Feedback Interactions between Dendritic Cells and $CD8^+$ T Cells during the Development of Type-1 Immunity

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$CD8^+$ T cell responses are crucial for immunity against intracellular infections and can mediate tumor regression. While $CD8^+$ T cells are widely recognized as cytolytic effector cells (cytolytic T cells; CTLs), little is known about their immunoregulatory functions and their impact on dendritic cells (DCs). A similar area of controversy is the role of DC in regulating the induction of $CD8^+$ T cell effector functions and $CD8^+$ T cell memory. This dissertation addresses the impact of bidirectional communication between DCs and $CD8^+$ T cells, during different phases of the immune response, upon the functions of both these cell types.

In order to reconcile the apparently contrasting notions that $CD8^+$ T cells perform both "suppressor" and "helper" functions, I compared the DC-modulating activity of $CD8^+$ T cells at different stages of activation. I observed that DC-killing and DC-activating (and protective) functions are exerted sequentially by activated $CD8^+$ T cells. In contrast to the effector cells that kill DCs in a granzyme B/perforin-dependent manner, memory $CD8^+$ T cells promote IL-12 production in DCs and support $CD4^+$ and $CD8^+$ T cell responses. Moreover, memory $CD8^+$ T cells instruct DC to over-express granzyme B inhibitor PI-9, protecting them from elimination by CTLs. I observed that the inclusion of "heterologous" $CD8^+$ T cell epitopes in cancer vaccines, promoting the interaction of vaccine-bearing DCs with large numbers of tumor-unrelated $CD8^+$ T cells, strongly enhances the immunologic and therapeutic activity of vaccination against established tumors that are resistant to standard vaccines.

Since the character of the vaccination-induced $CD8^+$ T cells is important for the efficacy of cancer immunotherapy, I have analyzed the role of DCs in influencing the cytolytic function and

peripheral tissue-homing ability of $CD8^+$ T cells. I observed that short-term-activated "inflammatory-type" DCs, capable of producing high levels of IL-12 and other pro-inflammatory cytokines, support induction of cytotoxic function and a switch from lymphoid to peripheral chemokine receptors in $CD8^+$ T cells. In contrast, "exhausted" DCs matured for extended periods of time or matured under the influence of the mediators of chronic inflammation, favor $CD8^+$ T cell expansion alone without acquisition of effector functions.

Collectively, the findings presented in this dissertation broaden our understanding of the feedback circuitry between $CD8^+$ T cells and DCs and will help us to design improved vaccines against cancer and chronic infections.

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PREFACE

I would like to sincerely thank my mentor, Pawel Kalinski, for his unwavering support and guidance throughout my tenure in laboratory. Your infectious passion and enthusiasm for science has always been a source of inspiration for me. Thank you for giving me the freedom to work on all the crazy observations and for being patient and optimistic about my work.

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1 INTRODUCTION

$CD8^+$ T lymphocytes are an important component of a host's immune response to intracellular infections and malignant tumors. $CD8^+$ T cells use multiple cytotoxic effector mechanisms such as perforin/granzyme and Fas-FasL to mediate the elimination of infected and transformed cells (1, 2). In addition, $CD8^+$ T cells exert non-cytotoxic effector pathway through the release of effector cytokines such as IFNγ and TNFα that block viral gene expression, recruit and activate macrophages and NK cells and upregulate antigen processing machinery and MHC expression in the infected cells(3, 4). Once a primary infection is resolved or brought under control, progeny of antigen-specific $CD8^+$ T cells develop into memory $CD8^+$ T cells. These memory cells have the ability to rapidly regain effector functions when re-exposed to antigen, thereby providing long–lasting protective immunity. The generation of a robust $CD8^+$ T cell response involves priming of naïve $CD8^+$ T cells by dendritic cells (DCs), the professional antigen presenting cells of the immune system. Activated DCs can provide naïve T cells with antigen-specific T cell activating "signal 1", costimulatory "signal 2" determining the magnitude of immune response and polarizing "signal 3" obtained from the peripheral environment activating the DCs and in turn affecting the character of the immune response. It is the dynamic interaction between naïve $CD8^+$ T cells and dendritic cells (DCs) that lead to generation of effector and memory $CD8^+$ T cells.

Until recently, the biology of $CD8^+$ T cells was focused on elaborating their role as cytotoxic effector cells in different infection models. Many questions remained unanswered

about the role of effector and memory $CD8^+$ T cells in regulating the immune response. In this dissertation, we investigate the functional impact of positive and negative feedback signals arising from memory and effector $CD8^+$ T cells on the magnitude and quality of DC-mediated immune responses. While the pathway of generation of effector and memory cells has been studied extensively, the models that have been proposed are apparently conflicting with regard to lineage relationship between effector and memory subsets. I attempt to address this problem and have focused on the conditions of DC maturation as a determining factor for regulating the acquisition of effector functions and tissue-homing ability. Before presenting a detailed exposition on the immunoregulatory functions of $CD8^+$ T cells and the influence of DC maturation on the differentiation of $CD8^+$ T cells, I briefly summarize the diverse functions of $CD8^+$ T cells performed in the course of an immune response.

1.1 $CD8^+$ T LYMPHOCYTES AS CYTOLYTIC EFFECTOR CELLS

Several studies collectively suggest that activation and expansion of naïve $CD8^+$ T cells can be initiated by a brief exposure to antigen (5, 6) (7, 8). For example, a two hour exposure of $CD8^+$ T cells to antigen *in vitro* is sufficient to induce proliferation and 24 hours of antigen exposure *in vivo* is sufficient to drive multiple rounds of divisions and induction of effector and memory populations. In addition to antigen, other signals such as inflammatory cytokines induced either by pathogen itself or by immunization in the presence of strong adjuvants can influence the differentiation program. The outcome of $CD8^+$ T cell differentiation program is the acquisition of cytolytic function and peripheral homing ability, and we discuss the regulation of these two aspects in the following subsections.

1.1.1 Role of signal 3 in development of effector functions

Previous studies have shown that generation of effective immune response against antigen requires co-injection of molecules with adjuvant activity (9). Adjuvants serve as danger signals, and the immune system has evolved to recognize and respond to such signals that indicate infection or cell damage (10, 11). Microbial stimuli (12) as well as pathogen-induced factors such as IFNγ and PGE_2 (13) can modulate the DC derived T-cell-polarizing signals (signal 3) such as IL-12 family members and type 1 interferons (13).

Both human *in vitro* and mouse *in vivo* studies have demonstrated that naïve $CD4^+$ T cell differentiation can be uncoupled from proliferation (14-16). The absence of *T-cell-polarizing signal 3* during the priming of naïve $CD4^+$ T cells resulted in $CD4^+$ T cell proliferation alone without differentiation into Th1 or Th2 pathway. While detailed study for $CD8^+$ T cells has not been done, there is increasing evidence suggesting the role of signal 3 in the development of cytolytic function. The study from Mescher's group indicates that exposure of naïve $CD8^+$ T cells to artificial APC (antigen and B7) and IL-12 enhances T cell cytolytic function compared to T cells stimulated in the absence of IL-12 (17, 18). *In vivo* studies with peptide immunization demonstrated that IL-12 worked as good as CFA in promoting full activation of $CD8^+$ T cells (19). Another inflammatory cytokine involved in promoting $CD8^+$ T cell expansion and survival is type I IFN (20). In addition, data from a human clinical trial using a combination of ALVAC vaccine and high dose IFNα also indicated the role of IFNα in the development of CTL function. The patients receiving high dose IFNα treatment following ALVAC vaccine showed enhanced *in vitro* anti-tumor responses and tumor regression *in vivo* (21). Together these observations suggest that in addition to antigen and co-stimulation, a third signal is required to induce a

complete differentiation program in $CD8^+$ T cells. Pathogen or pathogen-derived product can act on DCs and induce the release of cytokines (IL-12, IFN-α/β) which function as third signal, consequently making DCs a crucial link in the development of effector T cell responses.

1.1.2 Role of dendritic cells in the development of T cell effector functions

There are distinct DC subsets in both mouse and human and each subset exhibits a unique set of pathogen-recognition receptors (PRRs) allowing them to interact with distinct classes of pathogen. Several studies have shown that not all DCs are equally effective in inducing T cell differentiation. For example, results from mouse studies suggest that $CD4^+$ T cell differentiation is dependent on the subset of DC involved in priming (22)(23). It has been shown that the high IL-12 producing $CD8\alpha^+$DCs and lymphoid-related DC subset $CD11c^+CD11b^{dull}$ drive Th1 development while $CD8\alpha^-$DCs and myeloid-related DC subset $CD11c^+CD11b^{bright}$ induce Th2-type responses.

In addition to the intrinsic differences between subsets of DCs in their capacity to induce $CD4^+$ T cell differentiation towards Th1 and Th2 pathway, results from *in vitro* human experiments have shown that different levels of DC maturation can influence the levels of IL-12 production (14) and the outcome of T cell priming (15). Indirect evidence of the impact of DC maturation on $CD8^+$ T cell priming comes from *in vivo* mouse experiments involving immunization with live versus heat-killed or inactivated virus (24, 25). The use of inactivated virus was shown to be associated with reduced level of DC maturation, resulting in the inability to develop protective immunity to influenza (26). These results are likely to be explained by several phenomena: reduced TLR-mediated activation of innate immune responses (27), reduced TLR-mediated activation of DCs, insufficient DC maturation resulting in reduced expression of

CD80, CD86, CD40, MHC Class II and reduced production of IFNγ (25, 28, 29). Taken together, these observations imply that the extent of DC maturation qualitatively affects the pattern of T cell differentiation. Taking cues from these studies, I have developed an *in vitro* model that allows us to study the influence of degree of DC maturation (acute activation vs. chronic activation) on the development of $CD8^+$ T cell effector function and the generation of .memory population.

1.1.3 Regulation of peripheral–type chemokine receptors on effector $CD8^+$ T cells

The differentiation program of $CD8^+$ T cells induces two key changes: a) equips the cells with the necessary armament needed to perform effector functions and b) programs the cells to leave the lymph node (LN) and migrate appropriately to peripheral sites to carry out effector functions. Inflammation induced trafficking of effector cells requires imprinting of the information needed for migration, i.e. up-regulation of inflammatory chemokine receptors on T cells, to occur concurrently with activation of T cells.

While the exact mechanisms by which DCs regulate a switch in the T cell's responsiveness to LN-associated and peripheral tissue-associated chemokines is unclear, previous studies have shown that DCs act as source of signals that regulate the pattern of T cell migration. In mouse, DCs from Peyer's patches and mesenteric lymph nodes induce gut homing capacity in $CD8^+$ T cells whereas DCs from skin draining lymph node imprint skin homing-specific selectin ligands and chemokine receptors (30). These results demonstrate the role of DCs in influencing the expression of chemokine receptors, but it remains unclear how different levels and modes of DC maturation affect chemokine receptor programming.

1.2 IMMUNOREGULATION BY EFFECTOR $CD8^+$ T CELLS

Cytotoxicity is a defining characteristic of the effector $CD8^+$ T cells (31). Although previous studies indicate suppressor activity for $CD8^+$ T cells, the mechanism for such immunoregulatory function remains unclear (32, 33). One of the early experiments on suppressor activity studied the feed-back suppression by effector T cells as a way of regulating the primary response to ectromelia virus infection (34). More recent studies using mouse infection models (Listeria, LCMV, malaria and HSV) demonstrate that activated $CD8^+$ T cells limit the CTL response by regulating the duration of antigen display (35, 36). This self-limiting feature of activated $CD8^+$ T cell operates in murine models through elimination of DC in a perforin-dependent manner (37), although other mechanisms such as Fas-FasL mediated killing and the involvement of NK cell-like receptors NKp30 and NKp46 cannot be ruled out. The site of DC killing is debatable. Depending on the experimental setup both inflamed lymph nodes and peripheral tissues have been proposed to be the sites of DC killing (37, 38). Understanding the relation of CTL-mediated DC killing to the generation of secondary immune response will aid in designing vaccines for chronic infections and cancer.

1.3 DUAL ROLE OF EFFECTOR $CD8^+$ T CELLS

The cytolytic activity of effector $CD8^+$ T cells has two important roles: it is essential for the overall level of immunity by directly eliminating the infected or transformed cells, and also for suppressing immune responses by eliminating the antigen-carrying DCs. In this dissertation, I

will explore the mechanism of the regulatory function of cytolytic effector $CD8^+$ T cells and will analyze how DCs can resist such an attack.

1.4 TCR-INDEPENDENT FUNCTIONS OF PRIMED $CD8^+$ T CELLS

$CD8^+$ T cells are not generally associated with innate immune response and execute their effector functions only upon TCR triggering. Interestingly however, recent observations in murine infection models (*Listeria, Shigella, Burkholderia*) suggest the involvement of primed $CD8^+$ T cells in early phases of the innate immune response in a non-TCR dependent manner (39,40, 41). Direct evidence from the *Listeria* infection model indicate that TCR-independent but cytokine-mediated secretion of IFNγ by memory $CD8^+$ T cells can provide early protection against LM (42). Early innate response of $CD8^+$ T cells is induced by the concerted action of IL-12 and IL-18, both of which are secreted early on in the infection by infected macrophages and DCs (43, 44). Moreover, primed $CD8^+$ T cells have been shown to upregulate the receptors for IL-12 and IL-18 (45). Since the ability of primed $CD8^+$ T cells to participate in the innate response can be important for immunopathology and therapy, my objective in this thesis is to understand the regulatory mechanism of non-TCR inducible functions, and study their influence on promoting the local immune response.

1.5 IMMUNE MEMORY AND MEMORY CD8$^+$ T CELLS

CD8$^+$ T cells perform different functions at different time points of the immune response. Following the effector stage of the immune response, CD8$^+$ T cells progress towards a period of quiescence and homeostatic proliferation, leading to the development of a memory population.

Memory CD8$^+$ T cells are the antigen-experienced cells and are crucial for the success of T-cell based protective vaccination strategies. The cardinal features of memory CD8$^+$ T cells include their ability to persist over prolonged periods of time and to rapidly regain effector functions upon re-exposure to the same antigen (46, 47). Two questions essential for vaccine-related research are: a) identifying mechanisms by which memory CD8$^+$ T cells are formed and maintained over long periods of time and, b) the mechanism of their contribution to secondary immune responses.

Much effort has been devoted to understanding the differentiation pathway from naïve to memory CD8$^+$ T cells. Several conflicting models have been proposed to explain the T-cell differentiation. The first is a linear progression model (**Fig.1.1A**), where CD8$^+$ T cells differentiate from naïve to effector to memory CD8$^+$ T cells (48, 49). According to this model, memory cells represent the progeny of the effector cells that escape Activation Induced Cell Death (AICD). This model has been supported by data from adoptive transfer experiments using TCR transgenic CD8$^+$ T cells. The second is a branched model of CD8$^+$ T cell differentiation (**Fig. 1.1B**), where memory and effector cells simultaneously develop from a common precursor, but as separate lineages (50). Recent observations from Chang *et al* (51) provide a potential mechanism for the branched model of differentiation. Following a sustained interaction between naïve T cell and DCs, naïve T cells undergo an asymmetric first division giving rise to proximal daughter cell which can potentially receives more antigen and costimulation compared to the

distal cell. Accordingly the first proximal daughter cell could represent effector lineage while distal daughter cell can be poised to give rise to memory cells.

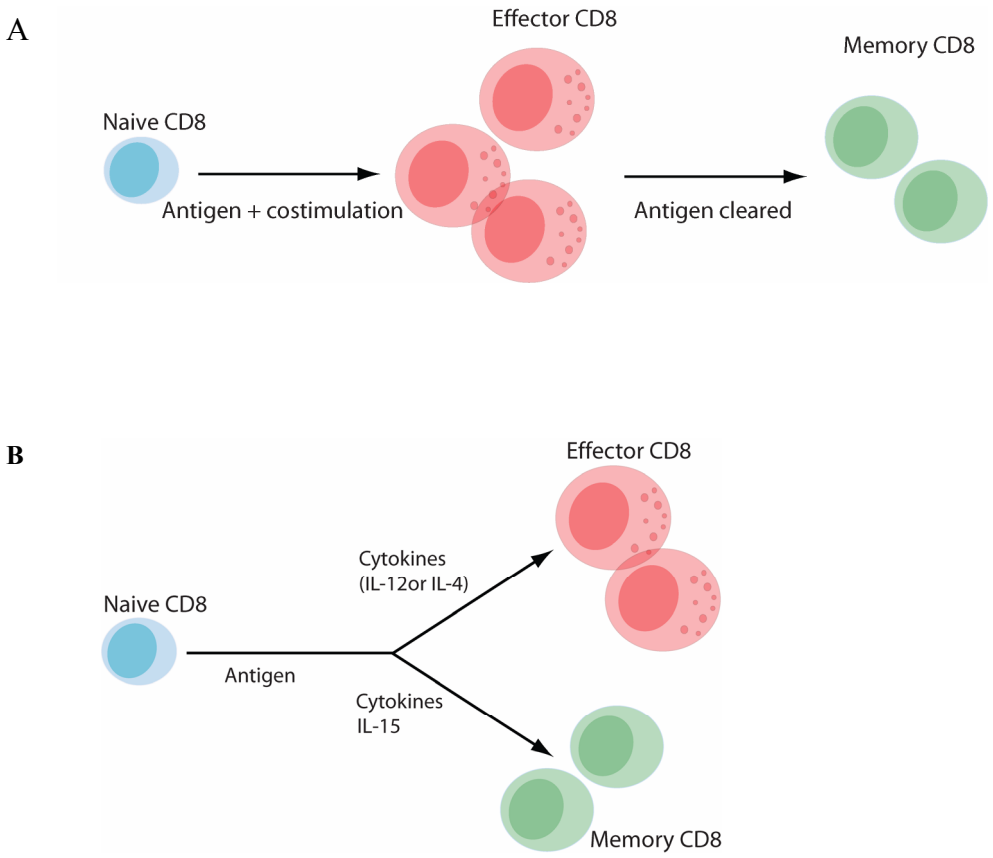

Figure 1.1 Linear progression and branched model of CD8$^+$ T cell differentiation (based on (49, 50).

(A) According to the linear model of differentiation, memory T cells are derived from effector cells that escape AICD and revert to quiescence state. (B) According to the branched model of differentiation, the duration of antigenic stimulation and type of cytokines present during priming lead to differentiation either into effectors cells or memory cells.

The progressive differentiation model was developed by Lanzavecchia and colleagues by incorporating signal strength as the factor governing differentiation of T cells (52) into effector or memory pathway. According to this model (**Fig. 1.2**), depending on the strength of TCR and costimulatory signals, responding T cells acquire effector functions, tissue-homing receptors and

responsiveness to homeostatic cytokines. While high signal strength allows acquisition of effector functions and gain of tissue-homing capacity, low signal strength results in retention of lymph node homing capacity without induction of effector functions.

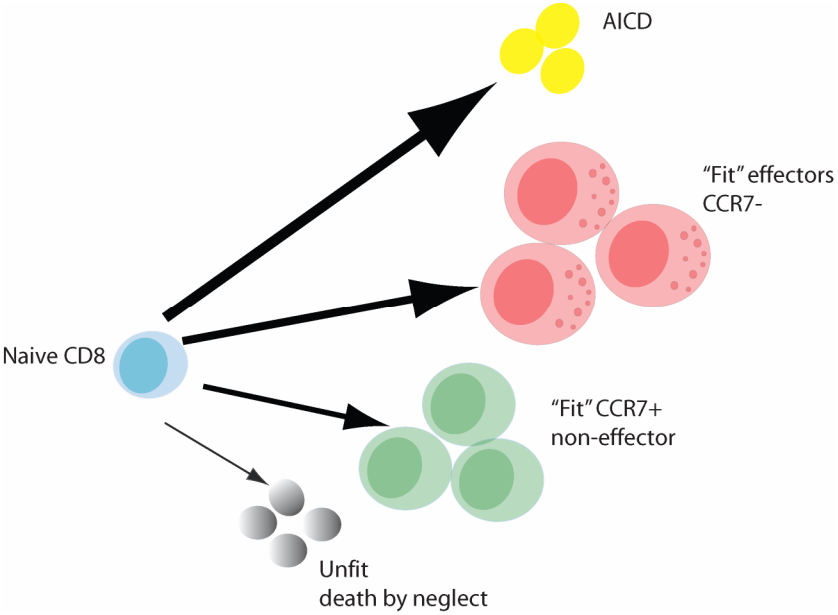

Figure 1.2 Progressive differentiation model for CD8$^+$ T cells (based on (52).

Hierarchical thresholds of signal strength induce naïve CD8$^+$ T cells to proliferate, acquire effector functions and to change their migratory capacity. The increasing signal strength is depicted by the increasing thickness of the arrow. At the increasing strength of stimulation, the responding T cells proliferate, acquire responsiveness to homeostatic cytokines, develop effector functions and lose lymphoid homing receptor (CCR7).

Similar to the signal strength-based model (52), *in vivo* observations using the model of chronic viral infections (53) resulted in formulation of the Goldilocks model of T cell differentiation (**Fig. 1.3**). According to this model, weak stimulation leads to expanded T cells that are unfit and rapidly die while strong stimulation allows differentiation of CD8$^+$ T cells and the development of memory cells. Furthermore, prolonged or very strong stimulation causes aberrant differentiation of cells without the development of memory cells.

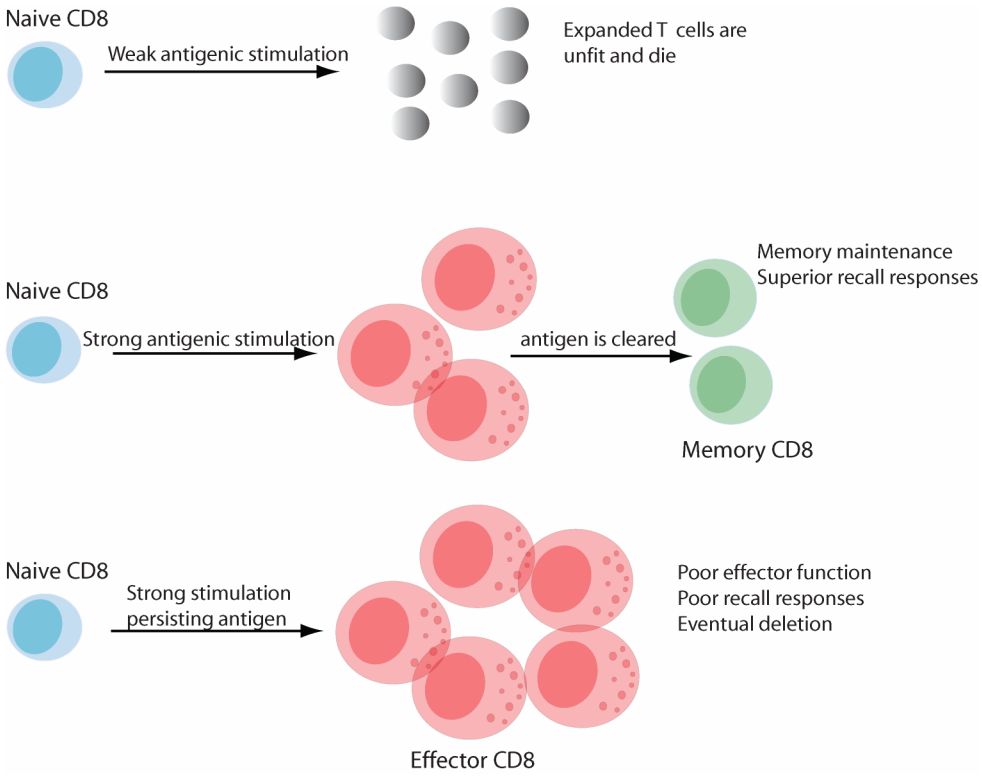

Figure 1.3 Goldilocks model for CD8$^+$ T cell differentiation (based on (53).

Weak antigenic stimulation leads to limited CD8$^+$ T cell expansion and poor memory development and attrition of unfit cells. Optimum memory development is favored when conditions are just right; that is strong antigenic stimulation leads to T cell proliferation and differentiation into tissue-homing effectors. Upon antigen clearance, the fittest expanded cells survive as central-memory and effector memory T cells. Chronic antigen exposure drives continuous proliferation of T cell and differentiation into effectors. The effector T cells progressively lose the ability to secrete cytokines and eventually get eliminated.

While these models explain the pathway of CD8$^+$ T cell differentiation, there are two parameters that are not explicitly taken into account in any of these models. The first parameter is the inflammatory milieu associated with differentiation of CD8$^+$ T cells. The second parameter is the time-point of the interaction of CD8$^+$ T cells with antigen presenting cells, and the potential difference in the outcome of priming occurring during early stages of infection

(antigen, costimulation, and inflammatory milieu) versus late stages of infection (antigen and inflammatory milieu disappearing).

1.5.1 Inflammation and development of memory CD8$^+$ T cells: an inverse relation

There is increasing evidence suggesting an inverse correlation between the level of inflammation and development of memory CD8$^+$ T cells (48, 54). Indirect evidence from mouse studies reveals the need for early inflammation following infection or DC immunization in order to generate a robust effector response. The antibiotic treatment (after infection), reducing the active phase of pathogen activity and the level of resulting inflammation (55) (56), has been shown to accelerate the formation of CD8$^+$ memory T cells in mouse. Depending on the nature of infection - acute versus persistent, varying differentiation phenotypes of CD8$^+$ T cells have been observed in humans. Following an acute infection characterized by transient inflammation, such as influenza or measles, long-lasting memory population of CD8$^+$ T cells is generated (57). In contrast, chronic infections such as CMV and HCV result in persistent inflammation and reduced development of functional memory cells (58). The involvement of inflammation in differentiation of CD8$^+$ T cell is also reflected in the results from several of the human cancer immunotherapy trials (59), where most DC- or peptide-based vaccinations induce massive expansion of tumor-specific CD8$^+$ T cells but modest induction of effector functions. More work is certainly required to broaden our understanding of the relationship between inflammation and generation of the memory population.

1.5.2 Novel function of memory $CD8^+$ T cells

Antigen-experience gives memory $CD8^+$ T cells, a unique ability to mediate rapid, stronger, and more effective response to secondary pathogen challenge than naïve cells. Protective immune memory can persists for years after initial antigenic exposure, in humans $CD8^+$ memory T cells can be maintained for up to 75 years after vaccination (60). Memory $CD8^+$ T cells are less dependent on co-stimulation and cytokines to reacquire effector functions, which partly explains their ability to respond to the pathogens more rapidly than naïve cells. Moreover, the ability of memory $CD8^+$ T cell to recognize the pathogen and secrete cytokines before the onset of infection-induced inflammation and damage can potentially be used as a *warning sign* and as an alternate source of pathogen information. The information directly provided by the pathogen through PRR is misleading for the immune system in certain instances for example; both *Candida albicans and Bordetella pertussis* use TLR2 signaling as an escape strategy to induce DC production of IL-10, resulting in the development of antigen-specific Tregs.

In contrast to the effector cells, memory cells have been shown to lack immediate DC-killing activity and there is no direct evidence that demonstrates the particular role of memory $CD8^+$ T cells in immunoregulation. However, observations from mice and human studies suggest that prior infection influences the character of the immune response generated by subsequent infection. For example, mice infected with murine helminth *Heilgmosomoides polygrus* prior to infection with *Helicobacter felis* have significant reduction in gastric expression of type 1 cytokines such as IFNγ and TNFα and higher expression of IL-4, IL-10 and TGFβ (61). There are several examples of human infections that can modulate the immune response elicited to subsequent infection, for example helminthic infections are associated with suppressed IFNγ

responses to tuberculin antigen (62). Similarly, infections with mycobacteria, measles virus or gastrointestinal pathogens have all shown to be inversely correlated with the risk of atopy and IgE-dominated responses to different allergens (63-65). These observations suggest the possibility of immune system learning from past infections and using that information to modulate subsequent immune responses. My objective in this dissertation is to explore the role of memory $CD8^+$ T cells and DCs in this learning process.

1.5.3 DC as mediator of $CD8^+$ T cell dependent immunomodulation

Dendritic cells are optimally positioned at the anatomical sites of potential pathogen entry allowing them to sense and capture the antigenic material (66). In addition, DCs are flexible mediators of environmental signals and the only cell type that is capable of effectively priming naïve T cells in the lymph nodes (67). Since DCs are both the carriers of pathogenic information and the orchestrators' of immune response, I explore the idea of memory $CD8^+$ T cells using their own antigen experience to *educate* the DCs about the pathogen location (extracellular or intracellular) and what effector mechanism to use to eliminate pathogen rather than what the specific pathogen may be. As discussed in the dissertation, this education ensures that the immune response initiated by DCs matches the character of pathogen and not pathogen-associated features.

1.6 SCOPE OF THIS DISSERTATION

The scope of this dissertation is to identify the immunoregulatory roles of effector and memory $CD8^+$ T cells in the development of DC-mediated immune responses and the role of DC in development of effector and memory $CD8^+$ T cells.

The mouse model of DC-based cancer vaccination described in ***Chapter 2*** was designed to segregate regulatory functions of $CD8^+$ T cells from their effector activities *in vivo*. We were able to demonstrate that regulatory *suppressor* and *helper* functions represent sequential phases of $CD8^+$ T cell responses to peptide antigens. Our data demonstrate the ability of resting $CD8^+$ memory T cells to act as helper cells, providing DC-mediated helper signals that support the development of functional CTL responses. The current results show that the inclusion in cancer vaccines of "heterologous helper epitopes", promoting the interaction of DCs with high numbers of tumor-unrelated memory-type $CD8^+$ T cells, enhances the immunologic and therapeutic effectiveness of vaccination against established tumors. In ***Chapter 3***, I used human *in vitro* models to study the mechanisms of the effector $CD8^+$ T cell-mediated *suppression* and memory $CD8^+$ T cell-mediated *helper function* during the *de novo* development of functional CTL and Th1 responses in naïve populations of $CD8^+$ and $CD4^+$ T cells. My data demonstrate that the helper function of memory $CD8^+$ T cell includes promoting the survival and type-1 polarization of DCs and with TNFα and PI-9 (endogenous granzyme B inhibitor) being two key factors.

In ***Chapter 4,*** I focus on the role of the timing of DC activation and the role of IL-12 and other DC-related factors in inducing the cytolytic versus non-cytolytic pathways of differentiation of naive $CD8^+$ T cells. In addition, I test whether the switch in the expression of central and peripheral chemokine receptors on activated $CD8^+$ T cells is regulated by the functional status of dendritic cells. I address these issues by maturing DCs for different periods

of time or in different inflammatory environments. My data demonstrate that DCs matured under acute inflammatory conditions (similar to acute viral infection) promote the effector pathway of $CD8^+$ T cell differentiation, followed by the development of memory cells. In contrast, DCs matured under chronic inflammatory conditions, or over-matured "exhausted" DCs favored the differentiation of naïve $CD8^+$ T cells directly into memory cells bypassing the effector stage. The results suggest that the differentiation of $CD8^+$ T cells can be modulated by the character of the DCs.

Since, the results of chapter 4 indicated the negative impact of the effector-type T cells upon DCs, in ***Chapter 5,*** I attempt to uncouple the regulatory and DC-killing functions of effector CTLs, by utilizing alternative means of CTL activation that are not associated with DC killing. I analyze the requirements for activation of primed $CD8^+$ T cells in a TCR independent manner, and observed that such "alternatively-activated" CTLs activate DC but cannot kill them. These data suggest an additional way of utilizing the modulatory activity of the effector cells.

In ***Chapter 6***, I combine the findings from this work with the latest reports from other groups, to develop the concept of temporal and spatial separation of the regulatory *suppressor* versus *helper* functions of $CD8^+$ T cells. Based on these data, I propose that the helper function of memory $CD8^+$ T cells provides DC-mediated help to naïve $CD8^+$ T cells in a manner similar to $CD4^+$ T cell help, favoring effective development of T cell effector functions during secondary immune responses. My results help to design DC-based vaccines and vaccination regimens that preferentially support the generation of either cytolytic or memory T cell populations, in order to achieve optimal efficacy of therapeutic and preventive vaccines.

HELPER FUNCTION OF MEMORY CD8$^+$ T CELLS: HETEROLOGOUS CD8$^+$ T CELLS SUPPORT THE INDUCTION OF THERAPEUTIC CANCER IMMUNITY

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The majority of this work was performed by Yutaro Nakamura. Payal Watchmaker contributed to the design of mouse and human studies, and to the performance of *in vitro* mouse studies.

2 HELPER FUNCTION OF MEMORY CD8$^+$ T CELLS: HETEROLOGOUS CD8$^+$ T CELLS SUPPORT THE INDUCTION OF THERAPEUTIC CANCER IMMUNITY

2.1 ABSTRACT

In contrast to well-established efficacy of preventive vaccines, the effectiveness of therapeutic vaccines remains limited. In order to develop effective vaccination regimens against cancer, we have analyzed the impact of effector and memory CD8$^+$ T cells upon the ability of dendritic cells (DC) to mediate the immunologic and anti-tumor effects of vaccination. We show that in contrast to effector CD8$^+$ T cells that kill antigen-carrying DCs, IFNγ-producing memory CD8$^+$ T cells act as "helper" cells, supporting the ability of DCs to produce IL-12p70. Promoting the interaction of tumor antigen-carrying DCs with memory-type "heterologous" (tumor-irrelevant) CD8$^+$ T cells strongly enhances the IL-12p70-dependent immunogenic and therapeutic effects of vaccination in the animals bearing established tumors. Our data demonstrate that suppressive and helper functions of CD8$^+$ T cells are differentially expressed at different phases of CD8$^+$ T cell responses. Selective performance of helper functions by memory (in contrast to effector) CD8$^+$ T cells helps to explain the phenomenon of immune memory and facilitates the design of effective therapeutic vaccines against cancer and chronic infections.

2.2 INTRODUCTION

Preventive vaccines, usually composed of "priming" and "booster" doses, have proven effective in controlling multiple infectious diseases, but the efficacy of current therapeutic vaccines remains low (68-71). Successful induction of immune memory is considered to be essential for vaccine effectiveness, but the exact pathways of development of memory $CD8^+$ T cells, and the features of their biology allowing them to mediate protection upon secondary antigen (Ag) challenge, remain unclear (72-75). Two long-recognized but poorly understood phenomena in vaccine biology include the importance of delayed application of booster doses of vaccines in order to achieve effective secondary T cell expansion (76) and the paradoxical contraction of the $CD8^+$ T cell pool when the second antigenic exposure occurs too soon (76, 77).

$CD8^+$ T cells are key to our ability to control intracellular infections and cancer. They act as cytotoxic T lymphocytes (CTL) (78) that eliminate the infected or transformed cells, but also perform regulatory functions, being capable of either suppressing (36, 37, 79, 80) or supporting (81-84) immune responses. While the mechanism of the suppressive activity of $CD8^+$ T cells is far from clear, it has been shown that perforin- and Granzyme-dependent elimination of Ag-carrying dendritic cells (DCs) by Ag-specific $CD8^+$ T cells (36, 37, 80, 85, 86) can act as a suppressive mechanism, providing a self-limiting character to CTL responses (36, 37, 80), and restricting the efficacy of vaccination (36, 37, 80, 85). In contrast to the long-known ability of $CD8^+$ T cells to inhibit immune responses (79), it only recently became apparent that $CD8^+$ T cells can also activate DCs (81-84, 87) and support type-1 immunity (81-84). Such "helper" function of $CD8^+$ T cells depends on their ability to produce IFNγ and to promote the DC production of IL-12p70 (83, 84), the key factor supporting Th1 and CTL responses (88).

The relationship between the "suppressor" versus "helper" functions of $CD8^+$ T cells remains unclear. Previous reports demonstrated that the suppressive effects resulting from the elimination of Ag-carrying DCs by $CD8^+$ T cells are mediated by the effector but not memory cells (37, 80). However, the possibility that it is the memory $CD8^+$ T cells that selectively play the reciprocal helper role, has never been explored.

Here, using the model of therapeutic vaccination with tumor-loaded DCs carrying additional tumor-unrelated ("heterologous") antigens, that allowed us to uncouple the regulatory functions of $CD8^+$ T cells from their effector activity, we demonstrate that the "suppressor" versus "helper" functions represent sequential phases of $CD8^+$ T cell responses. Our data show that promoting the interaction of tumor Ag-carrying DCs with memory-type $CD8^+$ T cells specific for tumor-unrelated Ags promotes the therapeutic activity of vaccination against the established tumors that are resistant to standard vaccines.

2.3 MATERIALS AND METHODS

Mice. Female 6-8-week-old C57BL/6, C57BL/6Tg (TcraTcrb)1100Mjb (OT-1), and C57BL/6-$IL12^{tm1Jm}$ (IL-12p40 knockout), and perforin-deficient (C57BL/6-*Prf1*^{tm1Sdz}/J), female mice purchased from Jackson Laboratories (Bar Harbor, ME) were maintained in micro-isolator cages and used for all experiments at 8-10 weeks of age. All experimental procedures were approved by our Institutional Animal Care and Use Committee.

Cell lines, cell isolation and culture. MC38 adenocarcinoma was provided by Dr DL Bartlett, University of Pittsburgh (originally from Dr SA Rosenberg, NCI). EL4 and EG7 (OVA-expressing EL4) cell lines were purchased from ATCC (Manassas, VA). Spleen $CD4^+$ and $CD8^+$

T cells have been negatively selected using StemSep^{TM} isolation columns (Stem Cell Technologies, Vancouver, BC) with 90-95% purity. In some *in vitro* experiments (*Fig. 1b)*, we performed additional anti-LyC6-mediated removal (89) of preactivated OT-1 cells or flow-sorted $CD44^{-}CD62L^{+}$ (naïve) and $CD44^{+}CD62L^{-}$ (memory) cells. All cells were maintained in RPMI1640 with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA), glutamine, streptomycin and penicillin (Invitrogen).

DCs. Bone marrow-derived DCs were generated in GM-CSF and IL-4 (both 1000 U/ml; Schering-Plough, Kenilworth, NJ)-supplemented cultures, as described (90). On day 6-7, $CD11c^{+}$ DCs were isolated using anti-mouse CD11c-coated magnetic beads (Miltenyi Biotech.). DCs expressed CD11c, CD40, CD80, CD86, and MHC I and II ((90) and *data not shown*).

Induction of IL-12p70. SEA ($CD4^{+}$ and $CD8^{+}$ T cell-activating superantigen (83, 91, 92); 1ng/ml)-loaded DCs ($2x10^{4}$ cells/0.2 ml/well) were co-cultured for 48h with $CD4^{+}$ Th cells (10^{5} cells/well), in the absence or presence of $CD8^{+}$ T cells (10^{5} cells/well). When indicated, (neutralizing) soluble (s)IFN-γ receptor or (s)IL-4 receptor were added (10 µg/ml; R&D Systems). Alternatively, Ag-free, $OVA_{257-264}$-, or SEA-loaded DCs were first cocultured for 48h with $CD8^{+}$ T cells ($0.75x10^{5}$ or 3×10^{5} cells; respectively from the spleens of OT-1- or wild-type mice), harvested, washed, counted, and stimulated (at $2x10^{4}$ DCs/0.2 ml) with $5x10^{4}$ CD40L-transfected J558 cells (83, 93) for 24h. IL-12p70 concentrations were determined by ELISA (Endogen).

***In vitro* DC killing**. $CD8^{+}$ T cells from wild-type or perforin-deficient mice were stimulated with SEA-loaded DC and IL-2 (20U/ml; Chiron Corp., Emeryville, CA) for 6 days. The resulting Granzyme $B^{high}/CD62L^{low}$ *(not shown)* effector cells were co-incubated with DCs (3h; 5:1 ratio) in the presence or absence of SEA. Induction of apoptosis in $CD11c^{+}$ DC was assessed by

staining for surface CD11c, followed by intracellular staining for active Caspase-3 (C92-605, BD Pharmingen).

DC elimination *in vivo.* DCs ($OVA_{257-264}$- or PBS-loaded; $1x10^6$) were labeled with CSFE (1 μM/L for 10 min at 37°C), washed 3 times and injected into the footpads. After 24h, single cell suspensions from popliteal lymph nodes were prepared and analyzed by flow cytometry. Total node cellularity was counted in hemocytometer.

Induction of LCMV- or OVA-specific immune responses. $LCMVgp_{33-41}$ peptide, (KAVYNFATC), the dominant H2-D^b/K^b-restricted epitope of LCMV, and dominant H-2K^b-restricted OVA epitope, $OVA_{257-264}$ (SIINFEKL), were synthesized by the University of Pittsburgh Peptide Synthesis Facility. Peptide-loaded DCs were washed twice and injected s.c. ($3x10^5$ DCs in 0.2 ml of PBS) twice with 1-week interval. The presence of effector and memory $CD8^+$ T cells in the spleens and lymph nodes of vaccinated animals was determined by 3-color flow cytometry after staining of isolated $CD8^+$ T cells, with CD62L (MEL-14; BD-Pharmingen), Granzyme B (GB12l; CalTag), and tetramer (iTAg^{TM}, Beckman-Coulter, Fullerton, CA).

Tumor vaccines. DCs were loaded overnight with MC38 tumor cells lysates (three freeze-thaw cycles; centrifuged, and supernatant collected), at three tumor cell equivalents to one DC, in the presence of LPS. DCs were resuspended in RPMI 1640 and loaded, with OVA $OVA_{257-264}$, LCMV gp_{33-41}, or PBS. For preparation of the EG7 vaccine, DCs were loaded with $OVA_{257-264}$ (alone or with $LCMVgp_{33-41}$). All vaccines were washed twice and suspended in PBS.

Tumor therapy models. Wild-type C57BL/6 mice (7-12/group; including 2 animals/group for CTL assays), naïve or carrying week-1 or week-4 immune responses against LCMV or OVA, were inoculated (right flank; s.c.; day 0) with high numbers of tumor cells ($3x10^5$ MC38 or $3x10^6$ EG7), to induce rapid tumor growth that was only marginally sensitive to standard therapeutic

vaccines (*see Fig. 3*). The mice were vaccinated ($3x10^5$ DCs, on the distant site on same flank, s.c.) on day 3, or on days 5–9-11, as indicated). Tumors were measured by vernier calipers every 3-4 days. Data are reported as the mean ± SEM of tumor area (product of the largest perpendicular diameters).

CTL activity. 10 days after vaccination, splenocytes were harvested from two (tumor-bearing) mice per group. They were restimulated *in vitro* ($1x10^6$ cells/well) with $1x10^5$ gamma-irradiated (10,000 R) MC38 or EG7 cells in the presence of 30 IU/ml rhuIL-2 in 24-well culture plates. Lymphocytes were harvested after 5 days and used in 5h ^{51}Cr release assays against MC38 and EG7 targets, with EL4 cells used as non-specific controls.

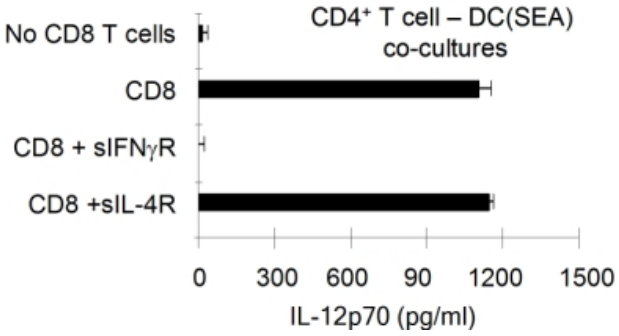
Statistical analysis. Data collected (day 4 till the last day of tumor measurement) were (natural) log transformed and used to fit a parametric mixed linear model that included animals as random effects with treatment group and day of measurement as fixed effects. If either group differences or group by time interaction were significant at $p<.05$ the analysis was applied to the last day of tumor area measurement. Data were (natural) log transformed when appropriate and a one way parametric analysis of variance (ANOVA) was used as an omnibus test of differences. Unless tests were significant at level $\alpha=.05$, no further testing of specific contrasts was conducted. Otherwise, individual pairwise comparisons were conducted with the t test. All tests were two tailed.

2.4 RESULTS

DC activating and DC-killing activities of resting versus effector $CD8^+$ T cells

In order to analyze the ability of mouse $CD8^+$ T cells to affect DC functions *in vitro*, we used the previously-established models of the superantigen (SEA) (83, 91)-driven or chicken ovalbumin (OVA) (89, 94)-driven stimulation of T cells from wild-type C57BL/6 mice or TCR-transgenic (94) OT-1 mice, respectively. These models allowed us to promote the interaction of DCs with high numbers of $CD8^+$ T cells without the need of prior T cell activation and clonal expansion. In accordance with our data showing that the superior ability of human $CD8^+$ T cells to produce IFNγ at early stages of activation allows them to co-stimulate IL-12p70 production by DCs interacting with $CD4^+$ T cells (83), freshly-isolated mouse $CD8^+$ T cells strongly supported IL-12p70 induction in co-cultures of SEA-loaded bone-marrow-derived DCs (90) with autologous $CD4^+$ T cells ***(Fig. 2.1A).*** They also primed the SEA- or $OVA_{257-264}$-loaded DCs for high IL-12p70 production during subsequent interaction with CD40L-transfected J558 cells, used as $CD4^+$ T cell surrogates (83, 93) (***Fig. 2.1B)***. Similar to the human system, neutralization of IFNγ, but not IL-4 (another IL-12-enhancing cytokine (92, 95)), abolished the IL-12-enhancing activity of $CD8^+$ T cells (***Fig. 2.1A)***. As expected (83), not only the simultaneous interaction of DCs with $CD8^+$ T cells and $CD4^+$ T cells (***Fig. 2.1A)***, but also their sequential interaction with $CD8^+$ T cells followed by CD40L stimulation (***Fig. 2.1B)*** resulted in the augmented IL-12p70 production, with naïve and memory $CD8^+$ T cells being similarly effective (***Fig.2.1B-top, inset)***. These data verified that, similar to human $CD8^+$ T cells, mouse $CD8^+$ T cells can act as "helper" cells, supporting IL-12 production by DCs.

A



B

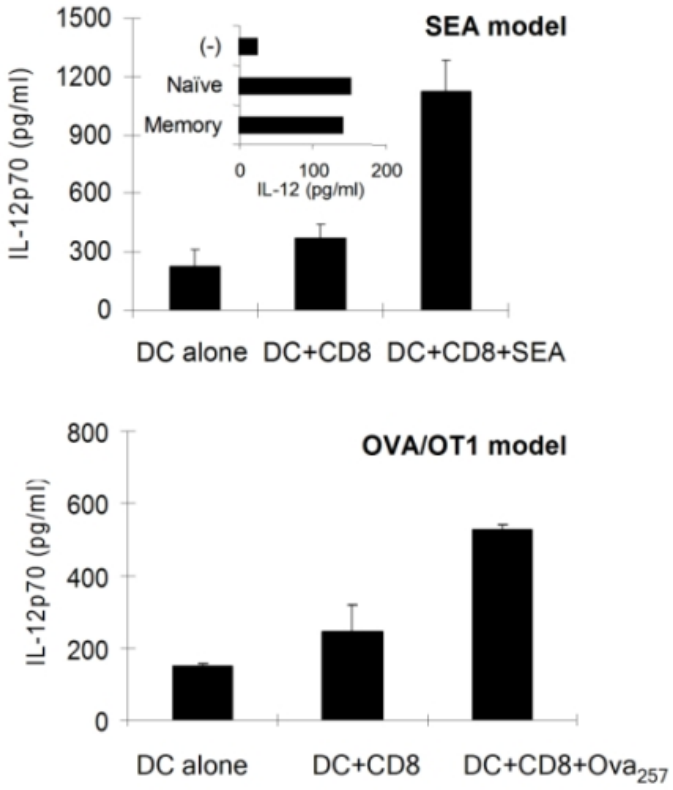


Figure 2.1 DC-activating function of resting CD8$^+$ T cells.

A, CD8$^+$ T cells support IL-12p70 induction in DC-CD4$^+$ T cell co-cultures. SEA-coated (83, 91) DCs (90) were co-incubated with syngeneic CD4$^+$T cells, in the absence or in the presence of (IFNγ-producing, *not shown*) spleen-isolated CD8$^+$ T cells. Soluble IL-4R or IFNγR were used selectively neutralize IL-4 or IFNγ , two cytokines with IL-12-enhancing activities (83, 92, 95). Data (mean ± SD) from one experiment of three that yielded similar results. B, Interaction with CD8$^+$ T cells primes DCs for high IL-12p70 production. (*top*) DCs were cocultured for 48h with CD8$^+$ T cells from wild-type B6 mice either in the absence or presence of SEA, prior to washing and stimulation with CD40L (83, 93). Addition of SEA alone (no T cells) had no or marginal effect ((83) a*nd data not shown*). *The inset:* equivalent effectiveness of naïve and memory CD8$^+$ T cells. (*bottom*) DCs were cocultured with H-2K^b–

restricted $OVA_{257–264}$ -specific $CD8^+$ T cells, freshly isolated from spleens of OT-1 mice, in the presence of $OVA_{257–264}$ peptide, prior to washing and CD40L stimulation.

In contrast to resting T cells, six-day preactivated effector $CD8^+$ T cells, efficiently killed Ag-carrying DCs (***Fig. 2.2).*** In accordance with the *in vivo*-demonstrated key role of perforin in CTL-dependent DC elimination (37)**,** $CD8^+$ T cells from perforin-deficient mice were defective in their ability to kill DCs (***Fig. 2.2)***.

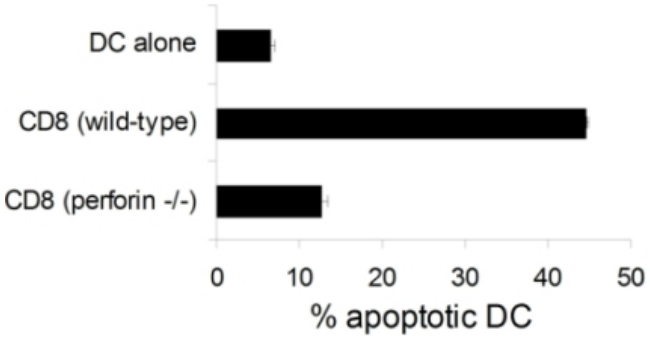


Figure 2.2 Effector T cells kill DCs *in vitro*.

SEA-loaded DC were co-cultured with pre-activated (Granzyme $B^{high}/CD62L^{low}$; *data not shown*) effector $CD8^+$ T cells from wild-type or perforin-deficient mice. Similar results were obtained in one additional experiment.

Taken together, these data verified that $CD8^+$ T cells can act both as activators and as killers of Ag-carrying DCs, but indicated that these two functions are performed at different stages of $CD8^+$ T cell activation. They suggested that non-cytotoxic naive or memory $CD8^+$ T cells versus the effector cells with CTL function may play opposite functions regulating the course of immune responses. In order to establish an *in vivo* model that would allow us to test in wild-type mice the regulatory impact of either the cytotoxic (effector) or non-cytotoxic (memory) $CD8^+$ T cells, we pre-immunized wild-type C57BL/6 mice with $OVA_{257-264}$ for 1 or 4 weeks. As expected, in 1 week-immunized animals we observed high numbers of tetramer-positive $CD8^+$ T cells with $CD62L^{low}$/Granzyme B^{high} effector phenotype. In sharp contrast,

$CD8^+$ T cells that were obtained 4 weeks after immunization demonstrated a selective presence of $CD62L^{high}$/Granzyme B^{low} memory-type T cells (***Fig. 2.3)***.

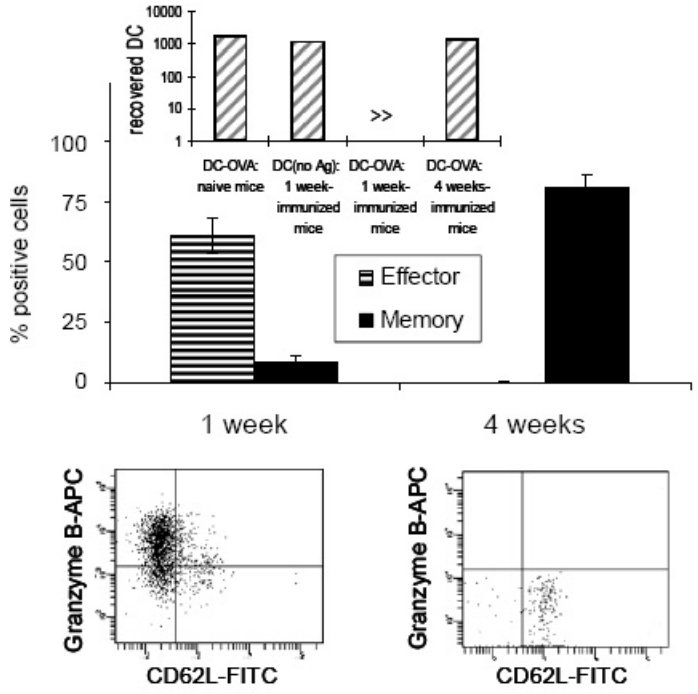


Figure 2.3 Predominance of $OVA_{257-264}$/H-$2K^b$-specific effector (1wk) versus memory T cells (4 wk).

Note the predominance of tetramer-positive Granzyme $B^+/CD62L^-$ effector cells in 1 week-immunized mice, as opposed to selective presence of Granzyme $B^-/CD62L^+$ memory cells in the spleens of 4 week immunized mice (n = 3 mice per group; *see the bottom for representative data from individual animals*). The frequencies of tetramer-positive $CD8^+$ T cells in 1 week- and 4 week-immunized mice were 4.3% ($^+$/- 1.5) and 0.7% ($^+$/-0.3), respectively. Data from one of two experiments that yielded similar results. *Inset:* Selective elimination of $OVA_{257-264}$-carrying CFSE-labeled DCs in 1-week-immunized, but not 4 week-immunized, mice. Naïve mice and 1-week-preimmunized mice receiving sham-loaded DCs served as control groups. Mice (3 mice/group) were injected with 10^6 DC and draining lymph nodes (37, 80) were removed after 16h.

Similar to previous reports (37, 80, 85), we observed that the animals harboring $CD62L^{low}$/Granzyme B^{high} effector $CD8^+$ T cells, induced by pre-immunization with $OVA_{257-264}$ one week earlier, rapidly eliminated Ag-loaded DCs (***Fig. 2.3, inset),*** while OVA-loaded DCs

could be readily recovered from the vaccine-draining lymph nodes of naïve mice or mice harboring 4 week old memory-type CD62L^{high}/Granzyme B^{low} T cell responses.

Memory CD8$^+$ T cells support the DC-mediated induction of tumor-specific CTL responses

In order to test the possibility that Ag-specific CD8$^+$ T cells at different stages of activation indeed play different regulatory roles, we have used a model of therapeutic vaccination with tumor-loaded DCs carrying additional, tumor-unrelated, MHC class I-restricted epitopes of $OVA_{257-264}$ or Lymphocytic Choriomeningitis Virus (LCMV) glycoprotein ($LCMVgp_{33-41}$) (***Figs. 2.4 and 2.5)***. We analyzed the impact of including tumor-unrelated "heterologous" class I-restricted peptide epitopes into DC-based vaccines (tumor-loaded DCs) upon the induction of CTL responses and the therapeutic activity of vaccination against established tumors (***Fig. 2.4A)***. In such models, the "heterologous" helper epitopes were only present in cancer vaccines and were not expressed by the tumor itself. Therefore, any "helper" or "suppressor" impact of ($OVA_{257-264}$-specific or $LCMVgp_{33-41}$-specific) CD8$^+$ T cells on the development of the immune responses against MC38 or EG7 tumors, could be analyzed in isolation from a possible indirect modulatory impact of CD8$^+$ T cells, mediated by tumor antigens and other tumor-derived factors differentially released from the CTL-targeted tumor tissues.

In order to model a clinically-relevant situation where significant numbers of resting T cells can be employed to interact with cancer vaccine in wild-type animals, we have used wild-type C57BL/6 mice harboring memory-type CD8$^+$ T cells against defined MHC class I-restricted epitopes of OVA or LCMV. For the same purpose, we have administered the vaccines to the animals bearing day 3-5 established tumors, the time point where, in analogy to the clinically-

applied therapeutic cancer vaccines (37, 80), standard vaccinations with tumor-loaded DCs were only marginally effective.

As shown in ***Figure 2.4B***, the inclusion of $OVA_{257-264}$ epitope in cancer vaccines composed of autologous DCs loaded with the relatively poorly immunogenic MC38 tumor lysate supported the generation of MC38-specific CTL responses in wild-type C57BL/6 animals. Similar data was also obtained in a model of wild-type mice harboring memory responses against $LCMVgp_{33-41}$, a dominant epitope of a natural mouse pathogen, where the inclusion of $LCMVgp_{33-41}$ peptide as a "heterologous helper epitope" strongly enhanced the induction of CTLs against established MC38 tumors (***Fig. 2.4B)***.

The vaccines including the $LCMVgp_{33-41}$ "CD8 helper" epitope not only showed strongly-elevated CTL-inducing function against the poorly-immunogenic MC38 adenocarcinoma, but also further enhanced the CTL responses against the highly-immunogenic (OVA-expressing) EG7 lymphoma, induced by $OVA_{257-264}$-loaded DCs (***Fig. 2.4D).*** The ability of the $CD8^+$ T cells specific for the same individual epitope ($OVA_{257-264}$), to both provide "CD8 helper" signals (***Fig. 2.4B***) and to benefit from such signals (***Fig. 2.4D***) indicates that $CD8^+$ T cell help is not restricted to responses against some unique antigens (e.g. responses to "strong" immunogens facilitating the responses to "weak" immunogens), but that any naïve $CD8^+$ T cells that can first receive the CD8 help and later, after becoming memory cells themselves, can provide helper signals to other cells.

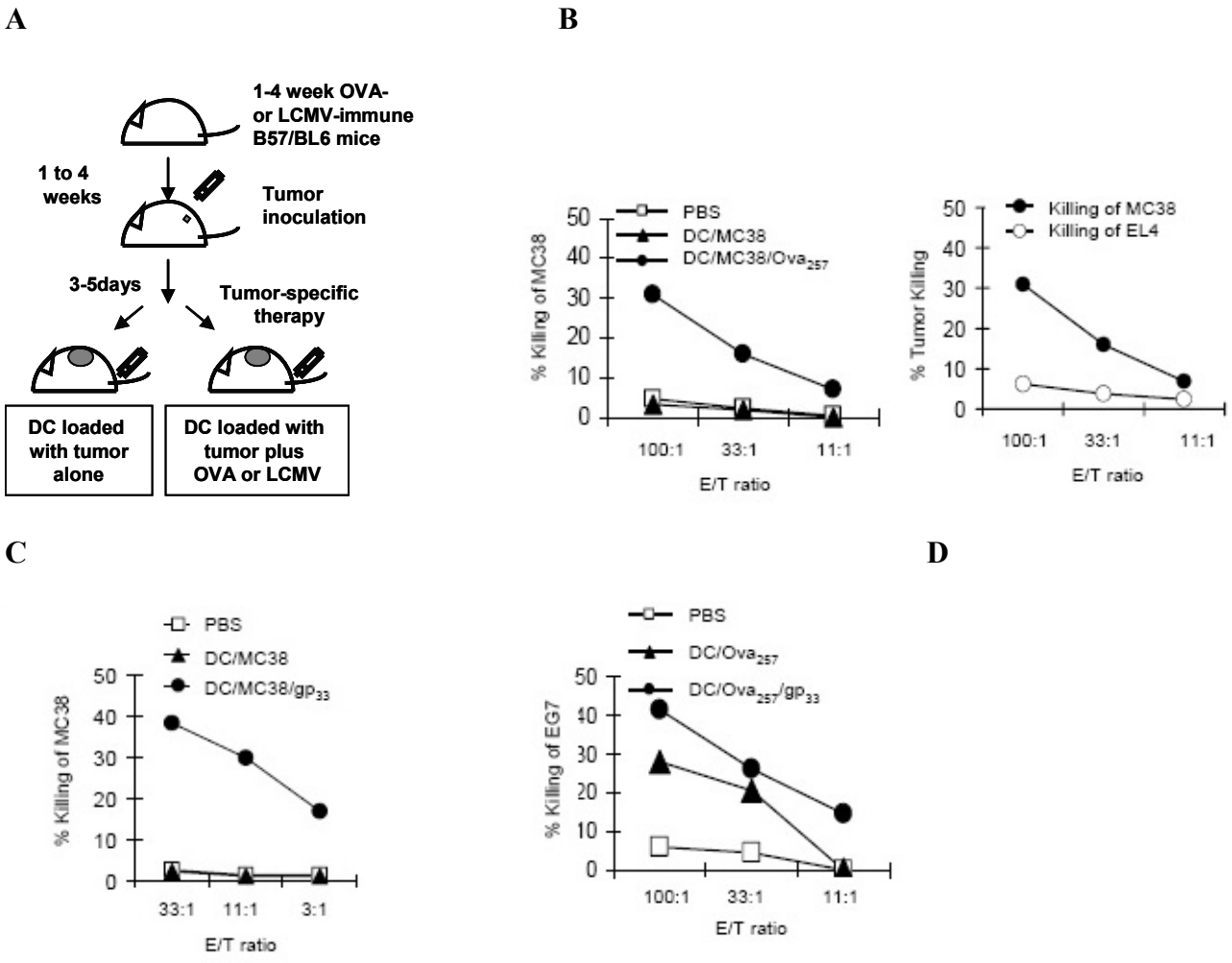


Figure 2.4 Memory CD8$^+$ T cells support the DC-mediated induction of tumor-specific CTL responses.

A, Schema of the experimental protocol. B, $OVA_{257–264}$-specific CD8$^+$ T cells support the induction of CTLs specific for MC38 adenocarcinoma. Mice carrying memory-type $OVA_{257–264}$-specific CD8$^+$ T cell responses, and inoculated with MC38 tumor (day 0), were treated (s.c.; day 3) with DCs loaded with MC38 lysate alone or with $OVA_{257–264}$ as tumor-unrelated "heterologous" helper epitope. *Left:* Induction of CTL activity in the spleens of the differentially-treated mice. *Right:* Comparison of CTL activity of the splenocytes from the DCs/MC38 lysate/LCMVgp_{33-41}-treated mice against the vaccine-relevant (MC38) and irrelevant (EL4) targets. Data from one of two experiments that yielded similar results. C, Memory-type LCMVgp_{33}-specific CD8$^+$ T cells support the induction of MC38 adenocarcinoma-specific CTLs. Tumor-bearing mice with memory-type LCMVgp_{33-41}-specific CD8$^+$ T cell responses were injected (s.c.) with DCs loaded with MC38 tumor lysate, alone or with LCMVgp_{33-41} as a "heterologous" helper epitope. The data from one of three independent experiments that all yielded similar results. D, LCMV-specific CD8$^+$ T cells support the induction of CTLs against $OVA_{257–264}$-expressing EG7 lymphoma. Tumor-bearing mice with memory-type LCMVgp_{33}-specific CD8$^+$ T cell responses were injected (s.c.) with DCs

loaded with $OVA_{257–264}$ peptide, as the EG7 tumor-relevant antigen, either alone or with the $LCMVgp_{33-41}$ peptide, as a tumor-unrelated "heterologous" helper epitope. Similar results were obtained in an additional experiment.

Heterologous $CD8^+$ T cell help supports the therapeutic effects of cancer vaccines

In order to test whether "helper" signals from memory $CD8^+$ T cells can enhance the therapeutic activity of vaccination against established tumors, we compared the therapeutic activity of the DCs loaded with tumor-relevant antigens, alone or with $LCMVgp_{33-41}$ peptide, as therapeutic vaccines against the established (day 3-5) MC38 and EG7 tumors in wild-type C57BL/6 mice, either naïve or carrying memory-type LCMV-specific $CD8^+$ T cells. As shown in ***Figure 2.5A,*** in such therapeutic settings, DCs loaded with tumor material alone had only marginal impact on the growth of established MC38 tumors. In LCMV-naïve mice, this outcome of was not improved by the inclusion of the $LCMVgp_{33-41}$ "helper" epitope in the vaccines (***Fig. 2.5A, left***).

However, in mice harboring memory-type responses against the MHC class I-restricted $LCMVgp_{33-41}$ epitope**,** the vaccination with DCs loaded with MC38 tumor lysate and $LCMVgp_{33-41}$, the "heterologous CD8 helper" peptide, resulted in a distinct therapeutic effect against day 5 established tumors that were resistant to treatment with standard DC-based vaccines (***Fig. 2.5A: right)***. These beneficial effects of "heterologous CD8 help" could not be mimicked by the vaccination with DCs loaded with $LCMVgp_{33-41}$ alone, demonstrating that the reduction in tumor growth is not due to nonspecific immunostimulatory effects of activation of high numbers of LCMV-specific $CD8^+$ T cells present in these animals.

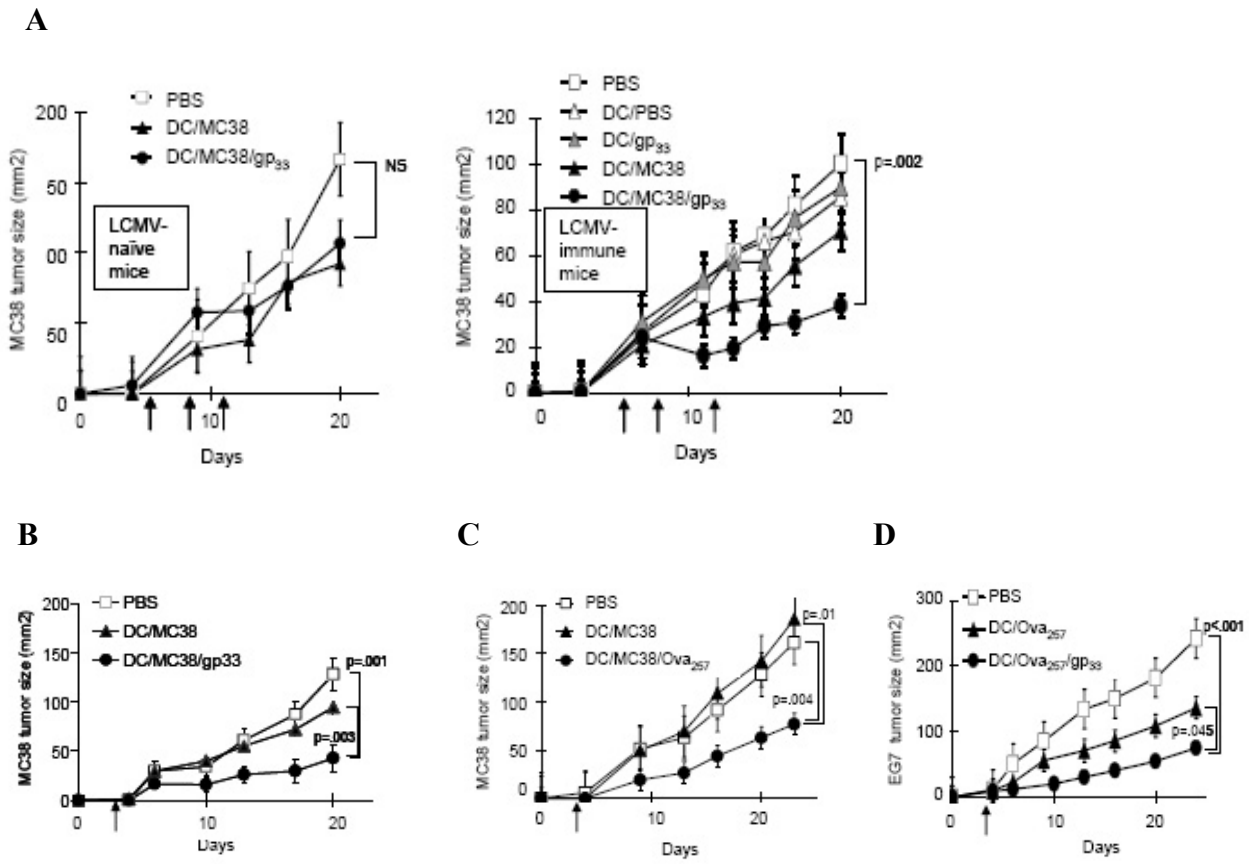


Figure 2.5 Heterologous CD8$^+$ T cells support the therapeutic activity of cancer vaccines.

A, Memory-type LCMVgp33-41-specific CD8$^+$ T cells promote therapeutic effects of vaccination against day 5 established MC38 tumors. MC38-bearing C57BL/6 mice (n=5 mice/group), either naive (left) or carrying memory-type (week 4) LCMVgp$_{33\text{-}41}$-specific CD8$^+$ T cell responses (*right)* were inoculated with MC38 tumors at day 0 and were treated on day 5, day 9, and day 11 after tumor inoculation. B-D, Day 3 tumor-bearing mice (B: n=10 per group; C-D: n=5 per group) with memory-type responses against tumor-unrelated "heterologous" helper antigens were injected (s.c.) with: PBS as a negative control (open squares), with DCs loaded with tumor antigen alone (MC38 tumor lysate or OVA$_{257\text{–}264}$ in the EG7 model), or with the relevant tumor antigen plus a tumor-irrelevant "heterologous" helper epitope (closed circles). B, Memory-type LCMVgp$_{33\text{-}41}$-specific CD8$^+$ T cells support the therapeutic activity of vaccination against MC38 adenocarcinoma. C, Memory-type OVA$_{257\text{–}264}$-specific CD8$^+$ T cells support the therapeutic activity of vaccination against MC38 adenocarcinoma. D, Memory-type LCMVgp$_{33\text{-}41}$-specific CD8$^+$ T cells support the therapeutic activity of vaccination against OVA$_{257\text{–}264}$-expressing EG7 lymphoma. Data (mean ± SEM) from one of two separate experiments in each model. The differences between the treatment groups were evaluated using ANOVA: NS: no statistically-significant differences (p>.05).

Similar enhancement of the therapeutic efficacy of vaccination has been observed in three additional models, when using LCMVgp_{33-41} or $OVA_{257-264}$ to enhance the antitumor effects of a single (rather than triple) dose of vaccine against the 3 day old MC38 tumors (***Fig. 2.5 B, C)***, or when using LCMVgp_{33-41} to boost the antitumor effects of vaccination against significantly more immunogenic EG7 lymphoma (***Fig. 2.5D)***. In accordance with the data on the induction of tumor-specific CTLs (***Fig. 2.4)***, the results of these functional tests of anti-tumor activity demonstrated that $OVA_{257-264}$-specific $CD8^+$ T cells at their naïve stage can benefit from "helper" signals delivered by memory T cells (in the current experiments: specific for LCMVgp_{33-41}), but at the memory stage they can themselves act as a source of $CD8^+$ T cell helper signals, verifying that the ability to receive and provide "CD8 helper" signals is a general feature of $CD8^+$ T cells.

Critical role of DCs in mediating "CD8 help"

While the inclusion of LCMVgp_{33-41} (or $OVA_{257-264}$) peptides strongly enhanced the immunologic and antitumor effects of vaccination in the animals harboring "memory-type" $CD8^+$ T cells specific for such "heterologous helper epitopes" (***Fig. 2.6)***, in accordance with the DC-killing activity of effector $CD8^+$ T cells (37, 80, 86), these positive effects were eliminated in the animals pre-immunized with LCMV at 1 week prior to tumor inoculation (***Fig. 2.6A)***. In further support of the central role of DCs and the DC-produced cytokines, the "heterologous help" from memory-type $CD8^+$ T cells could not be mediated by IL-12/IL-23-deficient DCs generated from the bone marrow of p40-knockout animals (***Fig. 2.6B***).

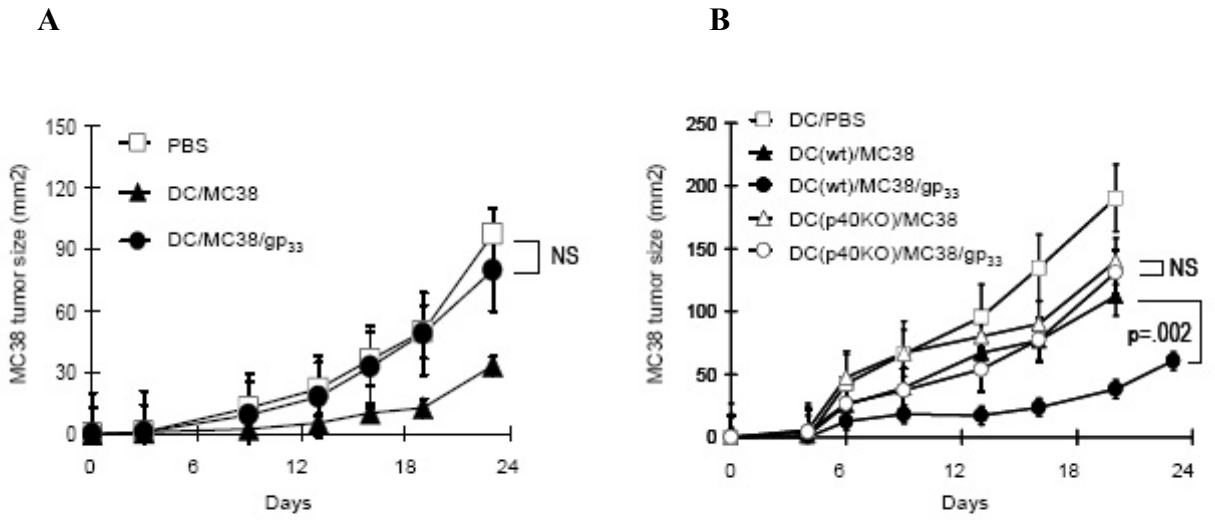


Figure 2.6 Critical role of DCs in mediating CD8 help for anti-cancer immunity.

A, Effector $CD8^+$ T cells do not deliver "heterologous CD8 help". Three days after MC38 tumor inoculation, C57BL/6 mice (n=5) with 1 week-old LCMV-specific responses received a single s.c. injection of MC38 tumor lysate with or without $LCMVgp_{33-41}$ peptide loaded on DCs from wild-type mice and monitored for the kinetics of tumor growth. B, IL-12p40-deficient DCs (unable to produce IL-12p70 and IL-23) do not mediate heterologous CD8 help. Three days after MC38 tumor inoculation, wild-type C57BL/6 mice (n=5) with 4 week-old LCMV-specific $CD8^+$ T cell responses were treated with the indicated vaccines. Mice received single s.c. injections of DCs (generated from wild-type or IL-12p40-knockout mice) loaded with MC38 tumor lysate alone or with $LCMVgp_{33-41}$ peptide. Data (mean ± SEM) from two independent experiments in each model.

2.5 DISCUSSION

The current data demonstrate that, in contrast to effector $CD8^+$ T cells that kill DCs and suppress Ag-specific immune responses (36, 37, 80, 85, 86), memory $CD8^+$ T cells can act as de facto "helper cells", supporting the DC-mediated immunologic and anti-tumor effects of cancer vaccines.

Our observations that $OVA_{257-264}$-specific $CD8^+$ T cell responses can both benefit from the "helper signals" delivered by memory cells (e.g. from LCMV-specific $CD8^+$ T cells) and can provide such signals, directly demonstrate that $CD8^+$ T cells of a single specificity can first benefit from the help delivered by memory T cells and, once expanded and having reached memory stage, they can themselves act as helper cells. The ability of memory $CD8^+$ T cells to support the DC-mediated activation of additional naïve $CD8^+$ T cells suggests that the phenomenon of immune memory, in addition to qualitative changes in the activation requirements of memory cells (72-75), may also involve a different pattern of interaction of $CD8^+$ T cells with Ag-carrying DCs.

Our findings help to explain the requirement for a delayed administration of booster doses of preventive vaccines to achieve the optimal expansion of pathogen-specific $CD8^+$ T cells and the optimal vaccine effectiveness (76, 77). Interestingly, recent evidence indicate that priming performed under non-inflammatory conditions allows for effective administration of booster vaccines substantially sooner than when priming with live pathogen (76). However, it remains to be tested whether such non-inflammatory priming conditions result in impaired induction of the effector (and thus suppressive) functions of the pathogen-specific $CD8^+$ T cells.

The existence of distinct "suppressor" and "helper" stages of $CD8^+$ T cell activation may help to explain the generally poor efficacy of therapeutic vaccinations of cancer-bearing patients

that show predominance of terminally-differentiated effector cells (96-98). It also helps to explain the high efficacy of prime-boosting vaccination strategies (75), when the first and second doses of vaccine are delivered using antigenically-distinct vectors.

The mechanism of helper function of memory $CD8^+$ T cells in the settings of established cancer is a subject of our current follow-up analyses. While our data demonstrate the key role of Ag-carrying DCs in this respect, it is unclear whether the "heterologous" memory $CD8^+$ T cells just hyper-activate DCs, allowing them to provide immunostimulatory signals to naïve/resting tumor-specific $CD8^+$ T cells prior to their destruction by effector cells, or whether memory $CD8^+$ T cells can also protect Ag-carrying DCs from CTL killing. It has been recently proposed that the $CD4^+$ T cell help for CTL responses is essential mainly for the secondary expansion of $CD8^+$ T cells, rather than for their effective priming (77). These observations raise the possibility that the protection of DCs from CTL killing (86, 99) may be as important as the originally proposed DC activation or "licensing" (100-102) in the overall mechanism of the $CD4^+$ T cell help.

The current data directly implicate the possibility of enhancing the effectiveness of therapeutic vaccination of cancer patients by incorporating tumor-unrelated "heterologous" epitopes that promote the interaction of the vaccine-carrying APCs with naturally occurring or artificially-induced memory-type T cells. While in the current studies, we have either used (*in vitro*) high numbers of resting $CD8^+$ T cells from TCR-transgenic animals, or have involved (*in vivo*) the memory $CD8^+$ T cells induced by a pre-immunization of wild type-animals, the most obvious source of "heterologous CD8 help" in cancer patients are the memory-type $CD8^+$ T cells induced naturally by past infections or by prior vaccinations against "childhood diseases" or such pathogens as influenza or hepatitis.

Our data showing that the helper functions are a selective feature of memory $CD8^+$ T cells, but not effector cells, indicate the existence of a novel mechanism contributing to the phenomenon of $CD8^+$ T cell memory. Our observations help to explain the benefits of delayed application of booster doses of preventive vaccines and high efficacy of prime-boost vaccination strategies, facilitating the development of effective strategies of therapeutic vaccination of patients with cancer and chronic infections.

MEMORY CD8$^+$ T CELLS PROTECT DENDRITIC CELLS FROM CTL KILLING

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All the experiments in this manuscript were performed by Payal Watchmaker, while the confocal microscopy images were taken by Julie Urban.

3 MEMORY CD8$^+$ T CELLS PROTECT DENDRITIC CELLS FROM CTL KILLING

3.1 ABSTRACT

CD8$^+$ T cells have been shown capable of either suppressing or promoting immune responses. In order to reconcile these contrasting regulatory functions, we compared the ability of human effector and memory CD8$^+$ T cells to regulate survival and functions of dendritic cells (DC). We report that, in sharp contrast to the effector cells (CTLs) that kill DCs in a Granzyme B- and perforin-dependent mechanism, memory CD8$^+$ T cells enhance the ability of DC to produce IL-12 and to induce functional Th1- and CTL responses in naïve CD4$^+$ and CD8$^+$ T cell populations. Moreover, memory CD8$^+$ T cells, that release the DC-activating factor TNFα prior to the release of cytotoxic granules, induce DC expression of an endogenous Granzyme B inhibitor PI-9 and protect DC from CTL killing with similar efficacy as CD4$^+$ T helper cells. The currently identified DC-protective function of memory CD8$^+$ T cells helps to explain the phenomenon of CD8$^+$ T cell memory, reduced dependence of recall responses on CD4$^+$ T cell help, and the importance of delayed administration of booster doses of vaccines for the optimal outcome of immunization.

3.2 INTRODUCTION

In addition to their function as cytotoxic T cells (CTLs) capable of killing transformed or infected cells in a perforin- and FasL-dependent mechanism (2, 103), $CD8^+$ T cells have also been shown to play a regulatory role, being able to suppress Ag-specific immune responses (79, 104). Their suppressor activity (32, 105, 106) involves the elimination of antigen-carrying DCs by effector $CD8^+$ T cells (80) in a perforin-dependent mechanism (37). Activated $CD8^+$ T cells have been shown to limit the CTL responses by restricting DC survival and the duration of antigen display *in vivo* in mice infected with Listeria, LCMV, HSV, and malaria (35, 36, 99). This self-limiting nature of $CD8^+$ T cell responses can be counteracted by $CD4^+$ T helper cells (86, 99), known to be important for the establishment of $CD8^+$ T cell memory and effective expansion of CTL precursors during recall responses (77, 107, 108). While the mechanism of helper activity of $CD4^+$ T cells was originally considered to involve Th cell-produced IL-2 (109) and the CD40L-mediated elevation of the stimulatory capacity of DCs (100-102), it was subsequently demonstrated that CD40L-expressing $CD4^+$ T cells can induce in DCs endogenous Granzyme B inhibitor (serpin SPI-6/PI-9) (86), rescuing the Ag-carrying DCs from killing by the same CTLs that they induce, and thus prolonging the time-frame of effective stimulation of the expanding population of Ag-specific $CD8^+$ T cells (86, 99). In contrast to the widely-studied suppressive activity of $CD8^+$ T cells, only recently it was demonstrated that $CD8^+$ T cells can also activate DCs and promote the Th1- and CTL-mediated type-1 immunity (82-84, 87, 110, 111).

In order to reconcile the paradoxical ability of $CD8^+$ T cells to act as both suppressor and helper cells, we addressed the possibility that helper and suppressor functions are selectively displayed by $CD8^+$ T cells at different stages of activation. Using the models of peptide-specific-

and superantigen-driven activation of $CD8^+$ T cells isolated from blood and tissues and the *in vitro* pre-activated $CD8^+$ T cells at different stages of activation, we show that the DC killing and DC-activating/protecting functions are exerted sequentially by human $CD8^+$ T cells: While effector $CD8^+$ T cells kill DCs in a Granzyme B- and perforin-dependent pathway, TNFα-producing memory $CD8^+$ T cells display an equivalent activity to $CD4^+$ Th cells in protecting DCs from premature elimination by the effector cells, supporting the induction of functional Th1 and CTL responses.

3.3 MATERIALS AND METHODS

Media, reagents, and cell lines. The cell cultures were performed using either IMDM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Hyclone, Logan UT) or serum-free AIM-V medium (Invitrogen). *rhu*GM-CSF and IL-4 were gifts from Schering Plough (Kenilworth, NJ). IL-2 was kindly provided by Chiron Corporation (Emeryville, CA). IFNγ, TNFα, and IL-1β were purchased from Strathman Biotech (Germany). Staphylococcus Enterotoxin B (SEB), used for priming high number of naïve $CD8^+$ T cells (83, 91), was obtained from Toxin Technologies (Sarasota, FL). CD40L-transfected J588 plasmacytoma cells were a gift from Dr. P Lane (University of Birmingham, UK) and JY-1 cells were a gift from Dr. Eddy Wierenga (University of Amsterdam). Granzyme B inhibitors IETD-CHO and Z-IETD-fmk were obtained from Calbiochem (San Diego, CA).

Isolation of the naïve, memory and effector T cell subsets from peripheral blood and tissues. Mononuclear cells, obtained from the peripheral blood of healthy donors, were isolated by density gradient separation using Lymphocyte Separation Medium (Cellgro Mediatech,

Hendon VA). Naïve $CD4^+CD45RA^+$T cells and naïve $CD8^+CD45RA^+$T cells were isolated by negative selection with the StemSep CD4 and CD8 enrichment cocktails, respectively (StemCell Technologies Inc, Vancouver, Canada). Biotinylated anti-CD45RO antibody was used in combination with enrichment cocktails for isolation of naïve population. The phenotype of naïve $CD8^+$ $CD45RA^+CCR7^+$ T cell population was confirmed by flow cytometry (***Supplementary Figure 1***).Tissue-type effector $CD8^+$ T cells were obtained from colorectal cancer patients, undergoing surgical resection of liver metastasis, and cultured overnight in low dose IL-2 to recover from the isolation-induced stress and possible effects of tumor derived factors (***Supplementary Figure S1: Appendix A).*** The memory subset $CD8^+CD45RA^-CCR7^+$ T cells from peripheral blood (***see Supplementary Figure S1: Appendix A***) was isolated using CD45RA-depleting/CD8 enrichment cocktail (StemCell Technologies).

Generation of dendritic cells. Day 6 immature DCs (used as a readout of functional activity of $CD8^+$ T cells), were generated from peripheral blood monocytes cultured ($5x10^5$ per ml) in IMDM/10%FBS supplemented with rhuIL-4 and rhuGM-CSF (both at 1000U/ml) in 24 well plates (Falcon, Becton Dickinson Labware, NJ). Type-1 polarized mature DCs, used for the generation effector- and memory-type $CD8^+$ T cells *in vitro*, were obtained in serum-free AIM-V medium with IL-4 and GM-CSF, and matured (days 6-8) in the presence of TNFα, IFNγ,IL-1β, IFNα, and poly I:C, as described (112).

In vitro **induction of effector- and memory-type $CD8^+$ T cells.** Naïve $CD8^+CD45RA^+CCR7^{high}$ (*see* ***Supplementary Fig. S1: Appendix A***) T cells ($5x10^5$ cells/well) were activated with SEB pulsed DCs ($5x10^4$ cells/well) in the presence of sCD40L (Alexis Corporation, San Diego, CA). Although $CD8^+CD45RA^+$ T cells may contain low frequencies of primed cells, our previous studies showed lack of differences between such cells isolated from adult or cord blood, when

using polyclonal models of activation (83). IL-2 (50U/ml) and IL-7 (5ng/ml) were added on day 3. Subsequently culture medium was replenished with fresh medium and cytokines every two days. Priming for 8 days resulted in $CD8^+$ T cells with high content of the cytotoxic granule components Granzyme B and perforin, referred to effector-type $CD8^+$ T cells in the current study. $CD8^+$ T cells primed and cultured for 15 days yielded a functional phenotype of memory cells with low Granzyme B and perforin content. The HLA-A2 restricted $CD8^+$ T cell clone (83) recognizing melanoma antigen gp100 (209-217) was cloned from TIL 1520 cell line provided by Drs Steven Rosenberg and John Wunderlich (National Cancer Institute, Bethesda, MD), and used either four days after Ag-specific restimulation, or after prolonged culture (> 4 weeks) in IL-2 (100U/ml), in the absence of stimulation.

Modulation of DC function by $CD8^+$ T cells. $CD8^+$ T cells (5×10^4 cells) were added to day 6 immature DC cultures with or without antigen SEB (or gp100 peptide). After 48 hours cells were harvested, washed and analyzed by flow cytometry or stimulated with CD40L transfected J588 cells (83) for 24 hours. For DC protection studies, memory-type $CD8^+$ T cells (5×10^4 cells/ml) were added to immature DC culture 6-8 hours prior to the addition of effector-type $CD8^+$ T cells(5×10^4 cells/ml). When indicated, day 8 primed $CD8^+$ T cells were pretreated with the perforin inhibitor (83) Concanamycin A (CMA: 100 nM) for 2 hours and then added to immature DC culture. The survival of DCs was assessed by staining with nonyl acridine orange dye (NAO; Sigma), as a marker of apoptosis (loss of mitochondrial potential: NAO which binds to mitochondrial cardiolipin in membrane potential dependent manner) (113). Light scatter properties and Annexin V staining have been used, yielding similar results (*see* ***Fig. 3.3*** *and* ***Supplementary Fig. S 3b).*** Briefly, DCs were stained with 0.2μM NAO in culture medium for 15 minutes at 37^0C. The cells were washed and immediately analyzed by flow cytometry. For

blocking TNFα release in day 14 memory type $CD8^+$ T cells, recombinant human soluble TNF RI (R&D Systems) and anti-human TNFα antibody Infliximab (a gift from Dr. Catharien Hilkens, NewCastle, UK) were added to culture wells with DCs and day 14 memory-type $CD8^+$ T cells.

Flow cytometry. Cell surface phenotype was analyzed by flow cytometry using Beckman Coulter XL. The FITC and PE-labeled isotype controls (mouse IgG1 and IgG2a), anti-human CD86, anti-human perforin were obtained from BD Pharmingen. CD83 monoclonal antibody was purchased from Immunotech and PE-labeled Granzyme B antibody was obtained from Cell Sciences. Goat anti-mouse IgG- FITC conjugated was obtained from Caltag Laboratories. For detection of intracellular PI-9, we used PI-9 specific mouse monoclonal IgG1 antibody as described (114). Briefly, for intracellular staining of PI-9, dendritic cells were washed and then blocked with human Ig for 10 minutes at room temperature. Subsequently, cells were permeabilized with 300μl of Permiflow (Invirion, Frankfort, MI) for 60 minutes at room temperature and then washed. The cells were stained with unconjugated anti human PI-9 antibody for 20 minutes at room temperature followed by staining with FITC conjugated goat anti-mouse IgG antibody. Granzyme B and perforin staining were performed according to manufacturer's protocol, using Permiflow as permeabilization reagent.

Cytokine detection. Concentrations of IL-12, TNFα, IL-5, and IFNγ were determined using specific ELISA, using matched antibody pairs from Endogen. Granzyme B was detected in the supernatants by ELISA (Diaclone, Besancon, France).

Microscopy. For TNFα, Granzyme B, and PI-9 visualization experiments, DCs were cultured on collagen coated cover glass (size 12RD, thinness 1, Propper Manufacturing Co., NY) placed in 24 well plates (Falcon). Before imaging, $CD8^+$ T cells, labeled with either CFSE (2.5μM) or

Calcein blue AM (10μM) (Molecular Probes) according to manufacturer's protocol, were added to SEB pulsed DCs (day 6) and incubated for 2 hours at 37^{o} C to allow conjugate formation. Following incubation, cells were fixed with 2% paraformaldehyde, permeabilized with Triton X, and were blocked with normal goat serum (Sigma). The primary antibodies were mouse anti-human granzyme B (Caltag), rat anti- human TNFα (Serotec), mouse anti-human CD11c – Cy5 (BD Pharmingen), and mouse anti-human PI-9. The secondary antibodies were goat anti-mouse Cy3 Fab 1 fragment and goat anti-rat Cy3 (Jackson ImmunoResearch). All the antibodies were used at final concentration of 5μg/ml. Fixed and stained DC-CD8 conjugates were imaged with BX51 upright epifluorescence microscope (Olympus) with a 60X objective and image capture was performed using Magnafire software (Optoronix). For PI-9 localization, DC-CD8 conjugates were imaged with Olympus 500 Scanning Confocal Microscope (Olympus) with 60X objective using Fluoview software. All image files were digitally processed using Metamorph or Adobe Photoshop.

3.4 RESULTS

$CD8^+$ T cells at different stages of activation selectively kill or activate DC.

In order to evaluate the DC-modulating functions of freshly-isolated effector and memory $CD8^+$ T cell subsets and *in vitro* differentiated naïve $CD8^+$ T cells at different stages of activation, we have used a superantigen (SEB) model (83, 115, 116). Similar to TCR transgenic mice, this model allows the activation of a high proportion of $CD8^+$ T cells (83, 91) without the need for prior cloning. In accordance with the observations from the *in vivo* TCR transgenic mouse models (37, 99), we observed that tissue-isolated effector $CD8^+$ T cells (but not memory or naïve $CD8^+$ T cells) rapidly killed immature DCs (***Fig. 3.1A: left, also see Supplementary Figs. S1 and S2: Appendix A***). In sharp contrast, blood-isolated memory $CD8^+$ T cells did not kill DCs (***Fig 3.1A: right***), but instead activated the DC, increasing their expression of the maturation-associated costimulatory molecules (***Fig. 3.1B left)***.

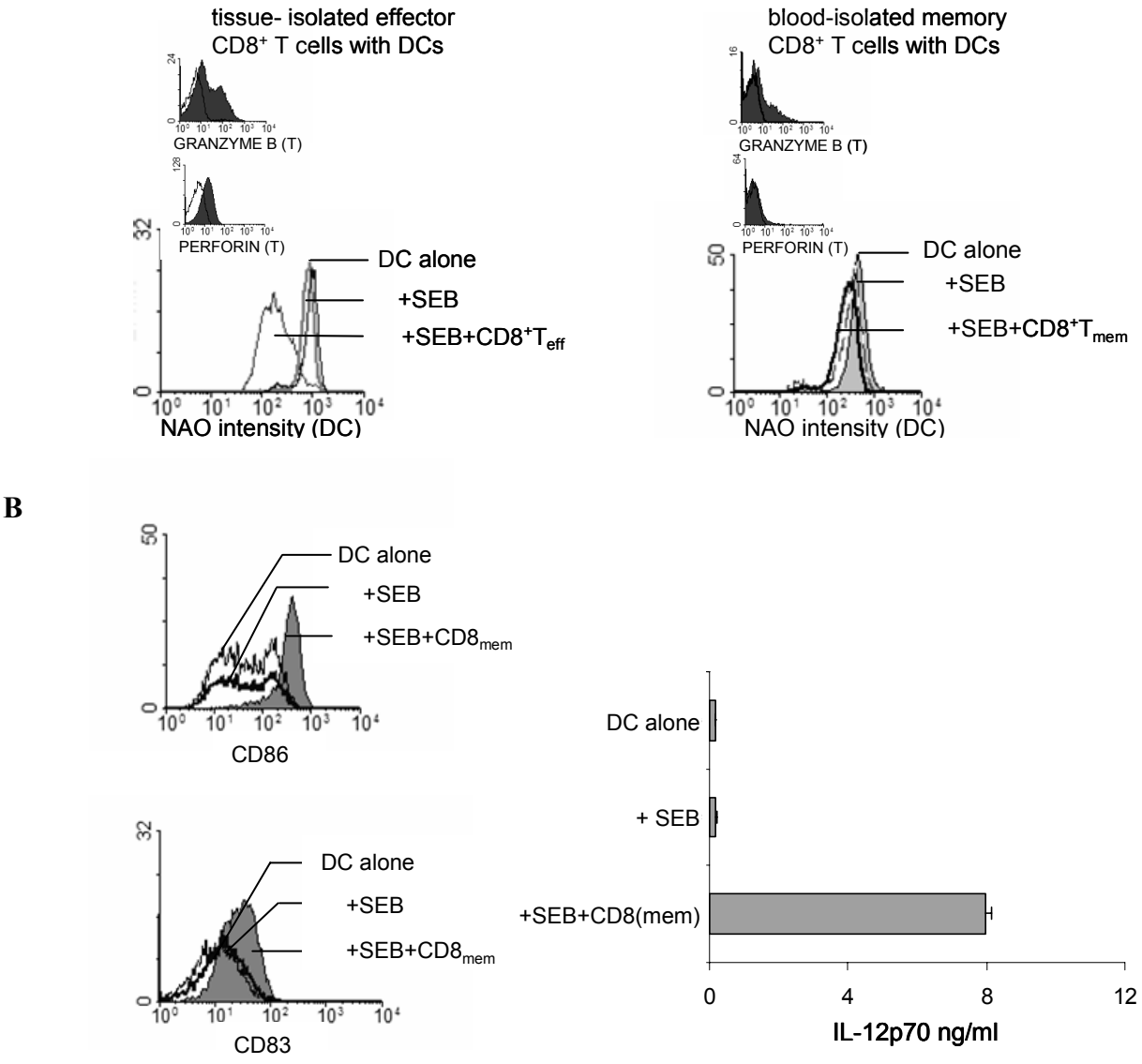


Figure 3.1 DC-killing versus DC-activating function of tissue–isolated effector and blood-isolated memory CD8$^+$ T cells.

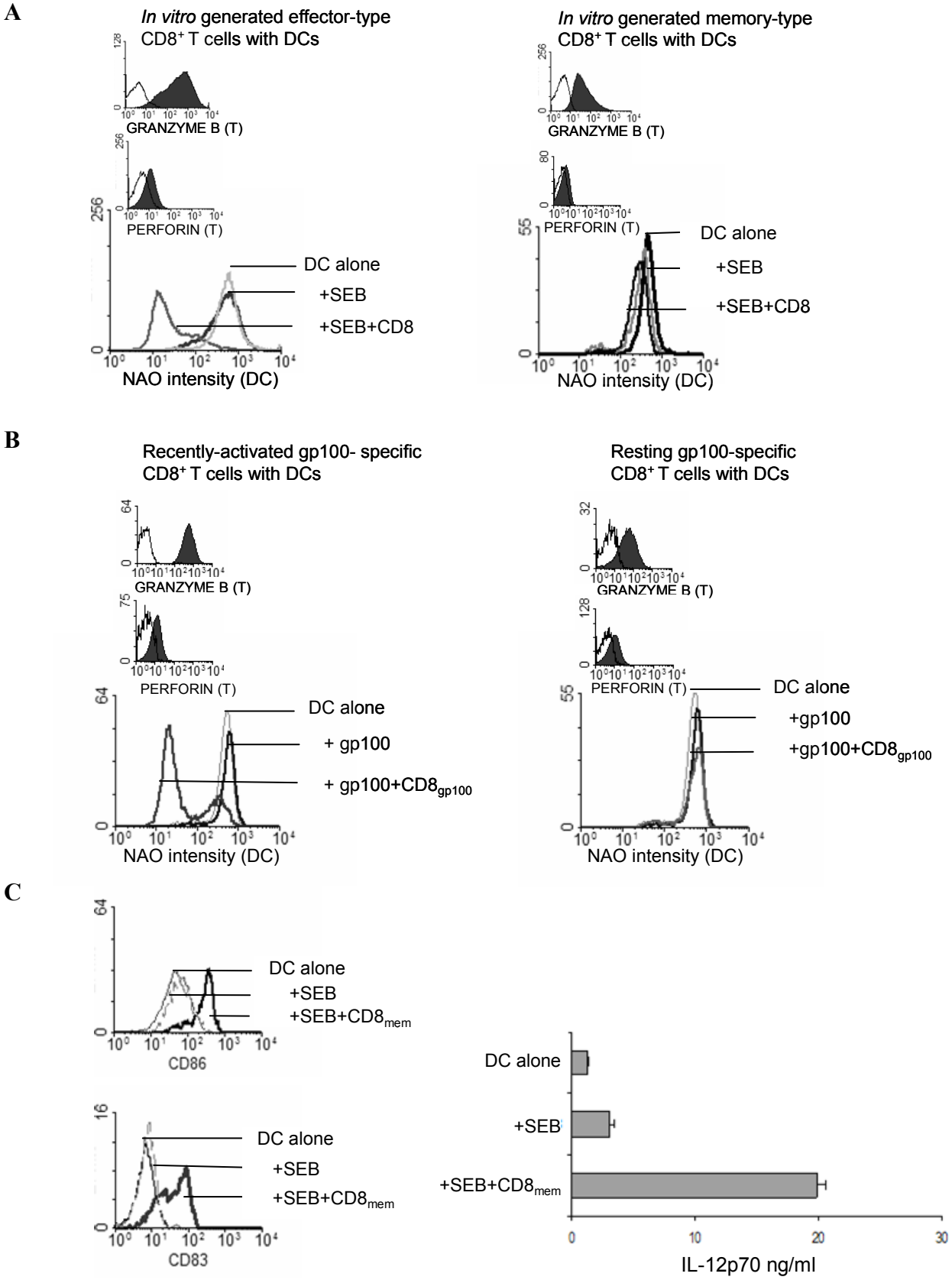
(A-B) Tissue-isolated effector CD8$^+$ T cells kill DCs while blood-isolated memory CD8$^+$ T cells induce DC maturation and prime them for high IL-12p70 production. SEB pulsed immature DCs (day 6) were co-incubated with blood-derived memory CD8$^+$ T cells or tissue-derived effector CD8$^+$ T cells for 48 hours. **(A)** DC viability was assessed by staining with NAO (113). *Left*: tissue-derived effector CD8$^+$ T cells eliminate DCs, as indicated by decrease in NAO staining intensity, demonstrating the loss of mitochondrial integrity. *Right:* blood-isolated memory CD8$^+$ T cells do not kill DCs as reflected by the maintained NAO staining pattern of the DCs. **(B)** *Left*: DC activation status was determined by flow cytometric analysis for surface expression of the co-stimulatory molecule CD86 and DC maturation associated marker CD83. *Right*: IL-12p70 production by DCs following stimulation with

J588-CD40L, as measured by ELISA. Results (mean +/- SD or triplicate cultures) are representative of three independent experiments.

Similar to the IFNγ-dependent ability of naïve $CD8^+$ T cells to elevate the IL-12 production in DCs (83), but in contrast to the effector cells, memory $CD8^+$ T cells primed DCs for high production of IL-12p70 upon subsequent stimulation with CD40L (***Fig. 3.1B: right***).

In order to verify that such reciprocal DC-modulating activities of tissue-isolated effector- and blood-isolated memory $CD8^+$ T cells indeed reflect their different stages of differentiation, we have used a SEB-based model of priming of blood-isolated $CD8^+CD45RA^+$ T cells (83), allowing us to study the regulatory functions of the same $CD8^+$ T cell cultures at different time points after activation. As shown in ***Figure 3.2A (left,*** *see the insets)*, similar to mouse *in vivo* models of $CD8^+$ T cell differentiation (117), $CD8^+$ T cells expanded for 6-8 days acquired a Granzyme B^{high}, $perforin^{high}$ phenotype, typical of cytotoxic effector cells, and acquired the ability to kill SEB-loaded tumor cells (***Supplementary Fig. S 3a: Appendix A)***. Similar to the tissue-isolated effector cells, such *in vitro* activated day 6-8 (effector-type) $CD8^+$ T cells effectively killed DC, as assessed by NAO staining (***Fig. 3.2A: left)*** or Annexin V ***(See Supplementary Fig. S 3b: Appendix A***).

Compared to the effector-type $CD8^+$ T cells, the cells activated for more than 14 days (memory-type) expressed reduced levels of Granzyme B and perforin. In contrast to the effector cells, such memory-type $CD8^+$ T cells no longer killed DCs (***Fig. 3.2A: right)***. Instead, similar to the blood-circulating memory cells, memory-type $CD8^+$ T cells induced DC maturation (manifested by up-regulation of CD86 and CD83) and primed DCs for high production of IL-12p70, the key Th1-and CTL-activating cytokine (118) (***Fig.3.2C***).


A
In vitro generated effector-type CD8+ T cells with DCs
GRANZYME B (T)
PERFORIN (T)
DC alone
+SEB
+SEB+CD8
NAO intensity (DC)
In vitro generated memory-type CD8+ T cells with DCs
GRANZYME B (T)
PERFORIN (T)
DC alone
+SEB
+SEB+CD8
NAO intensity (DC)
B
Recently-activated gp100- specific CD8+ T cells with DCs
GRANZYME B (T)
PERFORIN (T)
DC alone
+ gp100
+ gp100+CD8gp100
NAO intensity (DC)
Resting gp100-specific CD8+ T cells with DCs
GRANZYME B (T)
PERFORIN (T)
DC alone
+gp100
+gp100+CD8gp100
NAO intensity (DC)
C
DC alone
+SEB
+SEB+CD8mem
CD86
DC alone
+SEB
+SEB+CD8mem
CD83
DC alone
+SEB
+SEB+CD8mem
IL-12p70 ng/ml

D

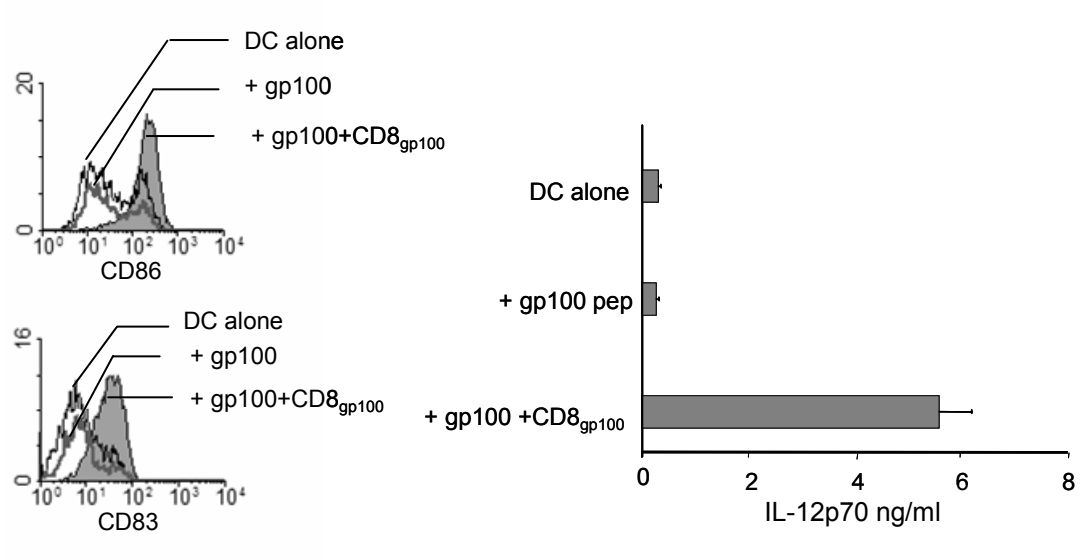


Figure 3.2 DC-killing versus DC-activating functions of *in vitro* generated effector-type CD8$^+$ T cells and memory-type CD8$^+$ T cells at different stages of activation.

Interaction of DCs with the *in vitro* generated effector (*left*)- or memory (*right*)-type CD8$^+$ T cells (A, C) or with melanoma (gp100)-specific CD8$^+$ T cells (B, D), at early or late stages of activation. (A, B): *Left: in vitro* generated Granzyme B^{hi}/ perforin^{hi} effector-type CD8$^+$ T cells and activated gp100 specific CD8$^+$ T cells kill DCs as evident from decrease in NAO staining intensity. *Right: in vitro* generated Granzyme B^{lo}/ perforin^{lo} memory-type CD8$^+$ T cells and resting gp100-specific CD8$^+$ T cells do not kill immature DCs as reflected by the maintained NAO staining pattern of the DCs. (C, D): Memory-type CD8$^+$ T cells at later stage of activation and resting melanoma (gp100) specific CD8$^+$ T cells induce DC maturation and prime DC for enhanced IL-12 production. Day 6, immature HLA-A2$^+$ DCs were co-cultured with melanoma gp100-specific HLA-A2 restricted CD8$^+$ T cells, in the presence of gp100$_{(209\text{-}217)}$ peptide (*shaded histograms*). Memory-type CD8$^+$ T cells were co-cultured with SEB loaded DCs for 48 hours. *Left:* Activation status of DCs (CD86, CD83) was assessed by flow cytometry. *Right:* IL-12p70 was measured in supernatants after stimulation of DCs with J588-CD40L. In contrast to the presence of live activated DC in cultures containing memory-type T cells, all DCs were rapidly eliminated from the cultures containing effector cells. Results are representative of three independent experiments. DC killing and DC activation required the presence of antigen in all the above systems (not shown).

Similar, stage of activation-dependent, differences were observed in case of CD8$^+$ T cells activated with a HLA-A2-restricted peptide antigen, gp100$_{(209\text{-}217)}$. As shown in ***Figures 3.2B and 3.2D***, human gp100-specific CD8$^+$ T cell clone rapidly killed immature DCs when being pre-activated with Ag-loaded DCs for four days, but lost such DC-killing function following

their prolonged culture in the absence of antigen. Similar to the blood-isolated memory $CD8^+$ T cells and day 14 SEB-activated memory-type cells, such "resting" clonal gp100 $CD8^+$ T cells efficiently induced DC maturation and primed DCs for high IL-12 production (***Fig. 3.2D)***.

The inability of the memory-type $CD8^+$ T cells to kill DCs did not result from any intrinsic defect resulting from long-term cultures, since they regained the ability to kill DCs upon short-term restimulation (***Supplementary Fig. S 3c: Appendix A***). These data, in conjunction with the results obtained using blood-isolated memory cells (***Fig. 3.1A***), indicate that after a transient period of DC-killing activity, activated $CD8^+$ T cells enter a "helper phase" of their activation cycle. Similar termination of the suppressor phase of activity has also been observed in case of tissue-isolated effector cells (*data not shown*).

Exogenous inhibitors of perforin- and Granzyme B restore DC-activating function of effector $CD8^+$ T cells

Prompted by the results of recent studies highlighting the role of perforin-/Granzyme B- and Fas/Fas-L pathways in CTL-mediated elimination of DCs in mouse (37, 119), we have analyzed the role of the perforin/Granzyme B- and Fas/Fas-L-mediated cytotoxic pathways in the killing of human DCs by effector $CD8^+$ T cells. As shown in ***Figure 3.3A***, DC killing was completely eliminated by the addition of EGTA or Concanamycin A (CMA), the inhibitors of the perforin-dependent (but not Fas/Fas-L-dependent) pathway of CTL-mediated killing (120, 121). In contrast, no inhibition of DC killing was observed in the presence of the Fas-L antagonist (***Supplementary Fig S4: Appendix A).***

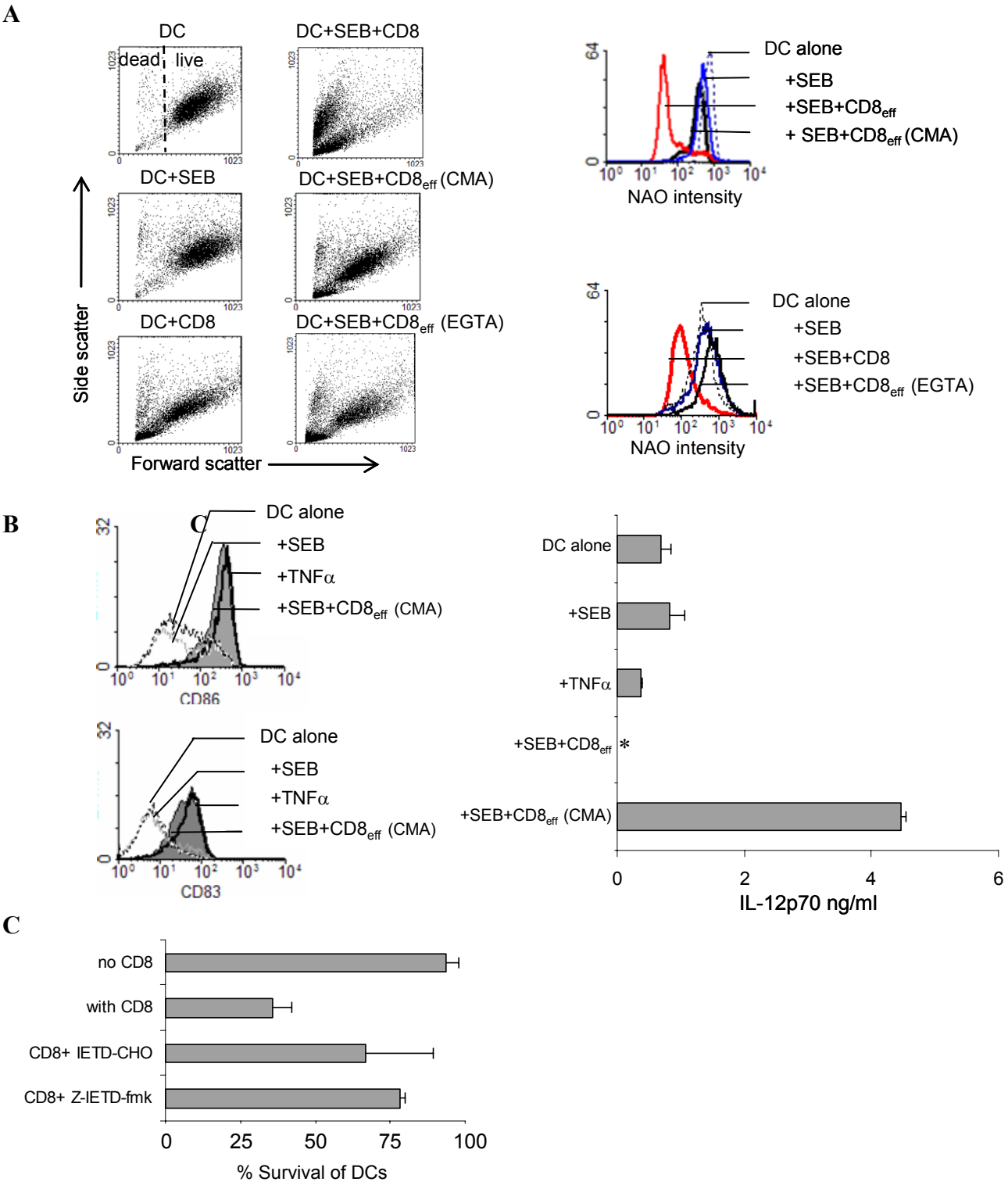


Figure 3.33 Exogenous inhibitors of perforin/Granzyme B pathway convert effector-type CD8$^+$ T cells into helper CD8$^+$ T cells.

(A) Inhibition of perforin in effector-type $CD8^+$ T cells results in the survival of the interacting DCs. Release of functional perforin was blocked by either pre-treatment of the effector-type $CD8^+$ T cells with CMA (100nM) or by the addition of EGTA (4mM) during DC-CD8 co-culture. The survival of DCs was analyzed by change in the light scatter properties (as indicated by dot plots: *left*), and verified using the NAO staining (*right*). Broken line within the dot plot separates live and dead cell populations. NAO analysis (*right*) included both regions (live and dead DCs). (B) Inhibition of perforin in effector-type $CD8^+$ T cells results in the induction of their helper function, manifested by DC activation. CMA-treated effector-type $CD8^+$ T cells were co-cultured with SEB loaded DCs for 48 hours. *Left:* CMA treated (perforin-blocked) effector-type $CD8^+$ T cells enhance DC activation. TNFα (50ng/ml) induced DC maturation was used as positive control. *Right:* CMA treated (perforin-blocked) effector-type $CD8^+$ T cells induce type-1 polarized phenotype in DCs, characterized by enhanced ability to produce IL-12p70. *: below detection limit. Results are representative of three independent experiments. (C) Inhibition of Granzyme B in effector-type $CD8^+$ T cells results in survival of interacting DCs. Dendritic cells pretreated (1 hour) with IETD-CHO (200μM) or Z-IETD-fmk (20μM), were co-cultured with effector-type $CD8^+$ T cells. Survival of DCs was analyzed after 10-12 hours. The data from two experiments are expressed as percent survival of DCs (mean ± SEM).

Taking advantage of the relative stability of the CMA-induced perforin inhibition in CTLs, without affecting the secretion of $CD8^+$ T cell-produced TNFα and IFNγ (***Supplementary Fig. S4b: Appendix A)***, we analyzed the outcome of the interaction of the CMA-pre-treated effector cells with immature DCs. As shown in ***Figure 3.3B***, pre-treatment of the effector cells with CMA abrogated their DC-killing ability, resulting in the induction of phenotypic maturation of the DCs (TNFα was used as a control for DC maturation) and their priming for high IL-12p70 production. Similar to the blocking of the perforin pathway, we also observed that pre-treatment of DCs with the specific Granzyme B inhibitors IETD-CHO or Z-IETD-fmk (122), abrogated the CTL-induced DC death ***(Fig. 3.3C)***.

These results indicate that the perforin- and Granzyme B-mediated cytolytic pathway is the principal mode of DC elimination by human effector $CD8^+$ T cells, and that, in its absence, the effector cells no longer suppress DC activity, but support it. These data also suggest that pharmacologic modulation of the perforin- or granzyme-mediated killing can be used to enhance

the effectiveness of active and adoptive immunotherapies performed in the setting of existing disease, where antigen-specific effector $CD8^+$ T cells predominate.

Memory $CD8^+$ T cells protect DCs from CTL-mediated killing: Equivalent induction of the endogenous Granzyme B inhibitor, PI-9, by memory $CD8^+$ and $CD4^+$ T helper cells

The inability of memory-type $CD8^+$ T cells to kill DCs even after 48 hour co-cultures was particularly intriguing in face of our observations that their killing function can be restored following re-activation (***Supplementary Fig. S 3c: Appendix A),*** and the data from mouse models that 30-72 hour-reactivated effector-memory and central-memory cells re-acquire their DC-killing potential (80, 123). These data suggested that the initial interaction of DCs with memory-type $CD8^+$ T cells may protect DC from the eventually-acquired CTL activity of the same cells. In order to test this possibility, we have sequentially exposed DCs, first to memory-type $CD8^+$ T cells, followed by co-culture with effector-type $CD8^+$ T cells.

As shown in ***Figure 3.4A,*** the DCs exposed to memory-type $CD8^+$ T cells became resistant to subsequent killing by CTLs. This protective effect of the memory-type $CD8^+$ T cells was similar to that exerted by activated $CD4^+$ T cells, the classical "helper T cells" (***Fig. 3.4A***) , which have been proposed to mediate their helper function by DC protection (86, 99). In further support of the similarity between the helper functions of $CD4^+$ T cells and $CD8^+$ memory T cells, DCs that interacted with either of these T cell subsets expressed similar levels of an endogenous Granzyme B inhibitor PI-9 (***Fig. 3.4B***), a human equivalent of murine serine protease inhibitor (SPI-6) (124) shown to mediate the protection of mouse DCs from CTL-mediated killing (86). The analysis of PI-9 expression at the per cell basis, using confocal microscopy, revealed that PI-9 is massively up-regulated in DCs within 2 hours following their interaction with memory-type

$CD8^+$ T cells, with PI-9 expression being detectable in the individual DCs interacting with T cells already within 15 minutes (***Fig. 3.4C***).

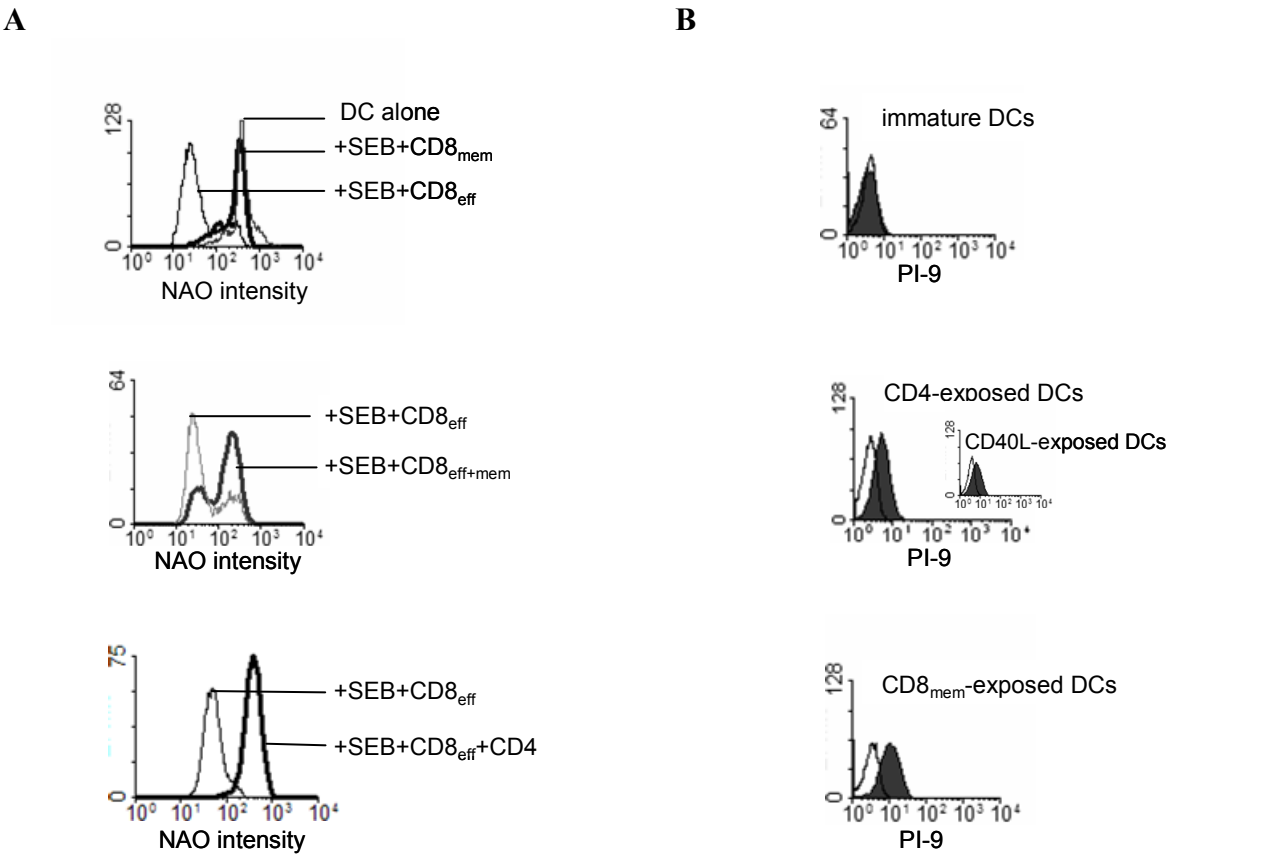


Figure 3.4 Memory-type $CD8^+$ T cells and $CD4^+$ T cells induce DC expression of endogenous Granzyme B inhibitor and protect DCs from CTL–mediated killing.

(**A**) Exposure of DCs to memory-type $CD8^+$ T cells confers protection from effector-type $CD8^+$ T cell mediated death, analogous to $CD4^+$ T cell mediated protection. Memory $CD8^+$ T cells or $CD4^+$ T_h cells were co-cultured with SEB-loaded DCs for 8-10 hours, followed by the addition of effector $CD8^+$ T cells. DC viability was assessed by

NAO staining at 24 hours. (**B**) Memory-type $CD8^+$ T cells induce uniform DCs expression of the endogenous Granzyme B inhibitor: PI-9 (10 hour co-culture). DCs exposed to memory-type $CD8^+$ T cells, $CD4^+$ T cells (or CD40L: *see the inset*) were stained for intracellular PI-9. (**C**) Rapid induction of PI-9 in DCs exposed to memory-type $CD8^+$ T cells, visualized by confocal microscopy: Localization of PI-9 (red) in DCs (blue), following the interaction with memory-type $CD8^+$ T cells (green). Please note lack of PI-9 expression in isolated DCs, and high levels of PI9 expression in DCs in 2 hour co-cultures.

The ability of CMA to abolish the DC-killing activity of CTLs without abrogating their ability to induce DC maturation, and our previous data that blocking of TNFα-RI (known to be triggered by TNFα and Lymphotoxin-β(LT)) abrogates DC maturation (83), prompted us to test if the CTL-protecting helper activity of memory-type $CD8^+$ T cells is mediated by TNFα and can be blocked by the addition of soluble TNF receptor I (blocking potential actions of TNFα and LT) or TNFα-specific antibody (Infliximab; blocking TNFα exclusively). In accordance with the key role of TNFα (rather than LT), both reagents proved equally effective in converting the memory T cell-induced DC activation into memory T cell-induced DC death (***Fig. 3.5A,*** *data not shown for sTNF-RI*). As expected, these effects were accompanied by the prevention of the induction of PI-9 in DCs (***Fig. 3.5B***).

In accordance with the different outcome of interaction of memory versus effector T cells with DCs, we observed a significant release of TNFα within two hours of interaction of DC with memory-type $CD8^+$ T cells, whereas the release of Granzyme B by memory-type $CD8^+$ T cells was significant only at later time points. Effector T cells, however, simultaneously released both TNFα and Granzyme B (***Fig. 3.5C, D***).

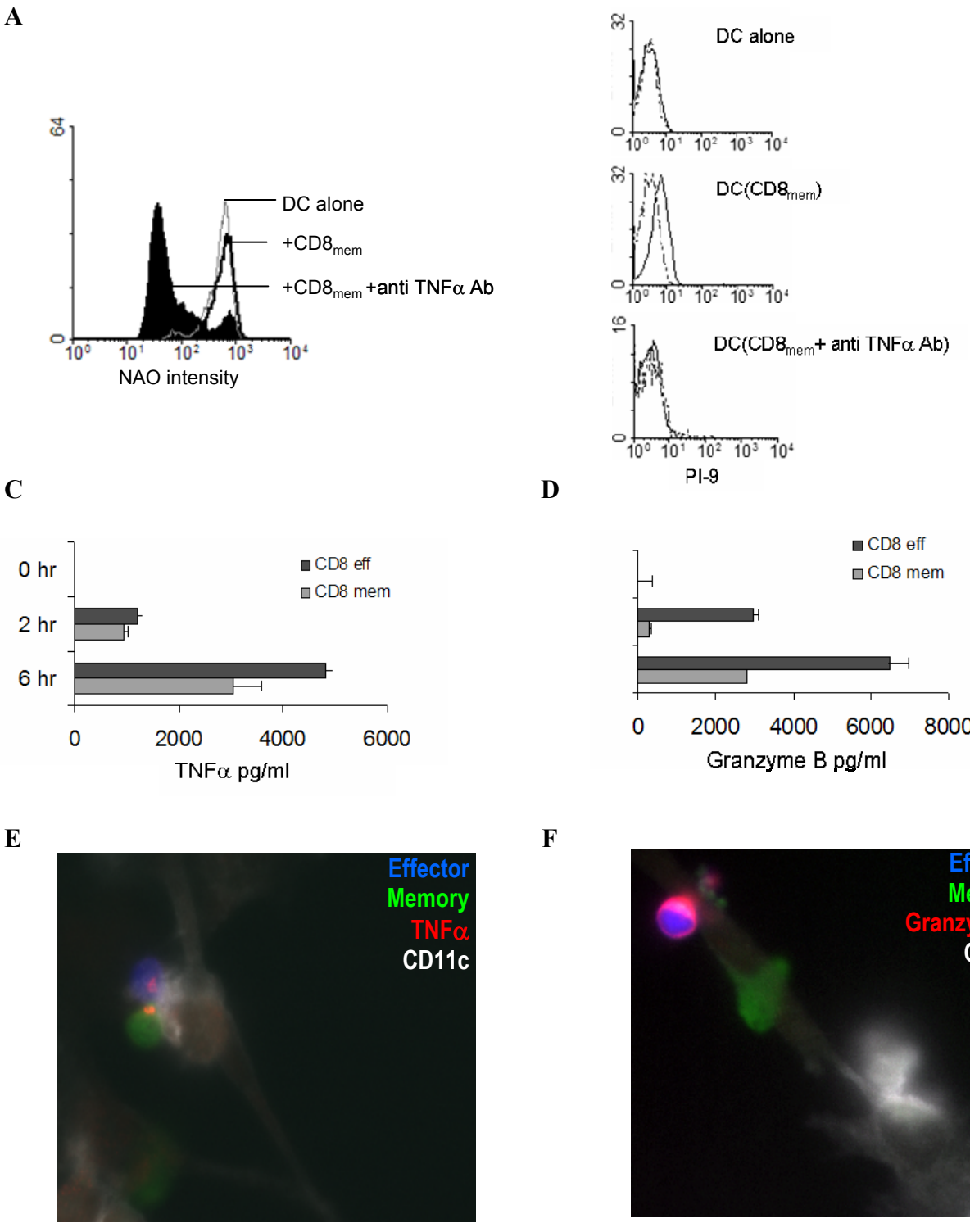


Figure 3.5 Memory-type CD8$^+$ T cell-derived TNFα plays a crucial role in helper function.

Inhibition of TNFα activity abrogates the helper function of memory-type CD8$^+$ T cells, resulting in DC killing. Neutralization of TNFα in the co-cultures of SEB-loaded DCs and memory-type CD8$^+$ T cells using anti-TNFα

antibody, reduces DC survival (24 hour cultures). **(B)** anti-TNFα antibody, blocks the memory-type $CD8^+$ T cell-induced induction of PI-9 in DCs (10 hour time point). **(C-D)** Different relative kinetics of the TNFα- versus Granzyme B release in memory and effector $CD8^+$ T cells during the interaction with DCs. Note that the release of TNFα precedes Granzyme B release in memory-type $CD8^+$ T cells, but not in effector cells. (**E)** Four-color fluorescent microscopy demonstrating the presence of high amounts of Granzyme B (red) in the effector $CD8^+$ T cells (blue) interacting (2 hours) with DCs (grey), but not in the memory $CD8^+$ T cells (green). **(F**) Presence of TNFα (red) in both effector (blue) and memory (green) $CD8^+$ T cells interacting with DCs (grey). Two hour cocultures.

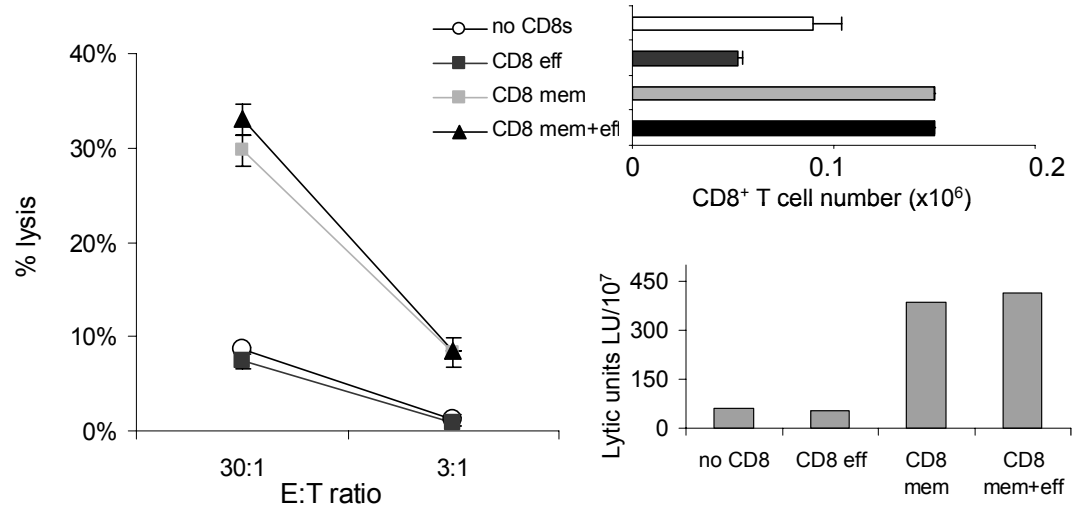
Furthermore, the microscopic analysis of the DC - T cell interactions (at the 2 hour time point, when PI-9 is induced in DCs: *see Fig. 3.4C*) demonstrated equivalent mobilization of TNFα in both memory-type and effector-type $CD8^+$ T cells (***Fig. 3.5E***), whereas exclusively the effector $CD8^+$ T cells, but not memory cells, directed Granzyme B-containing cytotoxic granules towards the contact zone with DCs (***Fig. 3.5F***). This sequence of events indicates that the early TNFα release by the memory $CD8^+$ T cells protects DC from the subsequently-released Granzyme B.

"CD8 to CD8 help" and "CD8 to CD4 help": Memory $CD8^+$ T cells support the de novo induction of CTLs and Th1 cells

In support of their ability to act as respective suppressor and helper cells during the *de novo* induction of type-1 immune cells, $CD8^+$ T cells at different stages of activation differentially regulated the expansion of naïve $CD8^+$ and $CD4^+$ T cells and the development of their respective CTL and Th1 functions (***Fig. 3.6A, B***). Importantly, in accordance with the dominant role of memory $CD8^+$ T cells in regulating the survival and function of DCs (***see Fig. 3.4***), the helper signals from memory $CD8^+$ T cells not only promoted the DC-driven functional differentiation of naïve T cells but were also able to fully counteract the suppressive activity of effector $CD8^+$ T

cells, resulting in the effective induction of functional CTL and Th1 responses even in the presence of effector $CD8^+$ T cells (***Fig. 3.6***).

A



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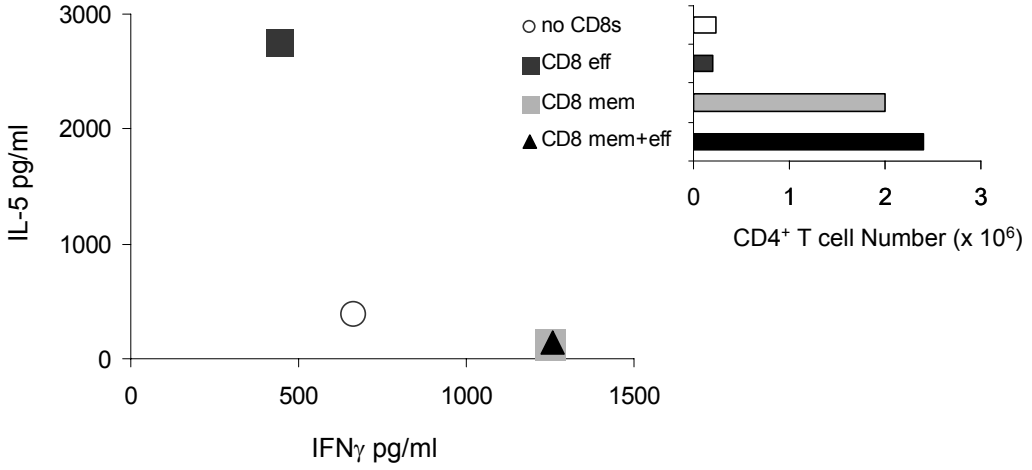

Figure 3.6 Memory $CD8^+$ T cells support *de novo* induction of functional CTLs and Th1 cells.

(A-B) Memory and effector $CD8^+$ T cells have reciprocal impact on the DC-driven expansion of CTL- and Th1 cell progenitors and the development of CTL and Th1 functions. Blood-isolated naïve $CD8^+$ or $CD4^+$ T cells were primed with the SEB-loaded immature DCs in the absence or presence of γ-irradiated memory-type or effector-type $CD8^+$ T cells. The expanding cultures of naïve $CD8^+$ or $CD4^+$ T cells were harvested, respectively at day 5 or day 10, counted and tested for their functional activity, using CTL assay or the analysis of their Th1/Th2 cytokine profiles. (A) Memory $CD8^+$ T cells support the expansion of CTL precursors and their acquisition of functional

activity. *Left:* CTL activity of $CD8^+$ T cell cultures performed in the presence or absence of memory or effector $CD8^+$ T cells was assessed by ^{51}Cr release assay, using SEB-loaded JY-1 cells as targets. Data from one of two independent experiments, that both yielded similar results. (B) Memory $CD8^+$ T cells support the expansion of Th1 cell precursors and the acquisition of Th1 cytokine production profiles. *Right:* Ability of memory-type $CD8^+$ T cells, but not effector-type $CD8^+$ T cells, to induce naïve $CD4^+$ T cell proliferation. *Left*: Naïve $CD4^+$ T cells primed in the presence of memory-type $CD8^+$ T cells develop a strongly polarized Th1 cytokine profile as determined by ELISA. Data from one of two independent experiments, that both yielded similar results.

3.5 DISCUSSION

We show that in contrast to effector $CD8^+$ T cells which rapidly eliminate antigen-carrying DCs in a perforin- and Granzyme B-dependent mechanism, human memory $CD8^+$ T cells protect DCs from CTL-mediated killing and exert DC-mediated helper function. These data indicate that the mechanism of $CD8^+$ T cell memory, in addition to the previously defined increased frequency of Ag-specific T cells and their rapid acquisition of effector functions, involves a novel $CD4^+$ T cell-like ability of memory $CD8^+$ T cells to prolong the life-span of antigen-carrying DCs.

Our data support the following functional model of $CD8^+$ T cell memory: During primary immune responses, DC activation requires their exposure to factors representing pathogen-associated- or tissue damage-associated molecular patterns (PAMPs versus “danger signals” or DAMPs) (10, 11), implicating the need for a significant pathogen load and tissue damage. In contrast, the activation of DCs during secondary responses can benefit from the activating signals released by high numbers of pathogen-specific memory-type $CD8^+$ T cells, even before the destruction of the infected cells and the release of additional copies of the pathogen. Such memory $CD8^+$ T cell-dependent early DC activation helps the immune system to respond to the pathogen at much earlier stages of (re)infection, limiting its early spread, the extent of tissue

damage and the activation of innate defense mechanisms, thus preventing the onset of disease symptoms.

Moreover, since during the primary responses, the PAMP- and DAMP-mediated DC protection from newly arising CTLs is limited to the period of active infection and ongoing tissue damage, the optimal activation of the T cells recruited to the lymph nodes at later stages of primary responses (needed for the optimal development of memory cells (125, 126)) is dependent on DC-protecting signals from $CD4^+$ T helper cells (99). In contrast, during secondary immune responses, antigen-carrying DCs can also interact with the $CD8^+$ memory T cells that are gradually recruited to the sites of infection or to inflamed lymph nodes, preventing premature DC elimination by the arising effector cells and limiting their dependence on the pathogen-related or $CD4^+$ T cell-related survival signals.

In addition to the currently-identified DC protection by memory $CD8^+$ T cells, recall immunity may also benefit from the IFNγ-dependent (83) ability of memory $CD8^+$ T cells to enhance the DC secretion of IL-12p70 (***Fig. 3.1B and 3.2B***), jointly contributing to the superior magnitude and quality (***Fig. 3.6***) of recall immune responses.

The current demonstration of the dominant impact of memory $CD8^+$ T cells in preventing DC killing by existing CTLs and promoting the optimal *de novo* induction of functional CTLs and Th1 cells facilitate the development of new vaccination strategies in therapeutic settings. In accordance with such a possibility, our *in vivo* observations in mouse demonstrate that the inclusion in tumor vaccines of tumor-unrelated helper epitopes, promoting the interaction of the vaccine-carrying DCs with tumor-unrelated memory $CD8^+$ T cells, can boost the immunologic and anti-tumor effects of vaccination against established tumors (*manuscript in press*). The

current data also help to explain the high efficacy of prime-boost vaccination strategies (75), when the first and second doses of vaccine are delivered using antigenically-distinct vectors and may help to further improve such strategies.

We are currently comparing the ability of different populations of memory cells to provide helper signals. While our preliminary data suggest that blood-isolated central-memory and effector-memory $CD8^+$ T cells are both effective in protecting DCs from CTL killing (*not shown*), we observed that the memory-type cells generated in our two week-long cultures (in contrast to blood-isolated memory cells, expressing low levels of Granzyme-B and perforin: *see Figs. 3.1 and 3.2*) can re-acquire the DC killing ability following short-term restimulation (*Supplementary Figure S 3c: Appendix A*). These data, and the recently-published observations that mouse effector-memory $CD8^+$ T cells can acquire DC-killing capacity *in vivo* within 30 hours of exposure to antigen-loaded DCs (123), suggest that an optimal long-term DC protection *in vivo* may require the presence of central memory cells. Since mouse long-term memory cells have been shown to kill DC only following 72 hours (but not 24 hours) of antigenic challenge (80) and human central memory $CD8^+$ T cells have been shown to need up to 72 hours of activation to re-acquire the cytotoxic function (127), the DC protection by central memory T cells during secondary immune responses is likely to have a substantial time-span.

An intriguing aspect of the current findings is the possibility of manipulating the respective suppressive and helper functions of effector and memory $CD8^+$ T cells, using pharmacologic agents. We observed that blocking perforin or Granzyme B activity results in the acquisition of helper functions by the effector cells, while blocking TNFα interaction with TNF-RI converts memory T cells into suppressive cells. These data have direct pathologic and therapeutic implications for autoimmunity, chronic infections and cancer. While the blockade of TNFα or

TNF-RI, using Infliximab or Eternacept, respectively, proved to be highly effective in the treatment of inflammatory bowel disease, rheumatoid arthritis, and other autoimmune diseases, a comparative evaluation of the role of these agents in limiting the DC-activating functions of $CD8^+$ T cells in these different disease settings may help to explain the differential efficacy of each of these agents in patients with different forms of autoimmunity, allowing further optimization of their treatment.

While the inability of perforin-deficient $CD8^+$ T cells (and possibly NK cells) to control $CD8^+$ T cell expansion has been proposed to explain autoimmune damage in perforin knockout mice infected with LCMV or Listeria (128), and the uncontrolled lymphoproliferation during viral infections in patients with perforin mutations (129), the current data provide the rationale for the evaluation of perforin or granzyme-targeting therapies as a tool to increase the efficacy of therapeutic vaccines in the settings of cancer and chronic infections, where terminally differentiated effector cells predominate (96, 98, 130). It also remains to be tested whether perforin- or granzyme-targeting approaches can be applied to enhance the long-term clinical effectiveness of adoptive immunotherapies with ex-vivo expanded tumor-infiltrating lymphocytes, where the long-term persistence of tumor-specific T cells and the positive clinical outcomes are tightly-correlated with the frequencies of the adoptively-transferred memory-type, expectedly non-DC-killing, $CD8^+$ T cells (131-133).

In summary, the current data help to reconcile the long-known paradoxical ability of $CD8^+$ T cells to play the reciprocal "suppressor" and "helper" roles, adding to our understanding of $CD8^+$ T cell memory, and facilitating the development of effective therapies of autoimmunity, cancer and chronic infections.

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DENDRITIC CELLS REGULATE THE LYMPHOID VERSUS PERIPHERAL/ EFFECTOR PATHWAY OF DIFFERENTIATION OF CD8$^+$ T CELLS.

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Manuscript in *preparation*

The majority of experiments in this manuscript were performed by Payal Watchmaker.

4 DENDRITIC CELLS REGULATE THE LYMPHOID VERSUS PERIPHERAL/ EFFECTOR PATHWAY OF DIFFERENTIATION OF $CD8^+$ T CELLS.

4.1 ABSTRACT

Effective $CD8^+$ T cell responses require T cell expansion and the development of the effector (CTLs) and memory cells. Here, we report that $CD8^+$ T cells stimulated by the "inflammatory-type" dendritic cells (DC) activated by interferons and TLR ligands, expand and acquire IL-7R and IL-15R, but also develop CTL functions and undergo a switch in chemokine (CK) responsiveness (from lymphoid to peripheral). In contrast, $CD8^+$ T cells activated by long-term-stimulated "exhausted" DCs or by DCs exposed to mediators of chronic inflammation, such as PGE_2, effectively expand and acquire IL-$7R^+$/IL-$15R^+$ phenotype while retaining the $CD62L^+CCR7^+CCR5^-$ central memory phenotype not associated with CTL functions. The "non-cytolytic/lymphoid" status induced in naive or memory (including tumor-specific) $CD8^+$ T cells by the PGE_2-matured DCs is fully reversed by the interaction with inflammatory-type DCs, suggesting that the non-cytolytic T cells are fully functional but undergo memory-type differentiation. The independent regulation of $CD8^+$ T cell expansion and their commitment to the peripheral/effector versus lymphoid/memory subsets helps to understand the phenomenon of preferential induction of memory $CD8^+$ T cells at late stages of immune responses and the negative impact of persistent inflammation on the development of immune memory. The limited

effectiveness of PGE_2-matured DCs in inducing peripheral-tissue-directing chemokine receptors on tumor-specific T cells helps to explain the limited clinical effectiveness of the current cancer vaccines, suggesting novel ways of enhancing their therapeutic effectiveness.

4.2 INTRODUCTION

Dendritic cells (DC) regulate the proliferation and differentiation of $CD8^+$ T cells. While the combination of "signal 1" (antigen) and "signal 2" (co-stimulation) is known to be essential for the induction of $CD8^+$ T cell proliferation and clonal expansion (134)(135)(136), it is less clear what signals govern their differentiation towards the effector cells (CTLs) with immediate cytolytic function, and tissue/peripheral homing capacity, as opposed to memory cells that retain the lymphoid/central homing pattern and lack immediate killing function (46, 47).

In case of $CD4^+$ T cells, studies in human and mouse models have demonstrated that the DC-induced expansion of naïve $CD4^+$ T cells can be uncoupled from their acquisition of Th1 or Th2 effector functions (13, 15, 16, 92, 115, 137), leading to the concept of "signal 3" (13) which selectively regulates the acquisition of T cell effector functions. While no similar data are available in the case of $CD8^+$ T cells, increasing evidence suggests a key role of inflammation in the development of cytolytic function and the requirement for its cessation in the effective transition from the effector to memory phase of immunity (138, 139). Several *in vivo* models of infections have demonstrated that inflammatory cytokines such as IL-12, IFNα and IFNγ, are involved not only in the regulation of proliferation of $CD8^+$ T cells but also in their acquisition of effector functions (140-142). There is also increasing evidence suggesting that active inflammation can delay the formation of memory $CD8^+$ T cells (48, 54), with the induction of

memory $CD8^+$ T cells being particularly effective at late stages of immune responses, following the termination of the inflammation-inducing active stage of the pathogenic infection. Moreover, the observations of prominent expansion of tumor-specific $CD8^+$ T cells in the blood of patients treated with current cancer vaccines in combination with limited induction of their effector functions or clinical activity of vaccination, together with the ability of inflammatory cytokines to convert such non-cytolytic T cells into cytolytic ones suggest a similar inflammation-dependent regulation of the effector versus memory phases of immune responses in humans (21, 131).

In order to address the role of dendritic cells in the development of human effector and memory $CD8^+$ T cells, we compared the magnitude and quality of $CD8^+$ T cell responses induced by the DCs activated in acute or chronic fashion by different inflammatory mediators. Our data indicate that DCs matured under conditions mimicking an early phase of immune response (short-term activation with inflammatory cytokines and TLR ligands) promote the effector pathway of $CD8^+$ T cells differentiation, followed by the development of non-cytolytic memory cells. In contrast, DC matured during the late phase of immune response (activation with inflammatory cytokines or prolonged exposure to inflammation) supported $CD8^+$ T cell expansion and direct differentiation into memory cells, without inducing their cytolytic function and a switch in chemokine responsiveness .

4.3 MATERIALS AND METHODS

Cell lines, media and reagents

Serum-free AIM-V medium (Invitrogen, Carlsbad, CA) was to used to generate DCs and IMDM (Invitrogen) with 5% human serum (Atlanta Biologicals, Norcross, GA) was used for *in vitro* sensitization (IVS) experiments. The following factors were used to generate mature DCs: *rhu* GM-CSF and IL-4 (gifts from Schering Plough, Kenilworth, NJ), IFNα (Intron A-IFNα-2b; Schering Plough, NJ), rhuTNFα, rhuIFNγ, rhu IL-1β (all Strathmann Biotech, Germany), rhuIL-6 (Genzyme, Cambridge, MA), lipopolysaccharide (Sigma, St. Louis, MO), PGE_2 (Sigma) and poly I: C (Sigma). IL-2 (Chiron Corp, Emeryville, CA) and rhuIL-7 (Strathmann Biotech) were used to support the $CD8^+$ T cell expansion.

Generation and maturation of dendritic cells

Peripheral blood mononuclear cells (PBMCs) were obtained from the blood of healthy donors or melanoma patients using lymphocyte separation medium (Cellgro Mediatech, Herndon, VA). Monocytes were isolated on density gradients, with Percoll (Sigma), followed by plastic adherence. Monocytes were cultured for 6 days in 24-well plates (Falcon) at $5x10^5$ cells per well in rhuGM-CSF and IL-4 (both 1000U/ml). The following combinations of maturation stimuli were added to DCs on day 6: LPS alone (250ng/ml), LPS and IFNγ (1000U/ml), LPS and PGE_2 (10^{-6} mol/ml), TNFα (100ng/ml) and IFNγ, TNFα and PGE_2, TNFα, IL-1β (25ng/ml), PGE_2, IL-6 (1000U/ml) and αDC1 maturation cocktail as described (112).

Isolation of Peripheral Blood $CD8^+$ T cell Populations

Naïve $CD8^+CD45RA^+$ T cells were isolated by negative selection with the StemSep and CD8 enrichment cocktails, respectively (StemCell Technologies Inc, Vancouver, Canada).

Biotinylated anti-CD45RO antibody was used in combination with enrichment cocktail for isolation of naïve population. $CD8^+CCR7^+CD45RA^-$ central memory T cell population was flow-sorted using MoFlo high-speed cell sorter (Dako Cytomation), after labeling with appropriate antibodies.

Flow cytometry

Two- and three-color cell surface and intracellular immunostaining analysis was performed using Beckman Coulter Epics XL flow cytometer. FITC and PE-labeled anti human CCR7 (R&D Systems), CCR5 (BD Pharmingen), CXCR3 (BD Pharmingen) and the corresponding isotypes IgG2a and IgG1 were used. PE-labeled anti-human Granzyme B and FITC-anti human perforin antibodies were purchased from Cell Sciences and BD Pharmingen respectively. HLA-A2/$MART1_{27-35}$ tetramer staining (Beckman Coulter, Immunomics) was performed according to the manufacturer's instructions.

In vitro sensitization

Naïve $CD8^+CD45RA^+CCR7^{high}$ T cells ($5x10^5$ cells/well) were activated with SEB-pulsed DCs ($5x10^4$ cells/well). On day 5-6, expanded $CD8^+$ T cells were used for immunostaining; alternatively the cultures were fed with low dose IL-2 and IL-7 (10ng/ml) every two days and analyzed for cell surface and intracellular markers on day 16-20.

4.4 RESULTS

Cytolytic and non-cytolytic differentiation of $CD8^+$ T cells induced by "inflammatory" versus "exhausted" DCs

In order to delineate the requirements for the effective DC-induced expansion of $CD8^+$ T cells and their acquisition of effector functions, we compared the outcome of naïve $CD8^+$ T cell priming by DCs induced to mature by the mediators of acute inflammation (combination of interferons and TLR ligands) or by mediators of chronic inflammation (presence of PGE_2), either for 24 hours or 96 hours.. The short versus prolonged maturation of DCs is associated with differential ability to produce IL-12p70 (13), the key mediator of inflammatory-type responses (143), and with different abilities to induce Th1 responses of $CD4^+$ Th cells (13, 15).

Naïve $CD8^+$ T cells primed by the short term-matured (24 hours) inflammatory-type DCs (high IL-12-producing activity; see the inset) demonstrated an effective induction of granzyme B (and Granzyme A; Fig S6) (***Fig. 4.1A left***), the marker of the effector pathway of T cell differentiation (31). Poor effectiveness of "exhausted" DC in inducing Granzyme B^+ T cells, did not simply result from an ineffective activation of naive T cells, since the proliferation of T cells in these cultures was similar or usually even higher compared to the T cells activated by "inflammatory" DCs (*Supplementary Fig. S6: appendix B)*.

In contrast to the situation with short term-activated DCs, long-term activated DCs (96 hours) generally induced much lower levels of granzyme B expression, and independently from the character of the maturation-inducing stimulus (***Fig. 4.1B right***). This observation is consistent with the exhausted status (13, 15) of such long-term-activated DCs, and their low activity in inducing functional Th1 responses (15).

These results indicate that the conditions of DC activation and its duration determine the ability of DCs to induce the cytotoxic function of $CD8^+$ T cells, while being less relevant for the induction of $CD8^+$ T cell proliferation.

Indeed, the induction of Granzyme B correlated with the superior cytolytic function of $CD8^+$ T cells primed by the inflammatory-type DCs (***Fig 4.1B, C***). In contrast, priming by the "exhausted" DCs led to low levels of granzyme B and perforin and the associated poor ability to kill antigen-pulsed target cells. The outcome of these experiments indicate that, in analogy to the DC-induced differentiation of $CD4^+$ T cells into Th1 and Th2 direction (13), the expansion of $CD8^+$ T can be uncoupled from their effector-type differentiation, and that $CD8^+$ T cell expansion induced by chronically stimulated "exhausted" DCs is not accompanied by the acquisition of their effector function.

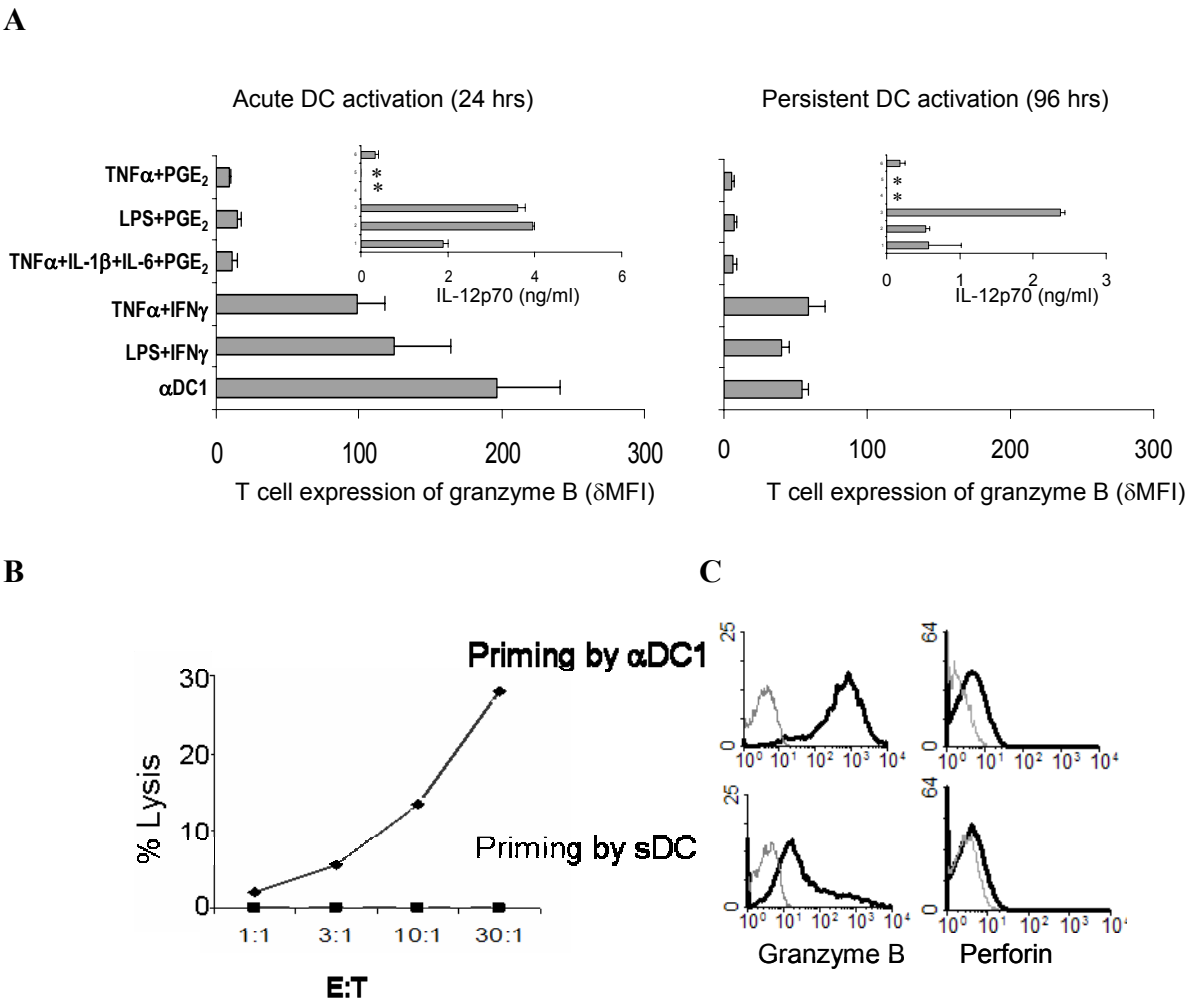


Figure 4.1 Induction of cytolytic and non-cytolytic pathway of $CD8^+$ T cell differentiation by, respectively, short-term activated and exhausted DCs.

(A) Immature DCs were activated with different combination of cytokines (details in Materials and Methods) for 24 hours. Naïve $CD8^+$ T cells were primed with activated DCs. On day 5, $CD8^+$ T cells were counted to assess proliferation (see appendix B for data on cell number) and intracellular staining for granzyme B was performed. Inset: IL-12p70 production from differentially activated DCs after CD40L stimulation, * denotes not detectable. Persistent activation (96 hours) of DCs with the different combinations of cytokines (see Materials and Methods). Naïve $CD8^+$ T cells were primed with chronically activated (96 hours) DCs. On day 5, $CD8^+$ T cells were counted to assess proliferation (see appendix B for data on cell number) and intracellular staining for granzyme B was performed. Inset: IL-12p70 production from differentially activated DCs after CD40L stimulation, * denotes not detectable. (B) Cytolytic function of $CD8^+$ T cells, primed with either inflammatory DCs (αDC1s) or exhausted DCs (sDCs), was assessed by standard ^{51}Cr-release assay. (C) Intracellular levels of cytolytic granules granzyme B and perforin were determined by flow cytometry.

Requirement for inflammatory-type DCs for the acquisition of peripheral chemokine responsiveness in activated CD8$^+$ T cells.

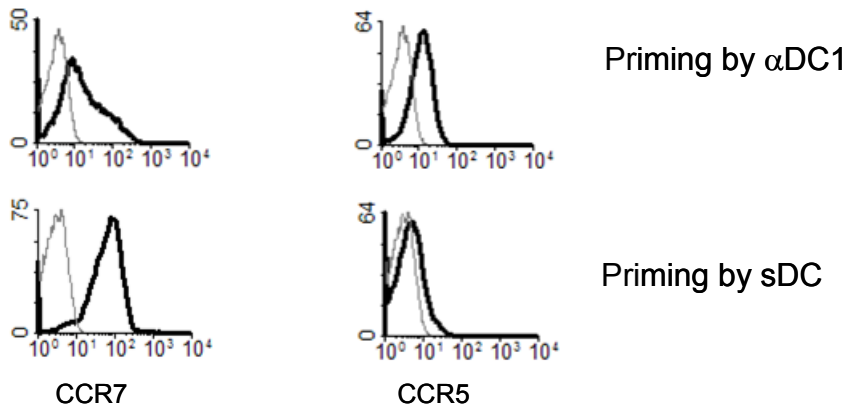
Since the inflammatory and exhausted DCs showed differential impact on the induction of CTL function in naive T cells, we tested their influence on the expression of the peripheral-type versus LN-type chemokine receptors on CD8$^+$ T cells.

As shown in ***Figure 4.2A***, inflammatory DCs induced the down regulation of CCR7 on 50-70% of CD8$^+$ T cells and their simultaneous acquisition of CCR5 expression (***Fig. 4.2A*** upper panel), the chemokine receptor involved in peripheral homing of effector CD8$^+$ T cells (144), Unexpectedly, CD8$^+$ T cells stimulated with the "exhausted" PGE_2-matured DCs retained high levels of CCR7 expression, a lymphoid homing receptor (and a marker of naïve and central memory T cells), and did not acquire CCR5 (***Fig. 4.2A*** bottom panel). We are currently analyzing the responsiveness of differentially primed CD8$^+$ T cells to chemokines CCL5 and CCL21.

In an attempt to determine the mechanism of the inflammatory-type DC-induced switch in the chemokine receptor pattern of CD8$^+$ T cells, we have analyzed the impact of several of the inflammatory-type cytokines differentially expressed by αDC1s and sDCs; such as IL-12p70, IL-23, IL-27, and IL-18 (112) and (***Supplementary Fig S8***). As shown in ***Figure 4.2B***, the addition of IL-12 during the priming of naïve CD8$^+$ T cells by the "exhausted" DCs resulted in the loss of CCR7 on 30-70% of the cells. In accord with the key role of DC-produced IL-12 in the induction of the lymphoid-to-peripheral switch in chemokine responsiveness, the neutralization of IL-12 during T cell priming abrogated the differences between αDC1 and sDC (***Fig. 4.2B-right***), preventing the down-regulation of CCR7. Our preliminary results indicate that other IL-12 family members by themselves do not have significant impact on the downregulation

of CCR7 on the $CD8^+$ T cells and we are currently analyzing the influence of the combination of IL-12 family members and IL-18.

A



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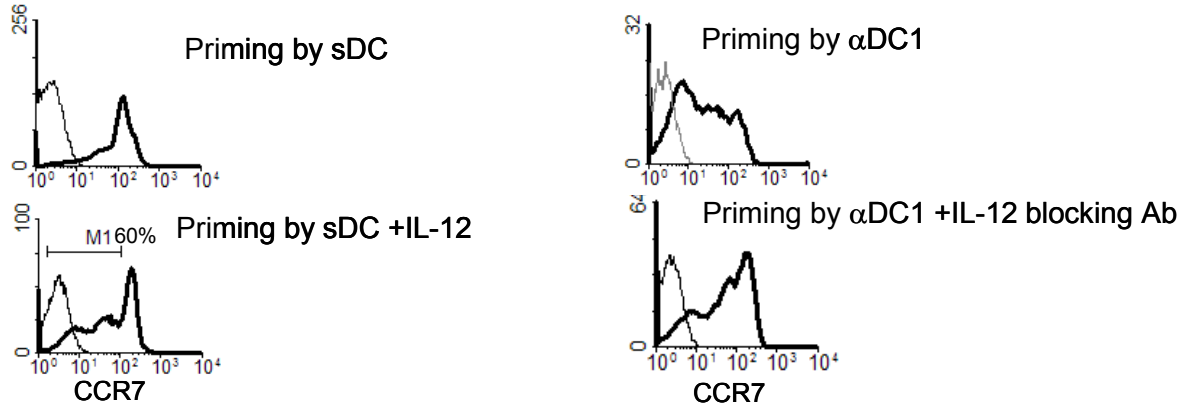

Figure 4.2 Induction of cytolytic function correlates with the switch in chemokine receptor expression.

αDC1-primed $CD8^+$ T cells and sDC-primed $CD8^+$ T cells were stained on day 5-6 for chemokine receptors CCR7, CCR5 and CXCR3 and flow cytometry was performed. (B) Recombinant IL-12 was added during the priming of naïve $CD8^+$ T cells by sDCs and CCR7 expression was assessed by flow cytometry on day 5. IL-12 blocking antibody was added during the priming of naïve $CD8^+$ T cells by αDC1 and CCR7 expression was assessed by flow cytometry on day 5.

"Exhausted" DCs induce CD8$^+$ T cells with central-memory phenotype and function

Since the priming of naïve CD8$^+$ T cells with exhausted DCs led to the expansion of CD8$^+$ T cells with persistent expression of CCR7 and CD62L and the absence of effector function, we asked whether such non-effector pathway of CD8$^+$ T cell activation represents an abortive pathway of differentiation, or an alternative pathway of differentiation of the functionally intact cells. As shown in ***Figure 4.3A***, in addition to CCR7 and CD62L, the sDC-primed T cells expressed typical markers of central-memory cells, such as IL-15Rα, and IL-7R (CD127), the receptors for the homeostatic cytokines mediating long-term survival of primed CD8$^+$ T cells (27, 145), making such cells indistinguishable from the αDC1-primed cells. In addition, the sDC-primed non-cytolytic CCR7$^+$ cells were capable of acquiring cytolytic activity and the peripheral-type chemokine receptor profile, upon restimulation with inflammatory DCs (***Fig. 4.3B***), sharing this ability with the peripheral blood-isolated central memory T cells (***Fig. 4.3C***).

As expected, similar to the *in vitro* generated non-cytolytic CD8$^+$ T cells induced by non-inflammatory DCs, blood-isolated central memory CD8$^+$ T cells acquired cytolytic effector function (and a shift in chemokine expression pattern) exclusively when stimulated with inflammatory DCs but not with "exhausted" DCs (***Fig. 4.3C***).

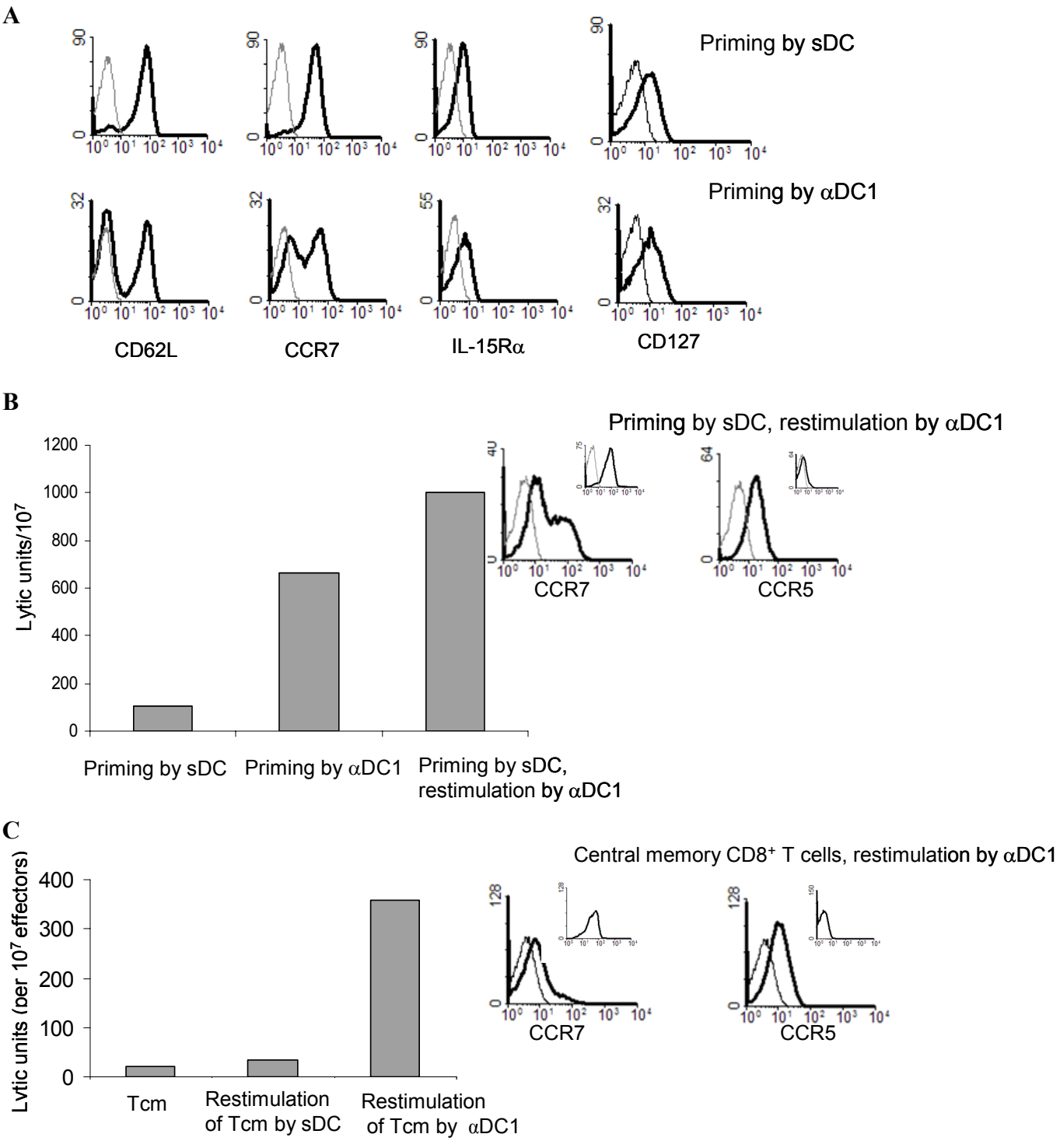

Figure 4.3 Induction of central-memory phenotype in CD8$^+$ T cells primed by exhausted DCs and their similarity to blood derived central memory T cells.

(A) Naïve CD8$^+$ T cells were cultured with αDC1s or sDCs. Expression of CCR7, CD62L, IL-15Rα and CD127 on primed CD8$^+$ T cells was measured on day 20-25. (B) CD8$^+$ T cells primed initially with sDCs, expanded for 14

days, were restimulated with αDC1s. After restimulation, on day 5 their cytotoxic ability was assessed by ^{51}Cr release assay and expression of CCR7 and CCR5 was measured by flow cytometry. (C) $CD8^+$ central memory T cells were sorted from peripheral blood and stimulated with αDC1s or sDCs. On day 5, cytolytic potential was assessed by ^{51}Cr release assay, and chemokine receptor expression evaluated by flow cytometry.

Inflammatory and exhausted DC differentially regulate CTL activity and tumor-relevant chemokine receptors on tumor-specific $CD8^+$ T cells

Prompted by the results of the experiments with polyclonally-activated naïve and memory $CD8^+$ T cells, we have compared the outcome of *in vitro* sensitization (IVS) of HLA-A2-restricted melanoma-specific $CD8^+$ T cells, using $MART1_{27-35}$-loaded autologous αDC1 or sDCs as the immunogen (112). In contrast to the short-term experiments performed in a polyclonal system, the generation of high numbers of MART-1-specific T cells required prolonged culture of the primed cells.

While in these culture conditions we could not detect the differences in CCR7 expression (data not shown), the IVS experiments performed using the pro-inflammatory DCs (αDC1s) resulted in the induction of CTL responses with strongly elevated CTL activity and led to the generation of $CD8^+$ T cells expressing high levels of CCR5. Since CXCR3, another peripheral-type chemokine receptor, has been implicated in the anti-tumor effectiveness of tumor-specific $CD8^+$ T cells (146) we also analyzed the expression of CXCR3 on tumor-specific $CD8^+$ T cells after restimulation with either inflammatory or exhausted DCs. As shown in ***Figure 4.4***, in accordance with the data obtained in polyclonal models, the inflammatory DCs proved superior in inducing Granzyme B expression in MART-1-tetramer-positive T cells (***Fig. 4.4A***), that were superior in their ability to kill MART-1- expressing tumor melanoma cell line (***Fig. 4.4B***). αDC1-stimulated $CD8^+$ T cells also induced elevated expression of CXCR3 and CCR5 on MART-1 tetramer-positive cells (***Fig. 4.4C***).

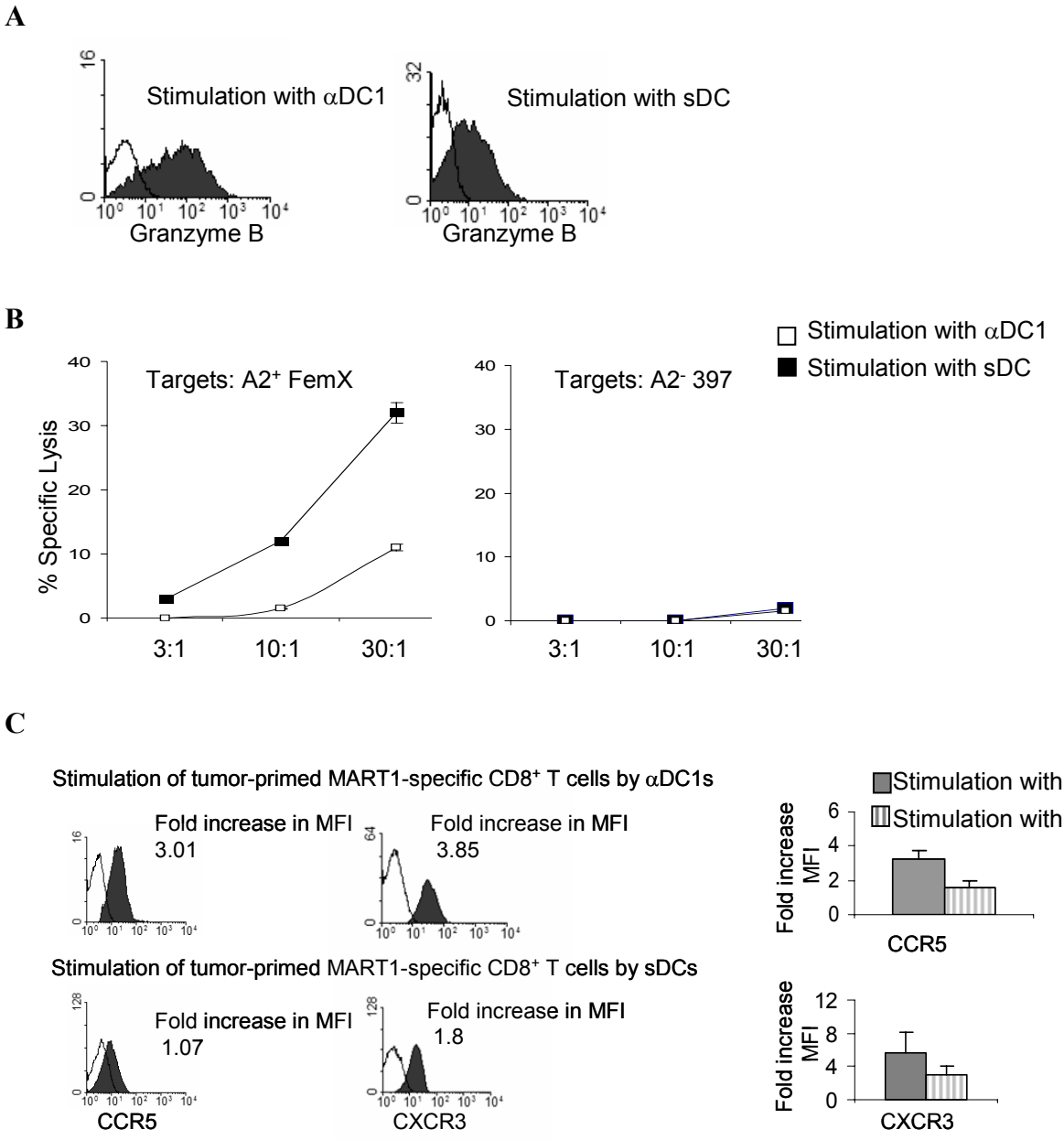

Figure 4.4 Inflammatory and exhausted DC induced differential expression of Granzyme B, CCR5 and CXCR3 on tumor-specific CD8$^+$ T cells.

αDC1s or sDC from HLA-A2$^+$ melanoma patients were pulsed with HLA-A2 restricted MART1 peptide and used to stimulate autologous CD8$^+$ T cells. (A, C) Expression of Granzyme B, CCR5 and CXCR3 was measured on MART1 gated CD8$^+$ T cells. (B) Cytotoxic activity of the CTLs induced by sDCs or αDC1 was measured against control tumor cell line (not expressing MART1) and MART1 expressing Fem X cell line. Similar results were obtained in experiments with two additional melanoma patients.

4.5 DISCUSSION

Our data demonstrate that in analogy to the DC-induced differentiation of $CD4^+$ Th cells into Th1 and Th2 direction, the functional differentiation of naïve and resting memory $CD8^+$ T cells is differentially regulated by the "inflammatory-type" and "exhausted" DCs. We observed that while the proliferation and expansion of $CD8^+$ T cells can be driven efficiently by the DCs undergoing maturation in a wide spectrum of inflammatory conditions, the induction of the $CD8^+$ T cell effector functions and a switch in their chemokine responsiveness was a sole property of the IL-12-producing DCs activated for a short period of time in the conditions mimicking acute inflammation (presence of interferons and TLR ligands).

Such an inflammatory pathway of activation of $CD8^+$ T cells resulted in roughly equal numbers of the cells acquiring the $CD8^+GrB^{lo}Prf^{do}CCR5^+CD62L^-CCR7^-$ effector-memory phenotype, and the $CD8^+GrB^{lo}Prf^{do}CCR5^+CD62L^+CCR7^+$ intermediate/central-memory phenotype. Interestingly, in accordance with the previously-reported long-lived potential of the cells activated by the pro-inflammatory high IL-12-producing DCs (112) and with the ability of recombinant IL-12 to promote CTL survival (39, 147), both these subsets expressed high levels of IL-7- and IL-15 receptors (***Fig. 4.3***), known to be essential for the homeostatic proliferation and long term survival of $CD8^+$ T cells *in vivo* (27, 145). In contrast to such cytolytic/effector pathway of differentiation associated with the termination of responsiveness to the lymph-node-associated chemokines and the acquisition of the responsiveness to the peripheral/inflammatory-type chemokines, the $CD8^+$ T cells activated by either long-term activated DCs, or the DCs induced to mature in the presence of PGE_2 showed high level of cell expansion, but failed to undergo the switch in chemokine responsiveness and to acquire CTL functions. Our data shows the functional and phenotypical similarity between such cells and central-memory cells isolated

from peripheral blood. In addition, high expression of IL-7R and IL-15R and the accelerated ability of such non-cytolytic cells to acquire effector function following (re)stimulation by the inflammatory DCs, suggest that such non-cytolytic pathway of $CD8^+$ T cell differentiation, induced by the "exhausted" DCs represents a direct pathway of development of the memory cells, an alternative to their "indirect" pathway of development via an intermediate effector stage.

We have shown that the differential ability of DCs to direct naïve T cells to either of these pathways (effector/effector-memory versus central memory) depends on the duration of DC activation and the character of the inflammatory environment driving DCs activation, with the differences in the DC-mediated IL-12 production representing the key underlying mechanism.

These data help to explain several of the phenomena relevant to the generation of $CD8^+$ T cell memory: mouse studies have revealed the need for early inflammation following an infection or DC immunization in order to generate a robust effector response (138). The application of antibiotics to shorten the duration of infection (55) (56) was shown to accelerate the formation of $CD8^+$ memory T cells.

The current debate centers on whether memory and effector cells represent alternative parallel pathways of T cell differentiation or sequential stages of T cell activation, with contrasting answers to this question obtained with different experimental systems (49, 50, 56).

Our current results help to reconcile this controversy, showing that differentiation of naïve $CD8^+$ T cells into memory cells can occur through either pathway, either directly into memory compartment, or through an indirect pathway associated with a (transient) acquisition of

the effector function by at least a proportion of the cells. This decision is dependent on the stage/character of DC activation.

The current *in vitro* results are also in accordance with the observed results of numerous clinical trials for cancer immunotherapy (59). Active immunotherapy approaches such as peptide or viral vector-mediated immunization result in massive expansion in the number of tumor-specific $CD8^+$ T cells but with negligible effector function and poor induction of clinical responses (21, 131), that could only be reversed into the effector-type responses, when followed by IFNα (21). Similar to vaccines (active immunotherapies), adoptive immunotherapy with ex-vivo expanded tumor-specific $CD8^+$ T cells has met with little success. While tumor-isolated T cells used in these trials show high *ex vivo* effector functions, the over-differentiation of the adoptively transferred $CD8^+$ T cells results in their relatively short half-life and can limit their clinical effectiveness, compared to less differentiated T cells (148-150) (151).

The current data demonstrating the possibility of exploiting the activation pathway of DCs in order to selectively promote either the effector or the memory pathway of $CD8^+$ T cells differentiation, facilitates the adjustment of the currently-used (active and adoptive) immunotherapeutic strategies, in order to maximize their clinical effectiveness.

ALTERNATIVE TWO-SIGNAL-DEPENDENT HELPER ACTIVITY OF CD8$^+$ T CELLS

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Manuscript in *preparation*

The majority of experiments in this manuscript were performed by Payal Watchmaker.

5 ALTERNATIVE TWO-SIGNAL-DEPENDENT HELPER ACTIVITY OF CD8$^+$ T CELLS

5.1 ABSTRACT

We have previously demonstrated the ability of resting CD8$^+$ T cells to program DCs for induction of type-1 immune response. This "helper function" of Ag-activated CD8$^+$ T cells requires TCR triggering and is mediated by TNFα and IFNγ. Here we show that in contrast to resting CD8$^+$ T cells, pre-activated CD8$^+$ T cells are no longer dependent on TCR ligation to exert DC-mediated immunoregulatory function, but can utilize the cues from the mediators of innate response such as IFNα and IL-18. Non-TCR dependent "alternative two-signal" activation is equivalent to TCR based signal with regards to induction of "helper activity" of primed CD8$^+$ T cells. Interestingly, the responsiveness of pre-activated CD8$^+$ T cells to non-TCR dependent stimuli is tightly regulated since each of the cytokine signals by itself is ineffective. Alternative two-signal-activated CD8$^+$ T cells induce DC maturation and prime them for enhanced IL-12p70 production following subsequent stimulation with CD40L. The current data indicate that while primed CD8$^+$ T cells can be activated by inflammatory cytokines in a TCR-independent manner to perform immunoregulatory role, such activity remains to be tightly regulated in a "two-signal" paradigm. TCR independent activation of CD8$^+$ T cells can allow local IFNγ delivery and DC activation during responses against MHC-deficient cells, and helps to explain the role of non-

antigen specific T cells within inflammatory infiltrates in tuberculosis, leprosy, and allergic reactions.

5.2 INTRODUCTION

The $CD8^+$ T cell mediated adaptive immune response plays a key role in elimination of transformed and virally infected cells (36). Naïve $CD8^+$ T cells require signal 1 (antigen), signal 2 (costimulation), and polarizing signal 3, in order to undergo clonal expansion and to differentiate into functional effector $CD8^+$ T cells (CTLs). In contrast, induction of the effector functions in antigen-experienced memory $CD8^+$ T lymphocytes requires lower activation threshold (47, 73), allowing them to respond to antigen already in the conditions of a limited tissue damage. In addition, memory cells also express increased levels of receptors for cytokines such as IL-15, IL-7, and IL-18 (45, 145, 152, 153).

Recent studies have shown that $CD8^+$ T cells are not only effector cells of adaptive immunity but also participate in early phase of the immune responses (154). Studies in mouse models of intracellular infections such as Listeria and melioidosis (39, 40) have shown that $CD8^+$ T cells can secrete IFNγ in response to pro-inflammatory cytokines such as IL-12, IL-18, and IFNα. IFNα, secreted by plasmacytoid DCs and other infected cells, plays both a pro-inflammatory effector role in innate responses by exerting a direct anti-viral effect, as well as can have an immunoregulatory role during adaptive immune responses (155, 156). Another innate cytokine is IL-18, which is secreted by macrophages, dendritic cells (DCs), keratinocytes, and epithelial cells (81, 157).

Prompted by the studies showing the contribution of IL-12, or IFNα, and IL-18 to the induction of IFNγ in NK cells and T cells (45, 158), in the current this study we addressed (i) the regulatory requirements for such cytokine mediated (TCR-independent $CD8^+$ T cell activation, (ii) impact of alternatively activated $CD8^+$ T cells on DCs.

The results presented here demonstrate that the previously activated $CD8^+$ T cells share the paradigm of two-signal activation with NK cells (158), being TCR-independent but requiring a simultaneous exposure to at least two different inflammatory signals. This alternative two-signal activation mechanism is an exclusive feature of $CD8^+$ T cells that have undergone prior TCR-mediated activation and allows the bystander cells to perform DC-mediated "helper" function.

5.3 MATERIALS AND METHODS

Culture media, reagents and cell lines. Cell lines and human DCs were cultured in IMDM (Invitrogen, Carlsbad, CA) containing L-glutamine and 10% FBS (Hyclone, Logan, UT). The following reagents were used to activate $CD8^+$ T cells: IFNα (Intron A-IFNα-2b; Schering Plough, NJ), recombinant human IL-18 (MBL International, Woburn, MA), recombinant human IL-15 (R&D Systems, Minneapolis, MN). Anti-human CD3 and CD28 stimulating mAb's were purchased from CLB. The following reagents were used to obtain immature DCs: rhuGM-CSF and rhuIL-4 (gifts from Schering Plough). The CD40L transfected J588 plasmacytoma cell line was a gift from P. Lane (University of Birmingham, UK).

Cell Isolation. Mononuclear cells from peripheral blood of healthy donors were isolated using Lymphocyte Separation Medium (Cellgro, Mediatech). Monocytes were isolated from the mononuclear fraction using Percoll (Sigma) density separation technique, followed by plastic

adherence, as described (137). Resting $CD8^+$ T cells were isolated by negative selection using Stemsep $CD8^+$ T cell enrichment cocktail (Stemcell Technologies Inc, Canada).

Cytokine production by primed $CD8^+$ T cells. Primed $CD8^+$ T cells (resting $CD8^+$ T cells were stimulated with CD3/CD28 activating antibodies for 6 days) were plated in 96-well flat bottomed plate at 10^5 cells/200µl/well. The untreated $CD8^+$ T cells or single cytokines –IL-18, IFNα or IL-15 were controls for spontaneous IFNγ and TNFα production. IFNγ and TNFα levels in 24h supernatants were measured by ELISA.

Activation and polarization of DCs by primed $CD8^+$ T cells. Primed $CD8^+$ T cells (2 $x10^5$ cells) were added to day 6 immature DC culture (2-3 x 10^5 cells/well) in the presence or absence of OKT3 (positive control) or IFNα (3000U/ml) and IL-18 (100µg/ml). After 48 hours, the cells were harvested and analyzed for the expression of maturation-associated surface markers and the ability to produce IL-12p70. To test IL-12p70 producing capacity of DCs, the harvested DCs were plated in 96-well flat-bottomed plate (2x 10^4 cells/ well) along with CD40L transfected J588 cells (5 x 10^5 cells/well) for 24 hours. Supernatants were tested for IL-12p70 by ELISA.

5.4 RESULTS

Primed $CD8^+$ T cells can be reactivated by proinflammatory cytokines: alternative two-signal paradigm of activation of $CD8^+$ T cells

The requirements for antigen specific activation of $CD8^+$ T cells are known, but regulatory requirements for cytokine mediated $CD8^+$ T cell activation have not been investigated till now.

We observed that the responsiveness of primed $CD8^+$ T cells to IFNα or IL-18 is tightly regulated, as single cytokine- IFNα or IL-18 by itself did not induce significant IFNγ production in primed $CD8^+$ T cells. The combination of IFNα and IL-18 had synergistic effect on activation and induction of IFNγ (***Fig. 5.1A right***). In contrast to primed T cells, resting $CD8^+$ T cells are not responsive to these inflammatory mediators, alone or in combinations (***Fig. 5.1A left***). Similar induction of IFNγ was observed, when combination of IL-15 and IL-18 was used to activate primed $CD8^+$ T cells (***Fig. 5.1C left***). In addition, IFNα and NK cell ligands present on the surface of K562 and Hela cells synergistically induce IFNγ secretion in primed $CD8^+$ T cells (***Fig. 5.1C right***). A similar two signal activation requirement has been observed in case of release of another DC-activating T cell product: TNFα (***Fig 5.1B***).

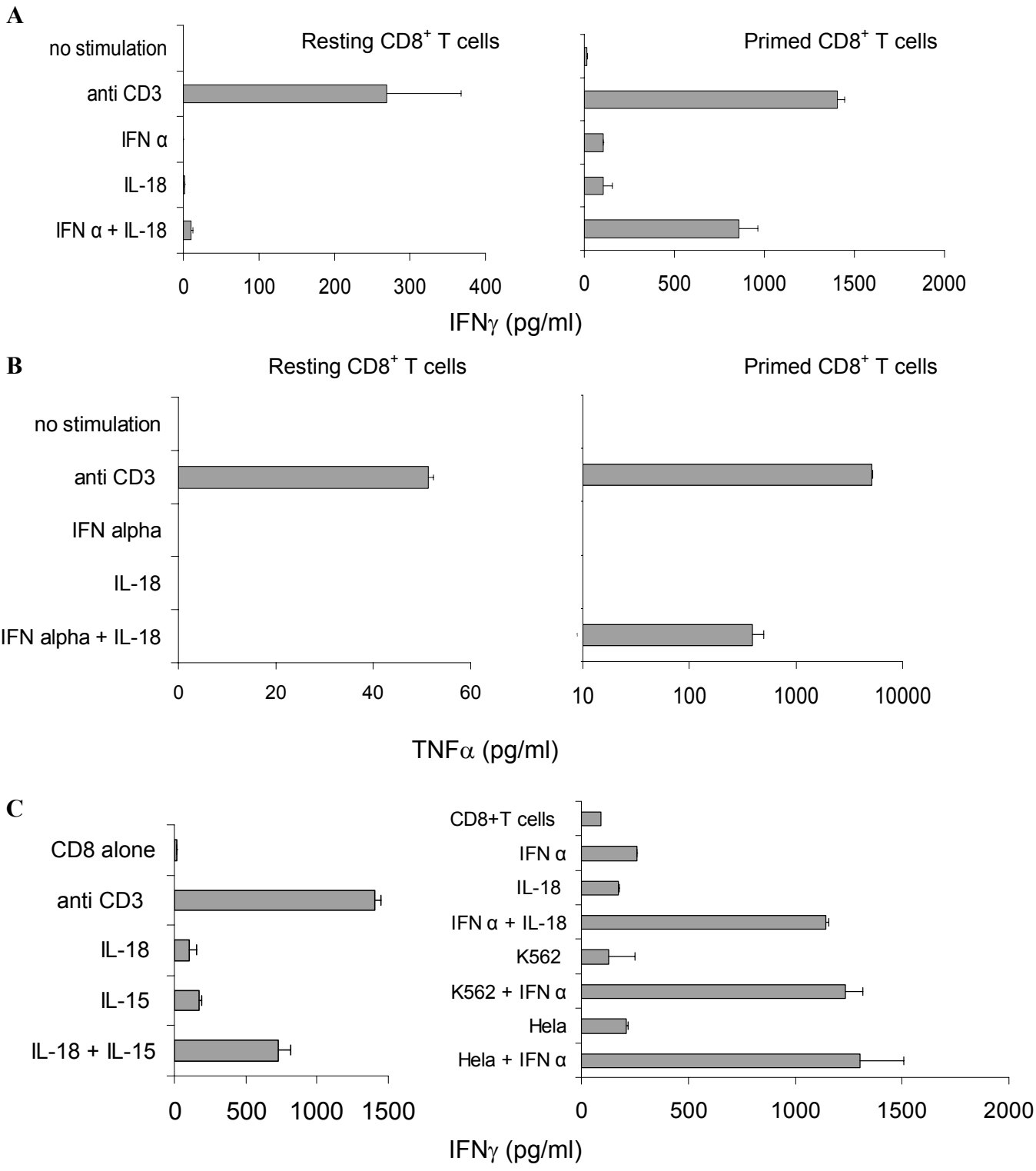

Figure 5.1 Two-signal requirement for the induction of IFNγ and TNFα production by primed CD8$^+$ T cells

(A, B**)** In contrast to resting CD8$^+$ T cells, primed CD8$^+$ T cells secrete IFNγ and TNFα in response to combination of IFNα and IL-18 *in vitro*. Resting or primed CD8$^+$ T cells were cultured for 24 hours with cytokines or TCR

stimulus in 96-well plate. Supernatants were assayed for IFNγ production by ELISA. (C, D) Similar to IFNα and IL-18, two other combinations-IL-15 and IL-18 and IFNα and target cell K562 or Hela cells also elicited IFNγ production in the primed $CD8^+$ T cells alone. Results are representative of four independent experiments.

"Two signal" activated $CD8^+$ T cells induce DC maturation

Having established that the previously-primed $CD8^+$ T cells can produce DC-activating cytokines in a non-TCR dependent manner (Fig. 1), we investigated the possibility of cross-talk between alternatively-activated $CD8^+$ T cells and immature dendritic cells (DCs). Addition of $CD8^+$ T cells alone (data not shown) or the combination of IFNα and IL-18 to DC cultures did not result in maturation of DCs. In sharp contrast, addition of primed $CD8^+$ T cells along with IFNα and IL-18, resulted in maturation of DCs (similar to TCR stimulation by antigen OKT3) as shown by induction of CD83 and high expression of CD86 (***Fig. 5.2***) These data demonstrate that in addition to the TCR-dependent activation, $CD8^+$ T cells which have been activated by cytokines alone can also induce bystander maturation of DCs.

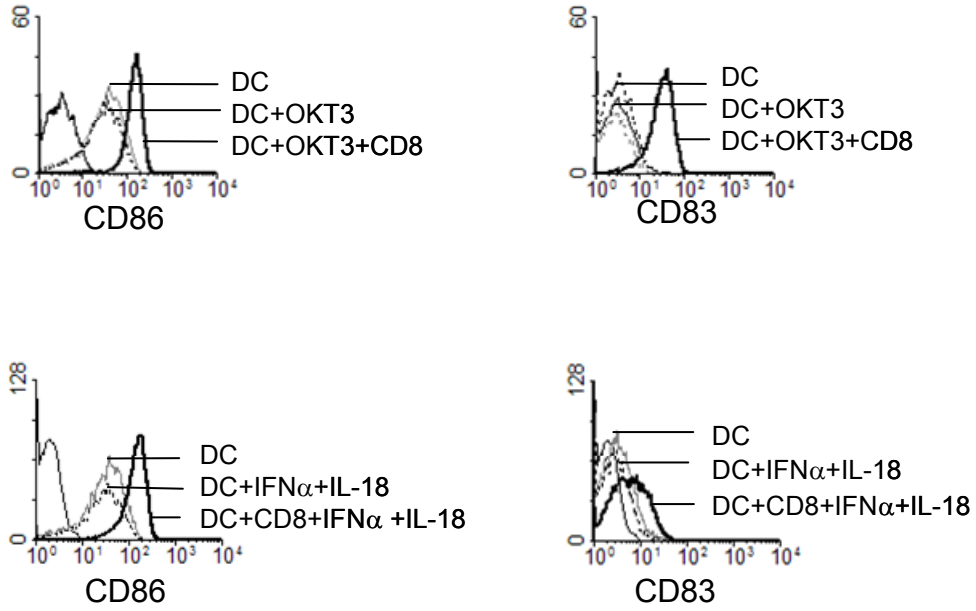

Figure 5.2 "Alternate" two-signal activated $CD8^+$ T cells induce DC maturation.

(A) IL-18 and IFNα stimulated $CD8^+$ T cells (similar to OKT3 stimulation) induced DC maturation manifested by induction of CD83 and upregulation of CD86 expression. IL-18 and IFNα or OKT3 was added along with primed

$CD8^+$ T cells to day 6 DC cultures for 48 hours. The cultures were harvested and stained for DC maturation markers CD86 and CD83. Similar result was obtained in two additional experiments.

Alternatively activated $CD8^+$ T cells induce type-1 polarized DC with enhanced IL-12p70-producing capacity

We next analyzed the impact of the alternatively activated $CD8^+$ T cells on DC function. To address this, immature DCs were cocultured with primed $CD8^+$ T cells in the additional presence of IFNα and IL-18. After 48 hours, the cells were harvested, washed and stimulated with CD40L transfected J588 cells. As shown in ***Figure 5.3*****,** DCs that were activated with combination of $CD8^+$ T cells, IL-18 and IFNα produced high amounts of IL-12p70 upon subsequent stimulation. Similar to induction of DC maturation, type-1 polarization of DCs was observed only when $CD8^+$ T cells were activated with IFNα and IL-18 or antigen OKT3. This observation demonstrates that alternative two signal-activated effector $CD8^+$ T cells can exert immunoregulatory "helper" function in a non- TCR dependent bystander fashion.

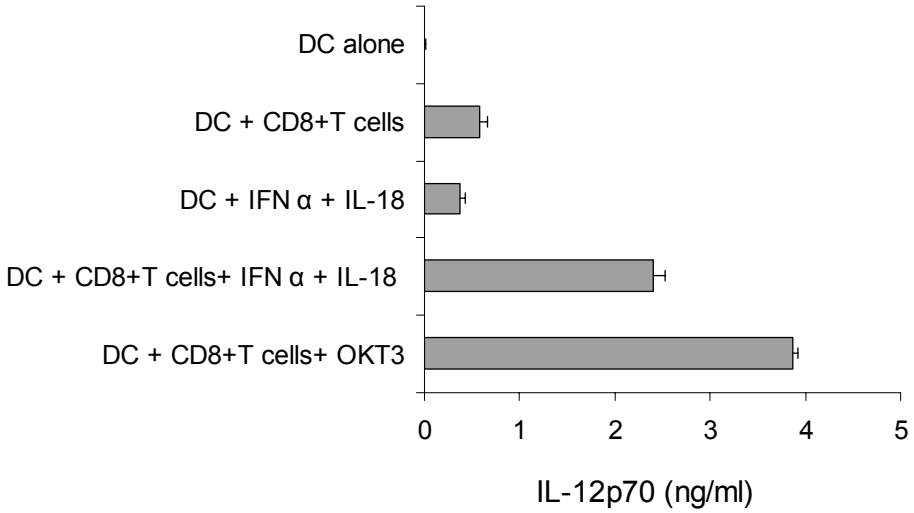

Figure 5.3 Alternatively activated $CD8^+$ T cells induce type-1 polarized DC with enhanced IL-12p70-producing capacity.

Day 6 DCs were cultured for 48 hours in the presence of $CD8^+$ T cells, OKT3, IL-18, IFNα or their combinations. At day 8, DCs were harvested, washed and stimulated with J588-CD40L. After 24 hours, supernatants were analyzed for IL-12p70 by ELISA. Results are representative for three independent experiments.

5.5 DISCUSSION

The current data indicate that primed $CD8^+$ T cells can be activated to perform immunomodulating activities in a TCR-independent fashion, their activation remains tightly controlled in an alternative two-signal activation paradigm. The efficacy of their alternative (cytokine-induced) "helper" function is dependent on the activation state of $CD8^+$ T cells. In case of primed $CD8^+$ T cells, the activation with "IL-18 + IFNα" was equivalent to the TCR-based activation. In contrast, no activation was observed when using unprimed $CD8^+$ T cells. In addition to the "IL-18 + IFNα" signal, two other combinations – IL-15 + IL-18 and IFNα + K562 cells (or IFNα+ tumor cells) also elicited helper response in primed $CD8^+$ T cells. In contrast to the effective activation of $CD8^+$ T cells with "two-signals", the same primed cells were not responsive to IL-15, IL-18 or IFNα when used as single agents. Resting $CD8^+$ T cells were resistant to all tested forms of the TCR-independent activation by IL-2, IL-18, and IFNα or their combinations. Such a strict regulation of primed $CD8^+$ T cells can reduce the risk of potential immunopathology caused by the presence of activated $CD8^+$ T cells in a cytokine-rich environment.

The phenomenon of alternative two-signal activation paradigm can partially explain the bystander activation and epitope spreading associated with autoimmune diseases, including multiple sclerosis, diabetes, or arthritis (159-162). Intracellular pathogens such as viruses and mycobacteria can be strong inducers of pro-inflammatory cytokines like IFNα, IL-18 and IL-12, and their combination can activate Th1 promoting function of $CD8^+$ T cells in a non-specific bystander fashion. The cytokine-activated $CD8^+$ T cells can secrete IFNγ and TNFα, creating cytokine environment that is conducive for maturation and type-1 polarization of DCs. This can

enhance the probability of presentation of self epitopes in type-1 cytokine milieu and promote anti-self epitope spreading.

The alternative two-signal activation paradigm can explain the role of the $CD8^+$ T cells of unrelated antigen specificity accumulating at the sites of infection and in allergic responses. The data from mouse model of allergen induced airway inflammation indicate that resident memory $CD8^+$ T cells of unrelated specificity can suppress allergic inflammation in the airways in an antigen independent but IFNγ-dependent manner and involving IL-12 and IL-18 (163). Data from murine respiratory viral infections suggest that during secondary infections, lung-resident memory cells are likely to represent an early defense mechanism (164). Early production of IFNγ by memory $CD8^+$ T cells may be an outcome of stimulation by cytokines, when the viral load is still low.

In summary, our data indicate that the NK cell-like activity of $CD8^+$ T cells involves DC-modulatory function. Importantly, it remains being tightly controlled by an alternative "two-signal" activation paradigm, depending on concomitant stimulation by at least two different cytokines. TCR independent induction of $CD8^+$ T cell helper function can help in maintaining localized pro-inflammatory IFNγ-rich milieu and activation of DCs and thus promote immediate immune response before antigen-specific T cells undergo clonal expansion to carry out specific immune reaction.

6 CONCLUSIONS

$CD8^+$ T cells are the effector cells essential for defense against intracellular pathogens and cancer. The priming of naïve $CD8^+$ T cells is a crucial step in the initiation of adaptive immunity, allowing the elimination of infected and transformed cells by the arising cytolytic effector $CD8^+$ T cells and leading to generation of long-lived memory $CD8^+$ T cells. While the functionality of activated $CD8^+$ T cell is most often defined in terms of their cytolytic activity, here we have focused on the role of DCs in regulating the acquisition of effector phenotype (cytotoxicity and peripheral-tissue homing ability) and the immunoregulatory functions of the effector and memory $CD8^+$ T cells.

The regulatory function of effector $CD8^+$ T cells involves the perforin/granzyme B-mediated killing of antigen-presenting DCs, as a negative feed-back mechanism limiting the magnitude of CTL responses. In contrast, memory $CD8^+$ T cells perform positive immunoregulatory functions, enhancing the DC expression of PI-9 (granzyme B inhibitor), thus promoting the survival of DCs by protecting them from CTL attack. This protective function of memory $CD8^+$ T cells, analogous to $CD4^+$ T cell-mediated DC protection, ensures that antigen presentation can continue in the presence of existing CTLs. Another important aspect of the "helper" function of memory $CD8^+$ T cells is to provide DC-mediated polarizing signals for the development of CTL and $CD4^+$ T cell responses. Our findings from mouse experiments (see Chapter 2) show that $OVA_{257-264}$-specific $CD8^+$ T cell responses can both benefit from "helper

signals" delivered by memory cells (e.g. from LCMV-specific $CD8^+$ T cells) and can act as a source of such signals. Consistent with this notion, the data indicate that $CD8^+$ T cells of the same specificity can first benefit from "helper signals" delivered by memory T cells and, once having been enriched in the host and having achieved the memory stage, they can themselves act as a source of $CD8^+$ T cell help. The current data indicate the possibility of enhancing the efficacy of the therapeutic vaccines for patients with cancer or chronic infections, by inclusion of pathogen/tumor-unrelated "heterologous" epitopes, that promote the interaction of vaccine-carrying APCs with naturally occurring (or possibly artificially-induced) memory-type T cells. While in the current studies we have either used (*in vitro*) high numbers of resting $CD8^+$ T cells from TCR-transgenic animals or have utilized (*in vivo*) the $Granzyme^{low}/CD62L^{high}$ memory $CD8^+$ T cells induced by a pre-immunization of wild type-animals, the most obvious source of "heterologous CD8 help" in cancer patients are the resting memory $CD8^+$ T cells resulting from the vaccination-induced responses to "childhood diseases" or the infections that the patients have undergone in the past such as influenza, or HBV. Our data imply that the patients enrolled for therapeutic protocols involving "heterologous" helper epitopes will need to be screened for particular $CD8^+$ T cell responses, to identify the appropriate memory-type $CD8^+$ T cells and to avoid targeting of effector cells. An alternative approach may be to pre-immunize patients with unique immunogens. In either case, our data implicate the need of targeting different populations of "heterologous" helper T cells during the consecutive courses of vaccination in order to assure that each dose of vaccination targets a high proportion of resting (rather than activated by a prior vaccine dose) T cells.

While helper function of memory $CD8^+$ T cells can be exploited as a therapeutic strategy for treating cancer, in case of autoimmune conditions, inhibiting either the production or function

of $CD8^+$ T cell derived type-1 polarizing factors may be beneficial to the patients. Blocking of TNFα, one of the components of $CD8^+$ T cell derived "helper signal", using anti-TNFα antibodies (Infliximab) or soluble TNF receptor (Etanercept) has been a common treatment approach for diseases such as rheumatoid arthritis, ankylosing spondylitis, Crohn's disease and psoriatic arthritis (165, 166). While the source of TNFα in these disease conditions is not clear, it remains to be tested whether the effectiveness of the drug is related to the inhibition of the helper activity of $CD8^+$ T cells. Although TNF blockers have helped to control chronic inflammatory conditions, they have also been shown to increase the risk of reactivation of tuberculosis, suggestive of the inability to promote and maintain effective type-1 immunity (167). Other potential strategies to inhibit $CD8^+$ T cell driven type-1 immune responses can be blockade of the actions of IL-18 and IFNα/β, combination of these two cytokines can activate $CD8^+$ T cells in the bystander fashion (see Chapter 5).

The existence of a "suppressor" stage of $CD8^+$ T cell activation helps to explain the poor efficacy of vaccinations performed in therapeutic setting for chronic infections (130, 168), the reason being the likelihood of clearance of antigen-pulsed DC by the circulating terminally-differentiated effector cells that predominate during chronic diseases. In addition, the current findings also help to explain the requirement for a delayed administration of booster doses of vaccination to prevent the phenomenon of CTL-mediated DC killing, thus achieving an optimal expansion of pathogen-specific $CD8^+$ T cells and the effectiveness of vaccines. Given the role of PI-9 (endogenous granzyme B inhibitor) in protecting DCs from CTL-mediated killing, it is of potential therapeutic value to design membrane-permeable peptide mimetics of the reactive center loop (binding site for granzyme B) of PI-9, which will inhibit the functional activity of granzyme B. This can be potentially administered along with DC- or peptide-based vaccines to

prolong the life span of antigen-presenting DCs in conditions like cancer or chronic infections where effector cells predominate. This strategy will also aid in reducing the time-periods between primary and subsequent booster doses.

The ability of memory cells to support DC-mediated activation of additional $CD8^+$ and $CD4^+$ T cells suggests that the phenomenon of immune memory, in addition to qualitative changes in the activation requirements of memory cells, may also involve a different pattern of interaction of the existing $CD8^+$ T cells with the antigen-carrying DCs. Our data support the following functional model of $CD8^+$ T cell memory (**Fig.6.1**). During primary immune responses, DC activation requires release of factors representing pathogen-associated- or tissue damage-associated molecular patterns (PAMPs versus "danger signals" or DAMPs) (10, 11, 69), implicating the need for a significant pathogen load and tissue damage. In contrast, the activation of DCs during secondary responses can benefit from the activating signals released by high numbers of pathogen-specific memory-type $CD8^+$ T cells, even before the destruction of the infected cells and the release of additional copies of the pathogen. Such memory $CD8^+$ T cell-dependent early DC activation helps the immune system to respond to the pathogen at much earlier stages of (re)infection, limiting its early spread, the extent of tissue damage and the activation of innate defense mechanisms, thus preventing the onset of disease symptoms.

Moreover, since during the primary responses, the PAMP- and DAMP-mediated DC protection from newly arising CTLs is limited to the period of active infection and ongoing tissue damage, the optimal activation of the T cells recruited to the lymph nodes at later stages of primary responses (needed for the optimal development of memory cells (126, 170) is dependent on DC-protecting signals from $CD4^+$ T helper cells (99).

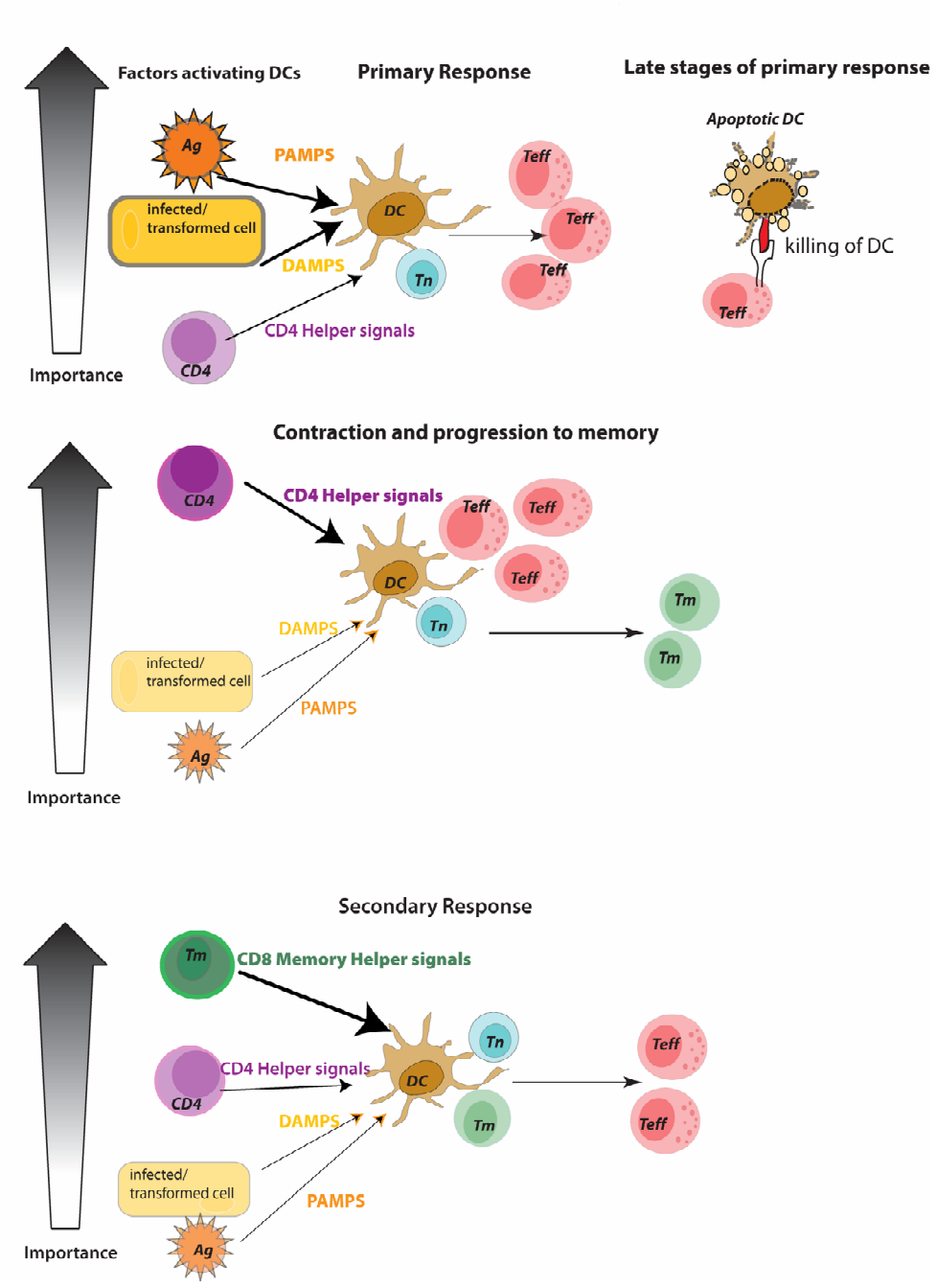

Figure 6.1 Emerging model of CD8 help in the establishment of $CD8^+$ T cell memory

Primary Response: Early stages of primary responses are characterized by significant pathogen load and consequently DCs are activated by PAMPS and DAMPS (the relative importance of DAMPS and PAMPS depicted by the thickness of arrow). In contrast, as primary response wanes and antigen presentation occurs in a reduced inflammatory environment, the absence of overt danger signals is compensated by $CD4^+$ T_{helper} cells taking over the role of protecting DCs from CTLs and ensuring antigen presentation. Recall response: During *secondary responses*,

high numbers of established pathogen-specific memory $CD8^+$ T cells can activate DCs at very early stages of infection prior to cell destruction and pathogen spread. In addition, memory $CD8^+$ T cells can protect DCs and thus allowing prolonged antigen presentation. This jump-starts the immune response and also prevents onset of disease symptoms. Although memory cells can revert to DC-killing effector cells within 30 hours in mice (38), and 3 days in humans (127), the continued recruitment of additional memory cells T cells to LN is likely to prolong the DC viability.Tn: naïve $CD8^+$ T cells, T*eff* : effector $CD8^+$ T cells, Tm: memory $CD8^+$ T cells.

In contrast, during secondary immune responses, antigen-carrying DCs can also interact with the $CD8^+$ memory T cells that are gradually recruited to the sites of (re)infection or to inflamed lymph nodes, limiting the dependence of antigen-carrying DCs on the pathogen-related or $CD4^+$ T cell-related survival signals. Since the acquisition of effector functions by human blood-circulating central memory $CD8^+$ T cells requires up to 72 hours of activation and several cell divisions, the memory T cell-dependent DC protection during secondary immune responses can have substantial time-span.

A recent report from Guarda *et al* (38) suggests that both effector and effector-memory populations of $CD8^+$ T cells can eliminate the DCs in the "reactive" lymph nodes. While the observation that effector $CD8^+$ T cells can eliminate antigen-carrying DCs supports similar earlier observations from several groups (80, 119), the location of DC killing is still unclear because of the conflicting data arising from different experimental models used to study the phenomena (37). The intravital two-photon microscopy used to image inflamed lymph nodes indicate that effector $CD8^+$ T cells traffic into "reactive" lymph node and kill antigen-bearing DCs. However, this study does not include a comparative analysis with peripheral tissues as a possible alternate site of killing, even though adoptive transfer of effectors is done prior to the injection of antigen-loaded DCs and thereby does not preclude DC-effector interaction in the peripheral tissues. In apparent contrast to our *in vitro* results in human models and the previous *in vivo* observations in mouse from Franca Ronchese's group (80), the same study also concludes

that effector-memory T cells (T_{em}) can kill antigen-bearing DCs with similar efficacy as the effector cells. However, taking into consideration recent observations indicating the conversion of T_{cm} to T_{em} and T_{em} to effectors under conditions that favor migration to non-lymphoid tissues such as adoptive transfers alone or adoptive transfer followed by infection (171, 172), there is a need to reevaluate whether DC killing is performed by effector memory cells or by the arising secondary effector population. Nevertheless, in view of recent data regarding the lineage relationship between central-memory and effector-memory cells especially after memory cell reactivation, it is essential to resolve the issue of relative contribution of different subsets of activated $CD8^+$ T cells towards DC killing.

The clarification of such relationship will allow reconciliation of the proposed killing of Ag-bearing DCs by memory $CD8^+$ T cells with ample evidence that pre-existing memory T cells accelerate and amplify secondary immune responses, instead of preventing such responses. Similarly, problem exists with regards to the well-established efficacy of booster doses of vaccines. The data from human and mouse studies suggest that there is a lag period before memory $CD8^+$ T cells can reacquire cytolytic activity (80, 127). In addition, using an *in vitro* human model, we have demonstrated that in memory cells granzyme B release is delayed and preceded by secretion of cytokine TNFα which upregulates the granzyme B inhibitor PI-9 in DCs and thus in our hands memory $CD8^+$ T cells did not exert immediate lytic activity against DCs. The existence of such a lag period, and the associated ability of recently activated memory $CD8^+$ T cells to exert a helper, rather than DC-killing activity, can help explain the mechanism of memory T cell function.

While the successful induction of immune memory is believed to be essential for vaccine effectiveness, both the exact pathways of development of memory $CD8^+$ T cells (in relation to

effector cells), and the features of their biology allowing them to expand and give rise to "effector" cells upon challenge with antigen, remain unclear. The current paradigm for successful vaccination is to include strong adjuvants along with peptide- or DC-based vaccines to induce a large effector T cell response, but it is not clear if this strategy will be equally successful for prophylactic and therapeutic vaccines. The reason being different requirements for prophylactic versus therapeutic vaccines, the goal of prophylactic vaccine is to generate large population of functional memory cells whereas the generation of a large effector T cell response is the immediate prerequisite for therapeutic vaccination. Following on this notion, our study identifies conditions of DC maturation that favor the cytolytic pathway of $CD8^+$ T cell differentiation followed by the development of memory cells versus conditions that support direct differentiation of naïve $CD8^+$ T cells into memory cells. Our results have implications not only for better design of vaccines and vaccination regimens but also for understanding the factors that govern $CD8^+$ T cell memory formation.

While most studies have focused on the dose and persistence of antigen as determinants of $CD8^+$ T cell activation and differentiation, there is considerable evidence from several groups suggesting that the $CD8^+$ T cell's potential to develop into effectors is influenced by the presence of inflammatory cytokines (IL-12, type-1 interferons) and the timing of naïve $CD8^+$ T cell priming (early or late in acute infection).

Recent studies by Harty and colleagues (76) have shown that coinjection of CpG ODNs (a potent inducer of inflammation) and an *in vitro* matured peptide-loaded DCs caused massive expansion of Ag-specific $CD8^+$ T cells and their delayed conversion to memory phenotype. In agreement with these results, using *in vitro* human model for $CD8^+$ T cell differentiation, we have found that induction of cytolytic function and tissue-homing ability required mature,

polarized DCs with the ability to produce high amounts of IL-12 family member cytokines. In contrast to the above scenario, we have also found that direct differentiation of naïve $CD8^+$ T cells into memory cells occurred when DCs were matured but chronically activated and characterized by low production of cytokines belonging to IL-12 family. In accordance with our results, studies from murine models (48, 54-56) also suggest that manipulations to decrease the duration of infection and thus cause reduction in the inflammatory milieu, favor accelerated formation of memory cells. Immunization with *in vitro*-matured peptide-loaded DCs alone causes rapid formation of memory cells. Thus the collective theme is that the presence or absence of inflammatory signals affects the differentiation of naive $CD8^+$ T cells.

Together these studies argue for the revision of the existing models of $CD8^+$ T cell differentiation. In the currently proposed model (**Fig 6.2**), the decision making will depend on the cumulative strength of three signals- TCR, costimulation and inflammatory factors or cytokines. While TCR trigger and costimulation are sufficient to drive T cell proliferation, inflammatory signals are required for acquisition of effector and tissue homing functions. Thus at low signal strength and the absence of inflammatory signals, the potential of the naïve $CD8^+$ T cell to develop into a memory cell is greatest. By contrast, at high signal strength combined with inflammatory signals, there is maximum potential for naïve cells to differentiate into effector T cells. During natural infections, the strength of inflammatory signal is related to the stage of infection. Early stages of infection are characterized by substantial amounts of PAMP-induced inflammatory mediators while waning stages of infection are associated with diminishing levels of inflammation. Moreover, DC populations vary greatly in secondary lymphoid tissues during infection (173, 174) and thus the combination of DC-derived costimulatory signals and inflammatory cytokines will qualitatively differ during the course of infection.

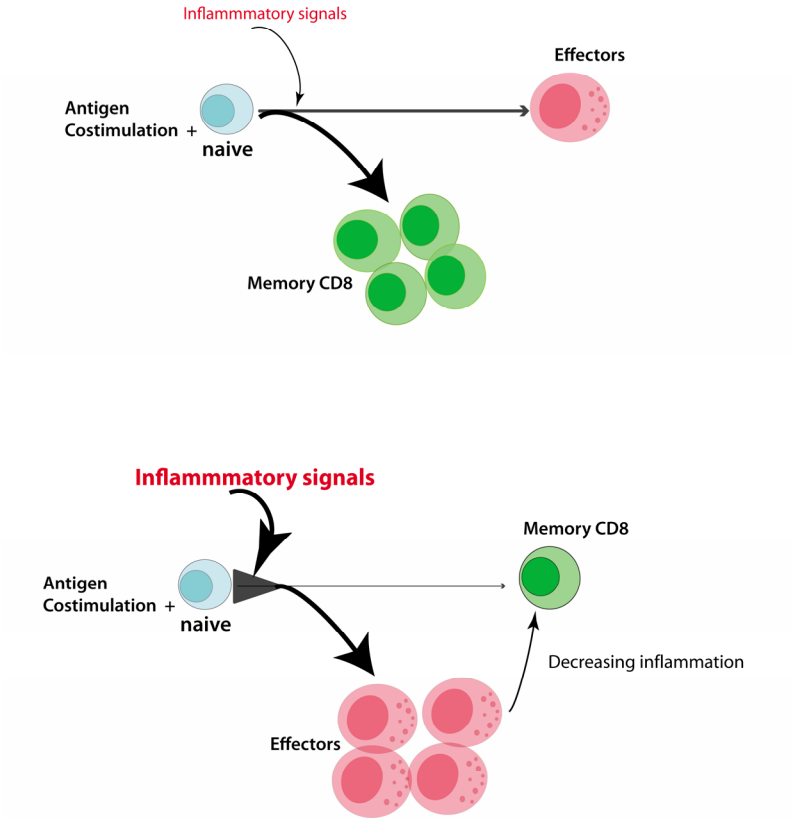

Figure 6.2 Emerging model of CD8$^+$ T cell differentiation based on the role of inflammatory cytokines

In the presence of both infection and inflammatory cytokines (during the early phases of primary immune response), there is a greater potential for naïve CD8$^+$ T cells to differentiate into effectors (thickness of the arrow denotes both increasing potential and inflammatory signals). In the absence or decreasing levels of inflammatory signals, the potential for naïve CD8$^+$ T cells to develop into memory cells is greatest.

We envisage a scenario in which naïve T cells recruited during early stages of infection will receive strong inflammatory signals and develop into effectors while those recruited in the later stages of infection could receive below-maximal stimulatory and inflammatory signals from exhausted DCs and thus be predisposed to give rise to long-lived memory cells (126).

In summary, existing and emerging data from human and mouse models of infection suggest that its possible to selectively prime either memory or effector cells depending on the type of vaccination– prophylactic or therapeutic, by increasing or limiting DC activation and production of polarizing cytokines. The challenge will be to devise vaccination regimens such that the efficacy of successive booster immunizations is not compromised by the phenomena of CTL-mediated killing of Ag-presenting DCs.

APPENDIX A: SUPPLEMENTARY FIGURES FOR CHAPTER 3

S 1a

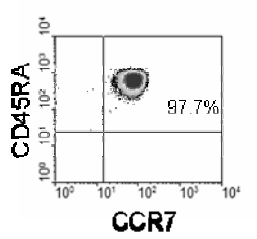

Figure S 1a. Co-expression of CD45RA and CCR7 on peripheral blood-isolated naïve CD8$^+$ T cells. Phenotype of naïve CD8$^+$ T cells isolated by negative selection.

S 1b

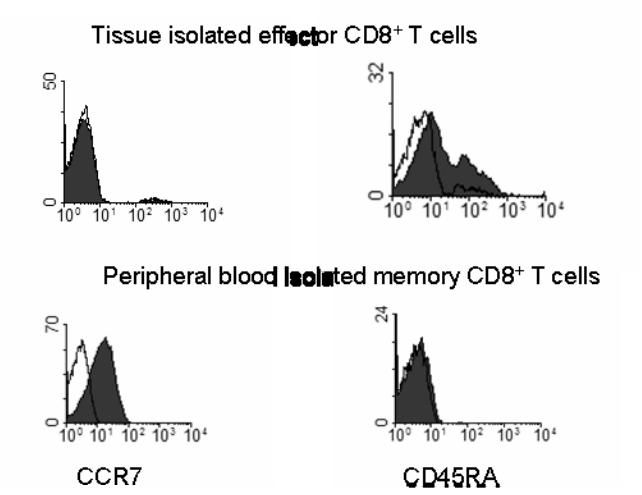

Figure S 1b. Characterization of tissue-isolated effector CD8$^+$ T cells and peripheral blood derived CD8$^+$ T cells. Effector CD8$^+$ T cells from tissue were CCR7$^-$CD45RA$^{+/-}$ while memory CD8$^+$ T cells from peripheral blood were CCR7$^+$CD45RA$^-$.

S 2

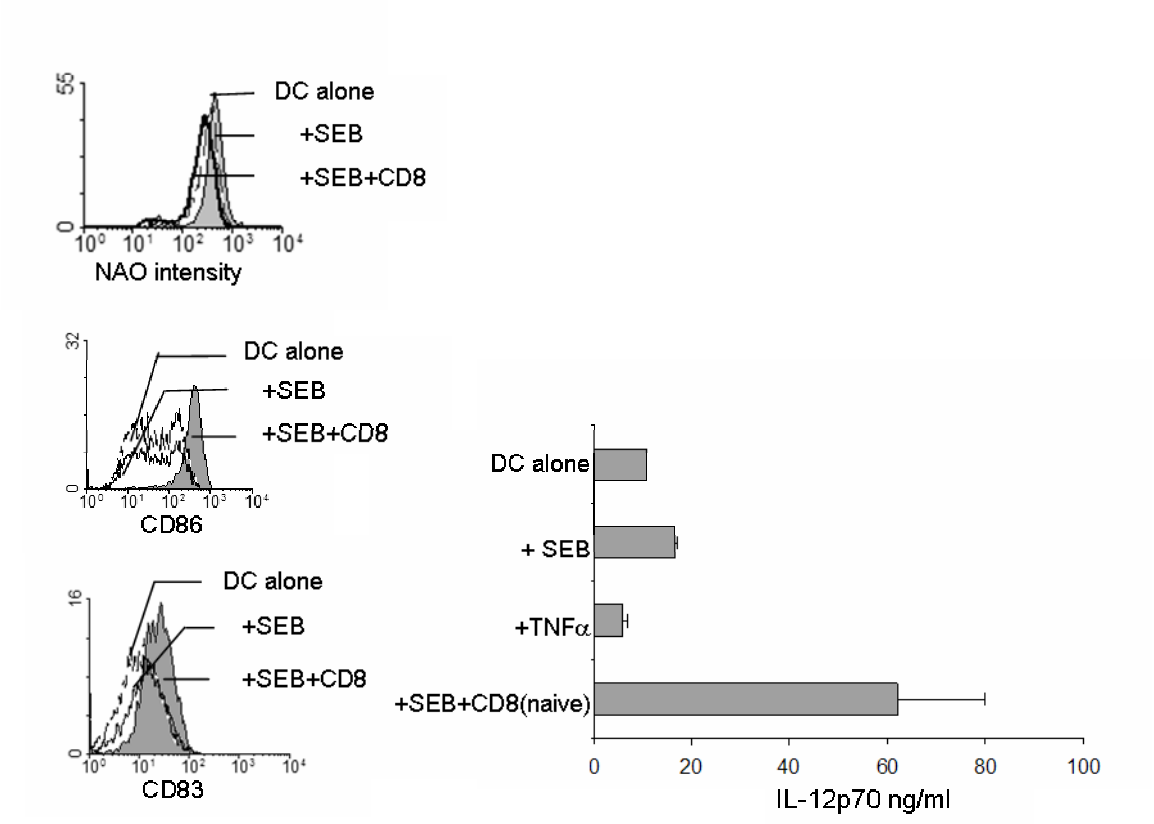

Figure S 2. Naïve CD8$^+$ T cells do not kill DCs but activate and induce maturation.

Blood-isolated naïve CD8$^+$ T cells induced DC maturation and primed them for high IL-12p70 production. SEB-pulsed immature DCs (day 6) were co-incubated with blood-derived naive CD8$^+$ T cells for 48 hours. Subsequently, DCs were harvested, their viability was assessed by staining with NAO and activation phenotype was analyzed by flow cytometry for the expression of co-stimulatory molecule (CD86) and maturation-associated marker (CD83). The DCs were also counted and stimulated with J588-CD40L to analyze their ability to produce IL-12p70.

S 3a

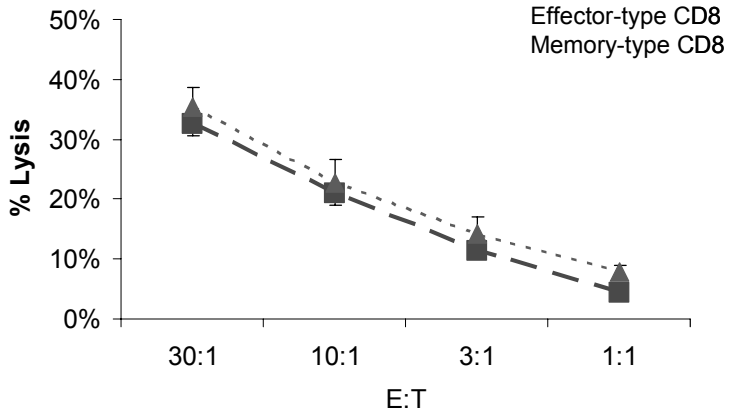

Figure S 3a. Both effector and memory type- CD8$^+$ T cells kill SEB-loaded tumor cells.

Effector-type and memory-type CD8$^+$ T cells were co-incubated for 4 hours with ^{51}Cr labeled JY-1 cells (standard Cr-release assay).

S 3b

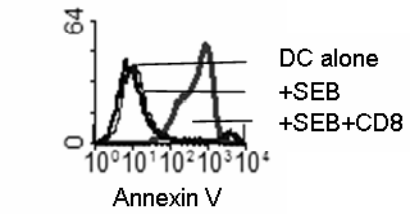

Figure S 3b. Effector-type CD8$^+$ T cells kill immature DCs as shown by Annexin V staining.

Effector-type CD8$^+$ T cells were co-incubated with SEB pulsed immature DCs (day 6) for 18-20 hours. Subsequently DCs were harvested, and their viability was assessed by Annexin V staining.

S 3c

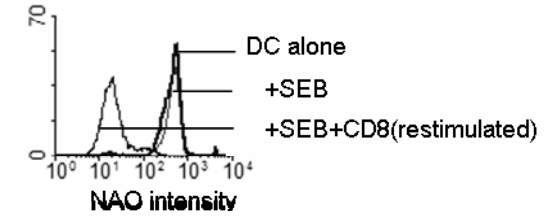

Figure S 3c. Short time restimulation of memory-type CD8$^+$ T cells results in re-acquisition of the ability to kill immature DCs. Memory-type CD8$^+$ T cells (day 14-16, no longer displaying DC killing function) were re-

stimulated (48 hours) and co-incubated with SEB-pulsed immature DCs (day 6) for 18-20 hours. Subsequently, DCs were harvested and their viability was assessed by NAO staining.

S 4a

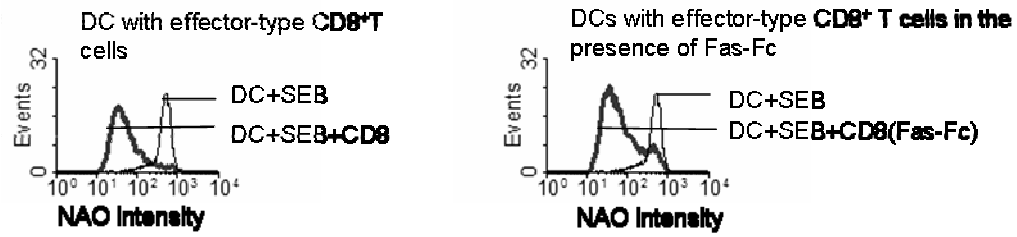

Figure S 4a. Fas-FasL interaction is not involved in CTL mediated DC killing.

The presence of FasL antagonist (Fas-Fc) during co-culture of effector-type $CD8^+$ T cells with immature DCs did not inhibit the killing of DCs as shown by NAO staining.

S 4b

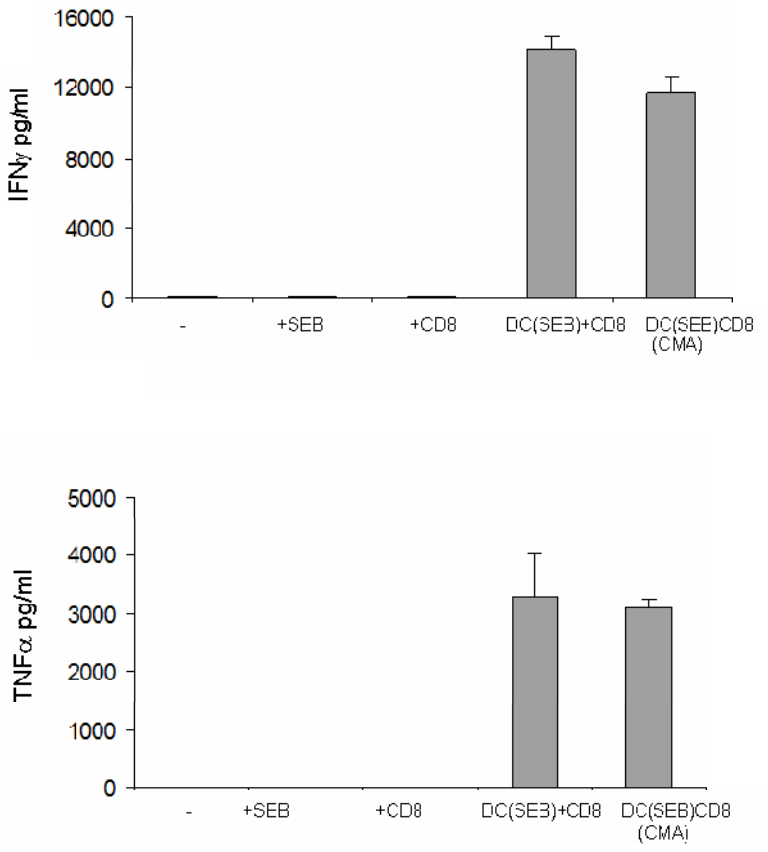

Figure S 4b. Unperturbed secretion of IFNγ and TNFα by effector-type $CD8^+$ T cells, pre-treated with the perforin inhibitor CMA. Effector-type $CD8^+$ T cells were pretreated with CMA and co-cultured with SEB-loaded DCs for 24-32 hours. Supernatants of CD8-DC co-culture were analyzed for cytokines IFNγ and TNFα by ELISA.

S 5

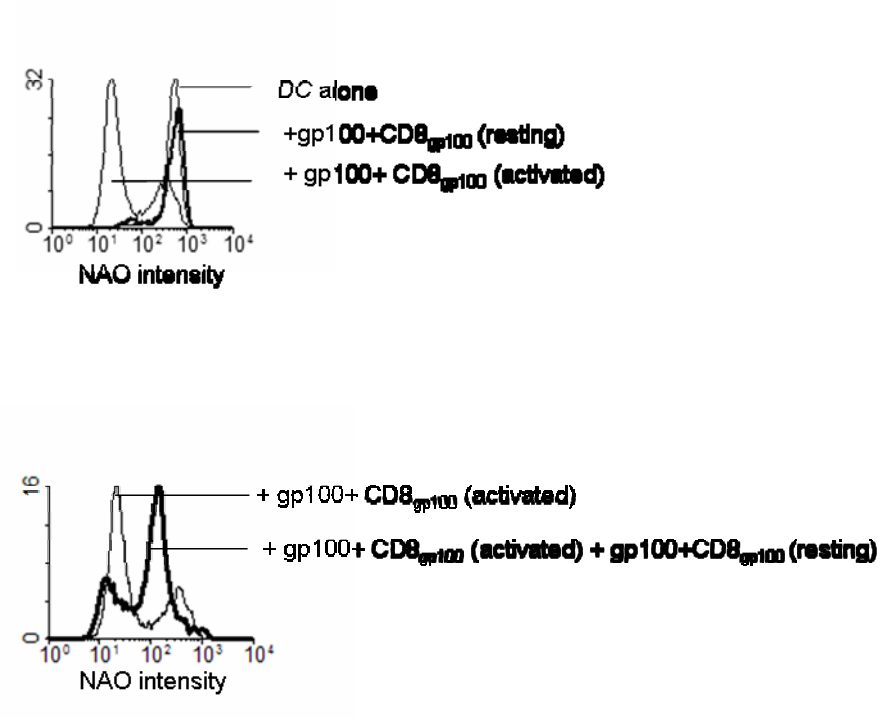

Figure S5. Class I-restricted melanoma (gp100)-specific CD8$^+$ T cells protect DCs from being killed by activated gp100-specific CD8$^+$ T cells. Resting HLA-A2 restricted gp100-specific CD8$^+$ T cells were co-cultured with immature DCs loaded with gp100 peptide for 8-10 hours, followed by the addition of activated gp100-specific CD8$^+$ T cells. DC viability was assessed by staining with NAO.

APPENDIX B: SUPPLEMENTARY FIGURES FOR CHAPTER 4

S 6

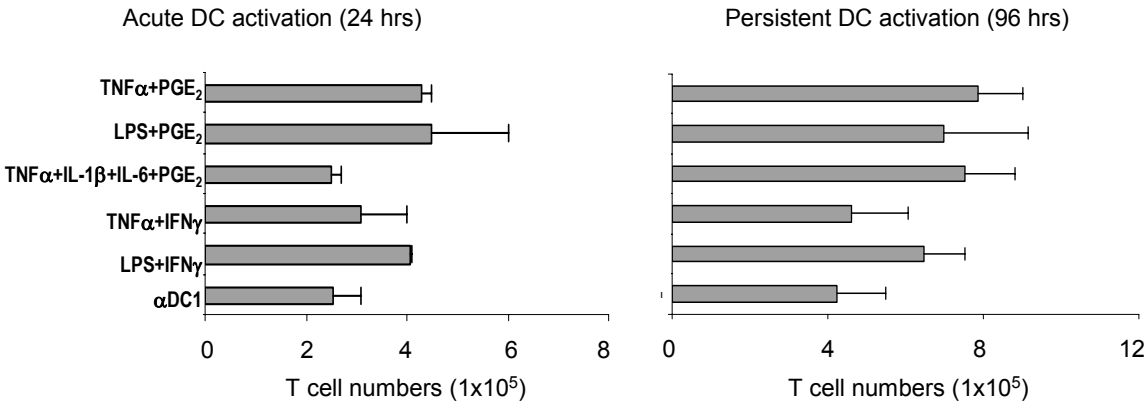

Figure S 6 CD8$^+$ T cell proliferation induced by short-time activated DC (24 hours) versus DCs activated for prolonged time period (72 hours)

S 7

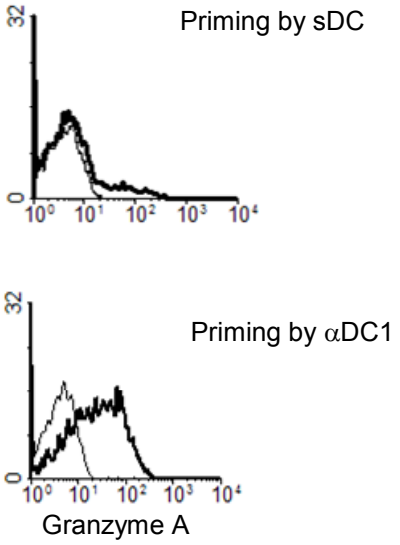

Figure S 7 Intracellular expression of Granzyme A in CD8$^+$ T cells primed by sDC and αDC1.

S 8

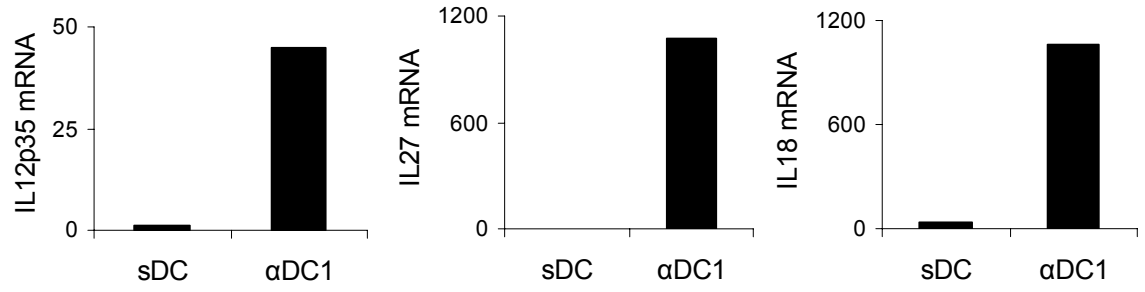

Figure S 7 Quantitative RT-PCR for IL-12p35, IL-27 and IL-18 genes in αDC1s and standard DCs

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