 Cytotoxicity assays

In the first *in vitro* cytotoxicity assay, $CD4^+$ Th-APC were used as effector cells, while ^{51}Cr-labeled DC_{OVA}, OVAII-pulsed LB27, and Th-APC were used as target cells in a chromium release assay (14). For testing the killing mechanisms, the effector cells were preincubated with CMA (1 μM) and emetin (5 μM) for 2 h before incubation with the target cells to prevent perforin- and Fas-FasL interaction-mediated cytotoxicity (15). In the second *in vitro* cytotoxicity assay, naive $CD8^+$ T cells (4×10^6 cells/ml, 100 μl/well) derived from OT I/B6.1 ($CD45.1^+$) mice were cocultured in 96-well plates with irradiated (4000 rad) DC_{OVA} (1×10^6 cells/ml, 100 μl/well) and $CD4^+$ Th-APC (2×10^6 cells/ml, 100 μl/well) in the presence of IL-2 (10 U/ml) for 3 days (8). After incubation, the active OT I/B6.1 $CD8^+$ T cells were harvested and then purified on density gradients followed by positive selection using the biotin-conjugated anti-CD45.1 Ab and anti-biotin microbeads (Miltenyi Biotec). These purified $CD8^+$ T cells were referred to as DC_{OVA}/OT $I_{6.1}$ and $CD4^+$ Th-APC/OT $I_{6.1}$, respectively, and used as effector cells. The ^{51}Cr-labeled EG7 and the control EL-4 tumor cells were used as target cells. Specific killing was calculated as: 100 × [(experimental cpm − spontaneous cpm)/(maximal cpm − spontaneous cpm)

as previously described (8). In the *in vivo* cytotoxicity assay, naive C57BL/6 mouse splenocytes were stained with either high (3.0 μM, $CFSE^{high}$) or low (0.6 μM, $CFSE^{low}$) concentrations of CFSE at 37°C for 20 min and then pulsed with OVAI and Mut1 peptide (10 μM) for 2 h, respectively. After washes with PBS, these target cells (2×10^6 cells each mouse) were i.v. coinjected at 1:1 ratio into the above different groups of mice 6 days after immunization. Sixteen hours later, the residual $CFSE^{high}$ and $CFSE^{low}$ target cells remaining in the recipients' spleens were sorted and analyzed by flow cytometry (8).

2.4.8 In vivo $CD8^+$ T cell survival

Naive C57BL/6 mice (six mice per group) were i.v. transferred with the above *in vitro* DC_{OVA}- and $CD4^+$ Th-APC-primed $CD8^+45.1^+$ T (DC_{OVA}/OT $I_{6.1}$ and $CD4^+$ Th-APC/OT $I_{6.1}$) cells (5×10^6 cells/mouse). The tetramer staining assay was performed to examine the presence of OVA-specific $CD8^+$ T cells in mouse peripheral blood 6 days and once a week for 3 months after the adoptive T cell transfer. The tail blood samples were stained with PE-H-$2K^b$/OVAI tetramer, FITC-anti-CD8, and ECD-anti-CD25, ECD-anti-CD44, ECD-anti-CD45.1, ECD-anti-CD62L, and ECD-anti-IL-7R Abs, respectively, and analyzed by flow cytometry.

2.4.9 Animal studies

Wild-type C57BL/6 mice (eight mice per group) were immunized with irradiated (4000 rad) DC_{OVA} (0.5×10^6 cells/mouse), the *in vitro* DC_{OVA}-activated $CD4^+$ Th-APC, ($K^{b-/-}$)Th-APC, (IL-$2^{-/-}$)Th-APC, (IFN-$\gamma^{-/-}$)Th-APC, (TNF-$\alpha^{-/-}$)Th-APC, ($CD40^{-/-}$)Th-APC, ($CD40L^{-/-}$)Th-APC, (4-1$BBL^{-/-}$)Th-APC, ($OX40L^{-/-}$)Th-APC, and ($CD80^{-/-}$)Th-APC (3×10^6 cells/mouse), or the *in vivo* DC_{OVA}-activated $CD4^+$ Th-APCvivo (5×10^6 cells/mouse), respectively. Six days subsequent to the immunization, each mouse was challenged i.v. with BL6-10_{OVA} or BL6-10 tumor cells (0.5×10^6 cells each mouse). The mice were sacrificed 4 wk after tumor cell challenge and the numbers of lung metastatic tumor colonies were counted in a blind fashion (8). Metastases on freshly isolated lungs appeared as discrete black-pigmented foci that were easily distinguishable from normal lung tissues and confirmed by histological examination. Metastatic foci too many to count were assigned an arbitrary value of >100.

2.5 Results

2.5.1 $CD4^+$ Th cells acquire costimulatory molecules and pMHC complexes by DC_{OVA} activation

We showed that OVA-pulsed bone marrow-derived DC (DC_{OVA}) expressed CD11c, Ia^b, CD40, CD80, 4-1BBL, OX40L, and pMHC I (Fig. 1*a*), indicating that they are OVA-specific mature DC. The *in vitro* DC_{OVA}-activated OT II $CD4^+$ T cells expressed CD4, CD40L, and CD69 (Fig. 1*b*), indicating that they are active OVA-specific $CD4^+$ T cells. In addition, they also expressed FasL and perforin. These active $CD4^+$ T cells secreted IL-2 (~2.5 ng/ml/10^6 cells per 24 h) and IFN-γ (~2 ng/ml/10^6 cells per 24 h), but not IL-4, indicating that they are Th1 cells. We previously demonstrated that these $CD4^+$ Th-APC acquired DC MHC class I and co-stimulatory molecules (CD54 and CD80) (8). In this study, we showed that the naive OT II $CD4^+$ T cells did not express any CD40, CD80, 4-1BBL, OX40L, and pMHC I. However, these *in vitro* DC_{OVA}-activated $CD4^+$ Th-APC did show some expression of CD40, CD80, OX40L, 4-1BBL, and pMHC I by using the anti-co-stimulatory molecule Abs and the specific anti-pMHC I Ab (Fig. 1*b*). Therefore, we termed these $CD4^+$ Th cells with acquired pMHC I and co-stimulatory molecules the $CD4^+$ Th-APC. Since active $CD4^+$ T cells do not express CD40, OX40L, and 4-1BBL, but do display some expression of endogenous CD80 by flow cytometric analysis (data not shown), the above results indicate that the expression of CD40, OX40L, and 4-1BBL on $CD4^+$ Th-APC should be derived from DC_{OVA} by DC_{OVA} activation, whereas CD80 molecules on $CD4^+$ Th-APC may be derived from either endogenous CD80 expression of the active $CD4^+$ Th cells or acquisition of CD80 from DC_{OVA} or both. This is confirmed by the evidence that 1) $CD4^+$ ($K^{b-/-}$)Th-APC derived from pMHC I-negative ($K^{b-/-}$)DC_{OVA} stimulation did not express any pMHC I complex (Fig. 1*c* and *d*) and 2) $CD4^+$ Th-APC with various co-stimulatory molecule gene KO did not express the respective co-stimulatory molecules such as CD40, 4-1BBL, and OX40L when these $CD4^+$ Th-APC were derived from the stimulation of ($CD40^{-/-}$)DC_{OVA}, (4-$1BBL^{-/-}$)DC_{OVA} and ($OX40L^{-/-}$)DC_{OVA}, respectively (Fig. 1*c*). $CD4^+$ Th-APC derived from OT II $CD4^+$ T cells activated by ($CD80^{-/-}$)DC_{OVA} still expressed some CD80 molecules (Fig. 1*c*), but in lower amounts than $CD4^+$ Th-APC derived from DC_{OVA} activation (Fig. 1*b*), indicating that some CD80 molecules expressed on $CD4^+$ Th-APC may be acquired from DC_{OVA}. To confirm it, we further analyzed the CD80 expression of the *in vitro* ($CD80^{-/-}$)DC_{OVA}-activated OT II/$CD80^{-/-}$ $CD4^+$ T cells with deficiency of endogenous CD80

expression by flow cytometry. As shown in Fig. 1*e*, the $CD4^+$ ($CD80^{-/-}$)Th-APC derived from naive OT II/$CD80^{-/-}$ $CD4^+$ T cells activated *in vitro* by ($CD80^{-/-}$)DC_{OVA} did not express any CD80 molecules, indicating that the CD80 expression on active $CD4^+$ Th-APC is derived partly from its endogenous CD80 expression and partly from acquired CD80 molecules from DC_{OVA}. To rule out the possibility of irradiated DC_{OVA} contamination in the $CD4^+$ Th cell population, we also analyzed them using the anti-CD4 and anti-CD11c Abs specific for $CD4^+$ T cell and DC markers, respectively, by flow cytometry. As shown in Fig. 1*f*, the original DC_{OVA} were $CD4^-CD11c^{high}$. The vast majority (99.98%) of $CD4^+$ Th-APC purified by positive selection using CD4 microbeads were $CD4^{high}CD11c^-$ T cells, indicating that there is no detectable DC_{OVA} in purified $CD4^+$ Th-APC population. This is because that any DC_{OVA} which survived after irradiation-induced apoptosis would still be eliminated by the killing activity of $CD4^+$ T cells activated by DC_{OVA} (16, 17). We noted that there was a very small amount (0.02%) of cells showing $CD4^{high}CD11c^{low}$ within the purified $CD4^+$ Th-APC population. These cells should still be considered to be $CD4^+$ Th-APC rather than $CD4^-CD11c^{high}$ DC_{OVA}, because they expressed CD4 and the T cell marker CD3. The small amount of CD11c on these $CD4^+$ T cells may be derived from its acquisition of DC's CD11c via a dissociation-associated pathway as we previously described (18). To assess pMHC I acquisition by *in vivo* DC_{OVA} stimulation, $CD4^+$ Th-APC were purified from OT II/$K^{b-/-}$ mice immunized with DC_{OVA} with pMHC I expression or ($K^{b-/-}$)DC_{OVA} without pMHC I expression and analyzed by flow cytometry. As shown in Fig. 1*g*, DC_{OVA}-stimulated $CD4^+$ Th-APCvivo, but not ($K^{b-/-}$)DC_{OVA}-stimulated $CD4^+$ ($K^{b-/-}$)Th-APCvivo cells, exhibited pMHC I expression, indicating that $CD4^+$ Th-APC can also acquire pMHC I by *in vivo* DC_{OVA} stimulation.

2.5.2 The acquired pMHC II complexes on $CD4^+$ Th-APC are functional

To examine the functionality of acquired pMHC II complexes, we initially assessed their ability to stimulate IL-2 secretion of T cell hybridoma MF72.2D9 expressing TCR specific for pMHC II. As shown in Fig. 2*a*, MF72.2D9 cells alone did not secrete any IL-2. However, the *in vitro* DC_{OVA}-activated $CD4^+$ Th-APC with acquired pMHC II, but not Con A-stimulated $CD4^+$ OT II T cells without pMHC II expression stimulated MF72.2D9 to secrete IL-2 (320 pg/ml) as did DCOVA (770 pg/ml), indicating that $CD4^+$ Th-APC express the functional pMHC II complexes. Furthermore, the *in vivo* DC_{OVA}-stimulated $CD4^+$ Th-APCvivo cells also stimulated

MF72.2D9 to secrete IL-2 (180 pg/ml), indicating that the acquired pMHC II complexes on $CD4^+$ Th-APCvivo are also functional.

2.5.3 $CD4^+$ Th-APC expressing TCR negatively modulate immune responses

To assess whether these $CD4^+$ Th-APC expressing FasL and perforin may kill pMHC II expressing DC_{OVA} and Th-APC, we performed an *in vitro* cytotoxicity assay. As shown in Fig. 2*b*, Th-APC exhibited cytotoxicity to OVAII-pulsed LB27 tumor cells expressing pMHC II (40% specific killing at E:T cell ratio of 50) and DC_{OVA} expressing endogenous pMHC II (25% specific killing at E:T cell ratio of 50), indicating that $CD4^+$ Th-APC after they were activated by DC_{OVA} can kill DC_{OVA}, which further supports our above data showing that there is no contaminating irradiated DC_{OVA} in the purified $CD4^+$ Th-APC population (Fig. 1*f*). Because $CD4^+$ Th-APC acquired the functional pMHC II, they may also become sensitive to neighboring Th-APC expressing TCR. Our data did display that Th-APC killed ^{51}Cr-labeled Th-APC (fratricide) (21% specific killing at E:T cell ratio of 50). In addition, they did not kill the original DC and LB27 cells, indicating that the killing activity is OVA specific. CTL are able to lyse target cells by two mechanistically distinct, but functionally similar mechanisms (19): a Ca^{2+}-dependent perforin and a Ca^{2+}-independent Fas/FasL mechanism. To assess which type of pathway is responsible for the cytotoxicity mediated by Th-APC expressing FasL and perforin, CMA and emetin were used to inhibit perforin- and Fas-FasL interaction-mediated cytotoxicity, respectively, in a chromium release assay. Our data showed that treatment of Th-APC with CMA (1 μM) and emetin (5 μM) resulted in ~85 and ~8% inhibition of its cytotoxicity to either Th-APC or DC_{OVA} target cells (Fig. 2*c*), indicating that the perforin-mediated pathway plays a major role in *in vitro* Th-APC-mediated cytotoxicity, which is consistent with some previous reports (20, 21). Our data thus indicate that $CD4^+$ Th-APC expressing OVA-specific TCR may negatively modulate immune responses by eliminating DC_{OVA} expressing endogenous pMHC II and Th-APC expressing acquired pMHC II, when the initial immune responses are too strong.

2.5.4 $CD4^+$ Th-APC with acquired pMHC II stimulate functional $CD4^+$ Th1 cell responses

Since $CD4^+$ Th-APC acquired the functional pMHC II complexes, we then assessed their ability to induce proliferation of naive OT II $CD4^+$ T cells *in vitro* and *in vivo*. Like the positive control stimulator DC_{OVA}, $CD4^+$ Th-APC with acquired pMHC II, but not Con A-stimulated

$CD4^+$ T cells without acquired pMHC II stimulated *in vitro* OT II $CD4^+$ T cell proliferation in a dose-dependent manner in a [^{3}H]thymidine incorporation assay (Fig. 2*d*). To confirm it, we first labeled the naive OT II $CD4^+$ T cells with CFSE. We then *in vitro* incubated these CFSE-labeled $CD4^+$ T cells with the stimulators or i.v. injected them into the wild-type C57BL/6 mice followed by i.v. immunizing the mice with these stimulators 1 day after the injection of CFSE-labeled T cells. This dye is equally partitioned upon cell division, allowing the generation of proliferation profiles. Three days after the incubation or immunization, the CFSE-labeled $CD4^+$ T cells in the culture wells or in the immunized mouse spleens were harvested and analyzed by flow cytometry. The flow cytometric analysis in Fig. 2*e* revealed substantial activation of naive $CD4^+$ T cells indicated by CFSE dilutions, indicating that DC_{OVA} and $CD4^+$ Th-APC stimulate both *in vitro* and *in vivo* CFSE-labeled $CD4^+$ T cell divisions. However, $CD4^+$ Th-APC-induced stimulations in the above experiments are less efficient than DC_{OVA}, possibly due to 1) fewer acquired pMHC II and co-stimulatory molecules on $CD4^+$ Th-APC than on DC_{OVA} and 2) lacking the third signal (DC-secreted IL-12) derived from $CD4^+$ Th-APC (22). To further assess whether Th-APC can stimulate *in vivo* OVA-specific $CD4^+$ T cell responses, we immunized C57BL/6.1 ($CD45.1^+$) mice with Th-APC and Th-APCvivo and DC_{OVA} derived from OT II and C57BL/6 mice ($CD45.2^+$) and then examined the OVA-specific $CD4^+$ T cell responses in these immunized mice by flow cytometry. The activation of $CD4^+$ T cells was measured by staining for CD44 surface expression as $CD44^{high}$ represents the activated T cell phenotype. As shown in Fig. 2*f*, DC_{OVA} stimulated proliferation of OVA-specific naive $CD4^+$ T cells and differentiation into active $CD4^+44^{high}45.1^+$ T cells that accounted for 37.5% of the total $CD4^+$ T cell population. Interestingly, $CD4^+$ Th-APC and Th-APCvivo with acquired pMHC II, but not Con A-stimulated OT II $CD4^+$ T cells without pMHC II expression also stimulated the responses of $CD4^+44^{high}45.1^+$ T cells that accounted for 35.1 and 26.6% of the total $CD4^+$ T cell population, respectively, indicating that both $CD4^+$ Th-APC and Th-APCvivo can stimulate *in vivo* OVA-specific $CD4^+$ T cell responses. To characterize Th-APC-activated $CD4^+$ T cells, we purified the *in vitro* Th-APC-activated $CD4^+$ T cells and analyzed them by flow cytometry. As shown in Fig. 2*g*, these *in vitro* $CD4^+$ Th-APC-stimulated OT II $CD4^+$ T cells displayed CD4, CD25, and CD69, indicating that they are active $CD4^+$ T cells. These active $CD4^+$ T cells also secreted IFN-γ (1.4 ng/ml/10^6 cells per 24 h) and IL-2 (1.9 ng/ml/10^6 cells per /24 h), but not IL-4, indicating that they are Th1 cells. Furthermore, immunization of mice with these $CD4^+$ Th-APC-stimulated

$CD4^+$ Th1 cells (Th-APC/OT II) alone did not induce any $CD8^+$ CTL responses (Fig. 2*h*), possibly due to lacking expression of pMHC I on these Th-APC-stimulated $CD4^+$ Th1 cells (Fig. 2*g*). However, the cotransfer of these $CD4^+$ Th1 cells into DC_{OVA}-immunized mice significantly enhanced the OVA-specific $CD8^+$ CTL responses from 2.0 to 3.1% ($p < 0.05$; Fig. 2*h*), confirming that these $CD4^+$ Th-APC-stimulated $CD4^+$ Th1 cells are functional.

2.5.5 The acquired pMHC I complexes on $CD4^+$ Th-APC are functional

To examine the functionality of acquired pMHC I complexes, we initially assessed their ability to stimulate IL-2 secretion of T cell hybridoma RF3370 expressing TCR specific for pMHC I. As shown in Fig. 3*a*, RF3370 cells alone did not secrete any IL-2. However, the *in vitro* DC_{OVA}-stimulated $CD4^+$ Th-APC with acquired pMHC I, but not Con A-stimulated $CD4^+$ OT II T cells without acquired pMHC I stimulated RF3370 to secrete IL-2 (100 pg/ml) as did DC_{OVA} (220 pg/ml), indicating that $CD4^+$ Th-APC express the functional pMHC I complexes. Furthermore, the *in vivo* DC_{OVA}-stimulated $CD4^+$ Th-APCvivo cells also stimulated RF3370 to secrete IL-2 (75 pg/ml), indicating that the acquired pMHC I complexes on $CD4^+$ Th-APCvivo are also functional.

2.5.6 $CD4^+$ Th-APC with acquired pMHC I stimulate $CD8^+$ CTL responses and antitumor immunity

The positive control DC_{OVA} strongly induced *in vitro* OT I $CD8^+$ T cell proliferation (Fig. 3*a*). $CD4^+$ Th-APC and Th-APCvivo with acquired pMHC I also stimulated *in vitro* OT I $CD8^+$ T cell proliferation in a dose-dependent fashion in a [^{3}H]thymidine incorporation assay (Fig. 3*b*). In addition, these $CD4^+$ Th-APC also stimulated both *in vitro* and *in vivo* CFSE-labeled $CD8^+$ T cell divisions (Fig. 3*c*). To assess their ability to induce *in vivo* $CD8^+$ T cell responses, we performed an OVA-specific tetramer staining assay (14). As shown in Fig. 3*d*, the positive control DC_{OVA} stimulated *in vivo* proliferation of OVA-specific $CD8^+$ T cells that accounted for 3.21% of the total $CD8^+$ T cell population. $CD4^+$ Th-APC and Th-APCvivo also stimulated *in vivo* proliferation of OVA-specific $CD8^+$ T cells that accounted for 2.14 and 1.05% of the total $CD8^+$ T cell population, respectively. These data clearly indicate that both $CD4^+$ Th-APC and Th-APCvivo can stimulate *in vitro* and *in vivo* $CD8^+$ T cell responses. To assess $CD4^+$ Th-APC-

induced $CD8^+$ T cell differentiation into effector CTL, we performed a cytotoxicity assay by adoptively transferring OVAI-pulsed/ $CFSE^{high}$-labeled and Mut1-pulsed/$CFSE^{low}$-labeled splenocytes into the immunized mice. We found that there was substantial loss of the $CFSE^{high}$-labeled OVA-specific target cells in DC_{OVA}-immunized (95%) and Th-APC-immunized (77%) or Th-APCvivo-immunized (52%) mice (Fig. 3*e*), indicating that both DC_{OVA} and $CD4^+$ Th-APC or Th-APCvivo can stimulate $CD8^+$ T cell differentiation into effector CTL with killing activity for OVA I-pulsed target cells. To assess the antitumor immunity, we performed animal studies by i.v. injecting OVA-expressing BL6-10_{OVA} tumor cells into mice immunized with DC_{OVA}, Th-APC, and Th-APCvivo. We also found that DC_{OVA} immunization protected all mice (eight of eight) from lung tumor metastasis, whereas PBS-injected control mice (eight of eight) all had large numbers (>100) of lung BL6-10_{OVA} tumor colonies (experiment 1 of Table I). All mice (eight of eight) immunized with $CD4^+$ Th-APC and Th-APCvivo also had no lung tumor metastasis. The specificity of protection was confirmed with the observation that $CD4^+$ Th-APC did not protect against BL6-10 tumors that did not express OVA, with all mice having large numbers (>100) of lung tumor colonies (experiment 1 of Table I).

2.5.7 $CD4^+$ Th-APCs' stimulatory effect on $CD8^+$ CTL responses and antitumor immunity is also mediated by its endogenous CD40L and acquired CD80 costimulations

To further elucidate the molecular mechanism of $CD4^+$ Th-APC's stimulatory effect in association with the co-stimulatory molecules, these $CD4^+$ Th-APC with respective co-stimulatory molecule gene deficiency were used in *in vivo* T cell proliferation and cytotoxicity assays and in animal studies as described above. As shown in Fig. 4*a*, the stimulation efficiency of OVA-specific $CD8^+$ T cell responses significantly decreased in the mice immunized with $CD4^+$ Th-APC with CD40L (0.37%) and CD80 (0.23%) gene KO ($p < 0.05$), whereas the OVA-specific $CD8^+$ T cell responses remained the same as seen in mice immunized with $CD4^+$ Th-APC with CD40 (1.87%), 4-1BBL (2.09%), and OX40L (2.05%) gene KO, indicating that the CD40L and CD80 costimulations greatly influence the $CD4^+$ Th-APC's stimulatory effect. To further confirm it, we assessed $CD4^+$ Th-APC's stimulatory effect in CD40 and CD28 gene KO mice by immunization of the mice with $CD4^+$ Th-APC. Similar to DC_{OVA}, $CD4^+$ Th-APC also failed in stimulating $CD8^+$ T cell responses in CD40 and CD28 gene KO mice (Fig. 4*b*), thus confirming that $CD4^+$ Th-APC-activated $CD8^+$ CTL responses are mediated by signaling derived

from CD40L-CD40 and CD80-CD28 interactions. In the cytotoxicity assay, we found that there was also a substantial decrease of the killing activity of $CFSE^{high}$-labeled OVA-specific target cells from 77% in Th-APC-immunized mice to 12 and 18% in ($CD40L^{-/-}$)Th-APC- and ($CD80^{-/-}$)Th-APC-immunized mice, respectively (Fig. 4*c*), whereas the killing activity of $CFSE^{high}$-labeled OVA-specific target cells remained the same in ($CD40^{-/-}$)Th-APC (76%)-, (4-$BBL^{-/-}$)Th-APC (74%)-, and ($OX40L^{-/-}$)Th-APC-immunized mice (73%) as in Th-APC-immunized mice (77%). In animal studies, we also found that all (eight of eight) ($CD80^{-/-}$)Th-APC- and 75% (six of eight) ($CD40L^{-/-}$)Th-APC-immunized mice lost their antitumor immunity (experiment 2 of Table I), whereas all ($CD40^{-/-}$)Th-APC-, ($OX40L^{-/-}$)Th-APC-, and (4-$1BBL^{-/-}$)Th-APC-immunized mice were alive as did the Th-APC-immunized mice. These data indicate that the $CD4^+$ Th-APC's stimulatory effect on $CD8^+$ CTL responses and antitumor immunity is mediated by its CD40L and CD80 costimulations.

2.5.8 $CD4^+$ Th-APC's stimulatory effect on $CD8^+$ CTL responses and antitumor immunity is mediated by its endogenous IL-2 secretion

To elucidate the molecular mechanism of $CD4^+$ Th-APC's stimulatory effect in association with cytokines, $CD4^+$ Th-APC with respective cytokine gene deficiency were used in *in vivo* T cell proliferation and cytotoxicity assays and in animal studies as described above. As shown in Fig. 4*a*, the stimulation efficiency of OVA-specific $CD8^+$ T cell responses also significantly decreased in the mice immunized with $CD4^+$ Th-APC with IL-2 (0.19%) gene KO ($p < 0.05$), whereas the OVA-specific $CD8^+$ T cell responses remained the same as seen in mice immunized with $CD4^+$ Th-APC with IFN-γ (1.95%) and TNF-α (1.92%) gene KO, indicating that IL-2 secretion influences $CD4^+$ Th-APC's stimulatory effect. In the cytotoxicity assay, we found that there was also a substantial decrease of the killing activity of $CFSE^{high}$-labeled OVA-specific target cells from 77% in Th-APC-immunized mice to only 8% in ($IL\text{-}2^{-/-}$)Th-APC-immunized mice (Fig. 4*c*), whereas the killing activity of $CFSE^{high}$-labeled OVA-specific target cells remained the same in ($IFN\text{-}\gamma^{-/-}$)Th-APC (75%)- and ($TNF\text{-}\alpha^{-/-}$)Th-APC-immunized mice (71%) as in Th-APC-immunized mice (77%). In animal studies, we also found that all (eight of eight) ($IL\text{-}2^{-/-}$)Th-APC-immunized mice lost their antitumor immunity (experiment 2 of Table I), whereas all ($IFN\text{-}\gamma^{-/-}$)Th-APC (75%)- and ($TNF\text{-}\alpha^{-/-}$)Th-APC-immunized mice were still alive as did the Th-APC-immunized mice. These data indicate that the $CD4^+$ Th-APC's

stimulatory effect on $CD8^+$ CTL responses and antitumor immunity is also mediated by its IL-2 secretion.

2.5.9 Targeting $CD4^+$ Th-APC's stimulatory effect to $CD8^+$ CTL responses and antitumor immunity via acquired pMHC I

To investigate the role of acquired pMHC I, we repeated the above assays using both DC_{OVA}-activated $CD4^+$ Th-APC with acquired pMHC I and ($K^{b-/-}$)DC_{OVA}-activated $CD4^+$ ($K^{b-/-}$) Th-APC without acquired pMHC I, respectively. We found that both *in vitro* and *in vivo* DC_{OVA}-stimulated $CD4^+$ Th-APC with acquired pMHC I stimulated *in vivo* proliferation of OVA-specific $CD8^+$ T cells that accounted for 2.14 and 1.12% of the total $CD8^+$ T cell population, whereas $CD4^+$ ($K^{b-/-}$)Th-APC without acquired pMHC I complexes completely lost their *in vivo* stimulatory effect (0.02%; Fig. 4*a*). In addition, the $CD4^+$ ($K^{b-/-}$)Th-APC-vaccinated mice also displayed little killing activity (5%) for the OVA-specific $CFSE^{high}$-labeled target cells in the *in vivo* cytotoxicity assay (Fig. 4*c*). Furthermore, all mice (eight of eight) immunized with $CD4^+$ ($K^{b-/-}$)Th-APC had lost their antitumor immunity against BL6-10_{OVA} tumor cells (experiment 2 of Table I). These data clearly indicate that the acquired pMHC I complexes play an important role in targeting the $CD4^+$ Th-APC's stimulatory effect mediated by IL-2 secretion and CD40L and CD80 costimulations to the *in vivo* OVA-specific $CD8^+$ T cell responses and antitumor immunity.

2.5.10 $CD4^+$ Th-APC with acquired pMHC I and costimulatory molecules stimulate $CD8^+$ T cell differentiation into $_{CM}$CTL

To further investigate the subset of $CD8^+$ CTL derived from *in vitro* $CD4^+$ Th-APC priming, we purified the DC_{OVA}- and $CD4^+$ Th-APC-primed $CD8^+$ T cells derived from OT I/B6.1 ($CD45.1^+$) mice using CD45.1 microbeads and then conducted phenotypic characterization of these $CD8^+$ CTL by flow cytometry. Our data showed that DC_{OVA}- and $CD4^+$ Th-APC-primed $CD8^+$ T cells displayed PE-tetramer staining (Fig. 5*a*), indicating that they are OVA-specific $CD8^+$ T cells. They also displayed the expected expression of CD25, CD44 (memory T cell marker) (16), and CD62L. Interestingly, $CD4^+$ Th-APC-primed $CD8^+$ T cells displayed higher CD62L expression than DC_{OVA}-primed ones, indicating that they may be prone

to becoming long-lived memory T (Tm) cells after i.v. transfer. To address this possibility, DC_{OVA}- and $CD4^+$ Th-APC-primed $CD8^+$ T cells derived from OT I/B6.1 ($CD45.1^+$) mice were i.v. transferred into C57BL/6 (B6, $CD45.2^+$) mice. The number of detected OVA-specific $CD8^+$ T cells derived from DC_{OVA} and $CD4^+$ Th-APC priming in the mouse blood accounted for 8.2 and 9.6% of the total $CD8^+$ T cell population at day 6 after the transfer (Fig. 5*b*). The majority of $CD8^+$ T cells were also CD45.1 positive, indicating that they are previously transferred $CD8^+CD45.1^+$ T cells. The numbers gradually dropped to 2.32 and 6.10% in the first month subsequent to, but stably maintained for at least 3 months after the transfer. Interestingly, 6 days after transfer, $CD4^+$ Th-APC-primed, but not DC_{OVA}-primed $CD8^+$ T cells became $CD44^+CD62^{high}IL\text{-}7R^+$ (Fig. 5*c*), a characteristic of central memory $CD8^+$ T cells (23). We then further examined whether $CD4^+$ Th-APC-primed CTL exhibited any other functional traits attributed to typical memory cells. These traits include 1) the enhanced survival and proliferation in response to IL-7 (24), 2) the capacity to generate Ag-specific CTL, and 3) to expand upon Ag stimulation. Our data showed that the *in vitro* $CD4^+$ Th-APC-primed CTL expanded similarly as DC_{OVA}-primed ones in the presence of IL-2. However, they expanded much better than DC_{OVA}-primed ones in the presence of both IL-2 and IL-7 (Fig. 5*d*). In the chromium release assay, $CD4^+$ Th-APC-primed CTL (Th-APC/OT $I_{6.1}$) showed cytotoxicity to OVA-expressing EG7 tumor cells, but at a lower level than DC_{OVA}-primed ones (DC_{OVA}/OT $I_{6.1}$; Fig. 5*e*). $CD4^+$ Th-APC-primed $CD8^+$ CTL expressing TCR also killed Th-APC expressing acquired pMHC I, which is consistent with a recent report of Cox et al. (25). In addition, the above *in vivo*-transferred CTL can be greatly expanded 3 mo after the transfer upon DC_{OVA} stimulation. As shown in Fig. 5*f*, the expanded $CD4^+$ Th-APC-primed $CD8^+$ Tm cells accounted for almost 95% of the total $CD8^+$ T cell population, which is 3-fold more than the expansion of DC_{OVA}-primed $CD8^+$ Tm cells (32%). These expanded $CD8^+$ Tm cells are $CD25^+44^+69^+$ effector CTL. Taken together, DC_{OVA}- and $CD4^+$ Th-APC-primed CTL have high and low cytotoxicity, but low and high survival capacity, representing the typical effector memory CTL ($_{EM}$CTL) and $_{CM}$CTL, respectively. Thus, our data indicate that $CD4^+$ Th-APC with acquired pMHC I and co-stimulatory molecules are capable of stimulating naive $CD8^+$ T cell differentiation into $_{CM}$CTL.

2.6 Discussion

According to the progressive linear differentiation hypothesis (26), T cell differentiation involves a phase of proliferation preceding the acquisition of fitness and effector function. Primed $CD8^+$ T cells reach a variety of differentiation stages that contain effector cells as well as cells that have been arrested at intermediate levels of differentiation. Thus, they retain a flexible gene imprinting. T cells that survive after the retraction phase of an immune response can be resolved into distinct subsets of either $_{CM}$CTL representing cells at the intermediate differentiation stage or fully differentiated $_{EM}$CTL with effector capacity (27). It has been shown that a strong Ag presentation stimulates development of effector CTL, whereas a less efficient Ag presentation can lead to the generation of $_{CM}$CTL responses (28). In this study, we demonstrated that $CD4^+$ Th cells were able to stimulate naive $CD8^+$ T cell differentiation into central memory $CD44^+CD62^{high}IL\text{-}7R^+$ T cells with less cytotoxicity and longer survival capacity, compared with DC_{OVA}-primed $CD44^+CD62^{low}IL\text{-}7R^-$ $_{EM}$CTL with high cytotoxicity and shorter survival capacity *in vivo*. This is consistent with another recent report by Adamopoulou et al. (7) that human $CD4^+$ T cells displaying viral epitopes elicited a functional virus-specific memory $CD8^+$ T cell response. Thus, it seems reasonable to conclude that due to the lower level of activation/costimulation signals provided by $CD4^+$ Th-APC as compared with DC, $CD4^+$ Th-APC-primed $CD8^+$ T cells would preferentially differentiate into $_{CM}$CTL, whereas DC_{OVA}-primed $CD8^+$ T cells would preferentially differentiate into $_{EM}$CTL. This finding is consistent with some recent reports that the "dilution" of pMHC complexes by DC division may regulate T cell fate, with more initial pMHC on DC stimulating the differentiation of T effector cells and less pMHC on daughter DC given rise to central memory T cells (29, 30) and that enhanced costimulation can lead to reduced Tm cell formation (31).

Previous experimental evidence showed that a provision of IL-2 dramatically augmented the efficiency of CTL expansion (32). However, one important question on how the $CD4^+$ Th-APC's IL-2 can be specifically delivered to Ag-specific $CD8^+$ CTL *in vivo* still remains puzzling. In this study, we clearly demonstrated that IL-2 secretion mediates the $CD4^+$ Th-APC's stimulatory effect to $CD8^+$ T cells *in vivo*, which is consistent with some recent reports showing that the help effect of transferred $CD4^+$ Th cells on adoptively transferred $CD8^+$ CTL responses and $CD8^+$ memory T cell expansion is mediated by its IL-2 secretion (33-35).

Costimulations derived from CD40, CD80, 4-BBL, and OX40L play an important role in APC-activated $CD8^+$ CTL responses (36). In this study, we found that $CD4^+$ Th-APC also acquired CD40, 4-1BBL, and OX40L by DC_{OVA} activation in addition to the previously reported CD80 co-stimulatory molecule (8). To assess the role of these acquired co-stimulatory molecules on $CD4^+$ Th-APC, we performed tetramer staining, *in vivo* cytotoxicity assays, and animal studies. We found that CD80, but not CD40, 4-1BBL, and OX40L co-stimulatory signaling on $CD4^+$ Th-APC is involved in mediating $CD4^+$ Th-APC-stimulated $CD8^+$ CTL responses and antitumor immunity. CD28-CD80 interactions are necessary to sustain late T cell proliferation (37). T cell-T cell Ag presentation, dependent upon $CD4^+$ T cells acquiring CD80 molecules from APC, has recently been reported. For example, $CD4^+$ T cell acquisition of CD80 from APC plays an important role in retaining $CD4^+$ T cell activation in the absence of APC via up-regulation of NF-κB and Stat5 (38). It has also been reported that $CD8^+$ memory T cells with acquired pMHC I and CD80 up-regulated caspase 3, bcl-x, bak, and bax, leading to cell apoptosis (39). In this study, we clearly elucidated another important role on $CD4^+$ Th-APC's acquisition of CD80 from DC_{OVA} by demonstrating that the acquired CD80 molecules on $CD4^+$ T cells are involved in stimulation of *in vivo* $CD8^+$ CTL responses by providing the critical signal 2 costimulation (40). CD40L-CD40 interactions play an important role in mediating CD4 helper functions for $CD8^+$ T cell responses. For example, DC present Ags to and activate $CD4^+$ T cells with its CD40 costimulation. In addition, when DC stimulate $CD4^+$ T cells, the stimulated $CD4^+$ T cells consecutively activate DC through CD40L, allowing DC to become competent to drive $CD8^+$ T cell responses (41-43). However, this concept was challenged by the finding that $CD8^+$ T cells transiently express CD40 after activation and they could receive CD4 help directly via CD40 (44), suggesting that the CD40L signals to $CD8^+$ T cells may be derived from active $CD4^+$ T cells. Recently, we have demonstrated that OVA-pulsed DC (DC_{OVA})-activated OT II $CD4^+$ Th cells acquired MHC I and co-stimulatory molecules from DC_{OVA} and acted as $CD4^+$ Th cells capable of stimulating OVA-specific $CD8^+$ CTL responses (8). In this study, we further demonstrated that $CD4^+$ Th-APC's CD40L signal plays an important role in initiation of $CD8^+$ T cell proliferation and differentiation into $_{CM}$CTL, clearly elucidating that the CD40L signaling to $CD8^+$ T cells is indeed derived from active $CD4^+$ Th-APC.

More recently, Cox et al. (25) have demonstrated that $CD4^+$ Th cells with acquired bystander pMHC I complexes from APC became susceptible to CTL killing in an Ag-specific

manner. However, the molecular mechanism for $CD4^+$ Th cell acquisition of the bystander pMHC I from DC by DC activation is unclear. We have recently demonstrated that pMHC I and II complexes colocalized in the same immunological synapse comprising co-stimulatory molecules CD40, CD54, CD80, OX40L, and 4-1BBL between a DC and a $CD4^+$ Th cell (18), leading to $CD4^+$ Th cell acquisition of the synapse-comprised bystander pMHC I along with pMHC I and co-stimulatory molecules via internalization and the recycling pathway (45, 46). In this study, we have further elucidated the critical role of the acquired pMHC I in regulation of immune responses. We note that it is the acquired pMHC I complexes on $CD4^+$ Th-APC that target the above stimulatory effects derived from IL-2 secretion and CD40L and CD80 costimulations to the Ag-specific $CD8^+$ T cells *in vivo*, leading to induction of OVA-specific $_{CM}$CTL responses and antitumor immunity. The targeting role of acquired pMHC I complexes is also supported by our recent report showing that the acquired pMHC I complexes on DC_{OVA}-activated $CD4^+$ Th cells can target its helper effect onto enhancement of adoptive $CD8^+$ Tc cell immunotherapy of cancer (26). The targeting role of the acquired pMHC I complexes on $CD4^+$ Th-APC may also be applied to interpret the generation of Ag-specific regulatory T (Tr) cells *in vivo* after an encounter with Ag presented by DC (47, 48). Zhang et al. (49) demonstrated that double- negative Tr (DN Tr) cells used their TCR to acquire allo-MHC peptides from APC and became Ag-specific Tr cells in suppression of graft rejection. Tarbell et al. (50) also showed that $CD4^+25^+$ Tr expanded *in vitro* by Ag-specific DC stimulation became 20-fold more efficient in suppression of autoimmune diabetes caused by diabetogenic T cells in NOD mice than polyclonal unexpanded $CD4^+25^+$ Tr cells. However, they did not elucidate the molecular mechanism on how the immune-suppressive effect of these Tr cells can be specifically or more efficiently delivered to the *in vivo* T cell-mediated graft rejections or autoimmune diseases. Based upon the above principle elucidated in this study, we assume that these $CD4^+$ or DN Tr cells may become Ag-specific after acquisition of pMHC I or pMHC II complexes by DC stimulation *in vivo*. This assumption is currently being assessed in our laboratory.

The molecular mechanism of *in vivo* $CD8^+$ T cell-mediated cytotoxicity still remains controversial although its *in vitro* cytotoxicity is mainly mediated by the perforin pathway (51). Winter et al. (52) demonstrated that the adoptive $CD8^+$ CTL's cytotoxicity against tumors is independent of perforin or Fas-FasL interaction. Later, it has been shown that $CD8^+$ T cells exerted its *in vivo* antitumor effector function via IFN-γ expression (53, 54). Recently, it has

been demonstrated that the host-derived TNF-α and IFN-γ were both involved in $CD8^+$ T cell-mediated tumor eradication (55, 56). Similar to $CD8^+$ T cells, $CD4^+$ T cells were able to kill Ia^+ target cells (15, 16, 57, 58). In this study, we demonstrated that $CD4^+$ Th-APC expressing OVA-specific TCR were able to kill pMHC II-expressing DC_{OVA} and Th-APC via the perforin-mediated pathway in an *in vitro* chromium release assay, indicating that $CD4^+$ Th-APC may negatively modulate *in vivo* immune responses by eliminating both pMHC II-expressing DC_{OVA} and neighboring Th-APC. However, the molecular mechanism of *in vivo* $CD4^+$ Th-APC-mediated cytotoxicity is still elusive. It has been previously demonstrated that TRAIL was found to be responsible for *in vivo* $CD4^+$ T cell-mediated cytotoxicity (59, 60).

Taken together, this study has greatly advanced our new concept of "sequential two-cell interactions by $CD4^+$ Th-APC" (8) by further demonstrating that 1) $CD4^+$ Th-APC can acquire co-stimulatory molecules (CD40, CD80, OX40L, and 4-1BBL) and functional pMHC I and II complexes by both *in vitro* and *in vivo* DC activation; 2) $CD4^+$ Th-APC with acquired pMHC I and II and co-stimulatory molecules can stimulate $CD4^+$ Th1 and central memory $CD8^+44^+CD62L^{high}IL\text{-}7R^+$ T cell responses, leading to efficient antitumor immunity; 3) $CD4^+$ Th-APC's stimulatory effect on $CD8^+$ T cell responses is mediated by IL-2 secretion and CD40L and CD80 signaling; and 4) this stimulatory effect is specifically targeted to the Ag-specific $CD8^+$ T cells *in vivo* via acquired pMHC I complexes (Fig. 6). In addition, $CD4^+$ Th-APC expressing OVA-specific TCR, FasL, and perforin may also negatively modulate *in vivo* immune responses by eliminating DC_{OVA} expressing endogenous pMHC II and neighboring Th-APC expressing acquired pMHC II, whereas $CD4^+$ Th-APC expressing acquired pMHC I can also be eliminated by Th-APC-activated $CD8^+$ CTL (Fig. 6). Therefore, these findings may have great impacts in not only the antitumor immunity, but also the regulatory T cell-dependent immune tolerance *in vivo*.

2.7 Acknowledgments

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2.8 Conflict of interests

The authors have no financial conflict of interest.

2.9 Table

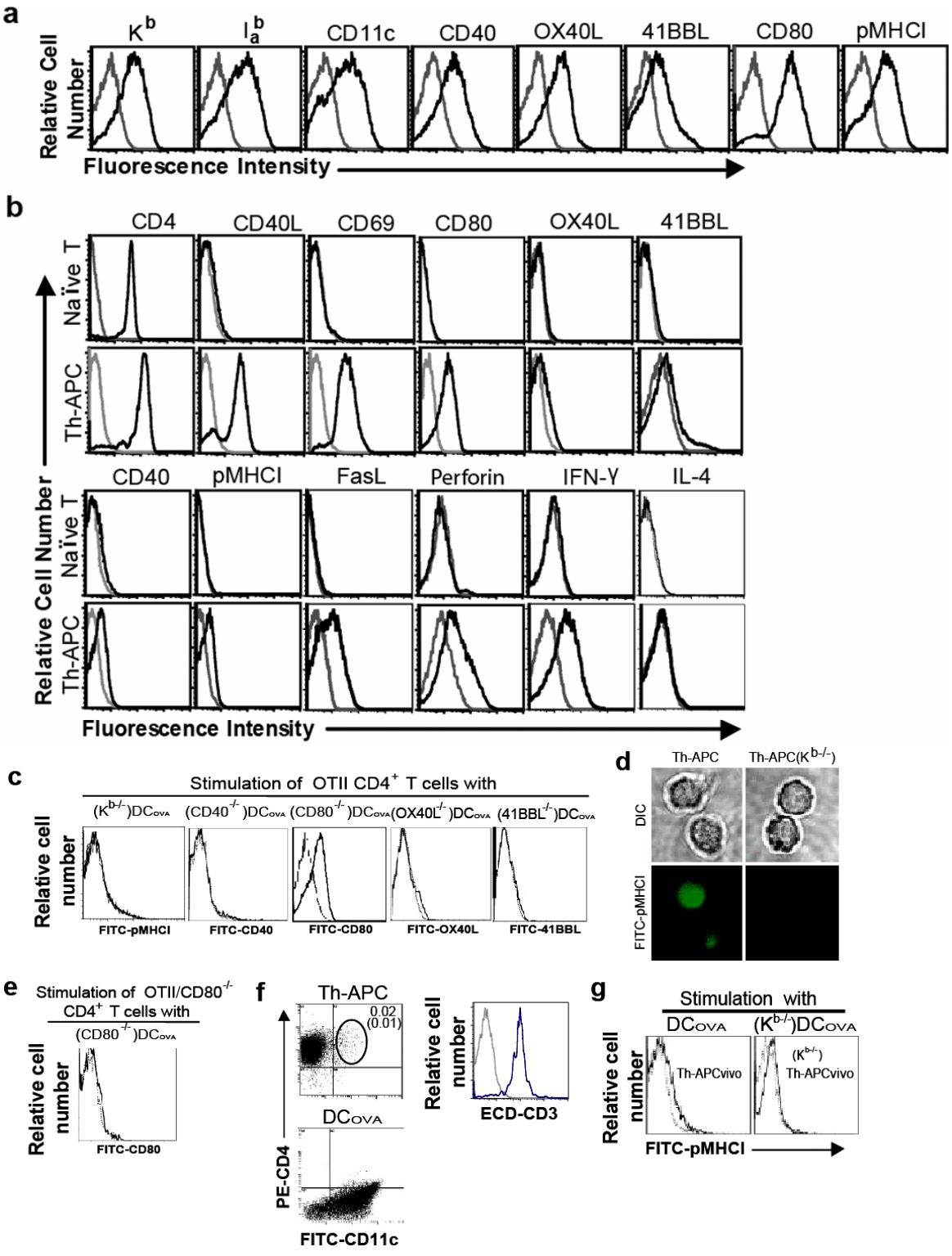
Table 1 Vaccination with $CD4^+$ Th-APC protects against lung tumor metastases in mice

Immunization[a]	Tumor Cell Challenge	Tumor-Bearing Mice (%)	Median No. of Lung Tumor Colonies
Expt. 1			
DC_{OVA}	$BL6\text{-}10_{OVA}$	0/8 (0)	0
$CD4^+$ Th-APC	$BL6\text{-}10_{OVA}$	0/8 (0)	0
$CD4^+$ Th-APCvivo	$BL6\text{-}10_{OVA}$	0/8 (0)	0
PBS	$BL6\text{-}10_{OVA}$	8/8 (100)	>100
$CD4^+$ Th-APC	BL6-10	8/8 (100)	>100
$CD4^+$ Th-APCvivo	BL6-10	8/8 (100)	>100
PBS	BL6-10	8/8 (100)	>100
Expt. 2			
DC_{OVA}	$BL6\text{-}10_{OVA}$	0/8 (0)	0
$CD4^+$ Th-APC	$BL6\text{-}10_{OVA}$	0/8 (0)	0
$CD4^+$ ($IL\text{-}2^{-/-}$)Th-APC	$BL6\text{-}10_{OVA}$	8/8 (100)	>100
$CD4^+$ ($IFN\text{-}\gamma^{-/-}$)Th-APC	$BL6\text{-}10_{OVA}$	0/8 (0)	0
$CD4^+$ ($TNF\text{-}\alpha^{-/-}$)Th-APC	$BL6\text{-}10_{OVA}$	0/8 (0)	0
$CD4^+$ ($CD40^{-/-}$)Th-APC	$BL6\text{-}10_{OVA}$	0/8 (0)	0
$CD4^+$ ($CD80^{-/-}$)Th-APC	$BL6\text{-}10_{OVA}$	8/8 (100)	78 ± 19
$CD4^+$ ($CD40L^{-/-}$)Th-APC	$BL6\text{-}10_{OVA}$	6/8 (75)	35 ± 11
$CD4^+$ ($OX40L^{-/-}$)Th-APC	$BL6\text{-}10_{OVA}$	0/8 (0)	0
$CD4^+$ ($4\text{-}1BBL^{-/-}$)Th-APC	$BL6\text{-}10_{OVA}$	0/8 (0)	0
$CD4^+$ ($K^{b-/-}$)Th-APC	$BL6\text{-}10_{OVA}$	8/8 (100)	>100
PBS	$BL6\text{-}10_{OVA}$	8/8 (100)	>100

[a]In experiments I and II, C57BL/6 mice (n=8) were immunized with DC_{OVA} and the *in vitro* irradiated DC_{OVA}-stimulated $CD4^+$ Th-, ($K^{b-/-}$)Th-, ($IL\text{-}2^{-/-}$)Th-, ($IFN\text{-}\gamma^{-/-}$)Th-, ($TNF\text{-}\alpha^{-/-}$)Th-, ($CD40^{-/-}$)Th-, ($CD40L^{-/-}$)Th-, ($OX40L^{-/-}$)Th-, ($41BBL^{-/-}$)Th- and ($CD80^{-/-}$)Th-APC, and *in vivo* DC_{OVA}-stimulated $CD4^+$ Th-ACvivo and ($K^{b-/-}$)Th-ACvivo cells, respectively. Following the immunizations, each mouse was challenged i.v. with OVA transgene-expressing $BL6\text{-}10_{OVA}$ or wild-type BL6-10 tumor cells. The mice were sacrificed 4 weeks after tumor cell challenge and the numbers of lung metastatic tumor colonies were counted. *, $P<0.01$ versus cohorts of $CD4^+$ Th group (Student's t-test). One representative experiment of two in the above animal studies is shown.

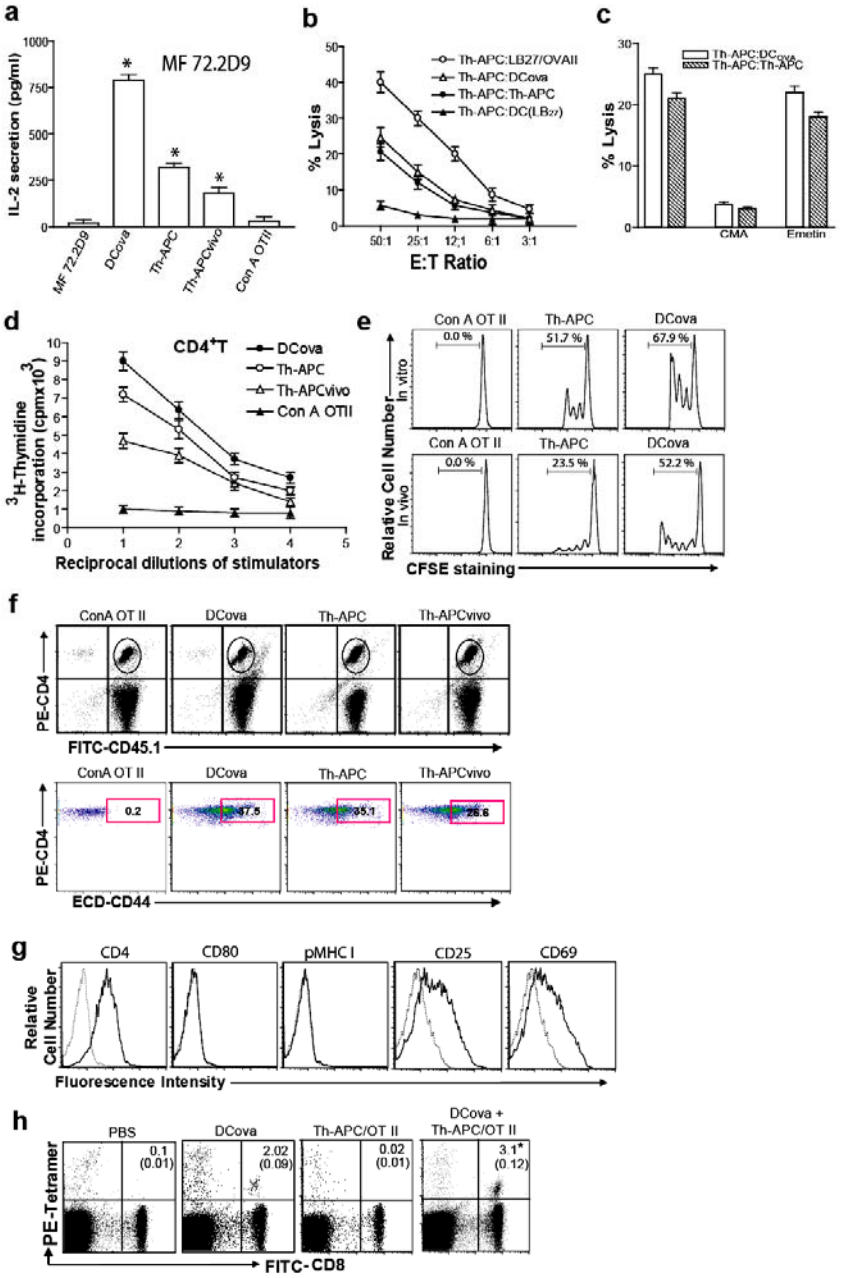
2.10 Figures

Figure 1



Flow cytometric analysis of DC_{OVA} and $CD4^+$ Th-APCs. (*a–c*) DC_{OVA}, naive $CD4^+$ T cells, $CD4^+$ Th-APC, and Th-APC with different gene KO were stained with a panel of Abs (black lines) and analyzed by flow cytometry. (*d*) $CD4^+$ Th-APC and ($K^{b-/-}$)Th-APC were stained with FITC-anti-pMHC I Ab and examined under differential interference contrast (DIC) by confocal microscopy. (*e*) ($CD80^{-/-}$)DC_{OVA}-activated $CD4^+$ Th-APC($CD80^{-/-}$) cells were stained with anti-CD80 Ab (solid line) and analyzed by flow cytometry. (*f*) DC_{OVA} cells and $CD4^+$ Th-APC were stained with PE-anti-CD4, FITC-anti-CD11c, and ECD-anti-CD3 Abs and analyzed by flow cytometry. The $CD4^{high}CD11c^{low}$ cells in the oval were grouped for ECD-CD3 analysis. The value in the panel represents the percentage of $CD4^{high}CD11c^{low}$ cells vs the total $CD4^{high}CD11c^{-}$ Th-APC population. The value in parentheses represents the SD. (*g*) The purified *in vivo* DC_{OVA}- and ($K^{b-/-}$)DC_{OVA}-stimulated $CD4^+$ Th-APC and ($K^{b-/-}$)Th-APC were stained with anti-pMHC I Ab (solid lines) and analyzed by flow cytometry. Irrelevant isotype-matched Ab was used as control (gray or dotted lines) in the above experiments. One representative experiment of two in the above experiments is shown.

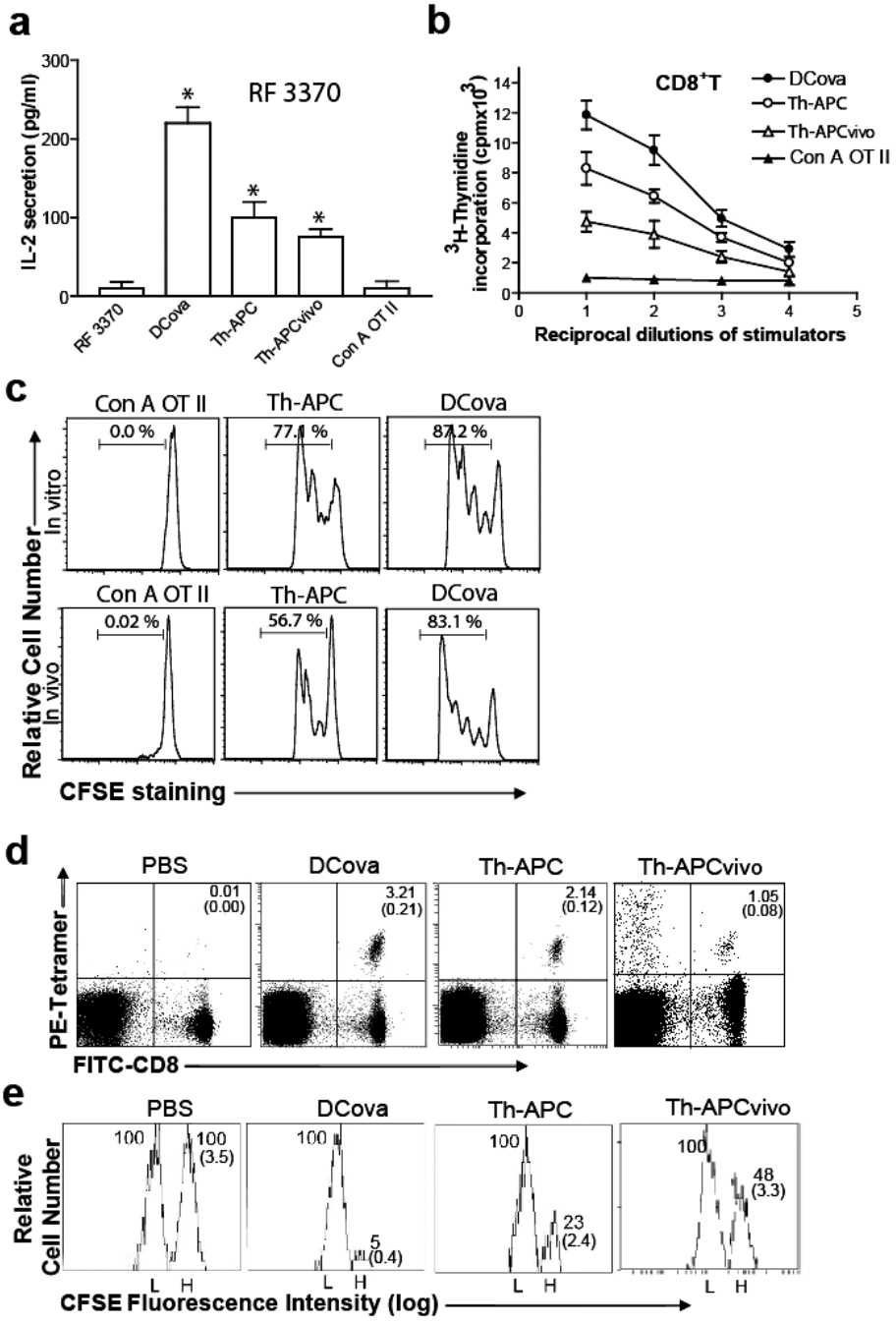
Figure 2



CD4$^+$ Th-APC with acquired pMHC-II stimulate CD4$^+$ Th1 cell responses. (*a*) The amounts of IL-2 in examining wells containing MF72.2D9 cells and various stimulators were substracted by the amount of IL-2 in control wells containing stimulator (DC_{OVA}, CD4$^+$ Th-APC, Th-APCvivo, Con A OT II cells) alone, and MF72.2D9 alone.*, $P < 0.05$ (Student's *t* test) vs cohorts of Con A OT II cells. (*b*) *In vitro* cytotoxicity assay. CD4$^+$ Th-APC were used as effector cells, while ^{51}Cr-labeled DC_{OVA}, OVAII-pulsed LB27, Th-APC, and the control DC and LB27 cells were used as target cells in a chromium release assay. (*c*) The effector cells were

preincubated with CMA (1 μM) or emetin (5 μM) for 2 h and used in the above cytotoxicity assay. (*d*) *In vitro* $CD4^+$ T cell proliferation assay. Varying numbers of irradiated DC_{OVA}, Th-APC, Th-APCvivo, and Con A OT II T cells and their 2-fold dilutions were cocultured with naive OT II $CD4^+$ T cells. After 2 days, the proliferative responses of the T cells were determined by overnight [^{3}H]thymidine uptake assay. *, $p < 0.05$ (Student's *t* test) vs cohorts of Th-APC themselves. (*e*) In the *in vitro* CFSE-labeled $CD4^+$ T cell proliferation assay, the CFSE-labeled OT II $CD4^+$ T cells were cocultured with irradiated DC_{OVA}, $CD4^+$ Th-APC, and Con A OT II T cells. Three days after culture, the cells were harvested to determine the number of divisions by flow cytometry. In the *in vivo* CFSE-labeled $CD4^+$ T cell proliferation assay, the CFSE-labeled OT II $CD4^+$ T cells were i.v. injected into C57BL/6 mice. Twelve hours later, each mouse was i.v. given with DC_{OVA}, Th-APC, and Con A OT II cells, respectively. Four days later, the number of division cycles of the CFSE-labeled $CD4^+$ T cells in the recipient spleens was determined by flow cytometry. Percentages denote the fraction of $CD4^+$ T cells that have undergone at least one division. (*f*) In the *in vivo* $CD4^+$ T cell proliferation assay, C57BL/6.1 mice were i.v. immunized with irradiated DC_{OVA}, Con A T, $CD4^+$ Th-APC, and Th-APCvivo. Four days after the immunization, the tail blood samples of immunized mice were stained with PE-anti-CD45.1, FITC-anti-CD4, and ECD-anti-CD44 Abs and then analyzed by flow cytometry. The PE-CD4- and FITC-CD45.1-positive T cells were gated for further analysis of ECD-CD44 and PE-CD4 expression. The values in each panel represent the percentage of $CD44^{high}$ $CD4^+$ T cells vs the total $CD4^+$ T cell pool. The values in parentheses represent the SD. (*g*) The *in vitro* $CD4^+$ Th-APC-activated $CD4^+$ Th1 cells (Th-APC/OT II) were stained with a panel of Abs (solid lines) and analyzed by flow cytometry. Irrelevant isotype-matched Abs were used as controls (dotted lines). (*h*) Tetramer staining assay. The tail blood samples of mice (six mice per group) immunized with irradiated DC_{OVA}, Th-APC/OT II cells, and DC_{OVA} along with Th-APC/OT II cells were stained with PE-H-$2K^b$/OVAI tetramer (PE-tetramer) and FITC-anti-CD8 Ab (FITC-CD8) and then analyzed by flow cytometry. The value in each panel represents the percentage of tetramer-positive $CD8^+$ T cells vs the total $CD8^+$ T cell population. The value in parentheses represents the SD. *, representing $p < 0.05$ vs cohorts of mice immunized with irradiated DC_{OVA} (Student's *t* test). One representative experiment of two in the above different experiments is shown.

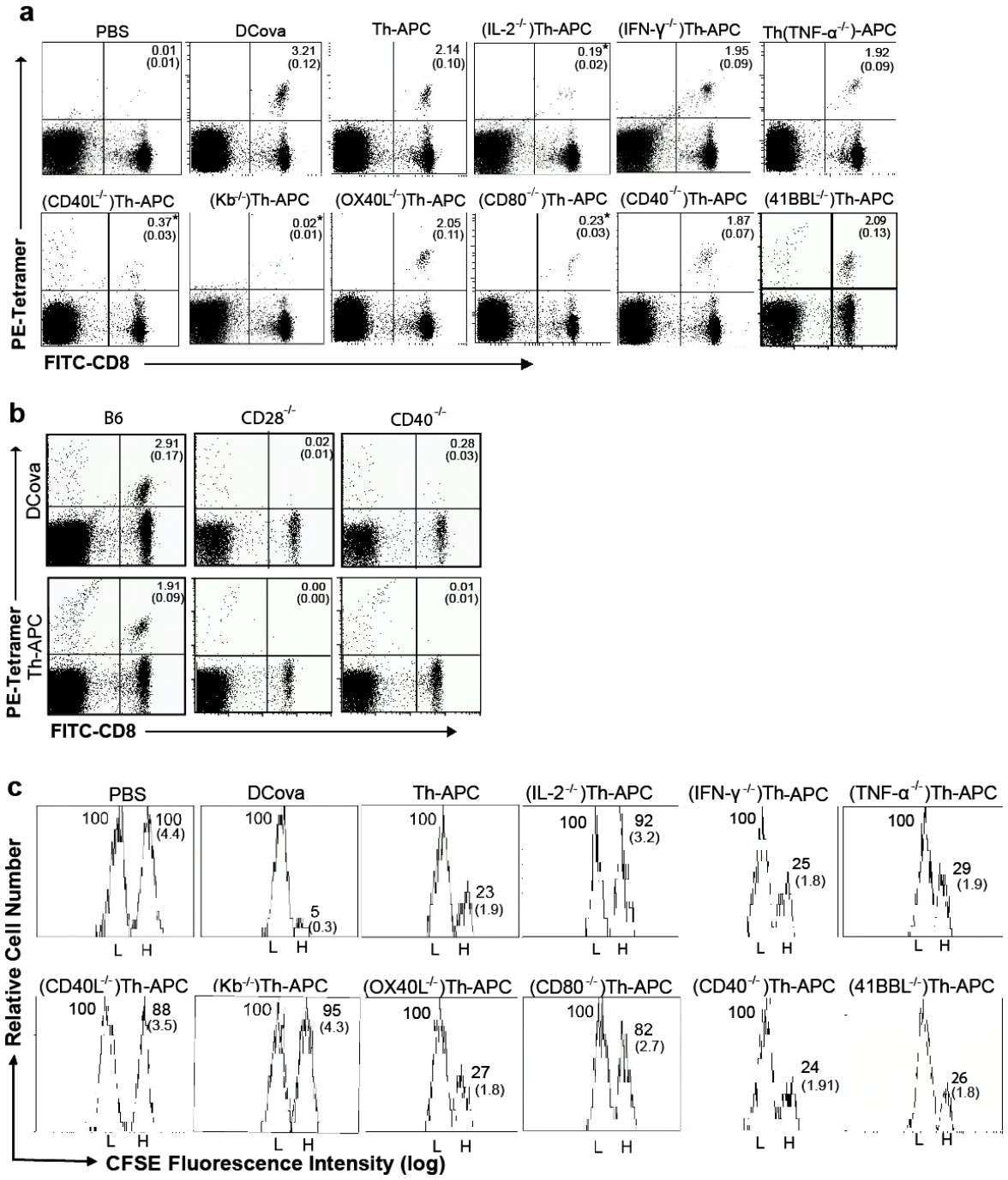
Figure 3



CD4$^+$ Th-APC with acquired functional pMHC I complexes stimulate CD4$^+$ T cell proliferation and the effect is mediated through IL-2 secretion. (*a*) The amount of IL-2 secretion in examining wells containing RF3370 cells and various stimulators were substracted by the amount of IL-2 in control wells containing various stimulators (DC_{OVA}, CD4$^+$ Th-APC, Th-APCvivo, and Con A OT II cells) alone. *, $p < 0.05$ (Student's *t* test) vs cohorts of Con A OT

II cells. The *in vitro* $CD8^+$ T cell (*b*) and CFSE-labeled $CD8^+$ T cell (*c*) proliferation assays were performed in a similar manner as the above *in vitro* $CD4^+$ T cell proliferation assays. (*d*) *In vivo* $CD8^+$ T cell proliferation assay. C57BL/6 mice were i.v. immunized with DC_{OVA}, $CD4^+$ Th-APC, and $CD4^+$ Th-APCvivo, respectively. Six days after the immunization, the tail blood samples of immunized mice were stained with PE-tetramer and FITC-CD8 Ab and then analyzed by flow cytometry. The value in each panel represents the percentage of tetramer-positive $CD8^+$ T cells vs the total $CD8^+$ T cell pool. The values in parentheses represent the SD. (*e*) *In vivo* cytotoxicity assay. Sixteen hours after target cell delivery, the residual OVA I-pulsed $CFSE^{high}$ and Mut1-pulsed $CFSE^{low}$ target cells remaining in the spleens of the above immunized mice were sorted and analyzed by flow cytometry. The value in each panel represents the percentage of $CFSE^{high}$ and $CFSE^{low}$ target cells remaining in the spleens. The values in parentheses represent the SD. One representative experiment of three in the above different experiments is shown.

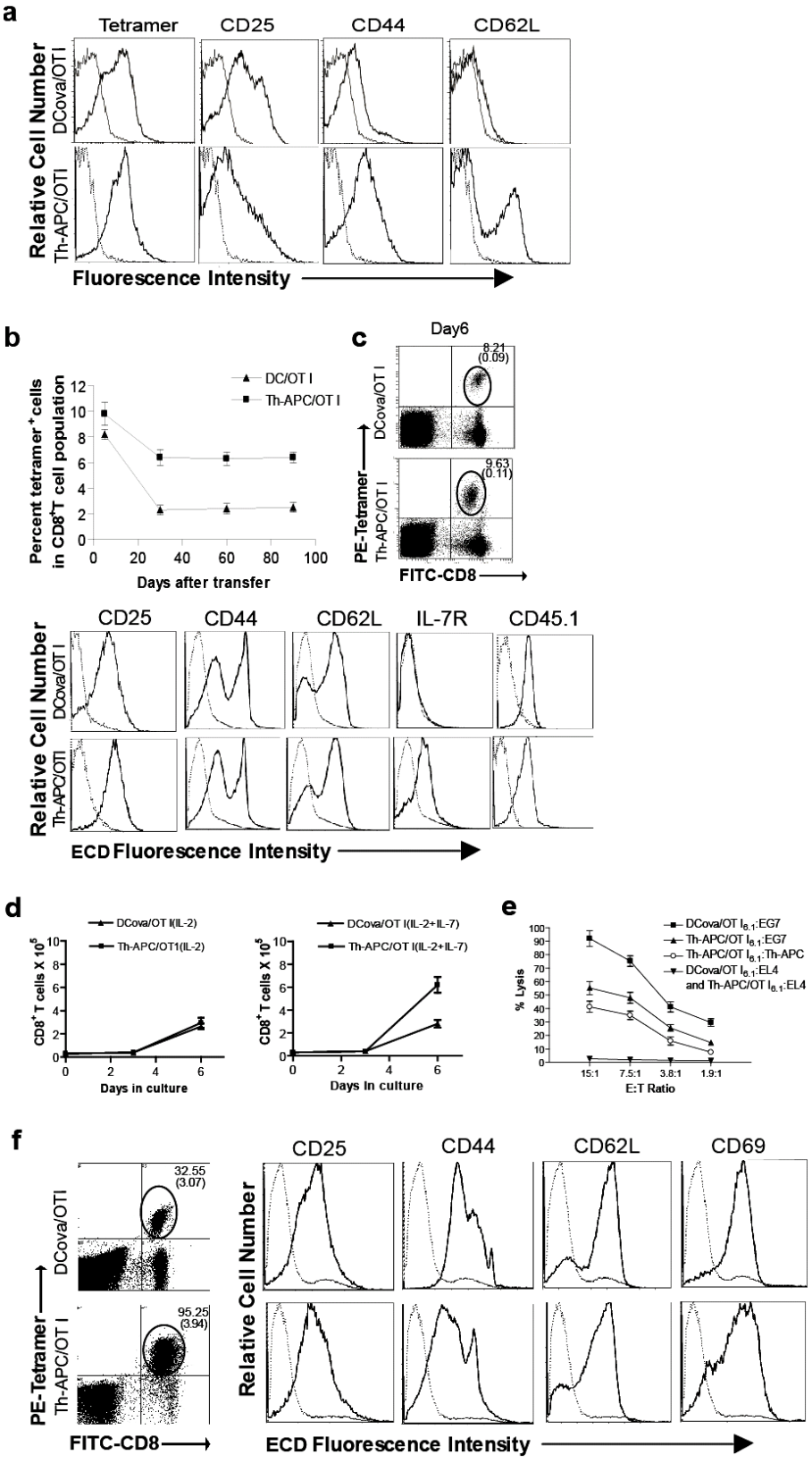
Figure 4



Th-APC's stimulatory effect on OVA-specific CD8$^+$ T cell responses is mediated by CD40L and CD80 signaling and acquired pMHC I targeting. (*a*) *In vivo* CD8$^+$ T cell proliferation assay. The tail blood samples of mice immunized with DC_{OVA}, CD4$^+$ Th-APC, and CD4$^+$ Th-APC with different gene KO were incubated with PE-tetramer and FITC-CD8 and then analyzed

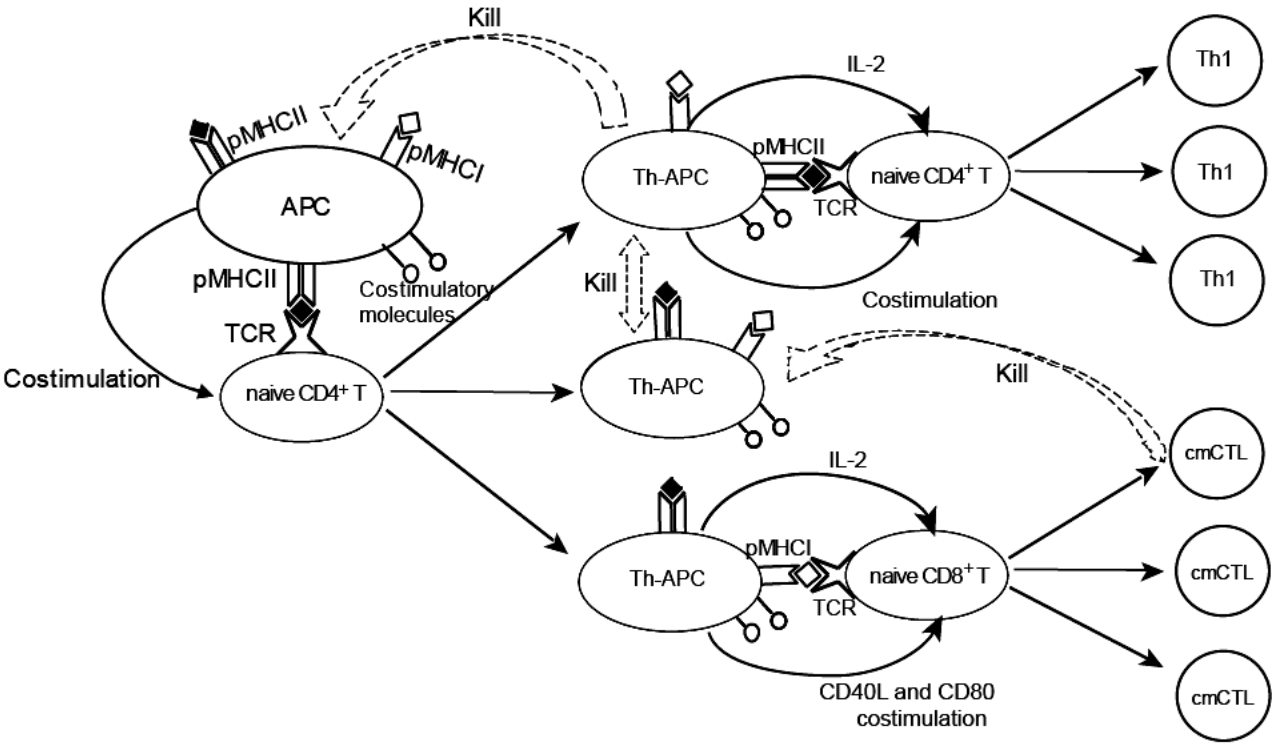
by flow cytometry. The value in each panel represents the percentage of tetramer-positive $CD8^+$ T cells vs the total $CD8^+$ T cell population. The values in parentheses represent the SD. *, Representing $p<0.05$ vs cohorts of mice immunized with irradiated $CD4^+$ Th-APC (Student's *t* test). (*b*) The tail blood samples of mice immunized with DC_{OVA} and $CD4^+$ Th-APC were incubated with PE-tetramer and FITC-CD8 and then analyzed by flow cytometry. The value in each panel represents the percentage of tetramer-positive $CD8^+$ T cells vs the total $CD8^+$ T cell population. (*c*) *In vivo* cytotoxicity assay. Sixteen hours after target cell delivery, the residual OVA I-pulsed $CFSE^{high}$ and Mut1-pulsed $CFSE^{low}$ target cells remaining in the spleens of the above immunized mice were sorted and analyzed by flow cytometry. One representative experiment of three in the above different experiments is shown.

Figure 5


a
Tetramer
CD25
CD44
CD62L
Relative Cell Number
DCova/OTI
Th-APC/OTI
Fluorescence Intensity
b
DC/OT I
Th-APC/OT I
Percent tetramer+cells in CD8+T cell population
Days after transfer
c
Day6
8.21 (0.09)
9.63 (0.11)
DCova/OT I
Th-APC/OT I
PE-Tetramer
FITC-CD8
CD25
CD44
CD62L
IL-7R
CD45.1
ECD Fluorescence Intensity
d
DCova/OT I(IL-2)
Th-APC/OT1(IL-2)
DCova/OT I(IL-2+IL-7)
Th-APC/OT I(IL-2+IL-7)
CD8+T cells X 10^5
Days in culture
e
DCova/OT I8.1:EG7
Th-APC/OT I8.1:EG7
Th-APC/OT I8.1:Th-APC
DCova/OT I8.1:EL4
and Th-APC/OT I8.1:EL4
% Lysis
15:1
7.5:1
3.8:1
1.9:1
E:T Ratio
f
32.55 (3.07)
95.25 (3.94)
DCova/OTI
Th-APC/OT I
PE-Tetramer
FITC-CD8
CD25
CD44
CD62L
CD69
Relative Cell Number
ECD Fluorescence Intensity

$CD4^+$ Th-APC stimulate $CD8^+$ T cell differentiation into central memory T cells. (*a*) Naive $CD8^+$ T cells derived from OT I/B6.1 mice were primed with irradiated DC_{OVA} and $CD4^+$ Th-APC, purified by using CD45.1 microbeads, stained with PE-tetramer and FITC-labeled Abs, respectively, and then analyzed by flow cytometry. PE-tetramer-positive $CD8^+$ T cells were gated for further analysis of FITC-CD25, FITC-CD44, and FITC-CD62L expression (solid lines). (*b*) *In vitro* DC_{OVA}- and $CD4^+$ Th-APC-activated $CD8^+CD45.1^+$ T cells derived from OT I/B6.1 mice were i.v. injected into C57BL/6 ($CD45.2^+$) mice (six mice per group). Mouse tail blood cells were stained with PE-H-$2K^b$/OVAI tetramer (PE-tetramer) and FITC-anti-CD8 (FITC-CD8) Ab and analyzed by flow cytometry at indicated time points after $CD8^+$ T cell transfer. In addition, 6 days after $CD8^+$ T cell transfer, mouse tail blood cells were also stained with PE-tetramer, FITC-CD8, and ECD-conjugated anti-CD25, anti-CD44, anti-CD45.1, anti-CD62L, and anti-IL-7R Abs, respectively, and analyzed by flow cytometry. The value in each panel represents the percentage of PE-tetramer-positive $CD8^+$ T cells vs total peripheral $CD8^+$ T cells. The values in parentheses represents the SD. (*c*) The OVA-specific $CD8^+$ T cells with PE-tetramer and FITC-CD8 staining were gated, and then assessed for CD25, CD44, CD45.1, CD62L, and IL-7R expressions (solid lines). (*d*) OTI $CD8^+$ T cells (0.4×10^5 cells/well) primed on day 0 with DC_{OVA} (▼) or $CD4^+$ Th-APC (■) were maintained in cultures for 1 wk with the indicated cytokines (IL-2 (50 U/ml) and/or IL-7 (10 ng/ml)) added on days 3 and 5. Live $CD8^+$ T cells with trypan blue exclusion for each culture done in triplicate were counted at the indicated time points. (*e*) The DC_{OVA}-primed and $CD4^+$ Th-APC-primed OT I/B6.1 $CD8^+$ T cells referred to as DC_{OVA}/OT $I_{6.1}$ and $CD4^+$ Th-APC/OT $I_{6.1}$, respectively, were used as effector cells, while ^{51}Cr-labeled EG7, Th-APC, and the control EL-4 tumor cells were used as target cells in a chromium release assay. (*f*) Memory T cell expansion. Three months after $CD8^+$ T cell transfer, mice were boosted by i.v. injection of DC_{OVA} (0.5×10^6). Four days subsequent to the boost, mouse tail blood cells were stained with PE-tetramer, FITC-CD8, and ECD-CD25, ECD-CD44, and ECD-CD69 Abs and analyzed by flow cytometry. The value in each panel represents the percentage of PE-tetramer-positive $CD8^+$ T cells vs the total peripheral $CD8^+$ T cell population. The OVA-specific $CD8^+$ T cells with PE-tetramer and FITC-CD8 staining were sorted and assessed for CD25, CD44, and CD69 expression (solid lines). ECD-labeled isotype-matched irrelevant Abs were used as controls (dotted lines) in the above experiments. One representative experiment of two in the above different experiments is shown.

Figure 6



A new view on the dynamic model of sequential two-cell interactions by $CD4^+$ Th-APC. In this model, APC license $CD4^+$ Th cells to act as Th-APC. By activation of $CD4^+$ Th cells, APC can also transfer the functional pMHC I and II and the co-stimulatory molecules such as CD80 onto an expanding population of CD40L-expressing and IL-2-secreting $CD4^+$ Th-APC. The $CD4^+$ Th-APC expressing TCR can negatively modulate immune responses by eliminating APC expressing pMHC II and neighboring Th-APC with acquired pMHC II. The Th-APC expressing acquired pMHC II and I complexes can also positively modulate immune responses by stimulating $CD4^+$ Th1 cell and $CD8^+$ $_{CM}$CTL responses via its endogenous IL-2 secretion and CD40L and CD80 signaling, respectively. The stimulatory effect of Th-APC is specifically targeted to Ag-specific naive $CD8^+$ T cells *in vivo* via its acquired pMHC I complexes. The $CD8^+$ $_{CM}$CTL expressing TCR can also eliminate Th-APC with acquired pMHC I complexes.

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CHAPTER 3

TH CELLS PROMOTE CTL SURVIVAL AND MEMORY VIA ACQUIRED PMHC-I AND ENDOGENOUS IL-2 AND CD40L SIGNALING AND BY MODULATING APOPTOSIS-CONTROLLING PATHWAYS

Channakeshava Sokke Umeshappa[¶,*], Shulin Xu[¶], Roopa Hebbandi Nanjundappa[¶,†] and Jim Xiang*[,¶,#]

[¶]*Cancer Research Unit, Saskatchewan Cancer Agency, Departments of* [¶]*Oncology, *Pathology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada*

Running Title: Molecular mechanisms of Th1 help in CTL fates

[#]**Correspondence:** Dr. Jim Xiang, Saskatoon Cancer Center, 20 Campus Drive, Saskatoon, Saskatchewan S7N 4H4, Canada, Tel: (306) 6552917, Fax: (306) 6552635, email: jim.xiang@saskcancer.ca

[†]Present address**:** Department of Microbiology, Immunology & Infectious Diseases, Health Research Innovation Centre, 3330 Hospital Drive NW, University of Calgary, Calgary, Alberta T2N 4N1 Canada.

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3.1 Brief introduction to Chapter 3

Although it has been frequently reported that $CD4^+$ T cell signals during priming (where $CD8^+$ T cells differentiate from naïve to effector cells) orchestrate memory CTL development, the role of these signals during effector/contraction phase (where differentiated effector CTLs eliminate invaded foreign Ags and destined to die or develop into memory CTLs) for memory CTL responses is less defined. In this chapter, using various monoclonal sources of T cells, and DCova immunization model, the study was designed to investigate how Th cell-derived signals modulate differentiated effector CTLs' survival and memory responses. This study showed the direct involvement of Th-APCs in modulation of effector CTL fate.

3.2 Abstract

Involvement of $CD4^+$ helper T (Th) cells is crucial for $CD8^+$ cytotoxic T lymphocyte (CTL)-mediated immunity. However, $CD4^+$ Th's signals that govern CTL survival and functional memory are still not completely understood. In this study, we assessed the role of $CD4^+$ Th cells with acquired antigen-presenting machineries in determining CTL fates. We utilized an adoptive co-transfer into $CD4^+$ T cell-sufficient or -deficient mice of OTI CTLs and OTII Th cells or Th cells with various gene deficiencies pre-stimulated *in vitro* by ovalbumin (OVA)-pulsed dendritic cell (DCova). CTL survival was kinetically assessed in these mice using FITC-anti-CD8 and PE-H-$2K^b/OVA_{257-264}$ tetramer staining by flow cytometry. We show that by acting via endogenous CD40L and IL-2, and acquired peptide-MHC-I (pMHC-I) complex signaling, $CD4^+$ Th cells enhance survival of transferred effector CTLs and their differentiation into the functional memory CTLs capable of protecting against highly-metastasizing tumor challenge. Moreover, RT-PCR and Western blot analysis demonstrate that increased survival of $CD4^+$ Th cell-helped CTLs is matched with enhanced Akt1/NF-κB activation, down-regulation of FasL and TRAIL, and altered expression profiles with up-regulation of prosurvival (Bcl-2) and down-regulation of proapoptotic (Bcl-10, Casp-3, Casp-4, Casp-7) molecules/genes. Taken together, our results reveal a previously unexplored mechanistic role for $CD4^+$ Th cells in programming CTL responses. The observed $CD4^+$ Th cell-effector CTL cooperation could explain why effector CTLs generated under cognate $CD4^+$ Th cell help show survival and recall advantages. This knowledge could also aid in the development of efficient adoptive CTL cancer therapy.

3.3 Introduction

$CD8^+$ T cells play a defensive role against viral infections and cancers. Following recognition of foreign Ag, they undergo 3 distinct phases (1): (i) a proliferation (primary) phase in which naïve $CD8^+$ T cells undergo autonomous clonal expansion and develop into effector cytotoxic T lymphocytes (CTLs); (ii) a contraction (transitional) phase, in which ~95% of effector CTL undergo activation-induced cell death (AICD) through apoptosis, allowing development of ~5-10% memory CTLs; and (iii) a maintenance (memory) phase in which memory CTL survive for prolonged duration. In subsequent Ag encounters, unlike their naive counterparts, memory CTLs respond swiftly by rapid proliferation and heightened effector functions in recall responses.

$CD4^+$ T cells have potential to influence multiple aspects of CTL responses. Their importance in primary CTL responses was first demonstrated in immunizations with non-inflammatory Ags such as male minor-HY and Qa-1 alloantigen (2). The requirement of cognate $CD4^+$ T cell help for different phases of CTL responses is frequently debated and shown to vary with type of immunizations. In the absence of inflammation, APCs have to be activated by $CD4^+$ T cells through CD40/CD40L interactions to prime $CD8^+$ CTL responses (3). Alternatively, cognate $CD4^+$ T cells have also been shown to provide direct signaling on CD40-expressing $CD8^+$ T cells through CD40L costimulation (4, 5). Although $CD4^+$ T cell help can be dispensable for primary CTL generation, it is prerequisite for programming memory CTLs in most situations (4, 6-8). As the effector phase constitute both AICD and memory CTLs development, APC-stimulated Th1 cells appear to play a critical role in effector CTL survival and functional memory development (9, 10). Recently, $CD4^+$ T cell help shown to be required for effector CTL survival via regulation of TRAIL and Bcl-x_L (6, 7, 11). However, the mechanism of $CD4^+$ T cell help which prevents AICD of effector CTL is still not completely understood. $CD4^+$ T cell help is also implicated in memory CTL recall responses (6). However, whether a cognate (6, 12-14) or heterospecific (15, 16) $CD4^+$ T cell help is required is still controversial. Although an important role of IL-2 during CTL priming has been suggested for full secondary expansion (17), the timing, and type of $CD4^+$ T cell helper-derived signals contributing to optimal recall responses is largely unknown.

Intercellular membrane transfer through trogocytosis, a wide-spread phenomenon in the immune system, plays a crucial role in immunomodulation (18-20). Recently, acquisition of APC's Ag-presenting machineries (APM) by $CD4^+$ T cells has attracted greater attention (1, 19, 21-28). Understanding the functional consequences of acquired APM on $CD4^+$ T cells is under intense

scrutiny. Although not efficient in soluble-Ag capture, $CD4^+$ T cells acquire APM from APCs, and present Ag to other naïve $CD4^+$ T cells, inducing activation and proliferation (20, 22, 28, 29). In contrast, their presentation to previously Ag-experienced, activated or memory $CD4^+$ T cells inhibits proliferation, thereby maintaining homeostasis of immune responses (1, 21, 22, 26). In various studies, we have demonstrated the role of APM on $CD4^+$ T cells in modulating naïve $CD8^+$ T cell responses (22, 24, 30). We showed that APC-stimulated $CD4^+$ type 1 helper cells (Th) are capable to stimulate naïve $CD8^+$ T cells via acquired peptide-MHC-I complexes (pMHC I), inducing central memory CTL stimulation and anti-tumor immunity. However, impact of these APM on Th cells in modulating previously Ag-experienced effector CTL responses is not well understood, a knowledge that is crucial for successful adoptive CTL therapy of cancers.

Here, we investigated thoroughly whether Th cells with its acquired APM and helper factors, IL-2 and CD40L, have any beneficial effects on adoptively transferred effector CTLs generated by *in vitro* cultivation of transgenic OTII $CD4^+$ and OTI $CD8^+$ T cells with ovalbumin (OVA)-pulsed DCs (DCova) into CD4-sufficient [wild-type (WT)] and -deficient [$Ia^{b-/-}$, knockout] mice. We demonstrated that Th cells enhanced CTL survival, transition to functional memory pool and protection against highly metastasizing tumor challenge. The Th's inherent ability to provide IL-2 and CD40L signals, and presence of low level of acquired pMHC I were found to be critical for such helper roles.

3.4 Materials and methods

3.4.1 Reagents, tumor cells and animals

The biotin- and fluorescent dye-labeled (FITC or PE) antibodies (Abs) specific for CD4 (GK1.5), CD11c (HL3), CD44 (IM7), H-$2K^b$ (AF6-88.5), Ia^b (KH74), CD80 (16-10A1), CD40 (3/23), CD40L (MR1), CD54 (3E2), CD62L (MEL-14), CD69 (H1.2F3) and IFN-γ (XMG1.2), and streptavidin-PE-Texas-Red or -FITC were purchased from BD-Biosciences. The biotin-anti-IL-7Rα (A7R34), -CCR7 (4B12) and -pMHC I (25-D1.16) Abs were purchased from eBioscience. The FITC-anti-perforin (CB5.4) Ab was obtained from Alexis Chemicals. The recombinant GM-CSF, IL-2, IL-4 and IL-12 as well as the anti-IL-4 Ab were obtained from R&D Systems. The FITC-anti-CD8 (KT15) and PE-anti-CD45.1 (A20) Abs, and H-$2K^b$/$OVA_{257-264}$ tetramer were obtained from Beckman Coulter. The mouse malignant melanoma (BL6-10) and OVA-transfected

BL6-10 (BL6-10_{OVA}) and EL4 (EG7) cell lines were cultured as described previously (22). The mouse B-cell-hybridoma cell line LB27 expressing both H-$2K^b$ and Ia^b was obtained from American Type Culture Collection. The age-matched WT [C57BL/6(B6, $CD45.2^+$)], $OVA_{257-264}$ and $OVA_{323-329}$-specific TCR-transgenic OTI and OTII, H-$2K^{b-/-}$, and $Ia^{b-/-}$ on WT background, and C57BL/6.1(B6, $CD45.1^+$) mice were purchased from Jackson Laboratory. The OTII/C57BL/6.1(B6, $CD45.1^+$), OTII/$CD40L^{-/-}$ and OTII/IL-$2^{-/-}$ mice were generated by backcrossing designated gene-deficient mice with OTII mice, and tested as described previously (22). All the experiments were performed as per the guidelines of University Committee on Animal Care and Supply.

3.4.2 Preparation and characterization of mature DCova

Bone-marrow-derived, DCova from WT mice were generated by culturing bone marrow cells for 6 days in medium containing IL-4 (20 ng/ml) and GM-CSF (20 ng/ml) and pulsing with 0.1 mg/mL OVA overnight at 37°C as described previously (24). OVA-pulsed DCs generated from WT B6 and H-$2K^{b-/-}$ mice were referred as DCova and DCova($K^{b-/-}$), respectively.

3.4.3 Preparation and characterization of naïve and effector $CD4^+$ or $CD8^+$ T cells

The naïve $CD4^+$ and $CD8^+$ T cells were isolated from WT, OTII or OTI splenocytes as previously described (22). To generate active, OVA-specific $CD8^+$ T (effector CTL) and Th cells, the OTI $CD8^+$ or OTII $CD4^+$ T cells ($0.75X10^6$cells/mL, 200µl/well) were respectively cultured with irradiated (4,000rads) DCova ($0.75X10^6$cells/mL, 50µl/well) at 1:4 ratio for three days as previously described (24). The proliferated $CD8^+$ or $CD4^+$ T cells were purified using using $CD4^+$ and $CD8^+$ MACS microbeads after Ficoll-Paque (Sigma-Aldrich) separation. The naïve, effector CTL and Th cells were stained with panel of cell-, naïve-, activation- or memory-specific markers, and characterized phenotypically by flow cytometry (Beckman Coulter). The Th cells derived from OTII/$CD40L^{-/-}$ and OTII/IL-$2^{-/-}$ mice were termed Th($CD40L^{-/-}$) and Th($IL\text{-}2^{-/-}$), respectively. DCova($K^{b-/-}$)-stimulated Th cells without acquired pMHC I molecules were referred as Th(pMHC $I^{-/-}$). For cytokine profiling, effector CTL and Th or Th($IL\text{-}2^{-/-}$) were respectively re-stimulated with irradiated EG7 and OVAII ($OVA_{323–339}$, Multiple Peptide Systems)-pulsed LB27, and culture supernatants were assessed using cytokine ELISA kits (R&D Systems) from previous description

with few modifications (31). Non-specifically-stimulated $CD4^+$ and $CD8^+$ T cells were obtained by culturing purified B6 mouse $CD4^+$ or $CD8^+$ T populations using ConA (Sigma-Aldrich) as previously described (22) followed by Ficoll-Paque separation. In priming studies, OTI $CD8^+$ T cells were co-cultured with irradiated DCova as above in the presence or absence of OTII $CD4^+$ T cells. Helped orl unhelped effector CTLs were purified by Ficoll-Paque separation and negative selection using anti-CD4 (L3T4) paramagnetic beads (Dynal).

3.4.4 In vivo $CD8^+$ CTL survival and recall studies

Approximately $5x10^6$ effector CTLs alone that received help during priming period or $5x10^6$ effectors CTLs with or without $2x10^6$ Th cells (i.e., help after CTL priming) were i.v. transferred WT mice. Similarly, effector CTLs alone that received help during priming period or effectors CTLs with or without effector Th, Th(IL-$2^{-/-}$), Th($CD40L^{-/-}$), Th (pMHC-$I^{-/-}$) cells ($2x10^6$each), naïve monoclonal OTII (naïve-OTII) or polyclonal B6 (naïve-WT) $CD4^+$ T cells ($15x10^6$ cells), or ConA-stimulated OTII (ConA-OTII) or ConA-stimulated B6 (ConA-WT) $CD4^+$ T cells ($2x10^6$ cells) were i.v. transferred to $Ia^{b-/-}$ mice. After confirming equal engraftment on the following day, the effector CTL survival was monitored 6 or 60 days later in peripheral blood by staining with H-$2K^b$/$OVA_{257-264}$ tetramer and FITC-anti-CD8 Ab (tetramer assay) (22). In recall studies, all the groups were boosted i.v. with $1x10^6$ DCova 60 days later and monitored on 4^{th} day for the expansion of memory CTL by tetramer assay. To characterize effector or memory CTLs phenotypically, the blood samples were collected at 6 or 60 days later, and stained for tetramer assay along with panel of biotin-conjugated Abs specific for effector or memory markers, and streptavidin-PE-Texas Red.

3.4.5 Tumor protection studies

To assess functional effect of memory CTLs, approximately $5x10^6$ effector CTLs alone that received help during priming period or $5x10^6$ effectors CTLs with or without $2x10^6$ Th cells (i.e., help after CTL priming) were i.v. transferred WT and $Ia^{b-/-}$ mice. In addition, to understand mechanism of $CD4^+$ T cells, some $Ia^{b-/-}$ mice groups were also transferred with effector CTLs with effector Th, Th(IL-$2^{-/-}$), Th($CD40L^{-/-}$) or Th (pMHC-$I^{-/-}$) cells ($2x10^6$each). Hundred days later, all the groups were i.v. challenged with highly-metastasizing BL6-10_{OVA} tumor cells ($0.5x10^6$/mice),

monitored for protection as described previously (22), and sacrificed later to determine the numbers of surface black tumor colonies in the lungs. The grading was done depending on numbers of metastatic tumor colonies as: -, no tumors; +, 1-25; ++, 26-50; +++, 51-75; ++++, 76-100; +++++, 101-250; ++++++, >250.

3.4.6 Intracellular IFN-γ staining

On 24th day of tumor challenge, the splenocytes of helped or unhelped mice were re-stimulated with OVAI ($OVA_{257-264}$, Multiple Peptide Systems) and subjected to intracellular IFN-γ staining (BD-Biosciences) as described previously (30).

3.4.7 RT^2 profiler PCR array system

The WT and $Ia^{b-/-}$ mice were i.v. injected with effector CTLs ($5x10^6$) with or without Th cells ($2x10^6$). The blood and lymphoid organs were collected 16 days later, and processed to remove RBC. The T lymphocytes were enriched in nylon wool columns (C&A Scientific). After labeling T lymphocytes with H-$2K^b$/$OVA_{257-264}$ tetramer and anti-PE microbeads (Miltenyi Biotec Inc), a highly purified population of OVA-specific CTLs was obtained by positive selection by passing in 2 separate columns sequentially. The expression of pathway-focused panel of 84 genes related to apoptosis in helped or unhelped CTL was examined using RT^2 *Profiler*^{TM} PCR array (SuperArray Bioscience). Total RNA was isolated using RNeasy extraction kit (Qiagen) and reverse transcribed using RT^2 First Strand Kit (SuperArray Bioscience). The mRNA expression of each gene in array system was performed using StepOnePlus thermocycler (Applied Biosystems) and analyzed using *Hprt1, Gapdh*, and *β-actin* as internal controls in web-based software as per manufacturer's instructions.

3.4.8 qRT-PCR analysis

The cDNA samples of helped or unhelped CTL were further subjected to quantitative RT-PCR (qRT-PCR) for validation of the array results. Sequence-specific primers for *β-actin, Bcl-2*, *FasL and Trail* were previously described (7, 32), and for *Trail receptor, Nfkb1, Bcl10, Akt1, Caspase-4* and *-7* were given in supplementary information (Table S1). qRT-PCR was performed using SYBR Green method following manufacturer's protocol. Briefly, 20ng of cDNA, 50nM of

each primer and 1X Master Mix (Applied Biosystems) were used in 25µl volume. The mRNA expressions were analyzed as described for PCR array using *β-actin* control.

3.4.9 Western blotting

Western blotting was performed in helped or unhelped CTL as described previously (22) with few modifications. The blots were stained with panel of monoclonal- or polyclonal-rabbit Abs specific for Bcl-2 (50E3), β-actin (13E5), Bcl10 (C79F1), Akt1 (2H10), phospho-Akt1 (S473), cleaved Caspase-3 (Asp175)(5A1E) and -7 (Asp198) (Cell Signaling Technology), NF-κB-p65 (C-20), phospho-NFkB-p65 (Ser536) (Santa Cruz Biotechnology) and NFATc1 (7A6) (BD Biosciences). The blots were incubated with goat anti-rabbit IRDyeR800/680CW, and band densities were quantified using ODYSSEY densitometer (LI-COR Bioscience).

3.4.10 Statistical analysis

The statistical analysis was performed using Graphpad Prism-3.0. The results are presented as mean ± SD. The statistical significance between two or more groups was analyzed by Student's *t*-test or analysis of variance, respectively. $p<0.05$ was considered statistically significant.

3.5 Results

3.5.1 Th's help during priming or transitional period enhance effector CTL survival and its transition to memory development

In line with the previous results (22), OVA-pulsed bone marrow-derived DCs (DCova) expressed all the maturation markers, such as Ia^b, CD40, CD80, and pMHC I (Supplementory Fig. 1a). Following *in vitro* stimulation of irradiated DCova with OTII $CD4^+$ or OTI $CD8^+$ T cells, the active OVA-specific $CD4^+$ or $CD8^+$ T cells were purified using CD4 or CD8 MACS microbeads after Ficol separation and the purified Th or effector CTLs had negligible DC contamination [(22, 33); Supplementory Fig. 1b] DCova-stimulated $CD8^+$ T cells displayed their subset marker (CD8), activation marker (CD69) and effector molecule (perforin), but not IL-7Rα or CD62L [(33); Supplementory Fig. 1a], and secreted IFN-γ (3.55±0.61ng/mL/10^6cells/24hrs) and TNF-α (1.15±0.13ng/mL/10^6cells/24hrs), but not IL-4 or IL-10, suggesting a functional effector CTL phenotype. DCova-stimulated $CD4^+$ T cells displayed their subset marker (CD4) and activation

markers (CD69) [(22); Supplementory Fig. 1a], and secreted IFN-γ (2.1±0.35ng/mL/10^6cells/24hrs) and IL-2 (2.75±0.47ng/mL/10^6cells/24hrs), but not IL-4 and IL-10, confirming a Th1 phenotype.

Initially, we sought to determine the functional consequences of CTL responses upon its interaction with Th, particularly focusing on their survival and development into functional memory. This interaction can either have immunoregulatory (1, 19, 21, 22, 25, 26, 28) or immunopotentiation (11, 22, 28, 31) effects as suggested from previous reports. Here, the equal numbers of effector CTLs with or without Th were adoptively transferred to WT or CD4-deficient mice where Th cells likely provide help or regulate during effector or transition phase of CTL responses (Fig. 1a). Because $CD4^+$ T cell help during priming phase is also shown to be critical for programming memory CTLs (4, 7, 8), we also tested this possibility parallelly in the current model using a simplified method, where naive OTI $CD8^+$ T cells were stimulated with DCova in the presence (allow $CD4^+$ T cell help during priming) or absence of naïve OTII $CD4^+$ T cells. These primarily helped or unhelped effector CTLs were adoptively transferred to WT or CD4-deficient mice (24). Using tetramer assay, all the mice were monitored for CTL survival at different periods, and memory responses after boosting with DCova (Fig. 1b). Primarily helped CTLs significantly survived and expanded upon boosting in both WT and CD4-deficient mice (P<0.05 or 0.01 versus unhelped CTL), corroborating previous observations (4, 5, 7, 8). Interestingly, transitionally helped effector CTLs also significantly survived and expanded both in WT and CD4-deficient mice (P<0.05 or 0.01 versus unhelped CTL) up to 3.0 times from their basal levels. Furthermore, increasing the dose of Th alone from $2x10^6$ to $5x10^6$ resulted in corresponding increase of memory CTL pool in both WT and CD4-deficient mice (P<0.01 versus low Th dose) (Fig. 1c), suggesting the dose of Th might directly influence memory pool generation.

3.5.2 Phenotypic profiling of transitionally helped versus unhelped CTLs during primary and memory stage

Because we observed reduced memory pool without CD4+ T cell help, we compared helped versus unhelped CTL's effector and memory phenotype 6 and 60 days later in peripheral blood in an attempt to correlate with differential survival rates. On day 6, both helped and unhelped CTLs showed almost equal expression of activation, but not memory, markers although IL-7Rα expression was slightly higher in helped CTLs (Fig. 1d). It is possible that a slight increase

in the IL-7Rα expression in the effector stage (day 6) might have played some role in subsequent survival of helped CTLs as IL-7Rα is known to influence CTL survival. On day 60, memory CTLs from both WT and CD4-deficient mice showed almost similar expression of memory-specific marker, including CD62L, CD44 and IL-7Rα (central memory CTL phenotype), but not CD25, suggesting surface markers expression may not significantly contribute to CTL survival.

3.5.3 Naïve or non-specifically stimulated CD4$^+$ T cell help is dispensable for effector CTL survival and recall responses

Next, we assessed the helper roles of other CD4$^+$ T cell types, such as naïve or non-specifically-stimulated cognate or polyclonal CD4$^+$ T cells on effector CTL fates. ConA-stimulated OTII and WT CD4$^+$ T cells represent non-specifically stimulated cognate and polyclonal CD4$^+$ T cells without acquired pMHC I (not shown) (22). Effector CTLs were transferred along with different CD4$^+$ T cell types into Ia$^{b-/-}$ or WT mice, and monitored for their survival and memory responses. Although the persistent presence of polyclonal CD4$^+$ T cells alone supported effector CTL survival and recall responses significantly in WT mice (P<0.01 versus effector CTL alone in Ia$^{b-/-}$), the reconstitution of all CD4$^+$ T cell types, except Th, failed to enhance effector CTL survival in Ia$^{b-/-}$ mice (Fig. 2a), possibly due to their inability to provide help, and poor survival in MHC-II deficiency environment. To confirm this, we tracked adoptively transferred naïve, Th or ConA-activated polyclonal CD4$^+$ CD45.1$^+$ T cells in the periphery of CD45.2$^+$ Ia$^{b-/-}$ mice. Interestingly, although the reconstitution was made substantially, the transferred CD4$^+$ T cells progressively declined, reaching almost 0.05-0.11% on day 60 (Fig. 2b), confirming CD4$^+$ T cells need self-MHC contact for their survival (34). Although Th declined in similar fashion (Fig. 2b), their help during transient contraction phase was sufficient to enhance CTL survival and recall responses (P<0.01 versus effector CTL alone in Ia$^{b-/-}$). These results suggest naïve CD4$^+$ T cell help can act synergistically, but dispensable, for CTL survival and recall responses in the presence of cognate CD4$^+$ T cell help.

3.5.4 Th cells program effector CTL fates directly via acquired pMHC I, and endogenous CD40L and IL-2 signaling

Because Th's helper molecules, CD40L and IL-2, and acquired pMHC I are known to influence CTL priming (22, 35), survival or memory development (4-6, 17, 22), we sought to determine whether these molecular signaling from Th modulate effector CTL fates. From various studies including ours, it is increasingly clear that DC-stimulated T cells acquire non-agonistic bystander pMHC complexes in addition to agonistic ones (22, 24, 25, 28). Acquisition of DC-derived bystander pMHC I by Th can facilitate their direct interaction in an Ag-specific manner, which tempted us to monitor these bystander complexes on DC-stimulated Th. Consistent with previous results (22, 24), we found low to moderate levels of bystander pMHC I molecules during DCova interaction (Fig. 3a). The transfer of bystander pMHC I on Th was likely from DCova as DCova($K^{b-/-}$)-stimulated Th failed to show expression of these molecules. In contrast to Th, the Th($CD40L^{-/-}$) and Th($IL\text{-}2^{-/-}$) respectively fail to show the CD40L expression and IL-2 secretion (Fig. 3a and 3b). Effector CTLs were co-transferred with Th, Th($CD40L^{-/-}$), Th($IL\text{-}2^{-/-}$) or Th(pMHC $I^{-/-}$) into CD4-deficient mice and monitored for survival and memory responses. During memory stage, effector CTL transferred alone or with Th($CD40L^{-/-}$), Th($IL\text{-}2^{-/-}$) or Th(pMHC $I^{-/-}$) significantly failed to survive, and expand upon boosting (P<0.01 versus Th helped CTL) (Fig. 3c). In contrast, the helped memory CTLs expanded up to 2 times from their basal levels, reaching almost 5.8±1.5% of total $CD8^+$ T population.

To further assess the impact of Th's molecular signaling on the functionality of memory CTLs, WT or CD4-deficient mice were adoptively transferred with effector CTLs alone or with Th($CD40L^{-/-}$), Th($IL\text{-}2^{-/-}$) or Th(pMHC $I^{-/-}$) and challenged with highly-metastasizing BL6-10_{OVA} tumor cells 100 days later (Table 1). In line with recall responses, the lungs of all the unhelped mice and those which were transferred with Th(pMHC $I^{-/-}$), Th($CD40L^{-/-}$) or Th($IL\text{-}2^{-/-}$) developed tumor colonies of variable size and numbers considerably compared to mice with transitionally or primarily helped CTLs. In contrast, the lungs of mice which received Th did not show any tumor colonies with the exception of few mice (4/12) which showed considerably low number of tiny colonies. The tumor colonies were further confirmed by histopathology (not shown). To further correlate tumor protection, we tracked OVA-specific IFN-γ^+ CTLs in spleens by intracellular staining. Interestingly, IFN-γ^+ helped CTLs were present in significant proportions (P<0.01 versus

unhelped CTLs) (Fig. 3d) in both WT and CD4-deficient mice. Unhelped memory CTLs, although survived better, almost waned, and completely failed to protect upon lethal tumor challenge.

3.5.5 Poorly surviving unhelped CTLs show differential expression of genes linked with apoptosis

Although both helped and unhelped CTLs showed almost similar memory marker expressions (Fig. 1d), helped CTLs survived considerably compared to unhelped CTLs. We hypothesized that distinct mechanisms under CD4-helper influence might influence CTLs survival. To gain mechanistic insights into enhanced survival of helped CTLs, we compared its mRNA profiles with that of unhelped CTLs. On days 13-18 of adoptive transfer, we found moderate contraction rate in effector CTLs. During this period, we harvested highly purified population (94-96%) of tetramer^{+} helped or unhelped CTLs (Supplementary Fig. 2) from blood and lymphoid organs of WT or CD4-deficient mice, and subjected to apoptosis pathway-focused PCR array, which analyzes TNF ligands and their receptors; members of the Bcl-2, caspase, IAP, TRAF, CARD, death domain, death effector domain, and CIDE families; as well as genes involved in the p53 and ATM pathways. Strikingly, two distinct patterns of gene expression were observed. First, unhelped CTLs generally showed up-regulation of pro-apoptotic and down-regulation of pro-survival genes (Fig. 4a, 4d, 4e; Supplementary Table 2a and 2b). Among up-regulated genes, caspase family genes, *Casp-7, -2* and *-3,* TNF family members, *Fas, FasL* and *Tnfrsf1a*, and *Trp53*, which mediate apoptosis induction, were prominent. Interestingly, 34 genes were found to be significantly down-regulated, of which 24 were pro-survival and 10 were pro-apoptotic genes. Some of the most prominent pro-survival genes down-regulated include *Akt1, Api5, Bag1, Bcl-2, Birc3 and 5, Nfkb1, Nol3, Pak7, Pim3, Traf1,* and *Zc3hc1*. The down-regulation of some pro-apoptotic genes, although surprising, may represent compensatory mechanisms that occur in cells experiencing various stressors to prevent cell death. Second, in contrast to unhelped CTLs, there was a notable shift in the gene-expression profile in helped CTLs, favoring their survival (Fig. 4b-e, Supplementary Table 2a, 2b). Interestingly, a considerable overlap was observed between up- and down-regulated genes in helped CTLs derived from WT and CD4-deficient mice (Fig. 4d and 4e). Among up-regulated genes, *Akt1, Xiap, CD40lg* and *Traf1*, which mediate signals involved apoptosis inhibition, were prominent. Additionally, helped CTLs from Ia$^{b-/-}$ mice exhibited up-regulation of an anti-apoptotic gene, *Dad1*, and pro-apoptotic genes, *Casp-2* and *Dapk1*. Among down-regulated genes, caspase superfamily genes, *Casp-4, -7* and *-3,* TNF superfamily members,

Fas, FasL and *Tnfrsf10b,* and Caspase recruitment domain superfamily member, *Card6,* and *Bcl-10* involved in apoptosis induction were prominent.

3.5.6 Helped CTLs survive better by down-regulating pro-apoptotic and up-regulating pro-survival molecules

To provide key insights into the above gene-expression studies, some of the key genes associated with cell apoptosis or survival, such as *TRAIL, TRAIL-rec, FasL, Caspase-4, Caspase-7, Bcl-10, Bcl-2, Akt1* and *Nfκb1,* were further analyzed individually in helped and unhelped CTLs by qRT-PCR using the same cDNA samples. We found that helped CTLs showed up-regulation of the prosurvival (*Bcl-2, Akt1 and Nfκb1*) and down-regulation of the proapoptotic (*FasL, TRAIL, TRAIL-rec, Bcl-10, Caspase-4 and Caspase-7*) genes (Fig. 4f). To further substantiate the above results, some of the key molecules or their active forms, such as Bcl-10, and caspase-3, and -7, Bcl-2, NF-κB, Akt1, and NFATc1 transcription factor involved in cell survival or apoptosis were analyzed in helped and unhelped CTLs by western blot. Supporting with differential survival and gene-expression profiles, unhelped CTLs showed increased expression of activated NFATc1, Bcl-10, and cleaved caspase-3 and -7, and reduced expression of Bcl-2, and phosphorylated-Akt1 and -NF-κB when compared to helped CTLs (Fig. 5). Although we could not rule out the discrepancy observed in the mRNA and protein levels of NF-κB and Akt1, it possibly represents posttranscriptional and posttranslational modifications that may interfere with direct mRNA to protein translation (36). Nevertheless, these data reveal striking evidence that Th cells rescue effector CTLs from AICD by mediating up-regulation of anti-apoptotic and down-regulation of pro-apoptotic genes, and regulation of various pathways involved in cell death or survival.

3.6 Discussion

Understanding factors derived from $CD4^+$ T cell help involved in the regulation of effector CTL responses is currently one of the most active areas of immunological research. In the last two decades, the role of CD40L and IL-2 signaling by Th1 cells for memory CTL development has been extensively studied, yet the means by which such signaling occurs is not completely known (3, 17). The use of an agonistic antibody to CD40 can substitute $CD4^+$ T cell help for inducing optimal primary CTL responses, which led to a conclusion that Th1 cells might license APC via CD40-40L signaling (3). Alternatively, Tanchot and his colleagues, and our previous works,

suggested that Th1 cells can directly provide CD40L (4, 5, 22) and IL-2 (22) signaling to naïve $CD8^+$ CTL for efficient memory CTL development. A more recent study suggested direct interaction between DC-stimulated memory $CD4^+$ and $CD8^+$ T cells via CD40-CD40L and/or IL-2 signaling for optimal recall responses (16, 37). Moreover, a direct cooperative role between Th1 and effector CTLs has also been frequently reported in the absence of DC (11, 30, 31, 38-40). In this study, we demonstrate that cognate $CD4^+$ T cell help required for effector CTL survival and memory programming is mediated via direct CD40L and IL-2 signaling, and identify various apoptotic pathways and transcription factors involved in CTL survival or apoptosis under helper influence, thus providing strong evidence to recently establishing notion that effector phase can be altered to enhance both quantity and quality of memory CTLs (9, 10).

The acquisition of pMHC I appears to be critical for Th to make efficient contact with and delivery of CD40L and IL-2 signaling to the effector CTLs in Ag-specific manner due to several reasons. First, in the absence of pMHC I, a reduced memory CTL pool exhibiting poor recall functions is observed. Second, the transfer of naïve or non-specifically-stimulated $CD4^+$ T cells without pMHC I to $Ia^{b-/-}$ mice fails to rescue effector CTLs even though they are TCR-specific in nature. Finally, the results in this study well support recent emerging evidence that Th cells substantially rescue effector CTLs from AICD and promote functional memory generation (6, 11, 39). Perhaps, even in three-cell interactions, Th cells, after detaching from DCs, might directly act on CTLs in the vicinity due to pMHC I and TCR avidity.

The functional consequences of trogocytosis could vary depending on the situations either to prevent self-tissue damage or to protect host from invading pathogens (1, 22, 23). We previously demonstrated a 7-fold Ag-specific targeting power of T cell suppression between $CD4^+$ Tr1 with acquired pMHC I and $CD4^+$ ($K^{b-/-}$)Tr1 without acquired pMHC I (35). Mostbock *et al.* (21) also showed memory CTL with acquired pMHC-1 complexes up-regulate *Caspase 3, bcl-X, bak* and *bax*, leading to apoptosis. Cox *et al.* (25) also demonstrated that Th with acquired bystander pMHC I complexes from APCs became susceptible to CTL killing in an Ag-specific manner, perhaps due to brief period of culturing, and the use of nonprofessional, MHCII-transfected 293T APCs, which provide poor costimulation, and non-specifically-stimulated $CD4^+$ T clones to generate Th cells. In contrast, here we use highly mature, professional APCs to stimulate TCR-specific $CD4^+$ T cells for prolonged period (3 days in culture) to generate Th cells. Nevertheless, although Th cells, with acquired pMHC I and -II complexes, positively modulate

effector CTL responses, they become susceptible to killing by other Th cells (fratricide) (22), supporting previous observations (1, 21, 26). Hence, these results provide convincing evidence that culturing conditions, extent of stimuli, and nature and type of immune cells might determine outcome of trogocytosis in the immune system.

The gene-expression profiling together with our Western blot results provide mechanistic insights into the understanding of $CD4^+$ T cell helper functions in CTL fates. The Fas-FasL–mediated pathway appears to be the key mediator in the contraction of adoptively transferred effector CTLs. Up-regulation of *Fas* and *FasL* along with various down-stream signaling molecules, such as *Tnfrsf1a*, *Casp-2, -3* and *-7,* are known to mediate apoptosis by activation of caspase-8 and -3 (41), consisting with the present results. Furthermore, unhelped effector CTLs also show enhanced expression of NFATc1 transcription factor, a key regulator of FasL expression (42), further confirming Fas-FasL pathway in AICD. Interestingly, the helped CTLs show down-regulation of various pro-apoptotic genes, which mediates not only FasL-, but also Trail-apoptotic pathways. These pathways mediate extrinsic-apoptotic pathway that are known to cause exhaustion of unhelped CTLs during secondary responses following re-stimulation (7, 43). Possibly due to these reasons, unhelped, but not helped, IFN-γ^+ CTLs are almost absent in tumor-challenged mice, irrespective of polyclonal $CD4^+$ T cell help, suggesting cognate $CD4^+$ T cell helper signals during the constraction phase can rescue memory CTLs from AICD upon re-stimulation with Ag.

Th cells also appear to mediate CTL survival by secreting its survival factors, such as IL-2, and by providing its costimulations necessary for survival gene expression. Recently, cognate Th cells through secretion of IFN-γ and chemokines were suggested in mobilizing effector CTLs, after their differentiation, into infected tissues (40). Perhaps these factors, together with pMHC I and TCR interactions with strong affinity, greatly favor Th's ability to deliver CD40L and IL-2 signaling directly and efficiently to effector CTLs *in vivo*. Interestingly, unhelped CTL's poor survival correlated well with significant down-regulation of key survival genes, which mediate various signals both at nuclear and cytoplasmic levels required for activation of pro-survival NF-κB and attenuation of pro-apoptotic JNK pathways (44). Consistently, helped CTLs showed decreased Bcl10 expression and increased Akt1 and NF-κB activation, which known to mediate transcription of various survival genes (44, 45). TCR together with CD28 and/or IL-2 signaling enhance T cell survival via Akt-mediated NF-κB activation and c-jun down-modulation (44, 46).

In addition to pMHC I, IL-2 and CD40L signaling, Th cells, with acquired CD80 (22, 23), might mediate CD28 signaling as unhelped, but not helped, CTLs show striking down-regulation and decreased activation of Akt1 and NF-κB. Interestingly, up-regulation of survival genes is not a feature of helped effector CTLs; however, their mRNA profiles are almost similar to those of naïve OTI $CD8^+$ T cells predominated by survival genes. The few up-regulated genes above naïve cell levels, such as *Akt1, Xiap, CD40lg* and *Traf1* genes, may play leading role in effector CTL survival.

Although cognate $CD4^+$ T cell help during priming is shown to be required for memory programming (4, 7, 8), the present results indicate that such help most likely occurs after $CD4^+$ T cells polarized to Th following APC stimulation where they acquire ability to secrete IL-2 and express CD40L. In support of this, our previous and present results showed that Th, after detaching from DCs, can directly stimulate naïve transgenic or endogenous $CD8^+$ T cells (22, 24), and effector or memory CTLs (30, 31). In acute infection, Sun *et al.*(47) showed that naïve, polyclonal, but not cognate, $CD4^+$ T cell help, are required to orchestrate size and quality of memory CTLs. In their study, effector CTLs are generated *in vitro* after increasing the precursor frequency by more than 50 fold of physiological levels, which likely preclude cognate $CD4^+$ T cell helper requirements for memory programming (48), while polyclonal $CD4^+$ T cell help still supporting the maintenance of memory CTLs. Our study indicates that non-specific $CD4^+$ T cell help, although enhance CTL survival and memory pool, cannot replace cognate Th's help in improving the quality of memories (Fig. 6) as memory CTLs developed under polyclonal $CD4^+$ environment, although survived better, completely failed in producing functional IFN-γ^+ CTLs, and waned following tumor challenge. Recently, de Goer de Herve *et al* demonstrated that hetero-specific Th cells can also rescue effector CTL from AICD, and promote recall responses by direct contact via CD40L and IL-2 signaling (16). In contrast, by detailed analysis, here we show that acquired pMHC I on Th are required to make direct contact in Ag-specific manner, and to deliver CD40L and IL-2 signaling efficiently, thus supporting previously well-appreciated results (6, 12-14, 39, 40). Perhaps, the discrepancy seen in de Goer de Herve's report and current study may be derived from quantitative differences in the requirement of $CD4^+$ T cells although CD40L and IL-2 signaling is commonly reported.

Based upon the present and previous supportive observations (4-6, 11, 12, 17, 22, 31, 38, 39, 49), we argue that Th interactions with effector CTL might be critical to generate functional

memory CTL (Fig. 6). Both $CD4^+$ and $CD8^+$ T cells follow asymmetric immune responses and require different activation signals (50). In contrast to $CD8^+$ T cells, $CD4^+$ T cells need to contact with DC multiple times (Ag persistence) to undergo activation and differentiation (51). As a result, Th cells might acquire sufficient bystander pMHC I, which not only make them undergo exhaustion by fratricide killing (1, 21, 22, 26), but also permit surviving Th cells to precisely target effector CTL pool. During the peak effector phase, a majority of the effector CTL which miss Th cell contact due to their predominance undergo death by fratricide mechanism (11, 52). As a result, only a fraction of the effector CTL pool that receives helper signals might survive and develop into a functional memory pool. In support of this hypothesis, we observed a dose-dependent increase of the memory CTL pool with Th increase. Williams and Bevan (49), after shortening infectious period of recombinant *Listeria monocytogenes*, observed diminished primary $CD4^+$ and memory CTL differentiation although primary CTL expansion was unaltered. Our study could explain these observations wherein insufficient Th generation due to shortened infectious period might result in poor signaling of effector CTL pool and hence poor memory differentiation. Indeed, during the effector phase, the transfer of Th, but not naïve OTII $CD4^+$ T cells into AdVova-immunized $Ia^{b-/-}$ mice greatly enhanced effector CTL survival and memory differentiation (Unpublished, Umeshappa CS and Xiang J). Whether pMHC I acquisition by Th for memory programming is common phenomenon in immunity involving to variety of Ags, including pathogen-derived and altered self-Ags, needs further detailed study.

Taken together, our results reveal a previously unexplored mechanistic role of Th in programming effector CTL responses. To date, adoptive CTL immunotherapy for cancer using *in-vitro* expanded tumor-infiltrating $CD8^+$ T cells has achieved some degree of success (13, 53). However, one of the major obstacles in this therapy is their poor survival and development of corrupted memories (13, 53) due to AICD and CD4-deficient environment associated with many primary therapies. In the present study, unhelped CTL's poor survival and their incomplete protection against lethal tumor challenge re-emphasize the importance of using Th in adoptive CTL therapy. Finally, this form of Th-effector CTL cooperation could explain why memory CTLs generated under cognate Th help show survival and recall advantages (4-8, 11, 12, 17), a phenomenon frequently observed in cancers, and could aid in development of efficient vaccines against cancers, perhaps by inclusion of T helper epitopes in the therapy.

3.7 Acknowledgments

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3.8 Disclosures

The authors have no financial conflict of interest

3. 9 Tables

Table 1 Adoptive transfer of Th with effector CTLs leads to functional memory CTL development and protection against lethal tumor challenge.

Mice[a]	Adoptive transfer	% tumor-bearing mice	Tumor grading
WT	PBS	10/10 (100)	(++++++)
	Effector CTL	12/12 (100)	(+)
	Effector CTL(*In vitro* helped)	00/12 (0)	0
	Effector CTL+Th	00/12 (0)	0
$Ia^{b-/-}$	PBS	10/10 (100)	(++++++)
	Effector CTL	12/12 (100)	(++)
	Effector CTL(*In vitro* helped)	05/12 (42)	(+/-)
	Effector CTL+Th	04/12 (33)	(+/-)
	Effector CTL+Th($CD40L^{-/-}$)	12/12 (100)	(+)
	Effector CTL+Th($IL\text{-}2^{-/-}$)	12/12 (100)	(++)
	Effector CTL+Th($pMHC\text{-}1^{-/-}$)	12/12 (100)	(+)

[a]Approximately $5x10^6$ effector CTLs were i.v. transferred with or without Th, Th($IL\text{-}2^{-/-}$), Th($CD40L^{-/-}$) or Th(pMHC $I^{-/-}$) cells ($2x10^6$each) to WT or $Ia^{b-/-}$ mice as indicated. After 100 days, all the mice were challenged i.v. with highly metastasizing BL6-10_{OVA} tumor cells. The mice were sacrificed on 24^{th} day of challenge, and the numbers of metastasized tumor colonies in lungs were counted and graded depending on numbers of metastatic tumor colonies. The data are cumulative of three independent experiments with three to four mice per group.

Table S1, related to Figure 4

Primers	Tm	Sequence (5' – 3')	GeneBank ID
Akt1	58.0	F - CTTCTATGGTGCGGAGATTGTG	NM_009652
	59.0	R - CCCGGTACACCACGTTCTTC	
Bcl-10	59.0	F - GAAAGCTGCCGACACACTCA	NM_009740
	59.0	R - CCCGACGGCTTCTCAGAAC	
Casp-4	60.0	F - CAATGGCCGTACACGAAAGG	NM_007609
	58.0	R - GCCCCATACCTCAGTGAGAGAT	
Casp-7	59.0	F - CCACCAGCGCCTTATAATTCC	NM_007611
	58.0	R - ATGGTCCCTAGGCCCTCACT	
Nfkb1	59.0	F - CCAGCTTCCGTGTTTGTTCAG	NM_008689
	60.0	R - TCAGGGTAGTAGAGAAAGGGTTTCG	
Trail-rec (Tnfrsf10b)	59.0	F - GGGCCTCACAGACAATCAAATC	NM_020275
	60.0	R - GCCTCACGTGTGACCAGTGT	

Table S2A, related to Figure 4. *Top genes uniquely up-regulated above three fold*

Gene Symbol	Fold Regulation	Key functions
Helped CTL in CD4-sufficient mice		
Akt1	4.212	Inhibit apoptosis via phosphorylation of components of apoptosis pathway
Xiap	9.412	Inhibit apoptosis via inhibition of caspase 3, 7 and 9
Prdx2	3.462	Antioxidant protective role in cells
Cd40lg	6.537	Expressed on activated T cells, exerts diverse effects depending on type of cells involved
Traf1	3.838	Activates MAPK8/JNK and NF-κB and mediates anti-apoptotic signals by inhibiting inhibitor-of-apoptosis proteins
Helped CTL in CD4-deficient mice		
Akt1	3.524	Inhibit apoptosis via phosphorylation of components of apoptosis pathway
Xiap	3.164	Inhibit apoptosis via inhibition of caspase 3, 7 and 9
Casp2	6.674	Induce apoptosis by associating with several pro-apoptotic proteins
Dad1	9.983	Inhibits apoptosis possibly by interacting with Mcl-1 (a bcl-2 family member) (54)
Dapk1	3.013	Positive mediator of IFN-γ-induced programmed cell death
Cd40lg	6.215	Expressed on active T cells, exerts diverse effects depending on type of cells involved
Traf1	4.424	Activates MAPK8/JNK and NF-κB and mediates anti-apoptotic signals by inhibiting inhibitor-of-apoptosis proteins
Unhelped CTL in CD4-deficient mice		
Casp2	7.114	Induce apoptosis by associating with several pro-apoptotic proteins
Casp3	3.246	Mediates apoptosis in both extrinsic (death ligand) and intrinsic (mitochondrial) pathways (41)
Casp7	9.764	Executioner protein of apoptosis
Fas (CD95)	3.275	Induces apoptosis on binding by FasL.
FasL (CD95L)	4.056	Induce apoptosis by binding to Fas receptor (Regulate immune system via inducing apoptosis)
Tnfrsf1a	3.193	Activate transcription factor NF-κB, mediate apoptosis, and function as a regulator of inflammation.
Trp53 (p53)	3.014	Mediate apoptosis following activation by myriad of stressors

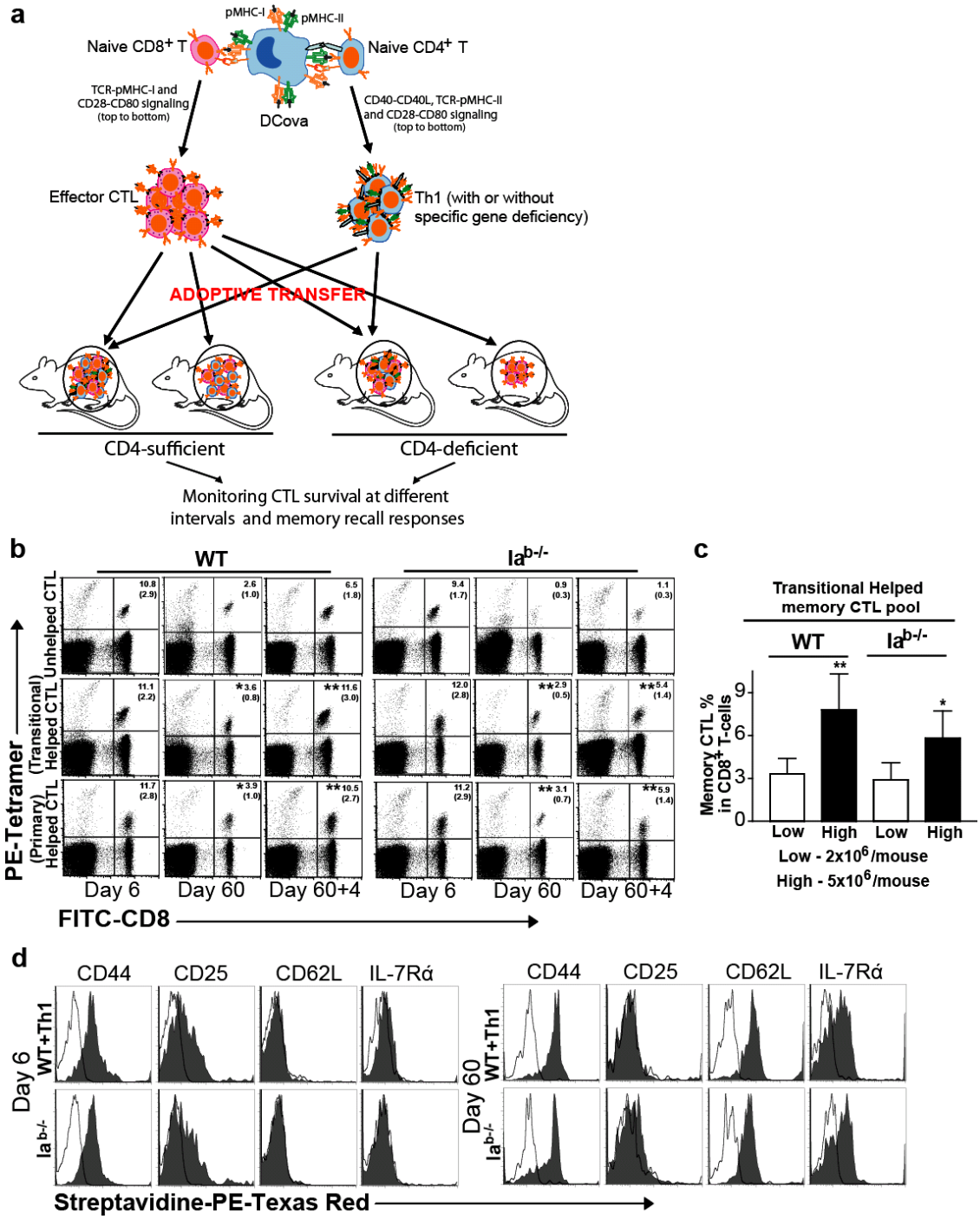
Table S2B, related to Figure 4. *Top genes uniquely down-regulated below three fold*

Gene Symbol	Fold Regulation	Key functions
Helped CTL in CD4-sufficient mice		
Bcl10	-14.122	Interact with CARD domain containing proteins including CARD9, 10, 11 and 14, and mediate apoptosis via NF-κB activation (45)
Card10	-8.342	Interact with Bcl10 and activate NF-κB to induce apoptosis (45)
Card6	-7.297	Interacts with Cardiac and Nod1 and specifically impairs their ability to induce the transcription factor NF-κB (55)
Casp3	-5.025	Mediates both extrinsic (death ligand) and intrinsic (mitochondrial) apoptotic pathways(41)
Casp4	-59.804	Inflammatory caspase, implicated in endoplasmic-reticulum stress-induced apoptosis
Casp7	-11.942	Executioner protein of apoptosis
Fas	-5.964	Induces apoptosis upon binding by FasL (11)
FasL	-13.781	Induce apoptosis by binding to Fas receptor (Immune regulation) (11)
Pycard	-10.111	Involved in inflammatory and cell death pathways in concert with Caspase-1
Tnfrsf10b (Trail-rec)	-7.399	Mediate apoptosis following activation by TRAIL (7)
Tnfrsf11b	-3.017	Exhibit paracrine survival functions on cells (56)
Helped CTL in CD4-deficient mice		
Bcl10	-3.059	Interact with CARD domain containing proteins including CARD9, 10, 11 and 14, and mediate apoptosis via NF-kappaB activation (45)
Birc5	3.01	Prevent apoptosis by inhibiting Caspase activation
Card6	-3.708	Interacts with Cardiak and Nod1 and specifically impairs their ability to induce the transcription factor NF-κB (55)
Casp3	-5.231	Mediates apoptosis in both extrinsic (death ligand) and intrinsic (mitochondrial) pathways (41)
Fas	-3.914	Induces apoptosis on binding by FasL (11)
FasL	-3.681	Induce apoptosis by binding to Fas receptor (Regulate immune system) (11)
Unhelped CTL in CD4-deficient mice		
Akt1	-14.993	Inhibit apoptosis via phosphorylation of components of apoptotic pathway (44, 46)
Api5	-9.923	Promote cell survival by inhibiting apoptosis
Atf5	-3.695	Promote cell survival by inhibiting apoptosis
Bad	-4.491	Involved in initiation of apoptosis
Bag1	-7.675	Enhances the anti-apoptotic effects of BCL2 and represents a link between growth factor receptors and anti-apoptotic mechanisms.
Bcl2	-11.071	Prevent apoptosis (57)

Bcl2l1	-10.706	Prevent apoptosis by controlling the production of reactive oxygen species and release of cytochrome C by mitochondria
Bcl2l2	-8.651	Prevent apoptosis
Birc3	-9.627	Prevent apoptosis by interfering with caspases activation
Birc5	-17.175	Prevent apoptosis by inhibiting Caspase activation
Bnip2	-5.851	Prevents apoptosis
Bnip3	-4.653	Exhibits pro-apoptotic functions
Bnip3l	-19.677	Promotes cell-death
Bok	-7.359	Pro-apoptotic molecule involved in regulation of cell cycle
Nod1	-5.493	Involved in triggering innate immune response that drives development of adaptive immune responses
Casp1	-3.262	Inflammatory caspsae involved in triggering apoptosis owing to pro-inflammatory cytokines
Cidea	-3.747	Involved in activation of apoptosis
Cideb	-3.602	Involved in activation of apoptosis
Dapk1	-3.833	Positively mediate IFN-γ induced programmed cell death
Ltbr	-3.132	Mediate apoptosis; involved in development and organization of lymphoid tissue and transformed cells
Mcl1	-7.914	A critical anti-apoptotic factor for the survival of T cells at multiple stages *in vivo*
Nfkb1	-28.496	Anti-apoptotic to T cells. MKP-1 is a NF-kappaB-mediated prosurvival effector in attenuating JNK-mediated pro-apoptotic response (44, 46)
Nol3	-9.897	Prevents apoptosis
Pak7	-7.727	Regulation of cytoskeletal dynamics, proliferation, and cell survival signalings
Pim2	-9.293	Promote the growth and survival of nontransformed hematopoietic cells
Polb	-4.186	Performs base excision repair (BER) required for DNA maintenance, replication, recombination, and drug resistance (anti-apoptosis)
Rnf7	-8.467	Anti-apoptosis (antioxidant)
Cd40	-4.395	Immunomodulation - exhibit diverse functions
Tnfsf12	-4.526	Known to mediate both apoptosis induction and suppression
Cd70	-6.649	CD27/CD70 interactions at the T cell/DC interface prime CD8(+) T cells to become tumor-eradicating cytolytic effectors and memory cells
Traf1	-18.465	Activates MAPK8/JNK and NF-κB and mediates anti-apoptotic signals by inhibiting inhibitor-of-apoptosis proteins (44)
Traf3	-4.016	TRAF3 potently suppresses canonical (p50-dependent) NF-κB activation and gene expression *in vitro* and *in vivo* (anti-apoptosis) (44)
Trp73 (p53)	-3.988	Induce apoptosis in mammalian cells (58)
Zc3hc1	-10.101	Anti-apoptotic role in NPM-ALK-mediated signaling events

3.10 Figures

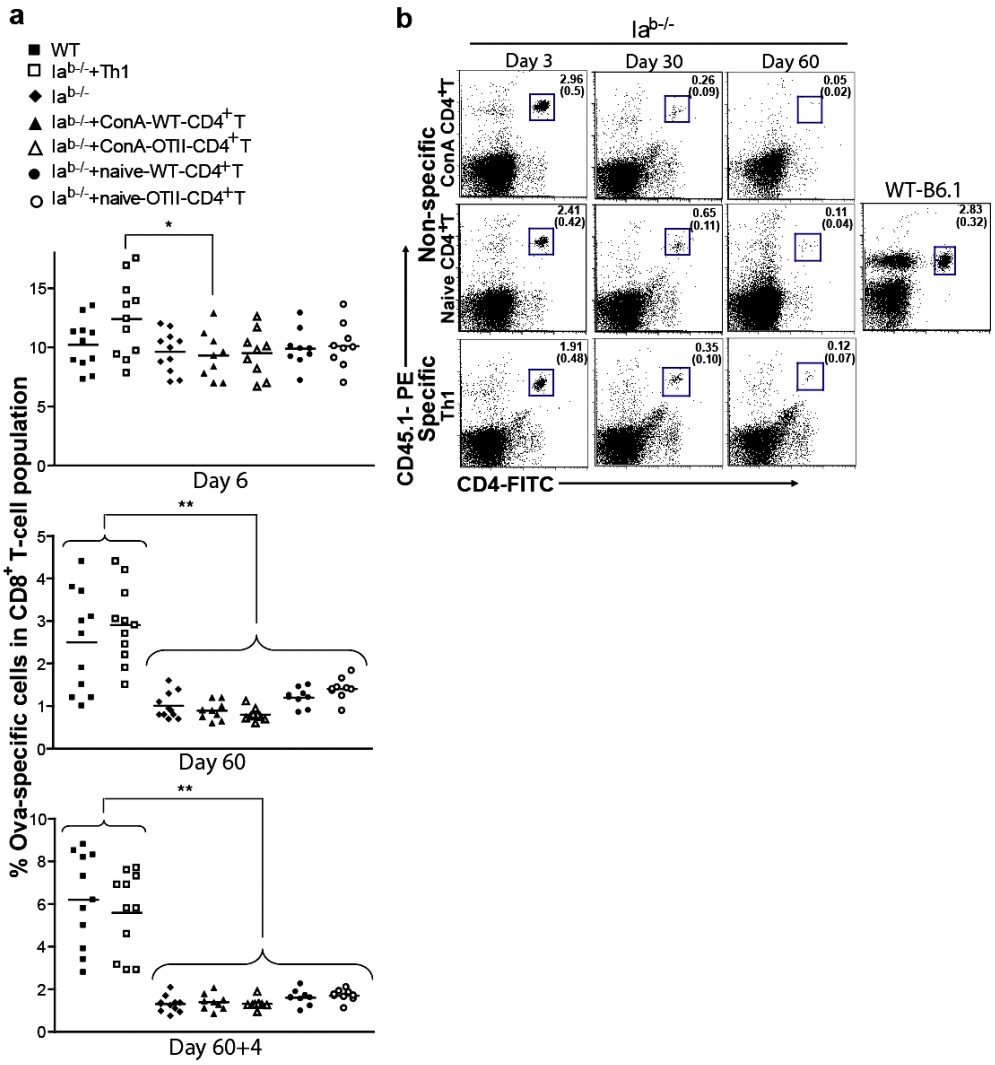
Figure 1



Th's help during priming or transitional period enhance effector CTL survival and transition to memory development. (a) Experimental Design. To determine direct impact of Th on effector CTL fates, effector CTLs and Th with or without specific gene deficiency were

adoptively transferred to WT or CD4-deficient mice**.** All these mice were monitored for CTL survival at different intervals and memory recall responses by boosting with DCova or challenging with highly metastasizing BL6-10_{OVA}. (b) Approximately $5x10^6$ OVA-specific transitionally helped effector CTLs with ($2x10^6$) or without Th were adoptively co-transferred to WT or CD4-deficient mice and monitored 6 and 60 days later in the peripheral blood by tetramer assay. OVA-specific effector CTLs generated in presence or absence of cognate $CD4^+$ T cells during priming (primary help) were also transferred and analyzed. On 60^{th} day, all the groups were boosted with $1x10^6$ DCova and monitored for memory CTL expansion after 4 days. The values represent mean%±(SD) of $tetramer^+$ CTLs in total $CD8^+$ T cell population, and are cumulative of three independent studies with three to five mice per group. * or **, $p < 0.05$ or 0.01, respectively, versus unhelped CTLs. (c) Influence of Th's dose on effector CTL survival and memory CTL development. Approximately $5x10^6$ effector CTLs with $2x10^6$ (low) or $5x10^6$ (high) Th cells were adoptively transferred to WT or CD4-deficient mice and monitored 60 days later in peripheral blood. The values represent mean%±SD of $tetramer^+$ CTLs in total $CD8^+$ T cell population, and are cumulative of two independent studies with four to five mice per group. * or **, $p < 0.05$ or 0.01, respectively, versus low Th dose. (d) The blood samples from WT mice which were transferred with effector CTLs and Th cells or from CD4-deficient mice which were transferred with effector CTLs alone were collected 6 and 60 days later. The samples were stained with H-$2K^b/OVA_{257-264}$ tetramer and FITC-anti-CD8 Ab along with panel of biotin-conjugated Abs specific for effector or memory markers, and streptavidin-PE-Texas Red. The marker expressions (histogram grey filled overlays) were analyzed in $tetramer^+$- and $CD8^+$-specific double population. Irrelevant isotype-matched Abs were used as control (dotted thin lines). One representative of the two independent experiments is shown.

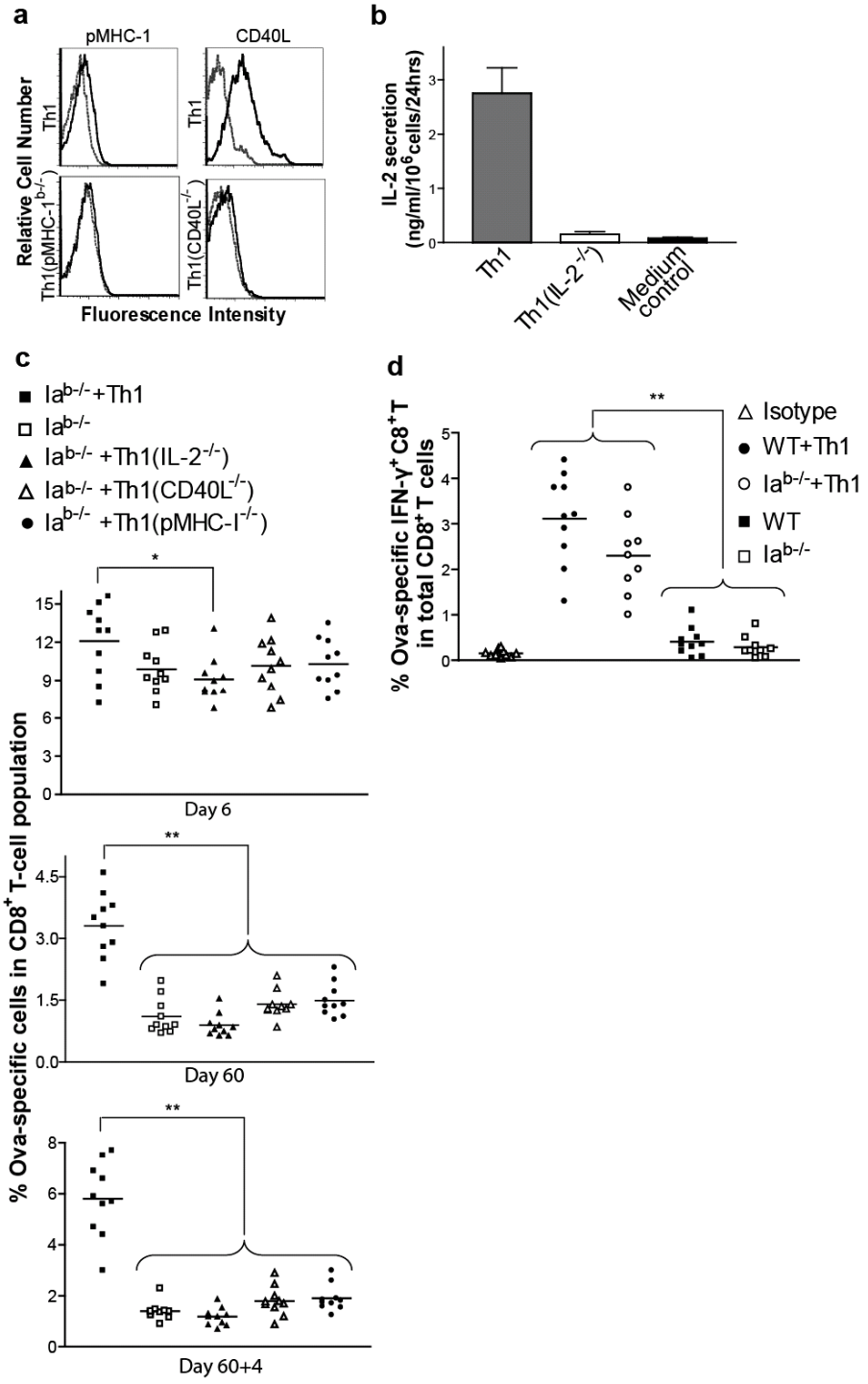
Figure 2



Nature of CD4$^+$ T cell help for CTL survival and recall responses. (a) Naïve or non-specifically-stimulated CD4$^+$ T cell help is dispensable for CTL survival and recall responses. Approximately 5x10^6 effector CTLs were i.v. transferred along with naïve-monoclonal or -polyclonal (15x10^6) or ConA-stimulated-monoclonal or -polyclonal (2x10^6) CD4$^+$ T cells, or Th cells (2x10^6) as indicated to WT or CD4-deficient mice and analyzed for survival 6 and 60 days later, and recall responses on 4th day of boosting with DCova (1x10^6). The values represent frequencies of tetramer$^+$ CTL in total CD8$^+$ T cell population, and are cumulative of three independent studies with three to four mice per group. The horizontal bars indicate means. * or **, $p < 0.05$ or 0.01, respectively, versus WT or Th-helped CTLs in Ia$^{b-/-}$. (b) Poor survival of CD4$^+$ T cells in CD4-deficient mice. Naïve (15x10^6), ConA-stimulated (10x10^6)-polyclonal or DCova-

stimulated Th cells on CD45.1^{+} background were i.v. transferred to congenic CD4-deficient mice and analyzed 3, 30 and 60 days later by flow cytometry. One representative figure from each group is shown in dot plot. The values represent mean%±SD of tetramer^{+} CTLs in total CD8^{+} T cell population, and are cumulative of two independent studies with two to three mice per group.

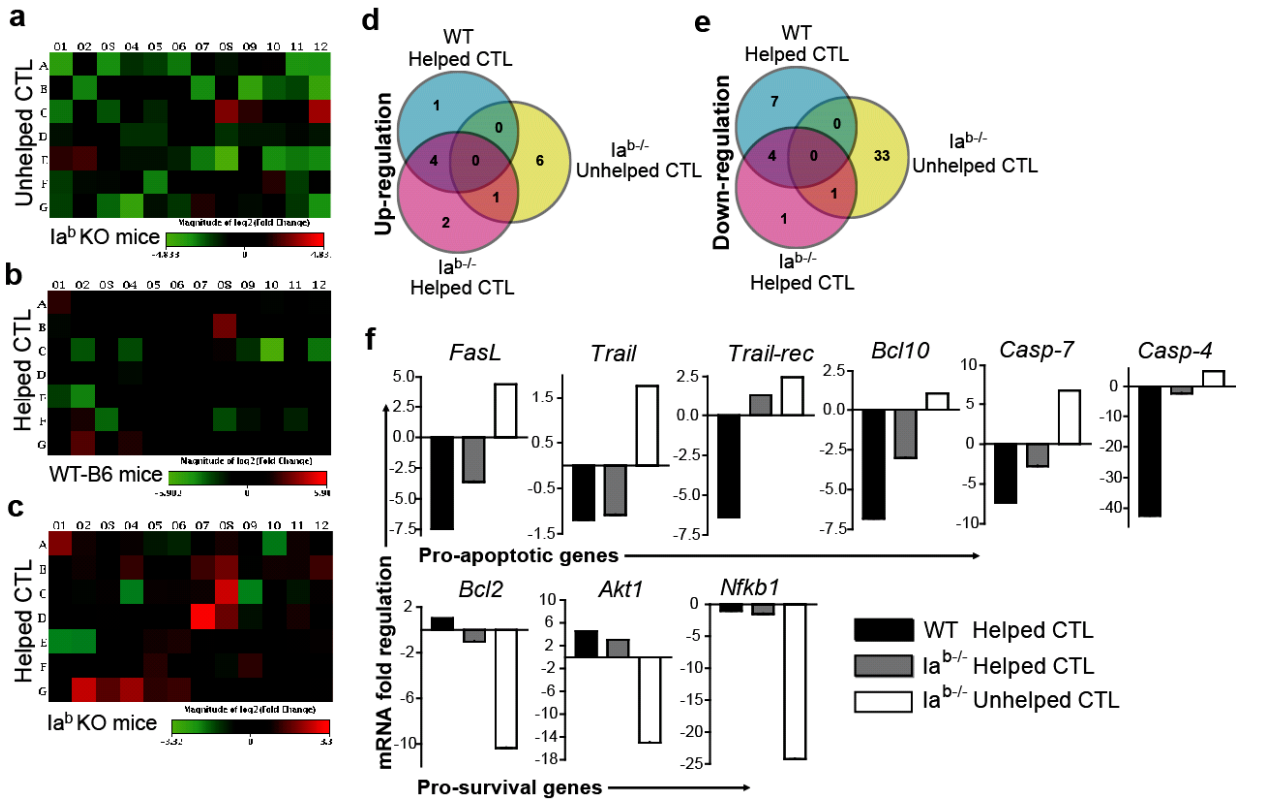
Figure 3



Molecular mechanisms of cognate Th's help for effector CTL survival and recall responses. (a) DCova($K^{b-/-}$)-stimulated Th(pMHC $I^{-/-}$) with anti-pMHC I Ab and DCova-stimulated Th($CD40L^{-/-}$) with anti-CD40L Ab (solid thick lines; bottom panels) were stained, keeping

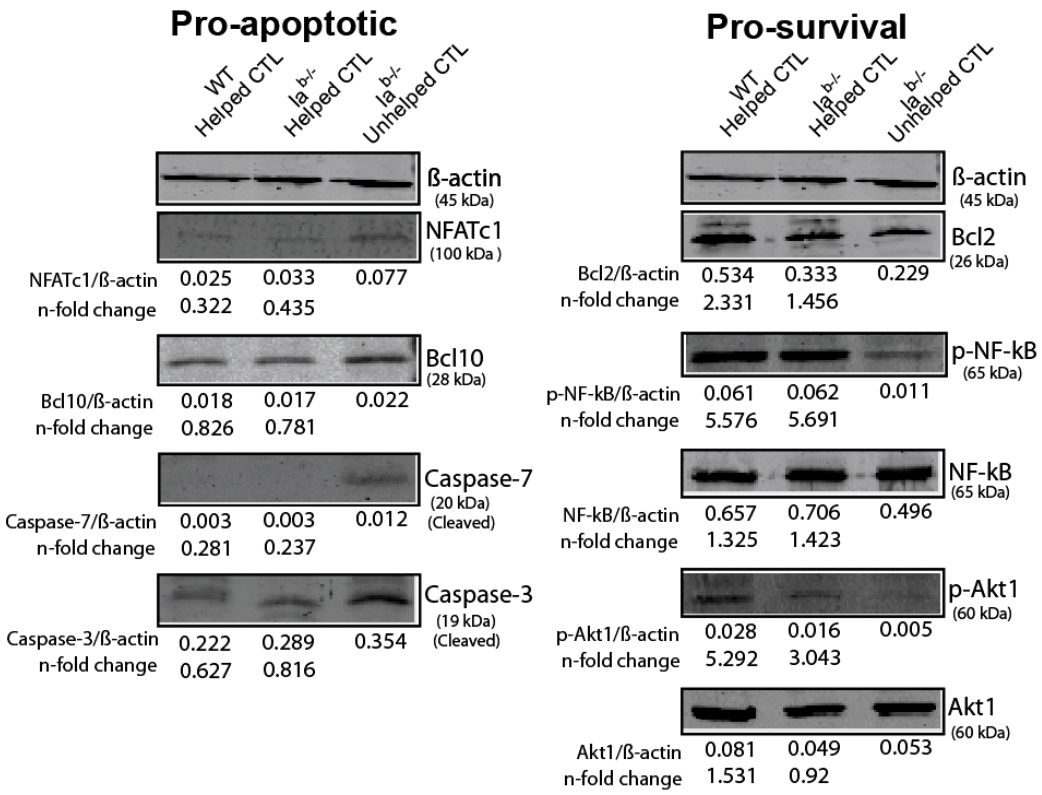
DCova-stimulated Th as positive control (solid thick lines; top panel) and analyzed by flow cytometry. One representative of the three independent experiments is shown. Irrelevant isotype-matched Abs were used as control (dotted thin lines) in the above experiments. (b) Both Th and Th(IL-$2^{-/-}$) were also re-stimulated with $OVA_{323-329}$-pusled LB27 cells and the extent of IL-2 secretion were analyzed in supernatants. The values represent mean%±SD of tetramer$^+$ CTLs in total $CD8^+$ T cell population, and are cumulative of two independent studies with four to five mice per group. (c) Approximately $5x10^6$ effector CTLs were i.v. transferred with or without Th, Th(CD40L$^{-/-}$), Th(IL-$2^{-/-}$) or Th(pMHC $I^{-/-}$) ($2x10^6$ each) to CD4-deficient mice, and analyzed for survival 6 and 60 days later, and recall responses on 4^{th} day of boosting. The values represent frequencies of tetramer$^+$ CTLs in total $CD8^+$ T cell population, and are cumulative of three independent studies with three to four mice per group. The horizontal bars indicate means. * or **, p <0.05 or 0.01, respectively, versus Th-helped CTLs in $Ia^{b-/-}$. (d) OVA-specific helped IFN-γ^+ CTLs infiltrate efficiently in tumor-challenged mice. Approximately $5x10^6$ effector CTLs with or without Th cells ($2x10^6$) were adoptively co-transferred to CD4-sufficient or -deficient mice. On 100^{th} day, all the groups were challenged with BL6-10_{OVA} tumor cells, and monitored for tumor colonies development. On 24^{th} day, the spleen samples were analyzed by intracellular IFN-γ^+ staining. The data represent cumulative frequencies of Ova-specific IFN-γ^+ CTL in total CD8 T cell population and are derived from three independent experiments with two to four mice per group. **, $p < 0.01$ versus unhelped CTLs in $Ia^{b-/-}$.

Figure 4



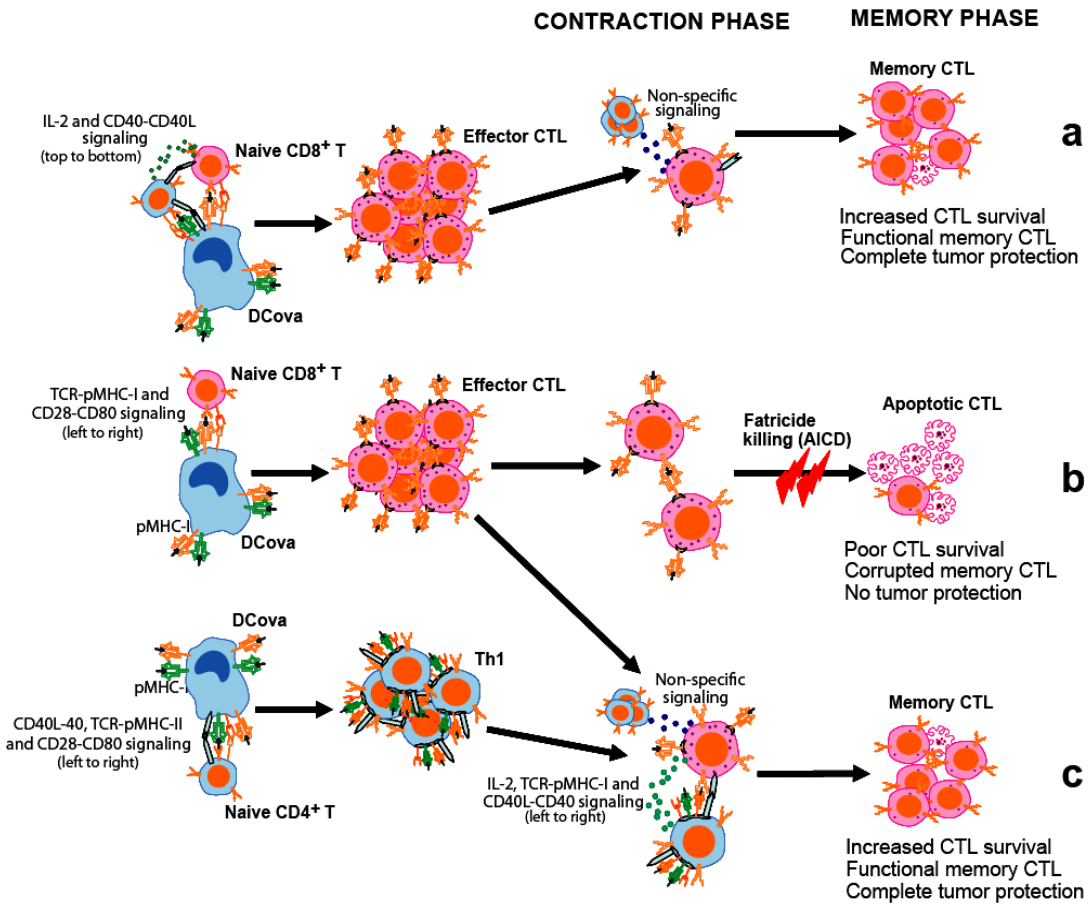
The provision of Th help causes distinct changes in expression of genes that regulate apoptosis, favoring effector CTL survival. (a-c) Apoptosis pathway-focused gene expression in helped versus unhelped CTLs. Total RNA from purified population of helped or unhelped CTLs was isolated, reverse transcribed to cDNA, and subjected to PCR array. Heat map showing relative gene expression in helped or unhelped CTLs where intensity of color towards red indicates up-regulation and green indicates down-regulation. One representative figure from each group of two independent experiments, each run on duplicates using pooled cDNA samples derived from two to four mice per group, is shown. (d-e) Statistically significant genes (a three-fold mRNA difference compared to naïve OTI $CD8^+$ T cells) that were up- (top) or down-regulated (bottom) and their overlapping between helped or unhelped CTLs purified from CD4-sufficient or -deficient mice are shown as indicated. See Table S2A and S2B for gene-expression data. (f) Validation of PCR array results by qRT-PCR. cDNA samples of helped or unhelped CTLs used for PCR array were further subjected to qRT-PCR using SYBR Green detection protocol. The genes up- or down-regulated in helped or unhelped CTLs purified from CD4-sufficient or -deficient mice are shown as indicated.

Figure 5



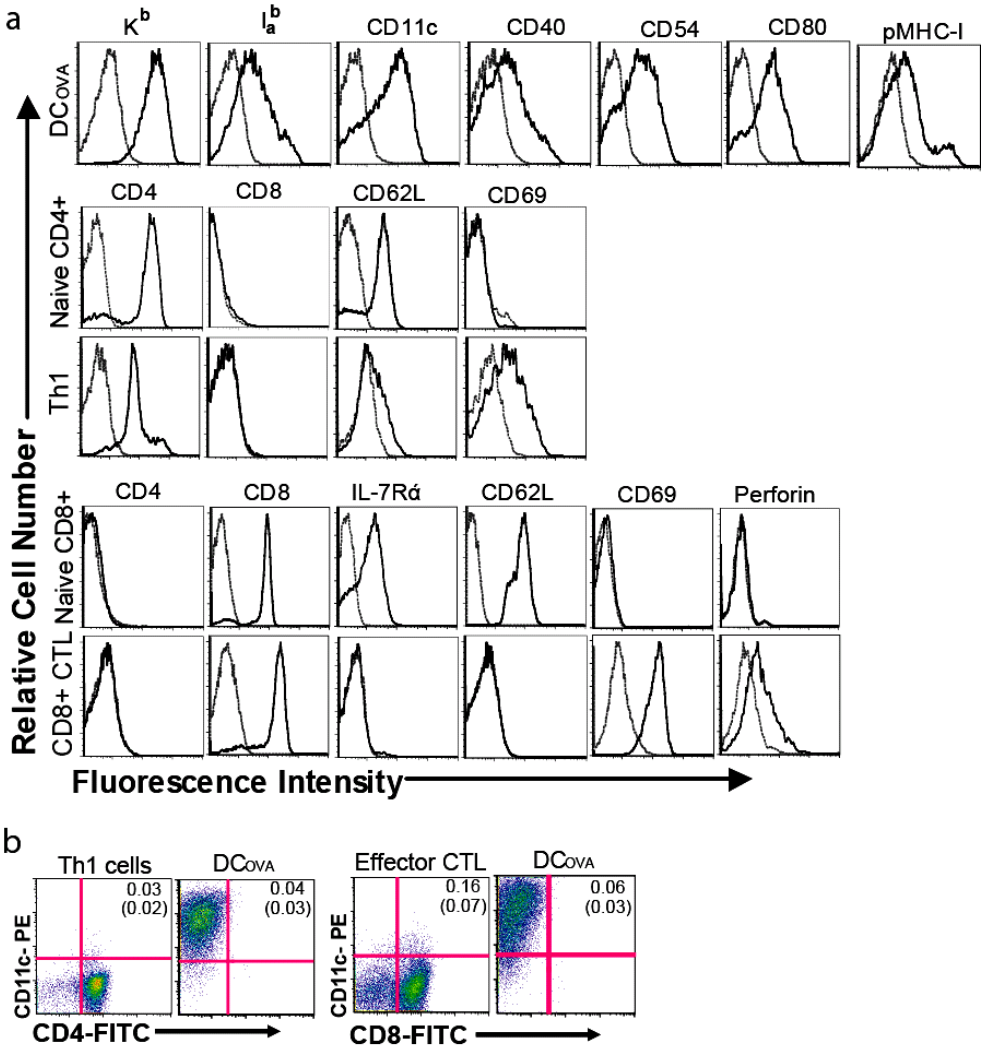
Immunoblot analysis of pro-survival and pro-apoptotic protein expressions or phosphorylations. The proteins in helped or unhelped CTL lysates were separated by SDS-PAGE, and the protein bands were transferred to nitrocellulose membrane blots. The blots were then stained with panel of Abs specific for β-actin, Bcl-2, Bcl10, Akt1, NF-κB-p65, phosphorylated-Akt1 and -NF-κB-p65, cleaved Caspase-3 and -7, or NFATc1 transcription factor and analyzed in ODYSSEY densitometer. Densitometric values normalized to β-actin expression as well as n-fold changes in normalized target molecule expression in the helped CTLs purified from WT or CD4-deficient mice are shown below the corresponding lanes. Data are mean derived from triplicate values and are representative of four to six mice in each group pooled from two independent experiments.

Figure 6



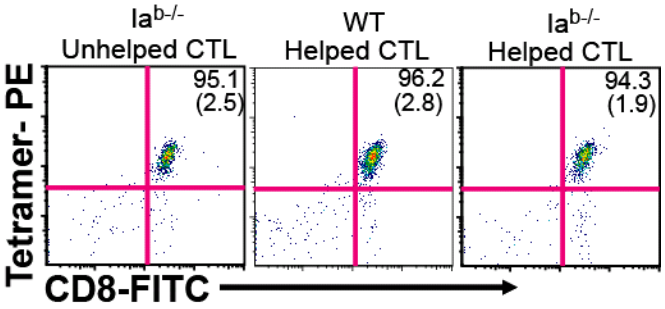
Fate of adoptively transferred effector CTLs under the influence of Th cells and/or naïve CD4$^+$ T cells. (a) Specific signals such as pMHC I, CD40L and IL-2 from Th cells alone program effector CTLs significantly for both survival and functional memory development. (b) Without specific or non-specific signals, effector CTLs survive poorly, possibly due to fratricide killing, and develop into defective memory. (c) Upon adoptive transfer, effector CTLs receive specific signals such as pMHC I (signal 1), CD40L (signal 2) and IL-2 from Th cells, and survive significantly. Non-specific signals from naïve, polyclonal CD4$^+$ T cells could also provide synergistic help for the survival. Furthermore, these signals in concert program development of fully functional memory CTLs.

Supplementary Figure 1



Phenotypic characterization of DCova, Th cells and effector CTLs. (a) DCova, naïve and DCova-stimulated $CD4^+$ (Th) and $CD8^+$ (effector CTL) T cells (solid thick lines) were stained with a panel of Abs specific for cell types, acquired pMHC I, and naïve, activation/maturation or memory status as indicated, and analyzed by flow cytometry. One representative of the two independent experiments is shown. (b) DCova and Th cells or effector CTLs were stained with PE-anti-CD11c and FITC-anti-CD4 or FITC-anti-CD8 Abs. The value in the panel represents the mean%±(SD) of $CD4^{high}CD11c^{low}$ (left) or $CD8^{high}CD11c^{low}$ (right) cells in the total population of $CD4^{high}$ Th or $CD8^{high}$ effector CTLs, respectively. One representative figure of three independent experiments is shown.

Supplementary Figure 2



Purity of tetramer-enriched CTLs. Approximately $5x10^6$ effector CTLs with or without Th cells ($2x10^6$) were adoptively co-transferred to CD4-sufficient or -deficient mice. On 16^{th} day, the transferred helped or unhelped effector CTLs were purified from blood and lymphoid organs of CD4-sufficient or -deficient mice by positive selection using PE-H-$2K^b$/$OVA_{257-264}$ tetramer and anti-PE microbeads. The purified CTLs were stained with FITC-anti-CD8 Ab and analyzed for purity by flow cytometry. The data represent mean% ±(S.D) and are cumulative of three independent experiments with two to six mice per group.

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CHAPTER 4

CD8$^+$ T CELL PRECURSOR FREQUENCIES INFLUENCE CD4$^+$ T HELP DEPENDENCE OF PRIMARY AND SECONDARY CTL RESPONSES AGAINST LUNG MELANOMA METASTASIS

Channakeshava Sokke Umeshappa[§,†], Roopa Hebbandi Nanjundappa[§,¶], Andrew Freywald[†] Qingyong Xu[†] and Jim Xiang[§,†,*]

[§]*Cancer Research Unit, Saskatchewan Cancer Agency, Departments of Oncology and* [†]*Pathology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada,*

Concise title: PF regulates CD4$^+$ T cell help for tumor-specific CTL responses

***Correspondence:** Dr. Jim Xiang, Cancer Research Unit, Saskatchewan Cancer Agency, 20 Campus Drive, Saskatoon, Saskatchewan S7N 4H4, Canada, Tel: (306) 6552917, Fax: (306) 6552635, email: jim.xiang@saskcancer.ca

[¶]Present address**:** Department of Microbiology, Immunology & Infectious Diseases, Health Research Innovation Centre, 3330 Hospital Drive NW, University of Calgary, Calgary, Alberta T2N 4N1 Canada.

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4.1 Brief introduction to Chapter 4

Recent studies have shown that natural differences in the size of Ag-specific $CD8^+$ T cell precursors can influence the magnitude of primary and memory CTL responses, suggesting altered PF could modify the requirement of regulatory factors for CTL responses. Since Th cells are considered to be the most important cells that regulate one or several phases of CTL responses, here we studied how altered PF impact $CD4^+$ T-helper signal requirements for functional primary and memory responses.

4.2 Abstract

$CD4^+$ T cell-derived help is dispensable for primary cytotoxic T lymphocyte (CTL) responses at increased $CD8^+$ T cell precursor frequency (PF). However, it remains unclear what role $CD4^+$ T cell action and related signals play in the development of memory CTL responses at higher PF. We addressed these questions using immunization with ovalbumin (OVA)-pulsed dendritic cells (DC_{OVA}) both at endogenous and increased PF, utilizing various gene-knockout and transgenic mice. We found that at endogenous PF, $CD4^+$ T-helper signals were required for both OVA-specific primary and secondary CTL responses. At increased PF, $CD4^+$ T cell help and associated CD40L, but not IL-2, signalling became dispensable for primary CTL responses. Furthermore, increased PF considerably enhanced DC_{OVA}-induced therapeutic immunity in mice with established OVA-expressing tumors independent of $CD4^+$ T cell help. In contrast, $CD4^+$ T cell help and related signals were indispensable for functional memory CTL development. Without these helper signals, memory CTLs failed in inducing efficient immune protection against tumor challenge, even when developed at increased PF. Our results demonstrate that alterations in $CD8^+$ T cell PF influence dependence of primary and secondary CTL responses on $CD4^+$ T helper factors and could impact the development of novel immune-based cancer therapies, since their efficacy would be determined in part by $CD4^+$ T-helper signals and partially by $CD8^+$ T cell PF.

4.3 Introduction

A hallmark of the cell-mediated immunity is the generation of millions of copies of effector $CD8^+$ cytotoxic T lymphocytes (CTLs) and a small fraction of memory CTLs arising from the proliferation of extremely low number of naïve $CD8^+$ T cell precursors (~10 of 3000) (1-3). This dynamic event is largely regulated by a multitude of factors during priming and effector phases of CTL responses (4). Among these factors, $CD4^+$ T cells are known to mediate helper effects either indirectly by inducing maturation signals to APCs via CD40L signaling (5-7), or directly by modulating $CD8^+$ T cell responses via cytokine (IL-2) and co-stimulatory (CD40L) signals (8, 9).

Recently, several studies have indicated that natural differences in the size of $CD8^+$ T cell precursor population for a given antigenic epitope can affect the magnitude of effector and memory CTL responses (2, 10-14). Even in genetically identical twins, variations in $CD8^+$ T cell repertoires impact the development of autoimmune diseases, such as type-I diabetes and multiple sclerosis (15). These observations inspired researchers to investigate, whether altered precursor frequency (PF) modifies the requirement for specific regulatory factors in the development of $CD8^+$ T cell responses. Studies in several models, have recently suggested that primary CTL responses at higher PF could occur independent of $CD4^+$ T cell help, even against minor-H Ag (11, 12, 16). We have recently demonstrated that, at higher PF, the primary CTL responses derived from ovalbumin (OVA)-pulsed dendritic cell (DC_{OVA}) stimulation occur in absence of $CD4^+$ T cell help (unhelped) in Ia^b knockout ($Ia^{b-/-}$) mice (17). However, whether these unhelped CTLs are functional effectors is unknown. While most of the studies have focused on the effect of PF on the primary CTL responses, its potential relation to the requirement of $CD4^+$ T-help and its helper signals mediated by CD40L for functional memory CTL development at higher PF is poorly understood. In addition, the situation is further complicated by observations that show significant differences in the requirement for $CD4^+$ T cell help in different types of infections or immunizations (5, 8, 9, 11, 12, 18, 19).

In our previous work, we demonstrated that, at higher PF, the primary CTL responses derived from DC_{OVA} stimulation occur in absence of $CD4^+$ T cell help in the gene knockout (KO) ($Ia^{b-/-}$) mice, lacking $CD4^+$ T cells, while also bypassing self-immune tolerance with the induction of type-1 diabetes in the transgenic RIP-mOVA mice (17). In this study, effects of immunizations with DC_{OVA} derived from wild-type (WT) or gene-KO ($CD40^{-/-}$ or $CD40L^{-/-}$) mice on primary and

memory CTL responses were assessed in $CD4^+$ T cell-sufficient WT mice or in $CD4^+$ T cell-deficient $Ia^{b-/-}$, or in help-signalling-deficient $CD40^{-/-}$ or $CD40L^{-/-}$ mice at endogenous PF, or at increased PF, assured by the transfer of OVA-specific naïve $CD8^+$ T cells derived from (WT)OTI-Ova Transgenic, ($CD40^{-/-}$)OTI or ($CD40L^{-/-}$)OTI mice. These experimental approaches allow us to monitor the requirement for specific $CD4^+$ T-helper signals in the primary and memory CTL responses at both endogenous and increased PFs.

4.4 Materials and methods

4.4.1 Reagents, tumor cells and animals

The biotin- or fluorescent-labeled (FITC or PE) Ab specific for CD11c (HL3), H-$2K^b$ (AF6-88.5), Ia^b (KH74), CD80 (16-10A1), CD40 (3/23), CD4 (GK1.5), CD44 (IM7), CD127 (A7R34), CD62L (MEL-14), CD69 (H1.2F3), Vβ5·1,5·2 TCR (MR9-4), IFN-γ (XMG1.2) and IL-2 (JES6-5H4), purified rat anti-mouse IL-2 (JES6-1A12), streptavidin-PE-Cy5 and streptavidin-FITC were purchased from BD-Biosciences (San Diego, CA). The PE-anti-CD8 (KT15) Ab from Serotec (Burlington, Ontario, Canada), and FITC-anti-CD8 (KT15) and H-$2K^b$/$OVA_{257-264}$ tetramer from Beckman Coulter (Miami, FL) were purchased. The recombinant GM-CSF, IL-2 and anti-IL-4 Ab were obtained from R & D Systems (Minneapolis, MN). The OVAI ($OVA_{257-264}$, SIINFEKL) peptide was synthesized by Multiple Peptide Systems (San Diego, CA). The OVA was obtained from Sigma-Aldrich Canada (Oakville, Ontario, Canada). The mouse malignant melanoma (BL6-10) (20) and OVA-transfected BL6-10 (BL6-10_{OVA}) (18) cell lines were cultured as described previously (8). The C57BL/6J (WT), $OVA_{257-264}$-specific TCR-transgenic OTI, IL-$2R\alpha^{-/-}$, $CD40^{-/-}$, $CD40L^{-/-}$ and $Ia^{b-/-}$ mice on C57BL/6 background were purchased from Jackson Laboratory or bred in University's animal resource center. The OTI/$CD40^{-/-}$ and OTI/$CD40L^{-/-}$ mice were generated by backcrossing designated KO mice with OTII mice, and tested as described previously (8). All the animal experiments were performed as per the guidelines approved by the University Committee on Animal Care and Supply, University of Saskatchewan.

4.4.2 Preparation of mature DC_{OVA}

Bone-marrow-derived DC_{OVA} from C57BL/6 mice were generated by culturing bone marrow cells for 6 days in medium containing IL-4 (20 ng/ml) and GM-CSF (20 ng/ml) and

pulsing with 0.1 mg/mL OVA overnight at 37°C as described previously (18). DC_{OVA} generated from $CD40^{-/-}$ and $CD40L^{-/-}$ mice were referred as $(CD40^{-/-})DC_{OVA}$ and $(CD40L^{-/-})DC_{OVA}$, respectively.

4.4.3 Isolation of mononuclear leukocytes from lung

The lungs were finely minced and digested for 30 min with collagenase D (1mg/ml) at 37°C. The cell suspension was incubated with 0.01M EDTA for 5 min and subjected to gradient centrifugation using Histopaque (Sigma, St. Louis, MO). The white buffy coat at the suspension and histopaque interface was collected for analysis.

4.4.4 Assessment of primary and memory CTL responses

C57BL/6 mice were i.v. immunized with DC_{OVA} ($1x10^6$/mice). In experiments involving endogenous PF, following DC_{OVA} immunization, blood samples from all the groups were collected 6 and 30 days later, stained with H-$2K^b$/$OVA_{257-264}$ tetramer and FITC-anti-CD8 Ab, and analyzed for CTL proliferation or survival (8). The naïve $CD8^+$ T cells were isolated from OTI, (OTI)$CD40^{-/-}$ or (OTI)$CD40L^{-/-}$ mouse splenocytes by enriching T lymphocytes in nylon wool columns (C&A Scientific, Manassas, VA), and negative selection using anti-CD4 (L3T4) paramagnetic beads (DYNAL, Lake Success, NY) as previously described (8). In experiments involving increased PF, the endogenous PF of mice was increased by i.v. transfer of $1x10^6$ OTI $CD8^+$ T cells one day before DC_{OVA} immunization. 6 and/or 90 days after DC_{OVA} immunization, the blood samples were collected, re-stimulated with OVAI and subjected to intracellular IFN-γ or IL-2 staining (BD-Biosciences) (21). Experiments were also performed to determine the presence of $tetramer^+$ or IFN-γ^+ CTL in lung or spleen during effector stage or 24^{th} day of tumor challenge.

4.4.5 In vivo cytotoxicity assay

The targets were prepared as described previously (8) by labeling splenocytes differentially with high (3.0 μM) or low (0.6 μM) concentrations of CFSE and by pulsing with OVAI or Mut1 peptide, respectively, and co-injected i.v. ($2x10^6$ cells/mouse) at 1:1 ratio into immunized or unimmunized mice. Sixteen hours later, the relative proportions of target $CFSE^{high}$ (H) and $CFSE^{low}$ (L) cells remaining in the spleens were analyzed by flow cytometry.

4.4.6 In vitro and in vivo CFSE proliferation assays

Both *in vitro* and *in vivo* CFSE proliferation assays were described previously (8). Briefly, for *in vitro* assay, OTI $CD8^+$ T cells were labeled with CFSE and co-cultured with irradiated (4,000 rad) DC_{OVA} at 1:4 ratios for 3 days. To study IL-2 requirements for priming, the culturing was done in IL-2 presence (20 U/ml) or absence of IL-2 but with different concentrations of neutralizing anti-IL-2 Ab (high, 200 ng/ml; medium, 50 ng/ml and low, 5 ng/ml) or isotype-matched control Ab. The cultured cells were purified by Ficoll-Paque (Sigma-Aldrich Canada) separation and analyzed for % labeled-cell division in flow cytometry. In *in vivo* assay, the WT or $Ia^{b-/-}$ mice were i.v. injected with CFSE-labeled OTI $CD8^+$ T cells ($2\text{-}3x10^6$cells/mouse) one day before immunizing with DC_{OVA} ($1x10^6$cells/mouse). The mice splenocytes were analyzed four days later for % labeled-cell division after labeling with PE-anti-CD8 Ab.

4.4.7 Phenotypic characterization of memory CTL

To phenotypically characterize memory CTL, the blood samples were collected 60 days after DC_{OVA} immunization, and stained with PE-tetramer, FITC-anti-CD44, a panel of biotin-conjugated antibodies (Abs) specific for effector or memory T cell markers and subsequently with streptavidine-PECy5. The relative expressions of surface markers were analyzed in $tetramer^+CD44^{hi}$ cell population.

4.4.8 Tumor protection studies

All the immunized mice were challenged with BL6-10_{OVA} or BL6-10 ($0.5x10^6$ cells/mice) on 30^{th} or 90^{th} day of immunization as shown in Table 1 and monitored for protection up to 24 days or earlier if the mice become moribund as described previously (8). The tumor grading was done depending on mean numbers of metastatic tumor colonies in lungs: -, no tumors; +, 1-24; ++, 25-49; +++, 50-74; ++++, 75-99; +++++, 100 to 250; ++++++, >250.

4.4.9 Statistical analysis

The statistical analysis were performed using Student's *t* or Mann–Whitney U test (Graphpad Prism-3.0); $*P<0.05$ and $**P<0.01$.

4.5 Results

4.5.1 Primary CTL responses require CD4$^+$ T cell help and associated CD40L signaling at endogenous PF

We investigated the roles of the CD4$^+$ T cell help and of the CD40L-initiated signal at endogenous PF by immunizing WT, Ia$^{b-/-}$, CD40$^{-/-}$ or CD40L$^{-/-}$ mice with mature DC_{OVA} expressing all the maturation markers, such as K^b, Ia^b, CD40 and CD80 (Fig. 1a and 1b). All these mice were analyzed for primary CTL proliferation in the peripheral blood using H-2K^b/$OVA_{257-264}$ tetramer staining and for CTLs' lytic functions in spleen using *in vivo* cytotoxicity assay. Compared to WT mice (Fig. 1c, panel b), all the other animals, deficient in CD4$^+$ T cell help or CD40L-induced signalling (Fig. 1c, panels c-e), showed significantly reduced CTL proliferation (P<0.01). To further assess the impact of CD40 or CD40L expression on DCs, we immunized WT mice with DC_{OVA} lacking these molecules. Compared to Ia$^{b-/-}$ and CD40L$^{-/-}$ mice, CD40$^{-/-}$ mice receiving wild-type DC_{OVA} (Fig. 1c, panel d), and WT mice receiving either (CD40$^{-/-}$)DC_{OVA} or (CD40L$^{-/-}$)DC_{OVA} (Fig. 1c, panels f and g) showed significantly higher CTL proliferation (P<0.01). Consistent with the results in blood (Fig. 1c, panel b), spleens and lungs of WT mice had considerably higher levels of primary CTLs compared to Ia$^{b-/-}$ mice (Fig. 1d; P<0.01). Correlating with the above tetramer-staining data (Fig. 1c), substantially stronger loss of the CFSE^{high}-labeled OVA-specific target cells was observed in the WT group (Fig. 1e, panel b), compared to all other groups, as determined by the *in vivo* cytotoxicity assay (Fig. 1e; panel c-g P<0.01). Collectively, these observations suggest that CD40L expression by host CD4$^+$ T cells is a prerequisite for optimal primary responses, and that the partial immune responses observed upon (CD40$^{-/-}$)DC_{OVA} or (CD40L$^{-/-}$)DC_{OVA} immunizations, or in CD40$^{-/-}$ mice may be due to compensatory mechanisms, as CD40-CD40L signaling can occur among CD8$^+$ T cells, DC and/or CD4$^+$ T cells since all these immune cells are known to express CD40 and/or CD40L molecules at least during some stages of immune responses (5, 9, 22-24).

4.5.2 The absence of $CD4^+$ T cell help and CD40L-derived signals lead to functionally defective memory CTL responses at endogenous PF

To assess the role of $CD4^+$ T cell-mediated help and the importance of the related CD40L-triggered signaling, we analyzed the size and quality of the memory CTL population generated at endogenous PF. Thirty days following immunization, all the groups were monitored for memory CTL survival by the tetramer assay and later assessed for anti-tumor protection (Fig. 1a; Table-1, Exp-I). Compared to WT mice (Fig. 2a), all the mouse groups ($Ia^{b-/-}$, $CD40^{-/-}$ and $CD40L^{-/-}$) deficient in $CD4^+$ T cell help and/or CD40L signalling showed significantly reduced memory CTL survival (P<0.05 or 0.01) (Fig. 2a). Although $CD40^{-/-}$ mice or WT mice receiving ($CD40^{-/-}$)DC_{OVA} had comparable primary CTL levels, they had lower memory CTL frequency, perhaps due to their poor survival rate. Furthermore, upon challenging with highly metastatic BL6-10_{OVA} tumor cells, $Ia^{b-/-}$, $CD40^{-/-}$ or $CD40L^{-/-}$ mice completely failed to provide anti-tumor protection, in contrast to the efficient tumor suppression in WT animals (Table-1, Exp-I). The complete protection observed in WT mice also correlated with high levels of CTLs in their lungs (Fig. 2b; P<0.05). On the other hand, WT mice receiving ($CD40^{-/-}$)DC_{OVA} or ($CD40L^{-/-}$)DC_{OVA} gave some degree of protection, particularly in the latter group, correlating with memory CTL levels in the blood. These results suggest the relative importance of CD40-CD40L signaling among DC, $CD4^+$ and $CD8^+$ T cells in modulating memory CTL survival and/or development. For instance, the reduced CTL survival and anti-tumor protection in $CD40^{-/-}$ mice could be due to lack of direct CD40L signaling to CD40-deficient $CD8^+$ T cells by DCova-stimulated Th1 cells (8, 9, 25). Similarly, the reduced anti-tumor protection in ($CD40^{-/-}$)DC_{OVA}-immunized mice compared to WT mice could be due to lack of CD40L signaling by endogenous Th1 cells to CD40-deficient DCova (5-7). It is possible that synergistically enhanced responses can occur by the combination of Th1-derived CD40L-initiated signaling triggered within CD40-expressing DCs and $CD8^+$ T cells; however, this intriguing possibility requires further investigation.

4.5.3 At higher PF, primary CTL response does not depend on $CD4^+$ T cell help and on associated CD40L stimulation, while requiring local $CD8^+$ T cell-derived IL-2 signals

In previous studies, we and others showed that $CD4^+$ T cell-independent CTL priming occurs not due to high-affinity CTLs, but possibly due to some unidentified cell-intrinsic or cell-

extrinsic factors resulting from higher PF (17, 26). Consistent with earlier reports (3, 17, 26), we observed an optimal CD4-independent primary CTL response upon transfer of $1x10^6$ naïve OTI $CD8^+$ T cells expressing TCR and CD62L, but not CD69 (activation marker) (Fig. 3a) to $Ia^{b-/-}$ mice. This optimized OTI $CD8^+$ T cell number was used to enhance endogenous precursor $CD8^+$ T cell populations in all the studies involving higher PF, unless otherwise indicated (Fig. 3b). In agreement with earlier reports (3, 17, 26), an efficient $CD4^+$-independent priming without obvious intraclonal competition was observed at this precursor level. To better understand memory CTL generation and its requirements at higher PF, we performed two different experiments after 90 days of DCova immunization as shown in Fig. 3b and Table-1, Expt-IIa and -IIb: one in the absence of $CD4^+$ T cell help and/or CD40L-induced signaling, by transferring $Ia^{b-/-}$ mice with (WT)OTI, ($CD40^{-/-}$)OTI or ($CD40L^{-/-}$)OTI $CD8^+$ T cells before immunizing with DC_{OVA}; and another in the presence of endogenous $CD4^+$ T cells, but in the absence of CD40L signaling by transferring WT, $CD40^{-/-}$ or $CD40L^{-/-}$ mice with (WT)OTI, ($CD40^{-/-}$)OTI or ($CD40L^{-/-}$)OTI $CD8^+$ T cells prior to immunizing with (WT)DC_{OVA}, ($CD40^{-/-}$)DC_{OVA} or ($CD40L^{-/-}$)DC_{OVA}. The initial experiment allowed us to determine whether $CD4^+$ T cell help and/or accompanying CD40L signaling were required for memory generation, whereas the latter experiment precluded possible CD40L signaling among $CD4^+$ T, DC and/or $CD8^+$ T cells (5, 9, 22-24), while allowing other $CD4^+$ T-helper signals. Initially, we performed both *in vitro* and *in vivo* CFSE proliferation assays as shown in Fig. 3c and 3d. Under both *in vitro* and *in vivo* conditions, in the complete absence of $CD4^+$ T cell help, DC_{OVA} stimulated proliferation of CFSE-labeled OTI $CD8^+$ T cells with almost equal efficiency (Fig. 3c and/or 3d) as in the presence of $CD4^+$ T cell help (Fig. 3d). Moreover, even in the complete absence of CD40L signaling, DC_{OVA} efficiently stimulated proliferation of CFSE-labeled OTI $CD8^+$ T cells, disregarding the presence or absence of $CD4^+$ T cell and its CD40L signals (Fig. 3c and 3d). Recently, it was shown that IL-2 signaling from $CD4^+$ T cells is very critical for primary CTL proliferation (27, 28). Consistent with these reports, IL-2R$\alpha^{-/-}$ mice with endogenous PF failed to show CTL proliferation upon DCova immunization (data not shown), suggesting IL-2 signaling may be indispensable for primary CTL proliferation. To ascertain this phenomenon at higher PF, we performed a simple *in vitro* study by co-culturing CFSE-labeled OTI $CD8^+$ T cells with DC_{OVA} in the presence of IL-2 or different concentrations of neutralizing anti-IL-2 Ab (Fig. 3e). Interestingly, although OTI $CD8^+$ T cells proliferated efficiently in the absence of both IL-2 and $CD4^+$ T cells, their proliferation was inhibited in a dose-

dependent manner when anti-IL-2 Abs were added to the cultures, suggesting attainment of local threshold levels of $CD8^+$ T cell-derived IL-2 and its role in driving primary response in an autocrine or paracrine fashion.

To further assess the proliferation and functionality of effector CTLs generated without $CD4^+$ T cell help and/or related helper molecules, we performed OVA-specific intracellular IFN-γ staining and *in vivo* cytotoxicity assays. At higher PF, intracellular IFN-γ-, rather than tetramer-staining, was used since it detects mostly Ag-experienced CTLs derived from transferred OTI as well as endogenous $CD8^+$ T cells, providing complete picture of overall $CD8^+$ T cell repertoire responses. In the absence of $CD4^+$ T cell help (Fig. 3f, panel c-e) and/or CD40L costimulation (Fig. 3f, panel f and g), DC_{OVA} immunization induced considerable proportions of IFN-γ^+ effector CTLs comparable to the induction in WT mice (Fig. 3f, panels a and b). Similarly, $CD4^+$ T cell-independent IFN-γ^+ CTL response was also observed in $CD4^+$ T cell-depleted WT mice (Fig. 3g, left panel). As *in vitro* assay revealed IL-2 requirement at higher PF (Fig. 3e), and $Ia^{b-/-}$ mice without $CD4^+$ T cell-derived IL-2 source and WT mice showed comparable IFN-γ^+ CTL proliferation *in vivo* at increased PF (Fig. 3f), we hypothesized that the attainment of local threshold levels of IL-2 could drive primary CTL proliferation in the absence of $CD4^+$-T cell-secreted IL-2 source. To verify this hypothesis *in vivo*, we tested IL-2 secretion at higher PF *in vivo* by assessing IL-2^+ CTLs in WT mice, $Ia^{b-/-}$ and $CD4^+$ T cell-depleted WT mice. Indeed, at higher PF, both $Ia^{b-/-}$ and $CD4^+$ T cell-depleted WT mice (Fig. 3e) showed the presence of IL-2^+ CTLs comparable to their presence in WT animals (Fig. 3g, right panel). We also did not observe any significant differences in the presence of IFN-γ^+ CTL levels in spleens and lungs of WT and $Ia^{b-/-}$ mice (Fig. 3h). Furthermore, correlating with the levels of IFN-γ^+ CTLs (Fig. 3f), the results from *in vivo* cytotoxicity assay also showed a substantial loss of the $CFSE^{high}$-labeled OVA-specific target cells in $CD4^+$ T cell-deficient mice (Fig. 3i, panels c-e) and in mice lacking CD40L-dependent signaling (Fig. 3i, panels f, g), similar to the situation observed in the WT mice (Fig. 3i, panel b). Collectively, these results suggest that, following DC_{OVA} immunization, effector CTLs generated at a higher PF, retain normal proliferative, cytokine-secreting and cytolytic functions, irrespective of the availability of $CD4^+$ T cell help.

4.5.4 Memory CTL responses are compromised by the absence of $CD4^+$ T cell help and/or CD40L-triggered signal even at increased PF

To better understand the functions of memory CTLs that generate at higher PF under $CD4^+$ T-helper influence, the intracellular staining assay was used to track the survival of IFN-γ^+ memory CTL population ninety days following immunization. Interestingly, when compared to WT animals, mice missing $CD4^+$ T cells and/or CD40L signaling showed significant decrease in the number of IFN-γ^+ memory CTLs in blood (Fig. 4a; $P< 0.01$). Furthermore, this decrease in the IFN-γ^+ memory CTL population was even strongly pronounced in $Ia^{b-/-}$ compared to the $CD40^{-/-}$ or $CD40L^{-/-}$ mice, lacking exclusively CD40L signaling. As there were considerable differences in memory CTL survival in $Ia^{b-/-}$ mice compared to WT mice, we sought to determine whether altered surface-marker expression correlates with survival rates. In both WT and $Ia^{b-/-}$ mice, the $tetramer^+$ population expressed high levels of CD44. Furthermore, the analysis of $tetramer^+CD44^+$ population revealed the central memory (T_{CM}) phenotype, showing considerable expression of CD62L and IL-7Rα, but not CD69 (Fig. 4b). However, there was no drastic difference in the expression of these markers between WT and $Ia^{b-/-}$ mice, suggesting other factors might influence the survival of helped versus unhelped memory CTLs.

To further analyze the survival and functionality of memory CTLs generated at higher PF under $CD4^+$ T-helper influence, all the immunized mice were challenged with BL6-10_{OVA} at ninety days following immunization and assessed for anti-tumor protection, as shown in Table-1, Exp-II. Interestingly, our preliminary attempts to challenge mice at 30 to 45 days post immunization induced anti-tumor protection irrespective of $CD4^+$ T cell help (data not shown), perhaps due to the prolonged maintenance of the transferred un-stimulated naïve (29) and stimulated effector CTLs or effector memory CTLs derived from higher PF. It was previously shown that the presence of pre-existing effector CTLs can boost responses of other naïve $CD8^+$ T cells in certain situations (26, 30). Consequently, survived residual DC_{OVA} might receive additional signals to prime remaining naïve OTI $CD8^+$ T cells, which escaped priming during early stage, and thus, prolong anti-tumor protective ability of primary CTLs. However, during later stages, the residual naïve OTI $CD8^+$ T cells, which escape DC_{OVA} priming, are unlikely to participate in protection against lethal tumor challenge, as we observed complete failure to protect against tumor challenge in both WT and $Ia^{b-/-}$ mice transferred with OTI $CD8^+$ T cells alone (Table-1, Exp-II). Challenging all the

immunized mice groups on the 90th day post-immunization revealed a strong requirement of CD4$^+$ T-helper signals for memory CTL responses. Thus, in contrast to WT, all the Ia$^{b-/-}$ mice, irrespective of the state of CD40L signaling, completely failed to protect against tumor challenge (Table-I, Exp-IIA). Consistent with these results, WT animals displayed much higher recruitment of IFN-γ^+ CTLs in lungs, compared Ia$^{b-/-}$ mice (P<0.01) (Fig. 4c). Similarly, although CD4$^+$ T cells were present in the experiment IIB, all mice missing exclusivelyCD40L developed tumors (Table-I, Exp-IIB). However, these mice had 2 to 5 fold lowered tumor burden, when compared to mice with the complete absence of CD4$^+$ T-helper signals. This implies that although CD4$^+$- and CD40L-independent primary responses occur, CD40L-induced signal alone or in concert with other CD4$^+$ T-helper signals appears to be essential for programming of memory CTLs for both survival and anti-tumor functions, even at higher PF.

4.5.5 Higher PF augments therapeutic efficacy of DC_{OVA} immunization in the CD4$^+$ T cell-deficient environment

As the success of cancer immunotherapy heavily depends upon its ability to induce protection against established tumors, we asked whether the functional CD4$^+$ T cell-independent primary responses observed at higher PF can be exploited to treat established tumors in DC_{OVA} immunization protocols. This approach could also be very beneficial in HIV patients, who suffer from CD4$^+$ T cell deficiency and often develop tumors. We challenged two groups of mice with BL6-10$_{OVA,}$ as shown in Fig. 5a. In the first group, three days after challenge (early tumor burden), PF was increased prior to immunizing with DC_{OVA}. In the second group, similar procedures were performed on the sixth day of challenge (established tumor burden). When compared to WT and Ia$^{b-/-}$ mice with endogenous PF, 3rd-day-tumor-bearing WT and Ia$^{b-/-}$ mice with increased PF showed nearly complete protection (Fig. 5b), which also correlated with efficient recruitment of IFN-γ^+ CTLs into lungs (Fig. 5c; P<0.01). Furthermore, even on day six of challenge, higher PF significantly decreased the tumor burden and incidence in both WT and Ia$^{b-/-}$ mice (Fig. 5d). In contrast, increasing PF (as high as 2-5×10^6 precursor cells/mice) alone, without DC_{OVA} immunization, failed to provide anti-tumor protection (data not shown). Collectively, these results suggest that it is possible to considerably enhance anti-tumor therapeutic efficacy of DC_{OVA} immunization by increasing PF even in the absence of CD4$^+$ T cell help.

4.6 Discussion

It has been demonstrated that altered $CD8^+$ T cell PF could affect effector and memory responses (2, 10-12, 15, 31-33). However, its relative contribution to the requirement for $CD4^+$ T cell help and for the related CD40L, and IL-2 helper signals for memory CTL responses, including survival, and anti-tumor functions, is still largely unknown. In this study, we report that it is possible to achieve primary CTL responses independent of $CD4^+$ T cell help by increasing PF in DC immunization protocol, which is consistent with some previously published observations in other models (11, 12, 17, 26). Mintern *et al.* showed that CD40L-deficient $CD8^+$ T cells proliferated well and exhibited cytotoxicity, when stimulated with Ag-coated splenocytes (26). Similarly, functional effector CTLs were observed in tissue transplantation model, even when CD40L or CD28 costimulation was blocked using specific Abs (11, 12). In the present study, we prevented any compensatory mechanisms that may occur in the absence of a single co-stimulatory molecule among DC, $CD4^+$ and/or $CD8^+$ T cells (5, 9, 22-24, 34) by using KO mice, and their immune cells, and confirmed that, even in the complete absence of CD40L costimulation, the primary CTL response occurs efficiently at higher PF. Interestingly, our work with *in vitro* IL-2 blocking assay and *in vivo* studies that demonstrated IL-2 secretion by $CD8^+$ T cells revealed that similar to the responses seen at endogenous PF, IL-2 signal may still be required for primary CTL responses at higher PF (27). However, it appears that the threshold IL-2 signal derived from $CD8^+$ T cells alone, which is capable of driving primary CTL proliferation, may still not be sufficient for programming memory CTL generation and survival, as $Ia^{b-/-}$ mice with higher PF failed to protect mice against tumor challenge during the CTL memory phase. Also, in relation to this response, a potential role of the deficient $CD4^+$ T cell-mediated CD40L signaling in $Ia^{b-/-}$ mice needs a further detailed investigation, since CD40L signaling can enhance IL-2 utilization in $CD8^+$ T cells by up-regulating IL-2Rα (19).

To date, factors that govern CTL memory development at higher PF has not been established. We demonstrate here that $CD4^+$ T-helper signals are indispensable for the development of the functional memory CTL population, even at increased PF. It has been recently reported that higher PF controls memory lineage development in infectious models, favoring the generation of the inter-convertible T_{CM} vs effector memory T cells (14). Our previous study showed that DC-stimulated Th1 cells with acquired Ag-presenting machinery can stimulate naïve

$CD8^+$ T cells, which in turn prone to become T_{CM} (8, 18), thus possibly explaining why skewing of T_{CM} generation occurs at higher PF. Various studies have suggested that primary and memory CTL responses are likely to operate within certain thresholds at higher PF. It is believed that above these threshold levels, diminished immune responses occur, since Ag-specific $CD8^+$ T cells compete for limited resources, such as antigenic stimulation by APC, costimulations, cytokines, and various $CD4^+$ helper factors (3, 35-37). Our results are indirectly suggestive of a model, where the provision of additional $CD4^+$ T cell help may decrease intraclonal competition for $CD4^+$ T-helper factors, and thus further enhance CTL immunity.

$CD4^+$ T cells contribute to memory CTL generation either indirectly, by modulating APCs (5, 19), or directly, by modulating cognate $CD8^+$ T cells (8, 9, 18) via CD40L signaling. Consistent with these data, we observed poor memory responses with inability to provide anti-tumor protection in the absence of CD40L signaling even at higher PF, though endogenous $CD4^+$ T cells retaining other helper factors were ensured. Nevertheless, the lack of CD40L signal alone resulted in a relatively low tumor burden, compared the situation with the complete absence of $CD4^+$ T cells, suggesting other $CD4^+$ T-helper factors are likely to contribute to memory generation. Whether the reduced protection in $Ia^{b-/-}$ mice or in mice without CD40-40L signaling alone is due to actual decrease of CTL survival rate as reported previously (29) or due to loss of memory functions needs further investigation. It is intriguing however, that anti-tumor protection retained for longer periods at higher PF in the absence of $CD4^+$ T helper signals, a phenomenon that can be utilized in therapeutic use in CD4-deficient conditions. It has recently been shown that Th1 license DCs to secrete IL-12, which in turn acts on $CD8^+$ T cells to boost IL-2 receptor expression for efficient IL-2 utilization (19). Consequently, IL-2-induced signaling might be able to reach threshold levels required for functional memory development only in the presence of endogenous $CD4^+$ T cells.

DC-based vaccines have been widely applied to induce therapeutic anti-tumor immunity (5-7, 19). Unfortunately, they often fail in treatment of malignancies due to inefficient CTL responses, resulting from tolerance induction, inhibitory receptor expression, lower-reactive PF and lack of Ag immunodominance (3, 38, 39). The present results are particularly relevant in the development of effective DC-based vaccines against established malignant tumors. For example, the frequency of pre-existing tumor-specific CTL precursors in mice represents a critical determinant of the quality of anti-tumor responses, in accordance with the already recognized role

that initial T cell numbers have in the functional immune response against pathogens (14, 40). Their cumulative frequency was found to be significantly higher in cancer patients and vary widely in relation to various tumor-Ag peptides (10). The detection of these CTLs in cancer patients is currently applied for evaluating tumor Ags for vaccination (41). Recent evidence suggests that Ag presentation by DCs and PF levels also determine the immunodominance, and, thereby, protection against foreign pathogens (42, 43). In support of this, we also observed an increase in the therapeutic anti-tumor efficacy of DC_{OVA} immunization for prolonged periods at higher PF, even in the absence of $CD4^+$ T cell help. Based on our current findings, we expect that it should be possible to achieve a robust therapeutic immunity for extended periods in chronic diseases with compromised $CD4^+$ T cell functions, including AIDS and malignancy, by increasing $CD8^+$ T cell PF in combination with immunization with mature DCs.

Taken together, our results demonstrate that altered PF influences the dependence of functional primary and memory CTL responses upon $CD4^+$ T cell help, and may thus, impact the development of novel cancer immunotherapies, whose efficacy is determined in part by $CD4^+$ T-helper signals and alterations in $CD8^+$ T cell PF. Based on the current findings, prolonged robust anti-tumor immune responses should be achievable through increasing $CD8^+$ T cell PF and immunizing with mature DCs, presenting both $CD8^+$ T cell and $CD4^+$ T-helper epitopes. Moreover, as this approach is also known to give prophylactical effectiveness in the lymphopenia-induced environment (3), it may potentially act as an excellent adjuvant in combination with other cancer therapies, such as chemotherapy and irradiation, which often deplete host lymphocytes. Finally, our observations imply that selective $CD4^+$ Th1 depletion or blockade of associated helper molecules may also potentially facilitate organ transplantations and support treatment of autoimmune disorders, which are often $CD4^+$ T cell help and costimulation dependent.

4.7 Acknowledgements

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4.8 Disclosure of conflicts of interest

The authors declare no competing financial interests.

4.9 Tables

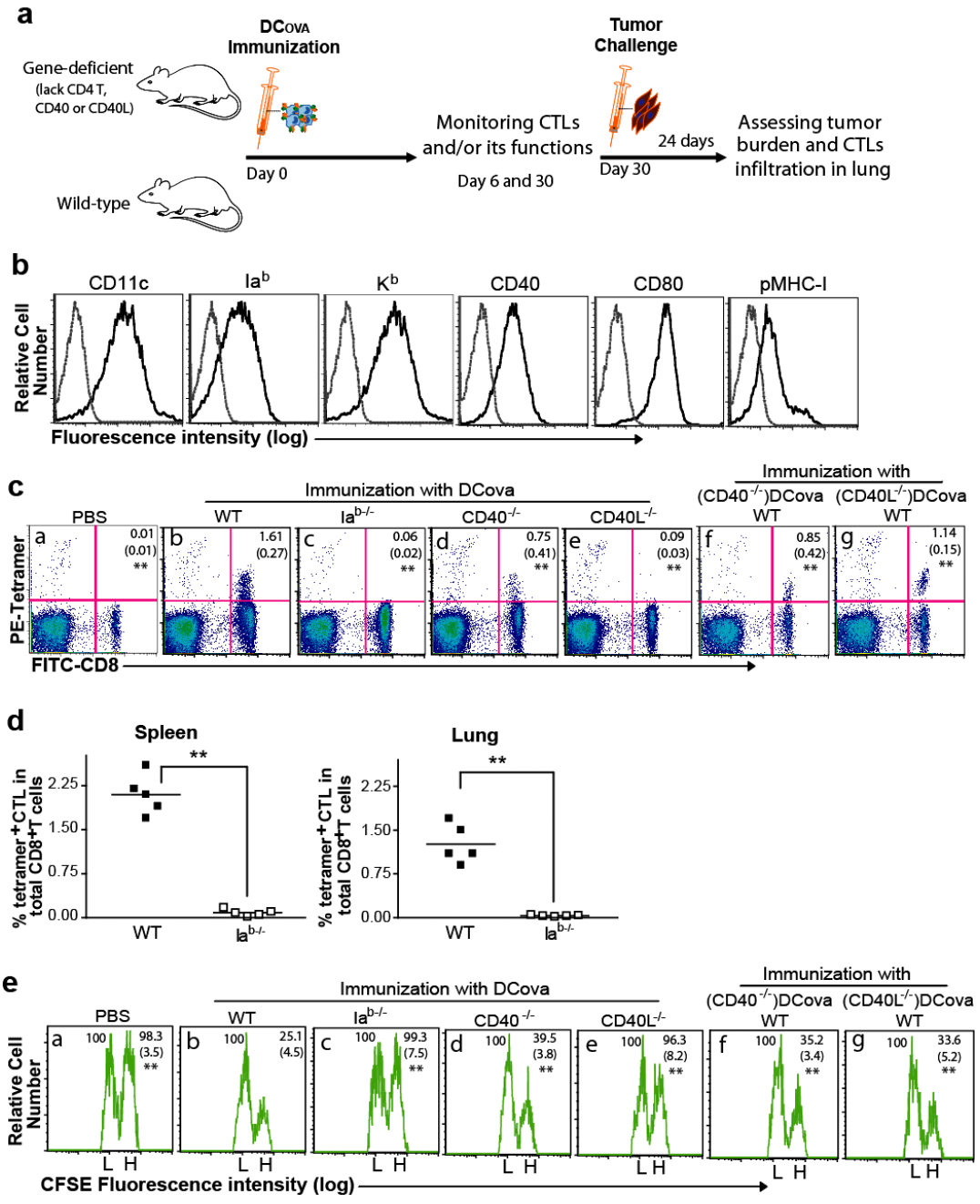
Table 1. $CD4^+$ help and its CD40L signaling are critical for functional memory development, irrespective of PF.

Mice[a]	Adoptive transfer (10^6)	Immunization	Tumor challenge	Tumor-bearing mice (%)	Lung tumor scoring
Experiment-I					
WT	Endogenous-PF	PBS	BL6-10_{OVA}	8/8 (100)	++++++
WT		DC_{OVA}	BL6-10	8/8 (100)	++++++
WT		DCova	BL6-10_{OVA}	0/12 (0)	-
WT		($CD40^{-/-}$)DC_{OVA}	BL6-10_{OVA}	5/10 (50)	++
WT		($CD40L^{-/-}$)DC_{OVA}	BL6-10_{OVA}	2/10 (20)	+
$Ia^{b-/-}$		DC_{OVA}	BL6-10_{OVA}	10/10 (100)	++++++
$CD40^{-/-}$		DC_{OVA}	BL6-10_{OVA}	10/10 (100)	++++
$CD40L^{-/-}$		DC_{OVA}	BL6-10_{OVA}	10/10 (100)	++++++
Experiment-II					
a) In the absence of endogenous $CD4^+$ T cells					
WT	OTI $CD8^+$ T	DC_{OVA}	BL6-10_{OVA}	0/12 (0)	-
$Ia^{b-/-}$	OTI $CD8^+$ T	DC_{OVA}	BL6-10_{OVA}	10/10 (100)	+++++
$Ia^{b-/-}$	($CD40^{-/-}$) OTI $CD8^+$ T	DC_{OVA}	BL6-10_{OVA}	10/10 (100)	+++++
$Ia^{b-/-}$	($CD40L^{-/-}$) OTI $CD8^+$ T	DC_{OVA}	BL6-10_{OVA}	10/10 (100)	+++++
b) In the presence of endogenous $CD4^+$ T cells					
WT	OTI $CD8^+$ T	DC_{OVA}	BL6-10_{OVA}	0/12 (0)	-
$CD40^{-/-}$	($CD40^{-/-}$) OTI $CD8^+$ T	($CD40^{-/-}$)DC_{OVA}	BL6-10_{OVA}	10/10 (100)	+++
$CD40L^{-/-}$	($CD40L^{-/-}$) OTI $CD8^+$ T	($CD40L^{-/-}$)DC_{OVA}	BL6-10_{OVA}	10/10 (100)	++++

[a]WT, $Ia^{b-/-}$, $CD40^{-/-}$ or $CD40L^{-/-}$ mice with endogenous- (Expt-I) or increased-PF (Expt-II) were immunized with DC_{OVA} as indicated. After thirty (Expt-I) or ninety (Expt-II) days, all the groups were i.v. challenged with highly metastasizing BL6-10_{OVA} or BL6-10. Twenty-four days later, the lung tumor colonies were counted and graded. The data are cumulative of two independent experiments, each comprising four to six mice per group.

4.10 Figures

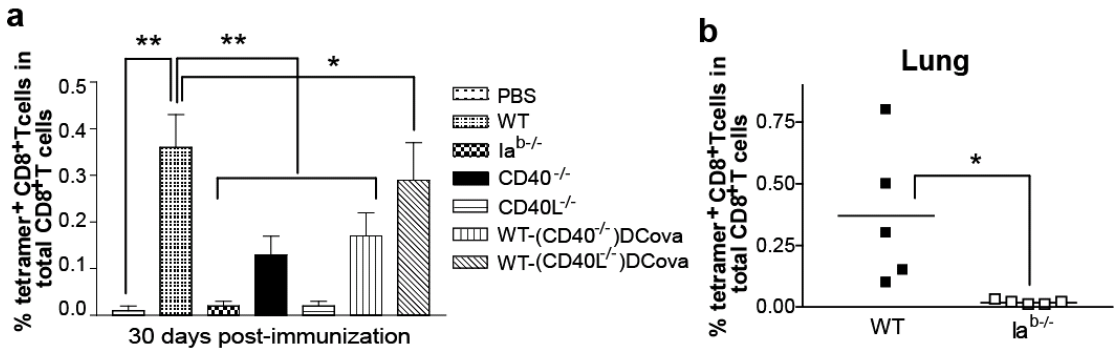
Figure 1



Primary CTL responses require $CD4^+$ T cell help and associated CD40L signaling at endogenous PF. (a) A schematic protocol. WT or KO mice were i.v. immunized with DC_{OVA} followed by assessment of CTL proliferation and/or its function at six and thirty days of immunization. During memory stage, all the groups were challenged with BL6-10_{OVA} tumor

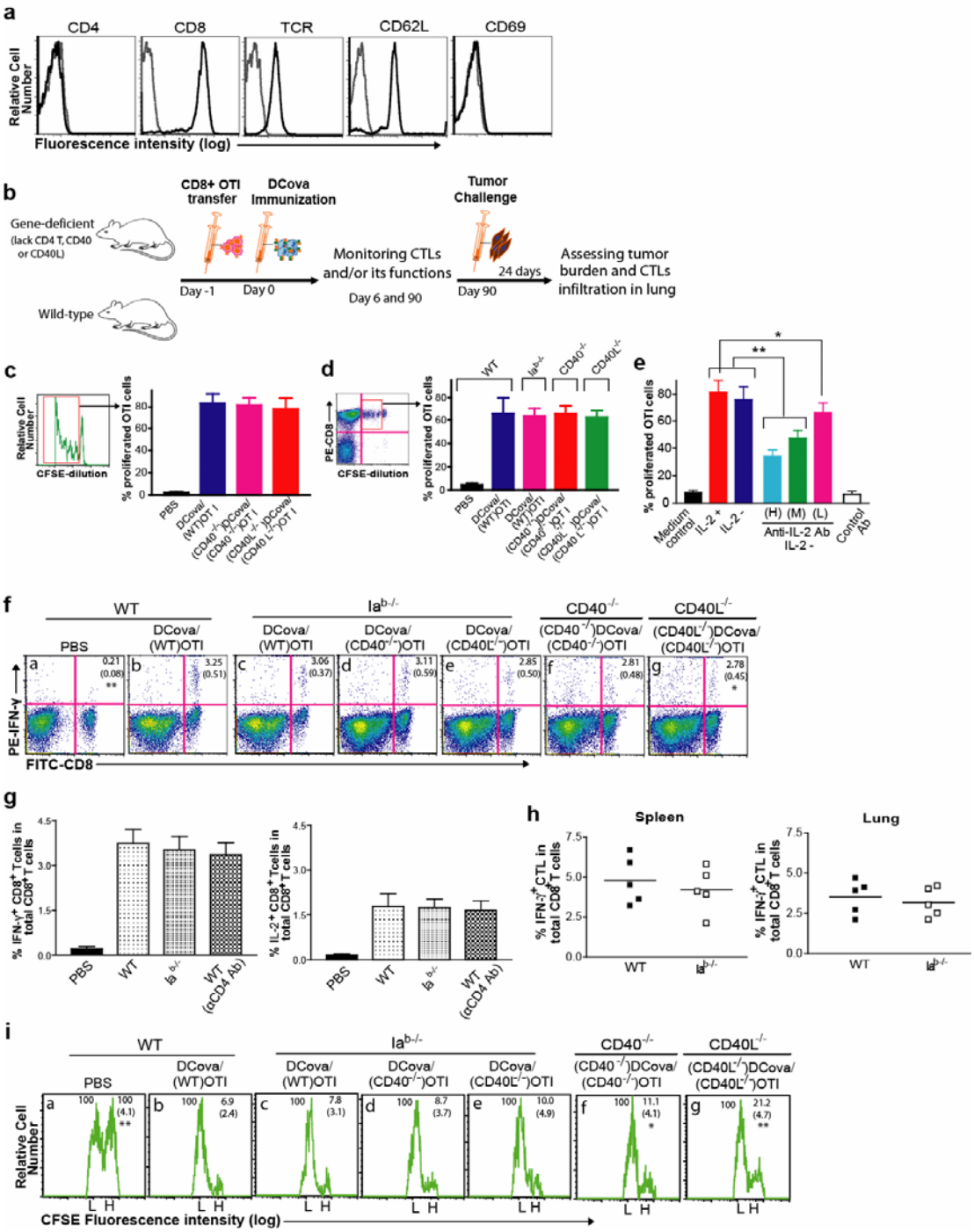
cells and assessed for tumor protection. (b) DCova were stained with a panel of Abs specific for DC and maturation markers (solid black lines), and analyzed by flow cytometry. Irrelevant isotype-matched Abs were used as control (dotted grey lines). (c) Six days after DC_{OVA} immunization, the proliferated CTLs were measured by tetramer staining in the peripheral blood. The values in each figure represent mean%±SD of $tetramer^+$ CTLs in total $CD8^+$ T cell population, and are cumulative of two independent studies with five to six mice per group. $^{**}P<0.01$, versus WT. (d) To determine recruitment/presence of $tetramer^+$ CTLs in spleens and lungs, WT- and $Ia^{b-/-}$-immunized mice were immunized and monitored by tetramer assay six days later. The values represent frequencies of $tetramer^+$ CTLs in total $CD8^+$ T cell population, and are cumulative of two independent experiments with two to three mice per group. The horizontal bars indicate means. $^{**}P<0.01$, versus WT mice. (e) In the above immunized groups shown in (c), the proportions of $CFSE^{high}$-OVAI-pulsed target cells lysed by effector CTLs were determined in the spleens by *in vivo* cytotoxicity assay seven days later. The values in each figure represent mean %±SD of targets remaining in the spleen relative to the controls. $^{**}P<0.01$, versus WT mice.

Figure 2



The absence of CD4$^+$ T cell help and CD40L-derived signals lead to functionally defective memory CTL responses at endogenous PF. (a) Thirty days after immunization, the blood samples were analyzed for the memory CTL survival by tetramer staining. The values represent mean %±SD of tetramer$^+$ CTLs in total CD8$^+$ T cell population, and are cumulative of two independent studies with four to six mice per group. *P<0.05 and **P<0.01, versus WT mice. (b) To determine infiltration of tetramer$^+$ CTLs in lungs of challenged mice, the WT- and Ia$^{b-/-}$-immunized mice were challenged thirty days later, and the lungs were monitored by tetramer assay after twenty-four days. The values represent frequencies of tetramer$^+$ CTLs in total CD8$^+$ T cell population, and are cumulative of two independent studies with two to three mice per group. The horizontal bars indicate means. **P<0.05, versus WT mice.

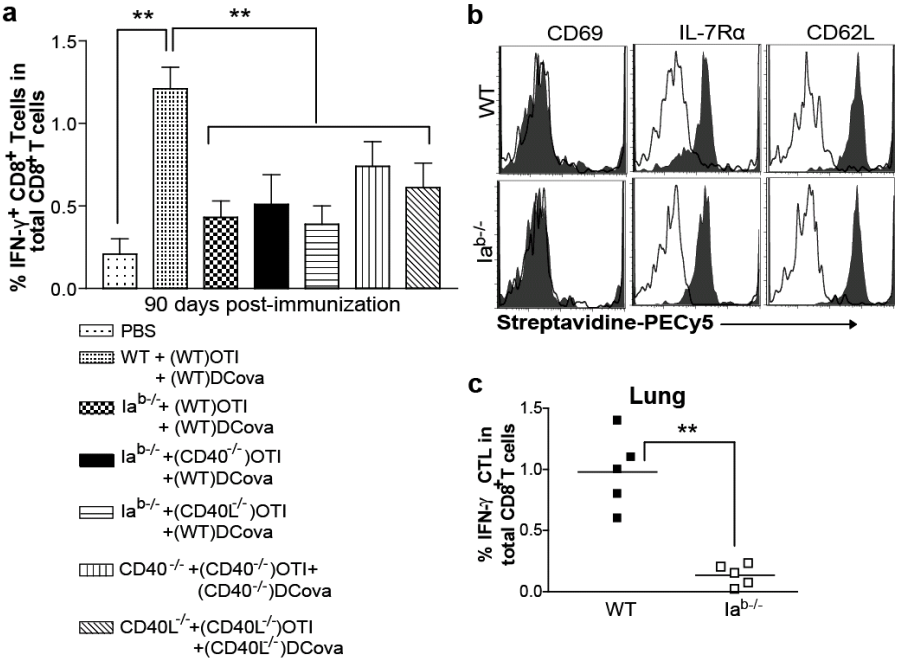
Figure 3



At higher PF, primary CTL response does not depend on CD4$^+$ T cell help and on associated CD40L stimulation, while requiring local CD8$^+$ T cell-derived IL-2 signals. (a) Naïve OTI CD8$^+$ T cells (solid black lines) were stained with a panel of Ab specific for naïve or activated status, and analyzed by flow cytometry. Irrelevant, isotype-matched Ab were used as

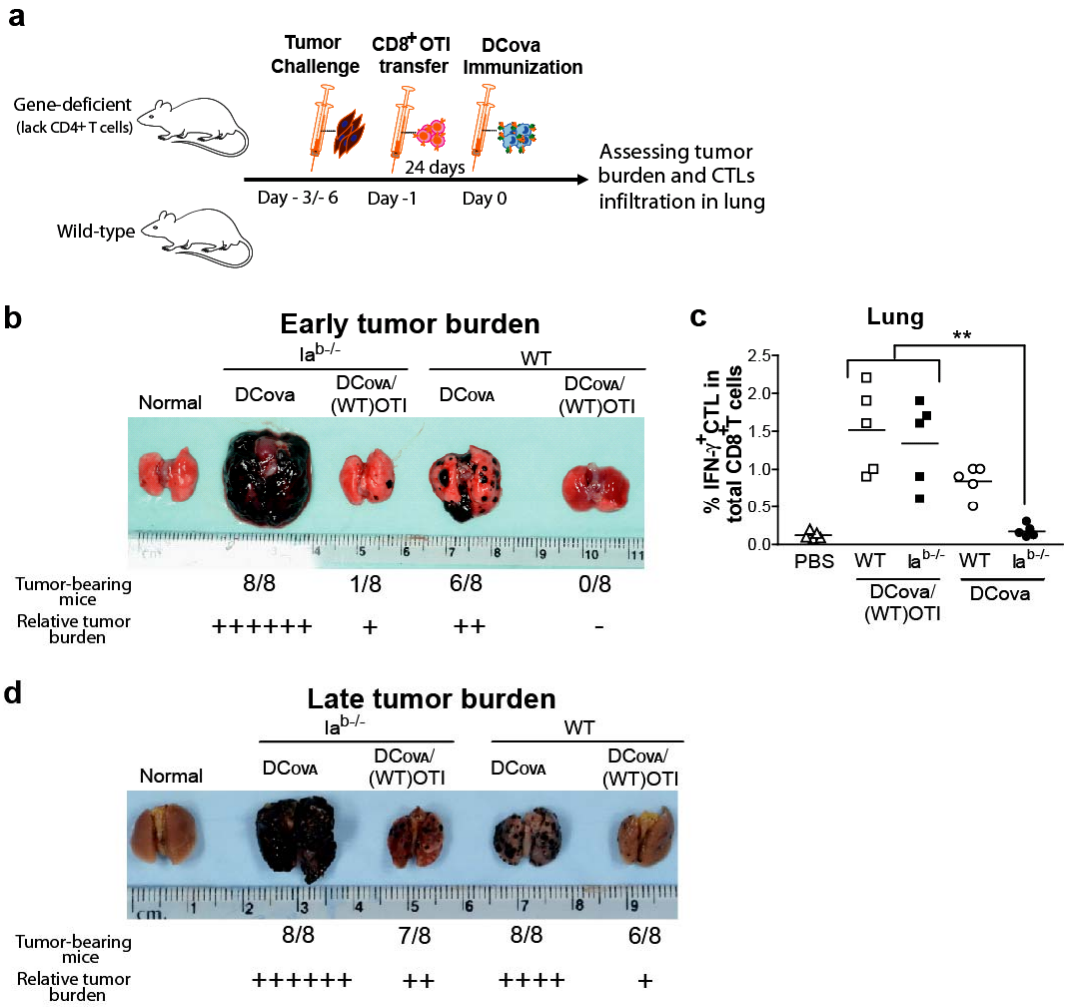
control (grey solid lines). (b) A schematic protocol. WT or KO mice were adoptively transferred with OTI $CD8^+$ T cells (with or without CD40 or CD40L molecules) and i.v. immunized with DC_{OVA} (with or without CD40 or CD40L). All the groups were monitored for the CTL proliferation, survival and/or its function after six and ninety days. During memory stage, all the groups were challenged with BL6-10_{OVA} and assessed for protection. (c and d) The extent of division of OTI $CD8^+$ T cells by DC_{OVA} in the absence of $CD4^+$ T cell help and/or CD40L signaling is shown by both *in vitro* (c) and *in vivo* (d) CFSE proliferation assays. (e) To determine IL-2 role in primary responses, the CFSE-labeled OTI $CD8^+$ T cells were co-cultured with DC_{OVA} in the presence of IL-2, or in the absence of IL-2 but with different concentrations of anti-IL-2 Ab (high (H), 200 ng/ml; medium (M), 50 ng/ml and low (L), 5 ng/ml), and percent proliferation of CFSE-labeled cells were determined. In experiments (c-e), the data are cumulative of three independent experiments, and presented as mean %±SD. (f and g) After immunizing mice with higher PF, the blood samples were analyzed by intracellular IFN-γ staining assay six days later. The values in each figure or bar diagrams represent mean %±SD of IFN-γ^+ CTLs in total $CD8^+$ T cell population, and are cumulative of two independent experiments with five to six mice per group. *P<0.05 and **P<0.01, versus WT. (h) The infiltration of IFN-γ^+ CTLs were also determined in spleens and lungs of WT- and $Ia^{b-/-}$-immunized mice. The values represent frequencies of IFN-γ^+ CTLs in total $CD8^+$ T cell population, and are cumulative of two independent studies with two to three mice per group. The horizontal bars indicate means. (i) In the above immunized groups shown in (f), the proportions of $CFSE^{high}$-OVAI-pulsed target cells lysed by effector CTLs were determined in the spleen seven days later. The values each figure represent mean %±SD of targets remaining in the spleen relative to the controls. *P<0.05 and **P<0.01, versus WT mice.

Figure 4



Memory CTL responses are compromised by the absence of CD4$^+$ T cell help and/or CD40L-triggered signal even at increased PF. (a) 90 days after DC_{OVA} immunization, the peripheral blood samples were analyzed for IFN-γ^+ memory CTL survival by intracellular staining assay. The values represent mean %±(SD) of IFN-γ^+ CTLs in total CD8$^+$ T cell population, and are cumulative of two independent experiments with five to six mice per group. $^{**}P<0.01$, versus WT. (b) Sixty days following immunization, the blood samples were stained for triple markers, and the expression of activation (CD69) and memory (CD62L and IL-7Rα) markers were analyzed in tetramer$^+$CD44^{hi} population (histogram grey filled overlays). Irrelevant isotype-matched Ab were used as controls (open black lines). (c) To determine recruitment and expansion of IFN-γ^+ memory CTLs in lungs of challenged mice, the WT- and Ia$^{b-/-}$-immunized mice were challenged ninety days later, and the lungs were assessed by intracellular IFN-γ assay after twenty-four days. The values represent frequencies of IFN-γ^+ CTLs in total CD8$^+$ T cell population, and are cumulative of two independent experiments with two to three mice per group. The horizontal bars indicate means. $^{**}P<0.01$, versus WT mice.

Figure 5



Higher PF augments therapeutic efficacy of DC_{OVA} immunization in the $CD4^+$ T cell-deficient environment. (a) An experimental design. WT or $Ia^{b-/-}$ mice were first challenged with $BL6\text{-}10_{OVA}$ three (early tumor burden) or six (late tumor burden) days before DC_{OVA} immunization. One day before immunizing, the endogenous PF of all these mice was increased by transferring OTI $CD8^+$ T cells. Twenty-four days after challenge, all the groups were assessed for tumor protection. (b) Impact of higher PF on the efficacy of DC_{OVA} immunization in early established tumors. Gross pathology of lungs showing relative surface tumor burden. (c) To determine whether protection is due to effector CTLs recruitment, the IFN-γ^+ CTLs in the lungs of some immunized mice were determined by intracellular staining assay. The values represent frequencies of IFN-γ^+ CTLs in total $CD8^+$ T cell population, and are cumulative of two independent studies with two to three mice per group. The horizontal bars indicate means.

**$P<0.01$, versus $Iab^{-/-}$ with endogenous PF. (d) Impact of higher PF on the efficacy of DC_{OVA} immunization in late-established tumors. Gross pathology of lungs showing relative surface tumor burden. The data in b and d are cumulative of two independent studies with four mice per group.

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CHAPTER 5

$CD4^+$ T CELLS PLAY CRITICAL ROLES VIA CD40L AND IL-2 SIGNALING IN MULTIPLE PHASES OF $CD8^+$ CTL RESPONSES FOLLOWING ADENOVIRUS VACCINATION

Channakeshava Sokke Umeshappa[1,2], Roopa Hebbandi Nanjundappa[1,¶], Yufeng Xie[1], Andrew Freywald[2], and Jim Xiang[1,2,*]

Cancer Research Unit[1], Saskatchewan Cancer Agency, Departments of Oncology[1] and Pathology[2], University of Saskatchewan, Saskatoon, Saskatchewan, Canada

Short title: "$CD4^+$ T cells' role in AdV-stimulated CTL responses"

***Correspondence should be addressed to:** J.X., Cancer Research Unit, Saskatchewan Cancer Agency, 20 Campus Drive, Saskatoon, Saskatchewan S7N 4H4, Canada, Tel: (306) 6552917, Fax: (306) 6552635, email: jim.xiang@saskcancer.ca

¶Present address**:** Department of Microbiology, Immunology & Infectious Diseases, Health Research Innovation Centre, 3330 Hospital Drive NW, University of Calgary, Calgary, Alberta T2N 4N1 Canada.

Key words: Adenovirus, $CD4^+$ T cell help, $CD8^+$ CTLs, CD40L and IL-2, and primary, maintenance and recall CTL phases

5.1 Brief introduction to Chapter 5

AdV vectors are at the forefront of vaccine development against cancers and chronic diseases, where the establishment of these diseases often reflected with poor functions or loss of $CD4^+$ T cells. Currently, how $CD8^+$ CTL responses are modulated by $CD4^+$ T cells following AdV immunization is less defined area although such an understanding is very critical for successful designation of AdV vaccines. Thus, while the above studies discussed in Chapter 2 to 4 addressed the importance of $CD4^+$ T cells in DC immunization model, the present study investigated the role of these cells in different phases of AdV transgene product-specific CTL responses following AdV immunization.

5.2 Abstract

Adenoviral (AdV) vectors represent most commonly utilized viral vaccines in clinical studies. While the role of $CD8^+$ cytotoxic T lymphocyte (CTL) responses in mediating AdV-induced protection is well understood, the involvement of $CD4^+$ T cell signals in the development of functional $CD8^+$ CTL responses remain unclear. To explore $CD4^+$ T helper signals required for AdVova-stimulated CTL responses, we established an adoptive transfer system by transferring $CD4^+$ T cells derived from various knock out and transgenic mice into wild-type and/or CD4-deficient animals immunized with recombinant ovalbumin (OVA)-expressing AdVova vector. Without $CD4^+$ T help, the survival of both primary and memory CTLs was greatly reduced, and memory CTLs were associated with increased PD-1 expression. The transfer of naïve OVA-specific $CD4^+$ T cells to $CD4^+$ T-deficient mice restored primary responses, and fairly supported survival and recall responses of memory CTLs. Furthermore, this help was specifically mediated by $CD4^+$ T cell-derived IL-2 and CD40L signals. Poor survival of adoptively transferred effector or memory CTLs in naïve $CD4^+$ T-deficient mice further revealed an additional role for polyclonal $CD4^+$ T cell environment in the survival of AdVova-stimulated CTLs, partially explaining protracted CTL contraction phase. Finally, during recall responses, $CD4^+$ T cell environment containing memory $CD4^+$ T cells greatly enhanced expansion of memory CTLs. Collectively, these results suggest a critical role for $CD4^+$ T help in multiple phases of AdVova-stimulated OVA-specific CTL responses, and could partially explain certain failures in AdV-based immunization trials against tumor and chronic diseases that are often associated with compromised $CD4^+$ T cell effect.

5.3 Introduction

$CD8^+$ T cells play a defensive role against viral infections and malignancies. Following recognition of a specific Ag, naïve $CD8^+$ T cells undergo 3 distinct phases (1): (i) a proliferation (or primary) phase in which naïve $CD8^+$ T cells undergo autonomous clonal expansion and develop into functional effector cytotoxic T lymphocytes (CTLs); (ii) a contraction (or effector) phase in which 90-95% of effector CTLs undergo activation-induced cell death (AICD) through apoptosis, allowing development of remaining effector CTLs into memory CTLs; and (iii) a maintenance (or memory) phase in which memory CTLs survive for prolonged duration. Upon subsequent Ag encounter, these memory CTLs respond swiftly by rapid proliferation and heightened effector functions leading to recall responses.

It is becoming increasingly clear that requirements for $CD4^+$ T cell help at different phases of CTL responses can vary, depending on a specific type of infection or immunization involved (2, 3). Primary CTL responses to infectious agents, such as *Listeria montocytogenes* (Lm), influenza and Lymphocytic choriomeningitis virus (LCMV), occur independent of $CD4^+$ T-helper signals (4-6). In contrast, primary CTL responses induced in noninfectious conditions by minor-H Ags, and cell-associated and protein-triggered immunizations (7, 8), and also CTL responses in infectious diseases, such as Herpes simplex (HSV), Viral encephalitis and Vaccinia virus (2, 3, 8, 9), heavily depend on $CD4^+$ T cell-produced signals. Requirement for cognate $CD4^+$ T cell signals during priming in functional memory CTL development has been frequently suggested (5, 6, 10). It has also been shown that signaling induced by $CD4^+$ T cell-expressed CD40L is needed for the generation of memory CTLs in the courses of the Lm or LCMV infections (11, 12), while it is not essential in influenza infections (13, 14). In AdV-mediated immunity, Yang et al have initially observed the importance of $CD4^+$ T cells in primary CTL responses in AdV immunization (15, 16). Subsequently, others also showed the importance of $CD4^+$ T cells in AdV-specific primary CTL expansion (17-19). However, the role of $CD4^+$ T cells in priming that modulates secondary CTL responses is still controversial. Yang *et al* (17, 20) reported that CTLs generated in $CD4^+$ T cell-deficient environment are less functional yet retain their proliferating ability during recall responses. On the other hand, Holst *et al* (18) showed generation of dysfunctional memory CTLs that neither provided the protection against a lethal virus challenge nor retained the ability to proliferate during recall responses in the absence of $CD4^+$ T cells. Mu

et al (19) reported that respiratory mucosal, which is in contrast to intravenous (i.v.) or intramuscular, route of AdV immunization can preclude $CD4^+$ T helper dependence for effective CTL responses. The molecular mechanisms involved in $CD4^+$ T-helper effects in the primary response that modulate different stages of CTL action are still unknown. Furthermore, the relative contributions of $CD4^+$ T cells during maintenance and recall phases respectively for CTL survival and expansion are yet to be determined. In this study, we addressed these issues by systematically investigating the requirement for $CD4^+$ T cells in multiple phases of ovalbumin (OVA)-specific $CD8^+$ CTL responses induced by recombinant OVA-expressing adenovirus (AdVova) vaccination.

5.4 Materials and methods

5.4.1 Reagents, tumor cells and animals

The biotin- or fluorescent-labeled (FITC or PE) Ab specific for CD4, CD44 (IM7), CD62L (MEL-14), CD69 (H1.2F3) and IFN-γ (XMG1.2), and streptavidin-PE Texas Red and streptavidin-PECy5 from BD-Biosciences, and PD-1 (J43) from ebiosciences were purchased. The FITC-anti-CD8 (KT15) and H-$2K^b$/$OVA_{257-264}$ tetramer from Beckman Coulter were purchased. The OVAI ($OVA_{257-264}$, SIINFEKL) and OVAII ($OVA_{265-280}$, TEWTSSNVMEERKIKV) peptides were synthesized by Multiple Peptide Systems. The OVA-transfected mouse malignant melanoma (BL6-10_{OVA}) cell lines were cultured as described previously (10, 21). The WT C57BL/6J, $OVA_{323–339}$-specific TCR-transgenic OTII, C57BL/6/B6.1 (B6.1, $CD45.1^+$), $CD80^{-/-}$, IL-$2^{-/-}$, $CD40L^{-/-}$ and $Ia^{b-/-}$ mice on C57BL/6 background were purchased from Jackson Laboratory or bred in University's animal resource center. The OTII/B6.1, OTII/$CD80^{-/-}$ and OTII/$CD40L^{-/-}$ mice were generated by backcrossing designated KO mice with OTII mice, and tested as described previously (10). All the animal experiments were performed as per the guidelines approved by the University Committee on Animal Care and Supply.

5.4.2 Generation of AdVova and mature DCova

The recombinant AdV-expressing OVA (AdVova) construction and its amplification were previously described (22). Bone-marrow-derived, OVA-pulsed dendritic cells (DC_{OVA})

from C57BL/6 mice were generated by culturing bone marrow cells for 6 days in medium containing IL-4 (20 ng/ml) and GM-CSF (20 ng/ml) and pulsing with 0.1 mg/mL OVA overnight at 37°C as described previously (21).

5.4.3 Animal studies - Adoptive transfer and AdVova immunization

In most experiments, $CD4^+$ or $CD8^+$ T cells were isolated from splenocytes and/or blood by enriching T lymphocytes in nylon-wool columns (C&A Scientific), and negative selection using anti-CD8 (L3T8) or anti-CD4 (L3T4) paramagnetic beads (DYNAL) as previously described (10). For immunization or boosting, $1x10^7$ pfu of AdVova was used for i.v. injection. The $CD11c^+$ DCs and, in some cases, $CD4^+$ T cells devoid of $CD11c^+$ $CD4^+$ DCs were purified from splenocytes of WT mice as per manufacturer's instructions (Miltenyl Biotec). In adoptive studies, to understand $CD4^+$ T-helper roles, ~$15\text{-}20x10^6$ polyclonal $CD4^+$ T cells or ~$1.5x10^6$ OTII $CD4^+$ T cells with or without designated gene deficiency and $0.5\text{-}1x10^6$ $CD11c^+$ DCs were transferred to $Ia^{b-/-}$ mice one day before immunization. For generating memory $CD4^+$ T cells, B6.1/OTII $CD4^+$ T cells were stimulated with DCova and resulting Th cells were adoptively transferred to congenic WT B6.2 mice. After 45 days, polyclonal $CD4^+$ T cells containing $CD4^+$ T memory cells were purified and transferred ($15\text{-}20x10^6$/mouse) to $Ia^{b-/-}$ mice for recall studies. In memory maintenance or recall studies, polyclonal $CD8^+$ T cells containing effector or memory CTLs (~$15x10^6$/mouse) were transferred to $Ia^{b-/-}$ mice.

5.4.4 Primary and memory CTL kinetics study by tetramer or intracellular IFN-γ staining assays

Following AdVova immunization, the blood and/or spleen samples were collected at different intervals, stained with H-$2K^b$/$OVA_{257\text{-}264}$ tetramer and FITC-anti-CD8 Ab, and analyzed for CTL proliferation or survival (21). For intracellular staining, the samples were collected, re-stimulated with OVAI or OVAII and subjected to intracellular IFN-γ assay (BD-Biosciences) for analysis of OVA-specific $CD8^+$ and $CD4^+$ T cell responses, respectively as described previously (23).

5.4.5 In vivo cytotoxicity assay

The targets were prepared as described previously (10) by labeling splenocytes differentially with high (3.0 μM) or low (0.6 μM) concentrations of CFSE and by pulsing with OVAI or Mut1 peptide, respectively, and co-injected i.v. ($2x10^6$ cells/mouse) at 1:1 ratio into immunized or unimmunized mice. Sixteen hours later, the relative proportions of $CFSE^{high}$ and $CFSE^{low}$ cells remaining in the spleens were analyzed by flow cytometry (10).

5.4.6 Phenotypic characterization of effector and memory CTLs, or memory $CD4^+$ T cells

To characterize memory CTLs phenotypically, the blood and spleen samples were collected 75 days after AdVova immunization, and stained with tetramer and Ab specific for PD-1 surface marker. The percentage of $tetramer^+$ CTLs that express PD-1 marker were determined by flow cytometry. To characterize memory $CD4^+$ T cells, the blood samples were collected 45 days of adoptive transfer, stained with PE-anti-CD4, FITC-CD45.1 and biotin-conjugated Abs specific for active or memory phenotype. The relative expression of surface markers in comparison to isotype control levels was determined in $CD4^+CD45.1^+$ population.

5.4.7 Tumor protection studies

All the immunized mice were challenged with BL6-10_{OVA} ($0.5x10^6$ cells/mice) on 120^{th} day of immunization as shown in Table 1 and monitored for protection up to 24 days or earlier if the mice become moribund as described previously (21). The tumor grading was done depending on mean numbers of metastatic tumor colonies in lungs: -, no tumors; +, 1-25; ++, 26-50; +++, 51-75; ++++, 76-100; +++++, 101-250; ++++++, >250.

5.4.8 Statistical analysis

The statistical analysis were performed using Student's *t* or Mann–Whitney U test (Graphpad Prism-3.0); $*P<0.05$ and $**P<0.01$.

5.5 Results

5.5.1 AdVova stimulates persistent CD4$^+$ and CD8$^+$ T cell responses

Initially, we assessed the kinetics of CD4$^+$ and CD8$^+$ T cell responses at different time points following AdVova stimulation by flow cytometry after staining cells with FITC-CD4/nonspecific PE-CD44 or FITC-CD8/OVA-specific PE-tetramer, respectively. In line with previous findings, upon AdVova immunization, we observed a peak of OVA-specific CD8$^+$ T cell responses on day 10 (21.2±3.7% of total CD8$^+$ CTLs) and a long-term maintenance of high levels of OVA-specific CTLs (as much as 6.5±1.1% of total CD8$^+$ CTLs) even 4 months later (Fig 1a), indicating that CD8$^+$ CTLs persist for prolonged period of time, consisting with earlier reports (17, 24). In contrast to the situation in blood, assessment of spleen samples revealed earlier responses, and was associated with relatively lowered levels (3 to 5 fold) of CTLs (data not shown). These results indicate that CD8$^+$ CTLs may persist for a very prolonged period of time, which is consistent with previously published reports (17, 24). In addition, our experiments also showed that AdVova-stimulated CD4$^+$ T cells persist in both peripheral blood and spleens. Strikingly, in contrast to CTL population that showed greater than 60% contraction 4 months later, AdVova-stimulated poly-specific CD4$^+$ T cells gradually increased their levels with time, representing 21.5±3.2% and 23.4±3.5% of total blood CD4$^+$ T cells as early as on 5 and 10 days post-immunization, respectively, and 42.2±5.7% in 4 months (Fig 1b). However, their levels in spleen leveled off by 4 months. In addition, we also demonstrated that frequency of IFN-γ-secreting CD4$^+$ T cells is significantly higher in the immunized mice than in control animals on both 5 and 10 days after immunization ($P<0.05$) (Fig 1c), confirming that AdVova immunization also triggered transgene-specific CD4$^+$ T cell responses.

5.5.2 CD4$^+$ T cells influence the kinetics of AdV transgene product-specific CTLs

To assess the influence of CD4$^+$ T cells in AdVova-stimulated CTL responses, we immunized Ia$^{b-/-}$ mice lacking CD4$^+$ T cells with AdVova and analyzed OVA-specific CD8$^+$ CTL responses at different time points by flow cytometry. Consisting with previous results (17), we found 4 to 6 fold decrease in primary CTL proliferation in Ia$^{b-/-}$ compared to WT mice (Fig. 2a). Furthermore, 90 days after immunization, WT mice showed more than ten-fold better

survival rate of CTLs compared to $Ia^{b-/-}$ mice. Although primary CTLs in $Ia^{b-/-}$ mice (unhelped) expanded less efficiently, they still appeared to show some degree of functions, such as IFN-γ-secreting ability (Fig. 2b) and cytotoxic functions (Fig. 2c). Owing to relatively prolonged persistence of AdVova in the body, it is possible that $CD4^+$ T cells could influence the exhaustion AdVova-specific CTLs. To rule out this possibility, we monitored the expression of PD-1, a well-established exhaustion marker, on helped versus unhelped CTLs (25). Indeed, the poor survival of AdV-specific CTLs in $Ia^{b-/-}$ mice correlated well with the presence of considerably higher numbers of PD-1-expressing memory CTLs in spleen compared to those of WT mice on day 75 post-immunization (Fig. 2d). These results strongly suggest $CD4^+$ T cells enhance AdV-stimulated CTL survival by rescuing them from exhaustion by inhibiting PD-1 expression.

5.5.3 Molecular mechanisms of $CD4^+$ T cell help in CTL primary responses and survival

To assess the molecular mechanisms of $CD4^+$ T cell help, we developed an immunization protocol with a combination of AdVova immunization and adoptive transfer of cognate OTII $CD4^+$ T cells in $Ia^{b-/-}$ mice (Fig. 3a). We first optimized the dose of purified monoclonal or polyclonal $CD4^+$ T cell requirement for efficient CTL responses (data not shown). A dose of polyclonal $CD4^+$ T (~15-20x10^6) or cognate OTII $CD4^+$ T (~1.5x10^6) cells was found to be efficient in reproducing primary CTL response in $Ia^{b-/-}$ comparable to WT mice (Fig. 3b). Since Ag-presenting cells (APCs) in these mice lack MHC-II molecules and unable to stimulate transferred $CD4^+$ T cells, $CD11c^+$ DCs (~1x10^6) purified from spleens were also necessary for adoptive transfer to initiate AdVova-stimulated CTL responses. The critical roles of $CD11c^+$ DCs in AdV immunization have been well demonstrated previously (26, 27). In the presence of polyclonal $CD4^+$ T cell help, perhaps due to endogenous levels of precursor $CD4^+$ T cells, the primary CTL expansion was considerably increased in $Ia^{b-/-}$ mice. Strikingly, in the presence of OTII $CD4^+$ T cells, the primary $CD8^+$ CTLs expanded even more efficiently (1.5 fold) in $Ia^{b-/-}$ than in WT mice, indicating that this immunization protocol can be used to assess the molecular mechanisms of $CD4^+$ T cell help, when $CD4^+$ T cells derived from IL-2-, CD40L- and CD80-deficient mice are transferred.

To address the involvement of specific molecular factors, we reconstituted $Ia^{b-/-}$ mice with polyclonal or monoclonal (OTII) $CD4^+$ T cells with or without a deficiency in selected

genes and with $CD11c^+$ DCs one day prior to the immunization. Once again, the reconstitution of polyclonal $CD4^+$ T cells in $Ia^{b-/-}$ mice considerably supported primary CTL expansion, compared to WT levels, and the CTL response was further increased, when endogenous levels of OTII $CD4^+$ T cells were additionally provided (Fig. 3c). Strikingly, the primary expansion was severely impaired in the absence of IL-2 signaling by polyclonal $CD4^+$ T cells, strongly resembling the situation in $Ia^{b-/-}$ mice. In addition, the primary expansion was significantly reduced when polyclonal $CD4^+$ T cells lacking CD40L ($P<0.01$) were transferred, while the absence of CD80 did not produce any negative effect, suggesting a potential role for CD40L in optimizing the primary expansion. During the memory phase, CTL survival considerably decreased in $Ia^{b-/-}$ compared to WT mice ($P<0.01$) irrespective of $CD4^+$ T cell transfer (Fig. 3c, right panel), perhaps due to the failure of $CD4^+$ T cells to survive in the MHC-II-deficient environment (28). Nevertheless, $Ia^{b-/-}$ mice reconstituted with polyclonal $CD4^+$ with or without OTII $CD4^+$ T cells had considerably higher frequencies of CTLs, when compared to $Ia^{b-/-}$ mice alone or to $Ia^{b-/-}$ mice transferred with polyclonal $CD4^+$ T cells lacking CD40L or IL-2 ($P<0.01$). Strikingly, the transfer of cognate $CD4^+$ T cells ($\sim 1.5 \times 10^6$) supported the primary CTL expansion, surpassing the WT levels (Fig. 3d). In contrast, the absence of co-stimulatory molecules, particularly CD40L, suppressed the ability of cognate $CD4^+$ T cells to support the primary CTL expansion, mimicking the response in untransferred $Ia^{b-/-}$ mice. As previously, CTLs in $Ia^{b-/-}$ mice survived poorly during the memory phase, irrespective of OTII $CD4^+$ T cell transfer. Nevertheless, $Ia^{b-/-}$ mice reconstituted with OTII cells with or without CD80 had relatively higher frequencies of CTLs, when compared to $Ia^{b-/-}$ alone ($P<0.01$) or to $Ia^{b-/-}$ animals reconstituted with CD40L-deficient polyclonal $CD4^+$ T cells ($P<0.05$).

To study the impact of $CD4^+$ T cell signals during priming for functional memory CTL responses, the above mice were challenged 120 days later with highly metastasizing, OVA-expressing mouse melanoma tumor cells. WT, but not $Ia^{b-/-}$ animals showed complete tumor protection (Table 1). However, $Ia^{b-/-}$ mice which received $CD4^+$ T-helper-produced signals during priming got protected to various extents, depending on signaling content. Although $Ia^{b-/-}$ mice with transferred polyclonal $CD4^+$ T cells developed tumors, they had very low tumor burdens compared to mice receiving CD40L-deficient or IL-2-deficient polyclonal $CD4^+$ T cells. Interestingly, 40% of the challenged $Ia^{b-/-}$ mice that were transferred with OTII cells, showed tumor protection. In contrast, mice transferred with OTII $CD4^+$ T cells missing CD40L remained

completely unprotected. Taken together, these data highlight the importance of cognate $CD4^+$ T-helper signals in AdV immunization, specifically CD40L and IL-2, in the optimization of the primary expansion and functional memory development.

5.5.4 Naïve polyclonal $CD4^+$ T cells support maintenance of AdV transgene product-specific CTLs

The observed faster decrease in the frequency of CTLs in $Ia^{b-/-}$ compared to WT mice, even in the presence of reconstituted $CD4^+$ T-helper population during priming, (Fig. 3c and 3d) suggests a possible role for polyclonal $CD4^+$ T cell environment in the maintenance of CTLs after priming. To examine this possibility, the effector CTLs from B6.1 mice ($CD45.1^+$) were purified on day 10 following AdVova immunization and transferred to naïve congenic WT or $Ia^{b-/-}$ mice ($CD45.2^+$) and tracked by the tetramer and congenic marker staining. This approach precludes detection of endogenous CTLs stimulated by any residual AdV-infected DCs following adoptive transfer. Consistent with our previous observations (Fig. 2a, 2b, 3c or 3d), the effector CTLs declined drastically starting from day 7 in $Ia^{b-/-}$ mice, while a significantly slower decline was characteristical for WT mice (Fig. 4a). In agreement, WT mice showed near complete protection against tumor challenge, while $Ia^{b-/-}$ remained practically unprotected (Fig. 4b). Furthermore, memory CTLs purified after 90 days following immunization behaved similar to effector CTLs, showing reduced survival rate in $Ia^{b-/-}$ mice (Fig. 4c). Again, WT animals received much better protection, when compared to $Ia^{b-/-}$ mice (Fig. 4d). In sum, these observations suggest that polyclonal $CD4^+$ T cell environment may be required for optimal maintenance of AdVova-specific CTLs.

5.5.5 $CD4^+$ T cell signals during priming and recall phase are required for optimal secondary responses

Recent studies in multiple models have shown that $CD4^+$ T cells are essential for optimal recall responses (1, 29-31). The results of our experiments (Fig. 3c and 3d) also suggest the role for cognate $CD4^+$ T cell signals, partly produced during priming, for functional memory responses, thus, supporting previously published data (5, 6). To further elucidate the relative importance for $CD4^+$ T cells in priming and/or recall responses, memory CTLs were purified

from splenocytes of WT (helped CTLs, TmA) or $Ia^{b-/-}$ (unhelped CTLs, TmB) mice after 90 days of AdVova immunization and adoptively transferred in equal quantities to the naïve secondary recipients, WT or $Ia^{b-/-}$ mice (Fig. 5a). Recently, Hutnick *et al* reported that AdV-stimulated memory CTLs can expand even in the presence of neutralizing Abs following boosting with similar strain of AdV (32). It appears the approach followed here further facilitated the expansion of AdV-stimulated memory CTLs following boosting in naive secondary recipients in the absence of neutralization of AdVova by Abs. Following AdVova boosting, helped memory CTLs robustly expanded in WT when compared to their expansion in $Ia^{b-/-}$ mice (Fig. 5b). Curiously, unhelped memory CTLs in WT showed increase in the expansion only slightly above the levels observed in WT without Tm transfer. Moreover, unhelped memory CTLs in $Ia^{b-/-}$ mice failed to expand and showed levels similar to $Ia^{b-/-}$ mice without Tm transfer. These results suggest possible synergistic role for $CD4^+$ T cell help in both priming and recall responses.

To further understand the nature of $CD4^+$ T cell helper requirement during recall responses, the adoptive transfer technology was employed again in $Ia^{b-/-}$ mice (Fig. 6a). Here, $Ia^{b-/-}$ mice, were transferred with $CD11c^+$ DCs and OTII cells, polyclonal $CD4^+$ T cells, or OVA-specific memory $CD4^+$ T cells after being adoptively transferred with helped memory CTLs (Fig. 6b) as shown in Fig. 6a. As expected, helped CTLs in the presence of $CD4^+$ T cells produced a modest increase in the expansion, but the response was considerably much higher in the presence of memory $CD4^+$ T cells (Fig. 6c). On the other hand, $Ia^{b-/-}$ reconstituted with OTII cells or polyclonal $CD4^+$ T cells in the absence of memory CTLs showed a response similar to that of unreconstituted $Ia^{b-/-}$ mice (not shown), suggesting the observed CTL responses were mainly due to memory CTL expansion. Overall, these results indicate the importance of $CD4^+$ T helper signals provided during the priming and recall phases for optimal AdV-stimulated memory CTL responses.

5.6 Discussion

In the absence of strong inflammatory signals, $CD4^+$ T cells enhance CTL immunity either indirectly by licensing DCs (7, 8), or directly by interacting with cognate $CD8^+$ T cells (10, 21, 33). Immunity to cancers, allogenic transplantations and autoimmune disorders thus requires $CD4^+$ T cells for optimal priming, maintenance and memory responses (1, 6, 33-36). Even in some infectious diseases, such as HSV, VSV, viral encephalitis and Vaccinia virus infections,

$CD4^+$ T cell help is still crucial for the induction of robust primary and functional memory responses (2, 3, 9, 37, 38) though the viral byproducts (such as DNA or double-stranded RNA) are capable of inducing inflammation. Interestingly, the present study also demonstrates the predominant role of $CD4^+$ T cells in multiple phases of AdVova-stimulated OVA-specific CTL responses, including the primary, memory maintenance and recall responses. We demonstrate that the cognate $CD4^+$ T cell help during priming is crucial not only for the primary expansion and memory CTL survival, but also for the fitness of memory CTLs. We also reveal that the differentiation of primary CTLs is considerably impaired in the absence of $CD4^+$ T cell-derived CD40L signaling. Similarly, functional primary, but defective memory CTL responses were also observed in association with LCMV, Pichinde virus and VSV infections in the absence of CD40L signaling (11, 39). It appears IL-2 signaling also represents a crucial event that not only supports primary expansion, but also helps in the generation of memory CTLs, providing survival advantages. The role of the CD40L and IL-2 factors derived from $CD4^+$ T cells in producing optimal primary and memory responses is well described in various models (2, 37, 40). It was shown that Th1 provide CD40L signals to DCs to induce IL-12 secretion and IL-12, in turn, acts on $CD8^+$ T cells to enhance IL-2R expression, thus enabling an efficient utilization of the IL-2 cytokine (2). Alternatively, it was shown that Th1 can directly provide CD40L, IL-21 and IL-2 signals to $CD8^+$ T cells in an Ag-specific manner (10, 21, 33, 40, 41). Interestingly, in both direct and indirect mechanisms, cognate $CD4^+$ T cells have been shown to modulate CTL responses in a CD40L- and IL-2- dependent manner. In $Ia^{b-/-}$ mice, the incomplete maturation of DCs without $CD4^+$ T cell signals, and/or absence of direct $CD4^+$ T cell signals might have led to poor primary and memory CTL responses since we were able to restore such defectiveness by provision of polyclonal or cognate $CD4^+$ T cells, suggesting critical roles for $CD4^+$ T cells in AdV-mediated CTL induction. .

The enhanced recall responses upon provision of naïve monoclonal or polyclonal, or memory $CD4^+$ T cells suggest yet another role for $CD4^+$ T cells in OVA-specific CTL responses. It is currently not clear, why memory CTLs required $CD4^+$ T cell environment, while receiving helper signals during priming and/or being maintained in polyclonal naïve and/or AdV-specific $CD4^+$ T cell environment. It was shown in various models that $CD4^+$ T helper signals operate similarly during recall responses (1, 31, 34, 36, 40). For instance, treatment with agonistic anti-CD40 mAb and IL-2 is known to decrease the expression of PD-1, a potent T cell inhibitory

molecule, on memory CTLs (31). Consistent with our present observations, memory $CD4^+$ T cells have also shown to enhance the primary responses of naïve and memory $CD8^+$ T cells in various models (30). Moreover, direct interactions between memory $CD4^+$ and $CD8^+$ T cells, involving CD40L and IL-2 signaling, and leading to enhanced recall responses have also been reported (34).

Considerable attention has been paid to understanding AdV-specific CTL persistency. Due to persistent transgene expression (20, 24, 42), the high levels of CTL maintenance for a long period of time might originate from reactivation of CTLs by APCs or AdV-transduced non-APCs (42). In this scenario, all these CTLs could exhaust over a period of time by losing their ability to secrete cytokines and protecting against viral or tumor challenges, as seen with chronic viral (LCMV and cytomegalovirus) infections (25). In AdV immunization, we demonstrate a drastic decrease of CTL persistency in the $CD4^+$ T cell-deficient environment, which may result from a poor activation of CTLs and a lack of $CD4^+$ T cell signals. It appears, in WT mice, both endured AdV-stimulated and naïve polyclonal $CD4^+$ T cells contribute to CTL persistency. Previously, involvement of nonspecific signals from naïve $CD4^+$ T cells in increasing fitness and quantities of memory CTLs in acute infections have been reported (43). Similarly, effects of signals from active polyclonal $CD4^+$ T cells on enhancing CTL survival have also been observed (44). On the other hand, cognate active $CD4^+$ T cells were able to support robust CTL priming. Perhaps due to the absence of some specific types of $CD4^+$ T helper signals, $Ia^{b-/-}$ animals demonstrate 2 to 3 fold increase in the $PD\text{-}1^+$ CTLs, when compared to WT mice. Recently, in Vaccinia virus model, Novy *et al* reported that $CD4^+$ T cells enhance memory CTL survival by providing IL-21 signals (1, 40). These signals were required to activate STAT1 and STAT3 pathways and subsequent increase of the prosurvival molecules, including Bcl-2 and Bcl-xL (40). This possibility could exist in AdV immunization owing to persistency of AdV-specific, active $CD4^+$ T cells in AdVova immunization. However, the precise relative contributions of transgene and non-transgene AdV-stimulated $CD4^+$ T cells vs naïve polyclonal $CD4^+$ T cells and associated molecular mechanisms for memory CTL survival need further investigation. Aubert *et al* demonstrated that $CD4^+$ T cells can rescue exhausted $CD8^+$ T cells during chronic viral and *Plasmodium* parasitic infections (45, 46). Similarly, exhausted $CD8^+$ T cells derived from progressive HIV patients underwent proliferation when co-cultured with $CD4^+$ T cells taken from acute HIV patients (45, 47), suggesting the provision of $CD4^+$ T helper factors could revert CTL

functions in chronic HIV infections. Previously, the use of autologous $CD4^+$ T cells in metastatic melanoma patient have also shown to induce prolonged clinical remission (48, 49). These results together with ours suggest that the use of supplemental $CD4^+$ T cell therapy or $CD4^+$ T helper factors may be beneficial in successful treatment of AdV-immunized patients with chronic infections or cancers.

Due to their efficiency in inducing strong innate as well as adaptive immune responses (50), AdVs have recently gained much attention as promising vaccination tools in treating intractable diseases such as cancers (51, 52) and chronic infectious diseases (53, 54). However, AdV-based vaccines often failed to give protection in clinical trials, partly due to the chronic nature of the pathogenic Ags and the associated imbalance in the host immune responses, such as the selective depletion or defectiveness of $CD4^+$ T cells in chronic diseases, such as HIV infection (55-58). Perhaps due to these reasons, our results could partially explain the failure in AdV-based vaccinations against these intractable diseases, warranting the development of novel modified AdV vaccines. The feasibility of this approach is further supported by observations demonstrating an improved $CD4^+$ T cell-independent CTL responses associated with linking of AdV-encoded Ag to invariant chain or β_2-microglobulin (18, 59-61) or altering route of immunization to certain pathogens (19).

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5.8 Disclosures

The authors have no financial conflict of interest

5.9 Table

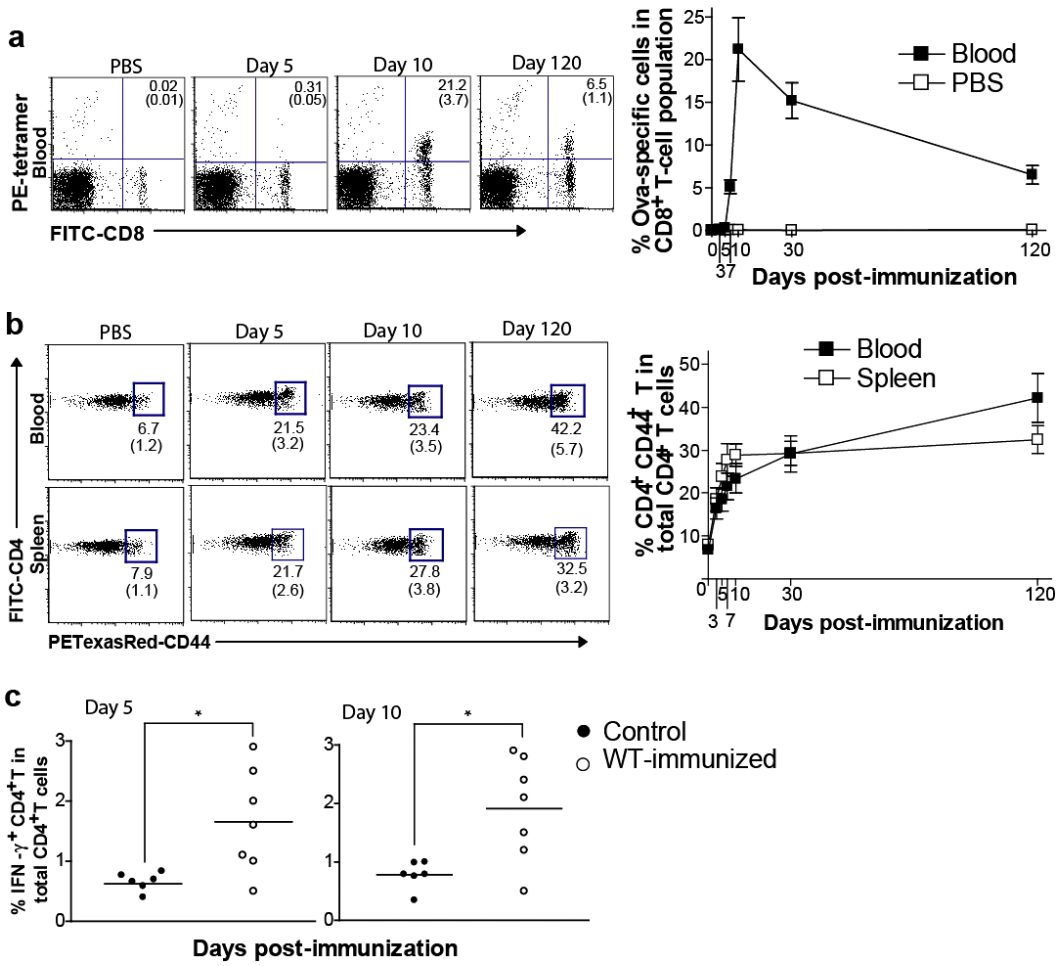
Table 1. Molecular mechanisms of $CD4^+$ T-helper signals required for functional AdV-specific memory CTL responses [a]

AdV_{OVA} immunization	% Tumor-bearing mice	Tumor metastasis grading
PBS	8/8 (100)	++++++
WT	0/12 (0)	-
$Ia^{b-/-}$	12/12 (100)	+++++
$Ia^{b-/-}$+polyCD4$^+$ T	10/10 (100)	+
$Ia^{b-/-}$+polyCD4(IL-2$^{-/-}$)	10/10 (100)	+++++
$Ia^{b-/-}$+polyCD4(CD40L$^{-/-}$)	10/10 (100)	++++
$Ia^{b-/-}$+polyCD4(CD80$^{-/-}$)	10/10 (100)	++
$Ia^{b-/-}$+OTIICD4$^+$ T	6/10 (60)	-/+
$Ia^{b-/-}$+OTIICD4(CD40L$^{-/-}$)	10/10 (100)	+++
$Ia^{b-/-}$+OTIICD4(CD80$^{-/-}$)	10/10 (100)	+

[a] One day prior to immunization, $Iab^{-/-}$ mice were adoptively transferred with $CD11c^+$ DCs and naïve polyclonal or OTII $CD4^+$ T cells with or without designated gene deficiency, as indicated. 120 days later, all the immunized mice were challenged with BL6-10ova tumor cells. Twenty-four days after the challenge, lung tumor colonies were counted and graded. The data are cumulative of two independent experiments, each comprising five to six mice per group.

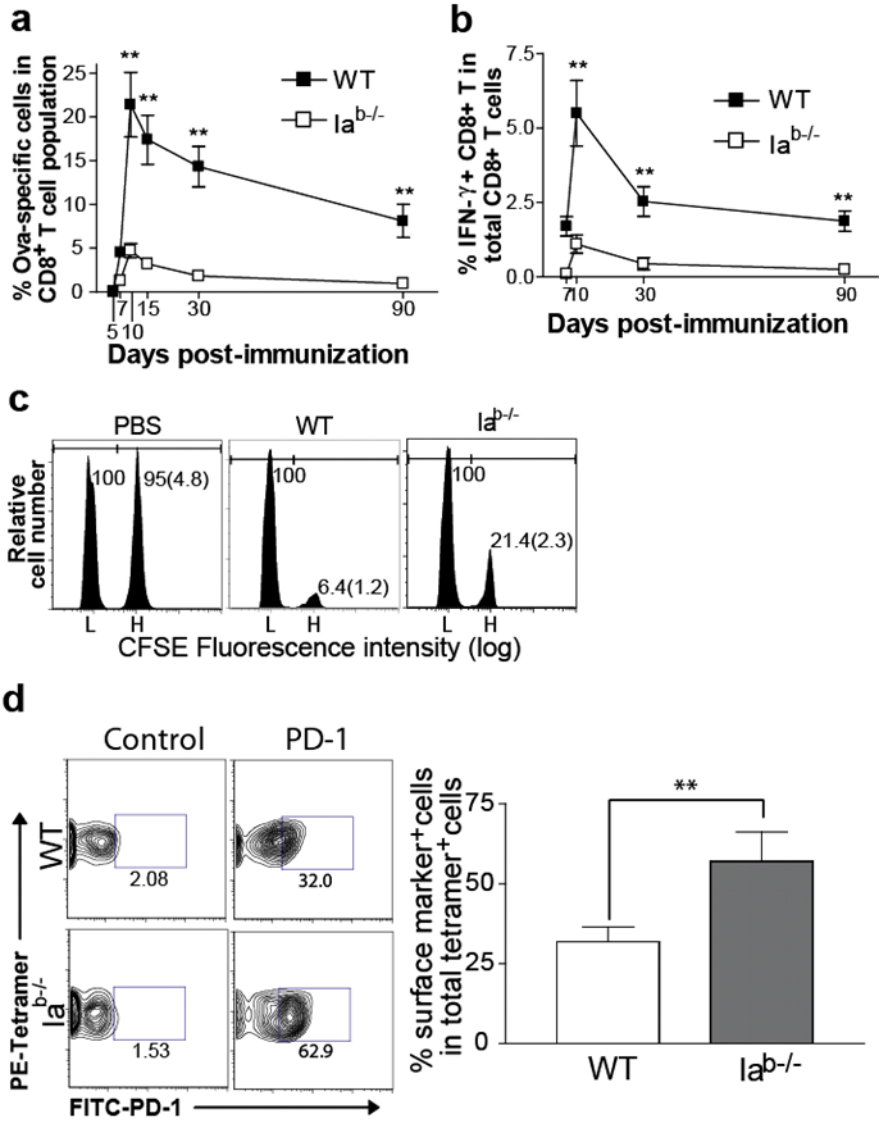
5.10 Figures

Figure 1



AdVova stimulates persistent $CD4^+$ and $CD8^+$ T cell responses. (a and b) Following immunization, the AdVova-specific CTLs (a) and $CD44^+CD4^+$ T cells (b) in the peripheral blood and/or spleen were analysed by flowcytometry at the indicated intervals after tetramer staining, and CD44 and CD4 double marker staining, respectively. The values in the figure (left panel) or line diagram (right panel) are presented as mean%±SD of OVA-specific $CD8^+$ CTLs in total $CD8^+$ T cell population (a) or of $CD44^+$ $CD4^+$ T cells in total $CD4^+$ T cell population (b), and are cumulative of three independent studies with three to five mice per group. (c) Following immunization, spleen samples were analyzed for AdVova-specific $CD4^+$ T cells by intracellular IFN-γ staining at the indicated intervals. The values (% frequencies) are cumulative of two independent experiments with three to four mice per group. **P*<0.05, versus matching controls.

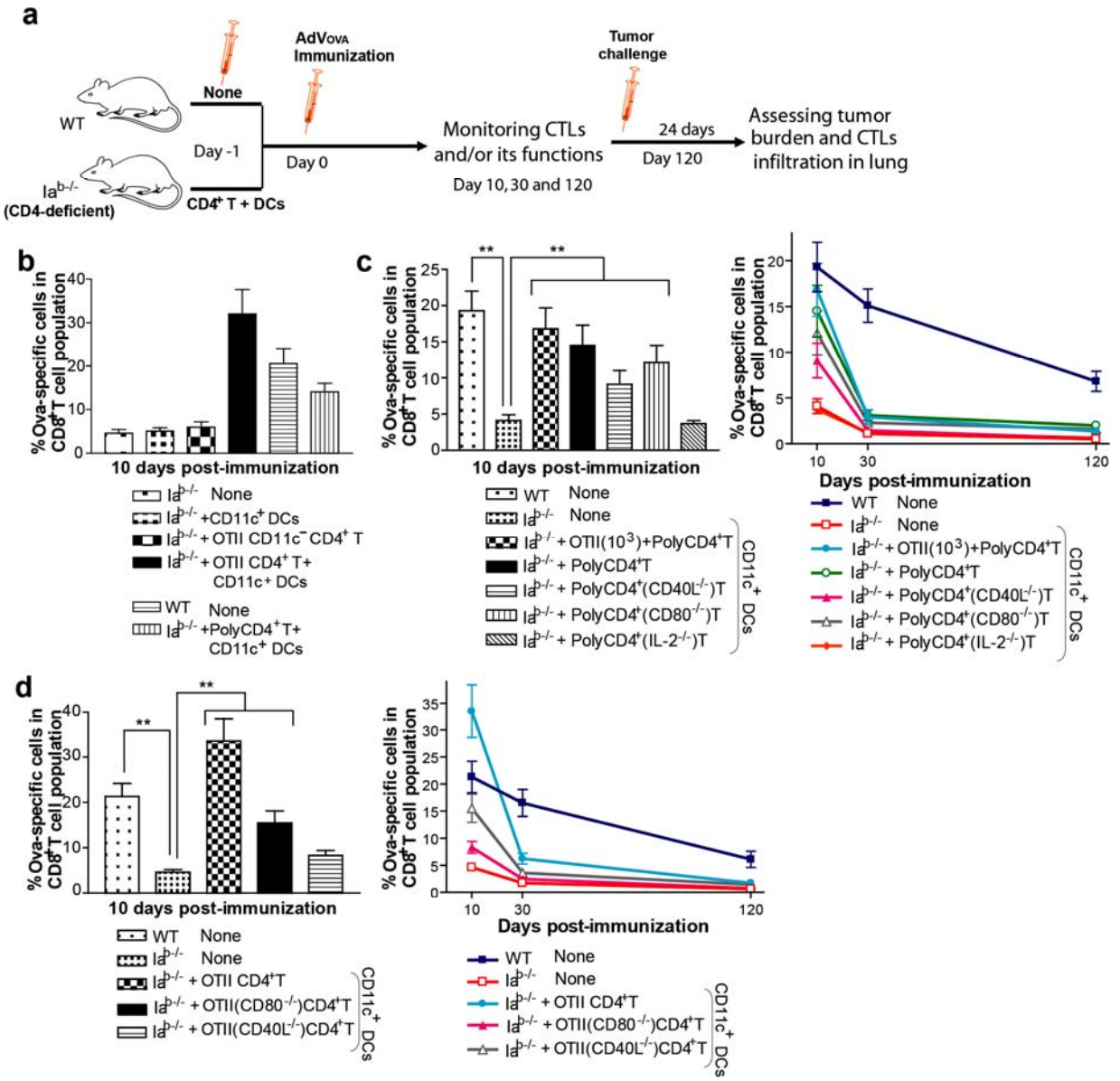
Figure 2



CD4$^+$ T cells impact the kinetics of AdV transgene product-specific CTL populations (a and b) Following immunization, AdVova-specific CTLs were analysed in the periphery at different intervals by tetramer (a) and intracellular IFN-γ (b) stainings. The values are presented as mean%±SD of OVA-specific tetramer$^+$ CTLs (a) or IFN-γ$^+$ CTLs (b) in total CD8$^+$ T cell population and are representative of two to three independent experiments with three to four mice per group. $^{**}P<0.01$, versus Ia$^{b-/-}$ mice. (c) Ten days following immunization, the proportions of CFSE^{high}-OVAI-pulsed target cells lysed by effector CTLs were determined in spleens by *in vivo* cytotoxicity assay. The values represent mean %±SD of targets remaining in spleens relative to controls and are representative of two independent experiments with three to

four mice per group. (d) On day 75, following immunization, OVA-specific memory CTLs were characterized in spleen for PD-1 expression by flow cytometry. A representative figure is shown on the left. The values in the bar diagram represent the mean %±SD of tetramer^{+} PD-1^{+} CTLs in total tetramer^{+} CTL population and are representative of two independent experiments with 3 to 4 mice per group.

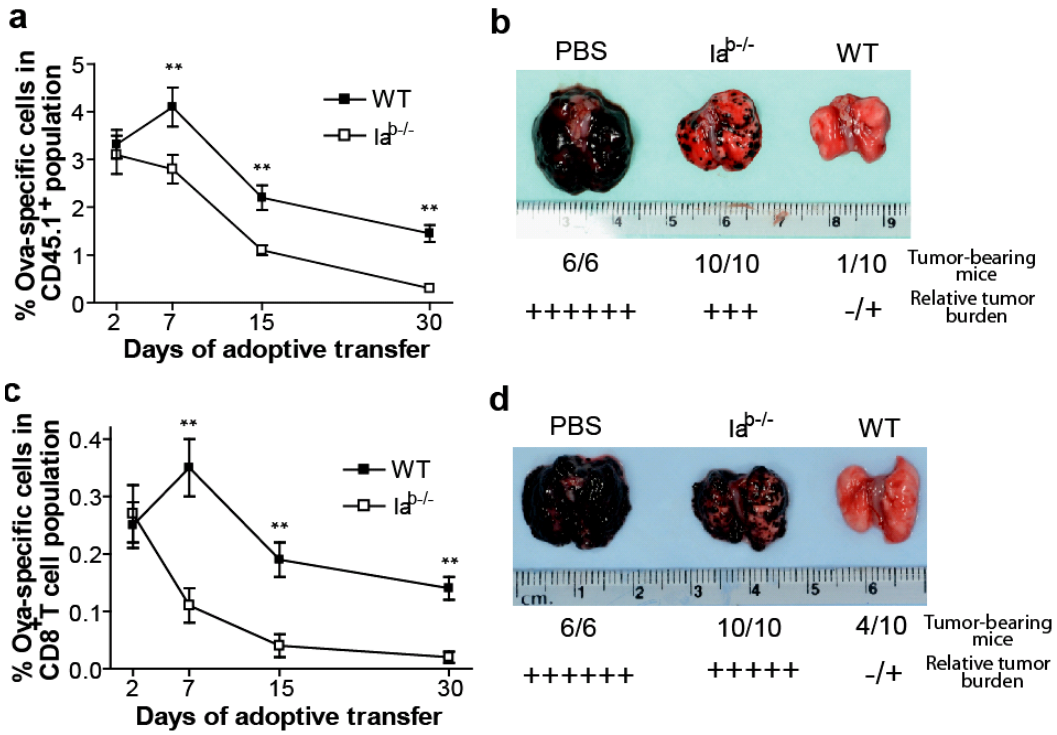
Figure 3



The molecular mechanisms of CD4$^+$ T cell help in CTL primary responses and survival. (a) A schematic protocol. Ia$^{b-/-}$ mice were adoptively transferred with monoclonal or polyclonal CD4$^+$ T cells with or without designated gene deficiency and supplied with spleen-derived CD11c$^+$ DCs. One day later, both Ia$^{b-/-}$ and WT mice were i.v. immunized with AdVova followed by assessment of CTL proliferation at the indicated intervals. After 120 days (memory stage) following the immunization, all the groups were challenged with BL6-10$_{OVA}$ tumor cells and assessed for tumor protection. (b) Optimized CD4$^+$ T-cell dose required for optimal CTL expansion in Ia$^{b-/-}$ mice. Ia$^{b-/-}$ mice were transferred with monoclonal (OTII) (~1.5X10^6) and/or

polyclonal (~15-20X10^6) $CD4^+$ T cells and $CD11c^+$ DCs (~0.5-1.0X10^6), as shown in the figure. One day later, all the groups were immunized and assessed for CTL proliferation by tetramer assay. The values represent mean %±SD of OVA-specific $tetramer^+$ CTLs in total $CD8^+$ T cell population and are representative of two independent experiments with three to four mice per group. (c and d) Molecular mechanisms of $CD4^+$ T cell help. $Ia^{b-/-}$ mice were transferred with monoclonal (OTII) or polyclonal $CD4^+$ T cells with or without designated gene deficiency and $CD11c^+$ DCs as indicated. One day later, all the groups were immunized and subsequently assessed for CTL proliferation by tetramer assay. The values represent mean %±SD of OVA-specific $tetramer^+$ CTLs in total $CD8^+$ T cell population on day 10 post-immunization (left panel) or at the indicated time points (right panel) and are representative of two independent experiments with five to six mice per group. $^{**}P<0.01$, versus $Ia^{b-/-}$ mice with no adoptive $CD4^+$ T cell transfer.

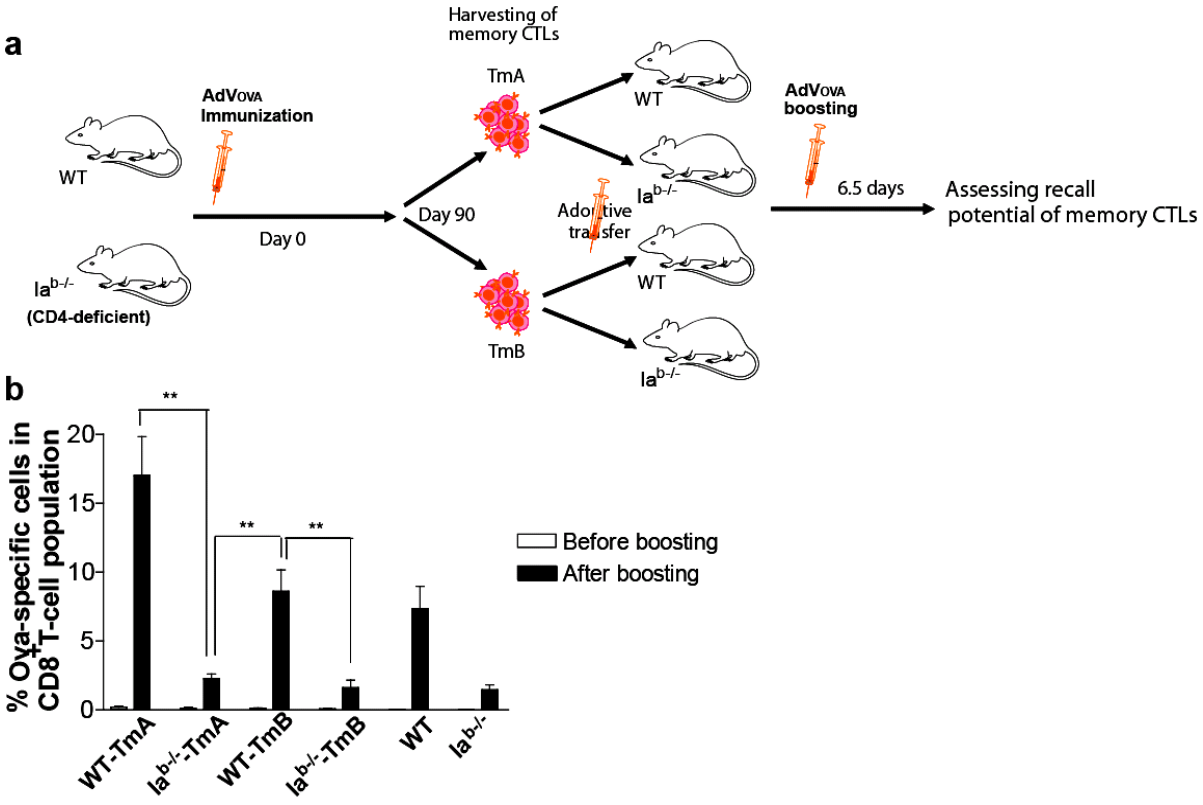
Figure 4



Polyclonal CD4$^+$ T cells support maintenance of AdVova transgene product-specific CTLs. (a) Ten days following immunization, total CD8$^+$ CTLs, containing AdVova-specific effector CTLs were purified from B6.1 mice (CD45.1$^+$ background) and adoptively transferred to naïve congenic WT and Ia$^{b-/-}$ mice (CD45.2$^+$ background). The OVA-specific tetramer$^+$ CTLs were tracked after staining peripheral blood samples with tetramer reagent and congenic marker up to 30 days post-adoptive transfer. The values represent mean %±SD of OVA-specific tetramer$^+$ CTLs in total CD45.1$^+$ adoptively transferred T cell population at the indicated intervals (right panel) and are representative of two independent experiments with five to six mice per group. $^{**}P<0.01$, versus Ia$^{b-/-}$ mice. (b) Forty-five days after adoptive transfer, the above mice groups were challenged with BL6-10$_{OVA.}$ Twenty-four days after the challenge, both groups were assessed for tumor protection. Images represent distorted pathology of lungs, showing relative surface tumor burden. (c) Ninety days following the immunization, total CD8$^+$ T cells containing AdVova-specific memory CTLs were purified from WT mice and adoptively transferred into naïve WT and Ia$^{b-/-}$ mice. The OVA-specific tetramer$^+$ CTLs were tracked in peripheral blood samples by tetramer staining. The values represent mean %±SD of OVA-specific tetramer$^+$ CTLs in total CD8$^+$ T cell population at the indicated intervals (right panel) and are representative of

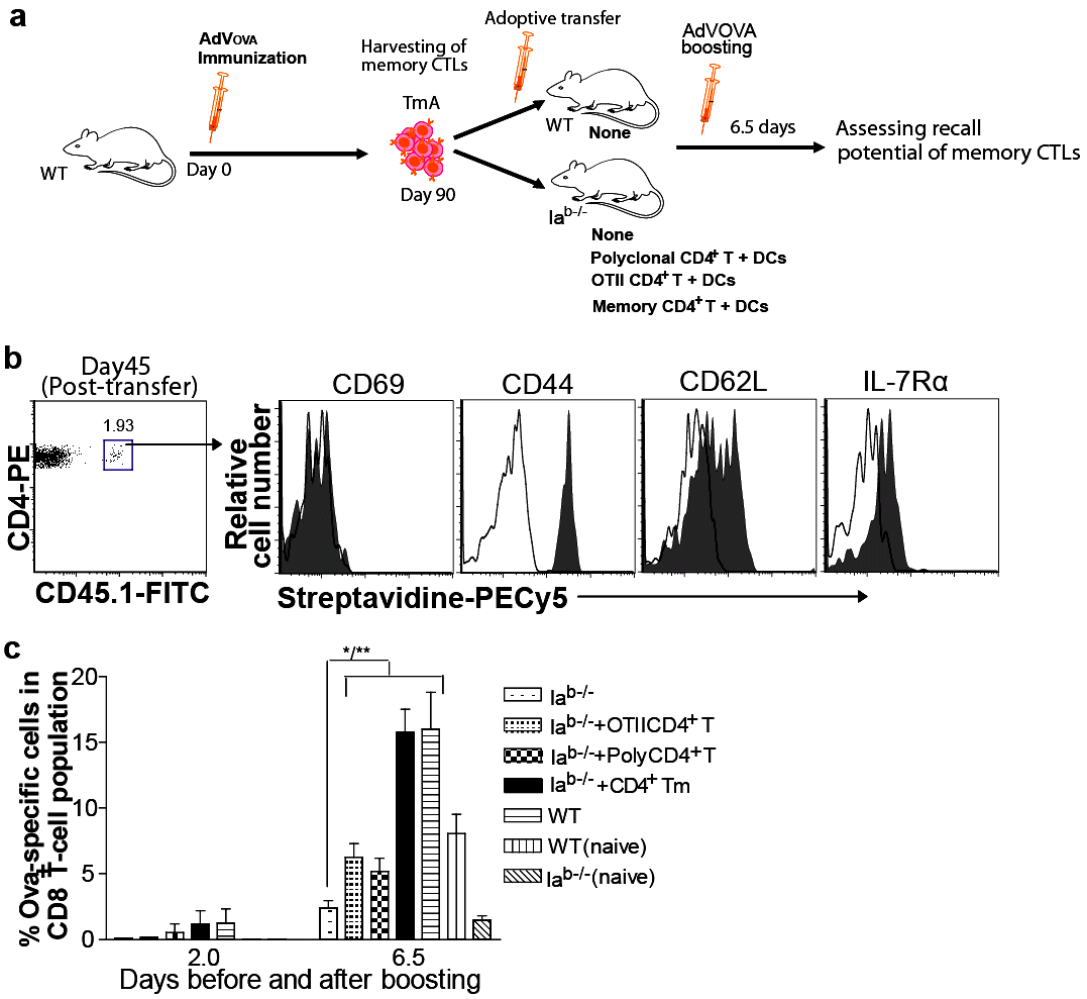
two independent experiments with four to six mice per group. $^{**}P<0.01$, versus $Ia^{b-/-}$ mice. (d) Thirty days after the adoptive transfer, the above mice groups (c) were challenged with BL6-10_{OVA} and the relative surface tumor burden was assessed 24 days after the challenge as detailed above.

Figure 5



CD4$^+$ T cell signals provided during priming and recall phase are required for optimal secondary responses. (a) A schematic protocol. After 90 days of immunization, total CD8$^+$ T cells containing memory CTLs were purified from WT (helped CTLs, TmA) or Ia$^{b-/-}$ (unhelped CTLs, TmB) mice, adoptively transferred in equal numbers into the naïve secondary recipients, WT and Ia$^{b-/-}$ mice, and assessed for recall potential after boosting. (b) Three days after adoptive transfer of helped (TmA) or unhelped (TmB) memory CTLs into naïve WT and Ia$^{b-/-}$ mice, all the mice groups were boosted with AdVova and monitored for the expansion of memory CTLs 6.5 days later. The values represent mean %±SD of OVA-specific tetramer$^+$ CTLs in total CD8$^+$ T cell population and are representative of two independent experiments with five to six mice per group. $^{**}P<0.01$, versus Ia$^{b-/-}$ mice with TmA or TmB.

Figure 6



CD4$^+$ T cell signals delivered during priming and recall phase are required for optimal secondary responses. (**a**) A schematic protocol. After 90 days following immunization, total CD8$^+$ T cells, containing memory CTLs were purified from WT (helped memory CTLs) mice, adoptively transferred equally to the naïve secondary recipients, WT and Ia$^{b-/-}$ mice. The Ia$^{b-/-}$ mice were additionally reconstituted with different types of CD4$^+$ T cells along with CD11c$^+$ DCs. Both groups were boosted with AdVova and assessed for memory CTLs expansion. (**b**) Naïve B6.1/OTII CD4$^+$ T cells were co-cultured with irradiated BM DCova, as detailed in material and methods to generate Th cells. Th cells were then transferred to naïve congenic WT mice. After 45 days, these cells were triple stained and phenotypically characterized by the presence of activation- and memory-specific markers, as shown in this figure. The value in the dot plot indicates % of OVA-specific CD4$^+$ T memory cells remaining in total CD4$^+$ T cell

population and is representative of two independent experiments with 3 to 4 mice per group. (**c**) $Ia^{b-/-}$ mice were adoptively transferred with helped memory CTLs (TmA) with or without $CD11c^+$ DCs and with OTII $CD4^+$ T cells, polyclonal $CD4^+$ T cells or OVA-specific memory $CD4^+$ T cells together with polyclonal $CD4^+$ T cells. Three days later, all the groups were boosted with AdVova and the recall potential of the memory CTLs were assessed 6.5 days later. The values represent mean %±SD of OVA-specific $tetramer^+$ CTLs in total $CD8^+$ T cell population and are representative of two independent experiments with four to five mice per group. $*P<0.05$ or $**P<0.01$, versus $Ia^{b-/-}$ mice with TmA alone.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

Although the discussions specific for Chapters 2-5 have been made, the intent of this Chapter is to provide a discussion of the thesis work in its entirety. The overall goal of the present work was to understand how $CD4^+$ T helper cells modulate different phases of $CD8^+$ CTL responses in order to provide effective anti-tumor immunity. The requirements of $CD4^+$ T helper cells for effective CTL responses are known to vary with the type of infections or immunizations. Hence, in the present work, two distinct immunization systems were chosen to understand the modulatory effects of $CD4^+$ T cells on cellular immunity. These included: 1) non-inflammatory DC immunization, which fails to provide inflammatory/danger signals; and 2) inflammatory AdV immunization, which provides profound inflammatory/danger signals. To assess the protective ability of memory $CD8^+$ CTLs developed under the influence of $CD4^+$ T help, a highly metastatic melanoma tumor cell line was used in challenge studies. Before the inception of the work in Chapters 2 and 3, the concept of a "new dynamic model of $CD8^+$ CTL responses via $CD4^+$ Th-APCs," proposed by Xiang *et al.* (1) was beginning to be understood. Hence, a sincere approach was made to further understand the novel role of $CD4^+$ T cells on $CD8^+$ CTL responses using DC immunization model. In Chapter 4, the regulatory roles of $CD4^+$ T cells were investigated under different $CD8^+$ T cell PF levels in DC immunization model. In Chapter 5, the study was focused on understand the behavior of cellular immune response under the influence of $CD4^+$ T cell signals following AdV immunization.

The immunologists' perception of the participation of $CD4^+$ T cells in various phases of CTL responses has changed over time. The role of $CD4^+$ T cells in modulation of $CD8^+$ CTL responses was observed originally in *in vivo* allograft rejection and *in vitro* studies of allogeneic mixed lymphocyte reactions (2). However, this notion of interaction between $CD4^+$ and $CD8^+$ T cells for effective CTL responses was again questioned when two recent reports showed the absolute $CD4^+$-independent CTL-mediated clearance of acute viral infections (3, 4). This finding led to the concept that $CD4^+$ T cell help is needed for generating primary CTL immunity to non-infectious Ags (such as minor histocompatibility Ags, tumor Ags or protein Ags) (5-7) and not for acute infections caused by live virus or bacteria (8). Live microbes were thought to directly

license the DCs to mature such that DCs acquire the ability to activate naïve $CD8^+$ T cells directly. Subsequently, the concept of pathogenic-mediated DC maturation/licensing and subsequent CTL responses was again subjected to skepticism. In the absence of $CD4^+$ T cells, it has been shown that the protection of the same virus or bacterial infection impairs during memory stage, and even during priming, the infection at high doses can persists without getting cleared from the body (9, 10). These findings suggest that $CD4^+$ T cells might play a crucial role in the survival/expansion of functional CTLs till the clearance of Ag occur (11, 12). Later, it was confirmed that, even in acute infections, $CD4^+$ T cell help is required for optimal memory CTL responses (4, 13, 14), suggesting they play a prime role in the memory phase irrespective of type of Ags (infection or noninfectious). For memory CTL responses, while these reports suggested the involvement of cognate $CD4^+$ T cell help during priming periods, a recent study implicated polyclonal $CD4^+$ T cells' role during memory maintenance phase (3). Based on the above observations, it appeared that the timing and nature of $CD4^+$ T cell's requirement was Ag/immunogen type dependent, providing the basis for choosing both inflammatory and non-inflammatory immunization models in the present work.

Although their involvement is much appreciated, $CD4^+$ T cell's conditional requirement for different CTL phases remains a long-standing paradox in cellular immunology. Previously, two models on how $CD4^+$ T help for $CD8^+$ CTL responses have been proposed. These include: 1) the passive model of three-cell interaction (5, 15); and 2) the dynamic model of sequential two-cell interactions by APCs (6). As reviewed in Chapter 1 (literature review), these two models do not convincingly explain how extremely rare number of $CD4^+$ and $CD8^+$ T cells, and/or Ag-presenting DCs interact simultaneously or sequentially, an event that has extremely low probability. Subsequently, Behrens et al (16) addressed this scarcity caveat partially in a diabetic model where they reported that $CD4^+$ T helper cells can convert the tolerogenic $CD8^+$ T cell response into one capable of causing destruction of the pancreatic islets which is in contrast to finding that help is only important for CTL memory (4, 13, 17, 18). They demonstrated that CD40 signaling on DCs is very important for generating effector CTLs. They also reported that Th cells, once activated, do not need recognize Ag on the same DCs they license and even may not require DC trafficking to LN. Rather, they will interact with various other DCs through CD40 signaling even without going for second cognate interactions (i.e. without awaiting second TCR signal via pMHC). In this way, the immune system can achieve efficient and rapid means

of amplifying CTL responses. Although one or several of these possibilities could exist *in vivo*, "new dynamic concept of Th-APCs" could solve the puzzle of long-standing paradox in cellular immunology. Contrary to $CD8^+$ T cells, $CD4^+$ T cells contact DCs multiple times (Ag persistence) to undergo activation and differentiation (19). As a result they are known to remain for longer periods in lymphoid organs and possibly acquire more and more pMHC-II as well as bystander pMHC-I complexes in addition to other APMs. Consequently, Th-APCs with relatively more number of pMHC I complexes can readily scan and activate rare number of cognate naïve $CD8^+$ T cells in addition to DCs, partially solving scarcity caveat of cellular interaction in cellular immunology. The work in the Chapter 2 and 3 further confirmed these observations.

Chapter 2 elucidated key regulatory events mediated by $CD4^+$ T cells that could physiologically occur following infections or immunizations. In different experimental systems, the multifaceted roles of $CD4^+$ T cells have been shown. In line with these observations, the work in Chapter 2 showed how $CD4^+$ T cells participate not only in augmenting CTL-mediated immune responses perhaps during early phase, but also in regulating/suppressing cellular immunity perhaps during later phase. Furthermore, it showed that Th-APC-stimulated $CD8^+$ T cells are more prone to become T_{CM} and the stimulation is mainly mediated via CD40L, IL-2, and acquired pMHC I signaling. The study also showed that Th-APC-stimulated $CD4^+$ T cells behave like Th cells in function, augmenting overall magnitude of CTL responses. Thus, these results greatly enhance our understanding of regulation of cellular immunity and could impact vaccine development. The cooperative role of Th and effector CTL have been frequently observed in adoptive CTL therapy of cancers, yet how synergistic response occurs from these two cells interaction is less understood. The work in the Chapter 3 partially explained how Ag-experienced Th cells can modulate CTL responses, such as apoptosis, survival, and functional memory CTL development, showing additional role of Th-APCs. This study could explain how millions of copies of effector CTLs generated *in vivo* destined to undergo death by apoptosis or their minute fractions develop into memory CTLs. This knowledge is particularly relevant in the context of adoptive CTL therapy of cancers and chronic diseases. From the current data, a successful adoptive CTL therapy should include the provision of Th help to effector CTLs. One of the greatest challenges associated with trogocytosis in immunological studies is the translation of *in vitro* findings to *in vivo* ones (20). So also here, although all these works performed

basically derived from a combination of *in vitro* and *in vivo* experiments and used transgenic mice systems, there remains to be determined the physiological relevance and *in vivo* implications of this concept.

The natural differences in the size of Ag-specific $CD8^+$ T cell precursors influencing the magnitude of primary and memory CTL responses in various types of disease conditions, including cancers, autoimmunity, infections and graft rejections, have attracted immunologists to investigate whether altered PF modifies requirement of regulatory factors for CTL responses. Several recent seminal reports published in high-rated journals suggested that, by increasing $CD8^+$ T cell PF, one could achieve $CD4^+$ T help- and CD40-40L-signaling-independent primary CTL responses even in immunity involving non-inflammatory conditions (21-25). However, how higher PF impact $CD4^+$ T-helper signal requirements for functional memory responses and its therapeutic usefulness are not understood. Hence, the work presented in the Chapter 4 addressed these issues using combined adoptive transfer technology and various KO systems in a non-infectious DCova immunization model. The key findings were: 1) At increased PF, $CD4^+$ T cell help and its CD40L but not IL-2 signal become dispensable for primary CTL responses; and 2) even at higher PF, memory CTL responses, perhaps both survival and functionalities require $CD4^+$ T cell signals, largely in the form of IL-2 and CD40L. Strikingly, $CD4^+$ T cell-independent primary CTL responses last of longer period. This phenomenon could be exploited in enhancing therapeutic efficacy of DC immunization protocols, which at present particularly failing in the treatment of cancers due to tumor-induced tolerance (26). Thus, these results could impact the development of immunotherapies for cancers, chronic diseases and autoimmune disorders, whose pathogenesis is determined in part by $CD4^+$ T cell helper signals and altered $CD8^+$ T cell PF. Previously, Marzo *et al.* reported that higher PF controls memory lineage development in infectious models, favoring the generation of the inter-convertible T_{CM} vs T_{EM} cells (27). In line with these results, the work of Chapter 2 also showed that DC-stimulated Th with acquired Ag-presenting machineries can stimulate naïve $CD8^+$ T cells, which in turn prone to become T_{CM} (1, 28), possibly explaining why skewing of T_{CM} generation occurs at higher-PF. Although these are possible speculations, future works should focus on understanding how Th-APC modulates T_{CM} generation under physiological conditions.

Having studied $CD4^+$ and $CD8^+$ T cell interactions in depth, the work in Chapter 5 was primarily focused at understanding CMI responses in the context of AdV immunization

protocols. Due to their exceptional ability to induce higher and sustained levels of transgene product-specific $CD8^+$ CTL responses (29, 30), Currently, AdV vectors are at the forefront of vaccine development against cancers and chronic diseases, such as HIV infection, where humoral immune response was shown to be less effective. However, how $CD8^+$ CTL responses are modulated by $CD4^+$ T cells is currently less defined area although such an understanding is very critical for successful designation of AdV vaccines. Hence, here an adoptive transfer system was undertaken in WT and CD4-deficient $Ia^{b-/-}$mice, and various KO and transgenic mice were exploited to investigate the $CD4^+$ T helper requirements for CTL responses. The key findings were: 1) without $CD4^+$ T help, both primary and memory responses were greatly reduced; 2) the transfer of Ag-specific $CD4^+$ T cells to $CD4^+$ T-deficient mice considerably restored primary and memory survival and recall responses, and such help was specifically mediated through $CD4^+$ T cell-derived IL-2 and CD40L signaling; 3) adoptive transfer of helped or unhelped effector or memory CTLs to naïve $CD4^+$ T-deficient mice revealed the additional role of polyclonal $CD4^+$ T-environment for AdV-specific CTL survival, partially explaining protracted CTL contraction phase; and finally 4) during recall phase, $CD4^+$ T-environment, particularly with memory $CD4^+$ T cells, greatly enhanced not only helped, but also unhelped, memory CTL expansion. In contrast to other infectious and noninfectious models, the present study in AdVova immunization model demonstrated the participation of both cognate $CD4^+$ T cells during priming and polyclonal $CD4^+$ T cells during maintenance phase. A similar synergistic response was also observed in DC immunization model (data not shown). Whether such $CD4^+$ T cell's requirement occurs synergistically in other disease conditions, such as cancers and intracellular infections, needs further investigation. Collectively, these results provided new dimension to our knowledge on $CD4^+$ T helper cells as an important modulators of cellular immunity in AdV vaccination procedures, and could partially explain certain failures in AdV-based immunization trials against cancers, and chronic diseases that are often associated with reduced $CD4^+$ T cell functions. Furthermore, these results warrant the development of novel modified AdV vaccines having ability to induce $CD4^+$ T cell-independent CTL responses.

Conclusions

Despite technological advances, including flow cytometry, knockout and transgenic mice, gene arrays, pMHC tetramer reagents and two-photon microscopy, our knowledge on cellular

and molecular mechanisms behind $CD8^+$ CTL responses only began to be understood in the last few decades. The requirements of $CD4^+$ T cells and molecular mechanisms for optimal CTL responses have puzzled immunologists for years. In the present thesis work, a sincere approach was followed to delineate the critical roles of $CD4^+$ Th cells in different stages of CTL responses and anti-tumor immunity using advanced technologies. The results presented here will significantly advance our current understanding of immunity to cancers, autoimmunity and chronic infections since pathogenesis of these diseases is largely determined by $CD4^+$ T helper functions. As most immunization procedures use the principle that is based on functions of memory T cells, the knowledge gained from this work will also have a major impact on designing vaccines against intractable diseases, including cancers and chronic infections such as hepatitis and AIDS. Moreover, in advanced tumors, vaccines developed using this knowledge may act synergistically with other cancer treatments such as irradiation, chemotherapy and microsurgery, minimizing their side effects and prolonging the lives of patients.

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