The function of CD27 costimulation in the activation and fate decisions of $CD8^+$ T cells

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Abstract

$CD8^+$ cytotoxic T lymphocytes are critical components of adaptive immunity against a variety of intracellular pathogens, and can play a key role in the control of tumors. Effective vaccination strategies against viral infections and tumors will likely require the development of potent $CD8^+$ T cell responses, which are constituted by the expansion of robust primary $CD8^+$ T cell populations and the establishment of long-lasting memory. Fully functional $CD8^+$ T cell responses are highly dependent upon $CD4^+$ helper T cells and Signal 3 inflammatory cytokine pathways. $CD4^+$ T cells have been demonstrated to play a critical role in inducing the expression of CD70, the ligand for CD27, on dendritic cells. However, it is not clear to what extent the 'help' provided by $CD4^+$ T cells is manifest via CD70, or how CD70-mediated stimulation of $CD8^+$ T cells is integrated with signals that emanate from Signal 3 pathways, such as type-1 interferon (IFN-1) and IL-12. In this work, by enforcing or abrogating CD27 function by genetic or protein intervention in murine models, we sought to identify the function of CD27 costimulation in the activation and fate decisions of $CD8^+$ T cells, to determine the extent it resembles $CD4^+$ T cell help, and how inflammation impacts the relative importance of CD70-CD27 interactions in $CD8^+$ T cell primary responses and $CD8^+$ T cell memory. Both subunit vaccine and replicating/non-replicating viral infection settings have been used to facilitate our comprehensive understanding of the role of CD27 costimulation in $CD8^+$ T cell responses, which has been previously complicated by its variable requirement during different stages of $CD8^+$ T cell responses, depending on the nature of the immunogen. We have demonstrated that in the low inflammatory setting of subunit vaccines, CD27 costimulation synergizes with IFN-1 at the level of $CD8^+$ T cells to achieve robust primary $CD8^+$ T cell responses; while in the high inflammatory setting of

replicating viral infection, CD27 costimulation antagonizes with impact of IL-12 to promote $CD8^+$ T cell memory. In addition, we identify CD70-CD27 interactions as one main downstream functional consequence of $CD4^+$ T cell help. Mechanistically, we demonstrate that CD27 costimulation supports $CD8^+$ T cell responses in part by modulating the expression of receptors for 'fate-decision' cytokines, including IL-7, IL-12 and likely IL-2. Particularly, IL-7Rα has been identified as a functional marker for memory precursor effector cells, and our data indicate that CD27 signals promote the re-expression of IL-7Rα on effector $CD8^+$ T cells via mRNA regulation. Furthermore, CD27 costimulation strongly induces transcription factor Eomesodermin (Eomes), which is a main contributor of effector function and memory differentiation. Based on this new information, we propose that the interplay between CD27 costimulatory and inflammatory cytokine pathways leads to delicate regulation of transcription factor pair Eomes and T-bet, a critical axis for the activation and fate decisions of $CD8^+$ T cells. In summary, our work has 1) identified complex interactions between Signal 3 and costimulatory pathways, 2) provided insights into the mechanistic basis by which CD27 costimulation influences $CD8^+$ T cell activation and fate decisions, 3) demonstrated a previously unappreciated role of CD27 costimulation as a positive regulator of IL-7Rα during CD8 T cell responses, and 4) highlighted the potential of targeting CD27-CD70 axis for antiviral/antitumor immunotherapy.

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List of Abbreviations

Ag, antigen;

APC, antigen-presenting cell;

α27 or αCD27, agonistic anti-CD27 monoclonal antibody;

BLIMP1, B Lymphocyte induced maturation protein 1;

B6, C57Bl/6;

cIg, control IgG;

CTL, cytotoxic T lymphocyte;

CTV, cell trace violet;

BMDC, bone marrow dendritic cell;

DAPI, 4',6-Diamidino-2-Phenylindole;

DC, dendritic cell;

Eomes, Eomesodermin;

FR70, antogonistic anti-CD70 monoclonal antibody

IFN-1, type-1 interferon;

IL-7, interleukin 7;

i.p., intraperitoneal;

i.v., intravenous;

LCMV, lymphocytic choriomeningitis virus;

MHC II, major histocompatibility complex, class II;

MPEC, memory precursor effector cells;

KLRG1, Killer cell lectin-like receptor subfamily G member 1;

OT-1, OVA-specific transgenic $CD8^+$ T cells

OVA, ovalbumin;

OVA-adeno, recombinant adenovirus expressing OVA;

OVA-vac, recombinant vaccinia virus expressing OVA;

PD-1, program death 1

Poly I:C, polyinosinic: polycytidylic acid;

SLEC, short-lived effector cells;

T-bet, T-box expressed in T cells;

T_{em}: effector memory T cells;

T_{cm}: central memory T cells;

TCR: T cell receptor;

TGF-β, tumor growth factor β;

TLR, Toll-like receptor;

TNFR, tumor necrosis factor receptor;

WT, wild-type.

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曰：善始善终。奈何潦草收尾，诸多遗憾。踉跄至终点，只觉掌声刺耳，前路晦暗。感谢父母和小弟无条件的爱与关怀，感谢友人们始终如一的鼓励与支持。一生不过十个六年，感谢自己接纳现下十分之一的失败并保持优雅。 岁月荏苒，莫忘初心。但行好事，莫问前程！

谨以此文纪念挚友王峥。

Chapter 1. Introduction

$CD8^+$ cytotoxic T lymphocytes (CTLs) are critical components of adaptive immunity against a variety of intracellular pathogens, and can play a key role in the control of tumors. Effective vaccination strategies against viral infections and tumors will likely require the development of potent $CD8^+$ T cells, which constitutes the expansion of robust primary $CD8^+$ T cell populations and the establishment of long-lasting memory. By defining the cellular and molecular determinants of naïve $CD8^+$ T cell activation, expansion and differentiation into either effectors or long-lived poly-functional memory cells, it can be anticipated that vaccine efficacy will be improved, and that preventive and therapeutic vaccines may be tailored according to the type of protection that is needed. In this chapter we focus on the role of $CD4^+$ T cell help both for primary $CD8^+$ T cell responses and for programming of $CD8^+$ T cell memory, and critically analyze existing data bearing on the question of CD70 involvement in the $CD4^+$ T cell help.. Further, we discuss the role of inflammatory cytokines in $CD8^+$ T cell responses, and consider how inflammation impacts the relative importance of CD70 in $CD8^+$ T cell primary responses and $CD8^+$ T cell memory. These together have motivated the studies in the following three chapters.

1.1 Definition of the stages of a $CD8^+$ T cell response [1, 2]

- **Primary response:** after engagement with antigen-presenting cells (APCs), naïve $CD8^+$ T cells are activated and start rapid proliferation and differentiation into functional CTLs, killing target cells by secreting cytokines and cell-lytic molecules such as granzymes.
- **Contraction and quiescent memory:** Following pathogen clearance, the majority of CTLs die via apoptosis during contraction stage, while ~5-10% of them are

maintained stably as long-lived memory cells largely via IL-7/IL-15-dependent homeostatic proliferation.

- **Secondary response:** once reinfection occurs, these quiescent memory $CD8^+$ T cells will rapidly proliferate and differentiate into functional secondary effectors and protect the host from severe infection. The secondary response has quantitative and qualitative differences from the primary response, which are attributed to the distinct characteristics of memory $CD8^+$ T cells from naïve ones.

1.2 Signal 1, 2 and 3 to induce $CD8^+$ T cell responses

To efficiently induce a primary $CD8^+$ T cell response and potentiate $CD8^+$ T cell memory, 3 signals need to be transferred to naïve $CD8^+$ T cells.

- Ag stimulation represents the first signal (**Signal 1**), which occurs through the interaction of specific T cell receptors (TCR) with pathogen-derived peptide-MHC class I complexes on APCs and initiates naïve $CD8^+$ T cell activation.
- Besides signal 1, a second signal (**Signal 2**) – co-stimulation by APC cell-surface molecules CD80/CD86 is passed on to naïve $CD8^+$ T cells by CD28 ligation and is necessary for their further activation and proliferation.
- However, optimal clonal expansion and specific differentiation of responding $CD8^+$ T cells in most cases cannot be efficiently induced by just signal 1 and 2. During and after initial activation, $CD8^+$ T cells receive a variety of stimuli elicited by immune cells that influence the programming of survival/proliferation, effector function and differentiation, which can be referred to as the third signal (**Signal 3**).

1.3 Signal 3 for $CD8^+$ T cell responses

Some Signal 3 stimuli are derived from the sensing and response to pathogenic infection, sensed by Toll-like receptors (TLRs) and other pattern-recognition molecules,

and are manifested in the form of inflammatory cytokines such as IL-12, type I IFN (IFN-1), IFN-γ and IL-6. In particular, **IFN-1** and **IL-12** have been described as classic signal 3 stimuli that promote effector expansion and function and direct cell differentiation. Other Signal 3 stimuli that influence $CD8^+$ T cell fate are derived from components of adaptive immunity, and are primarily orchestrated by **helper $CD4^+$ T cells**. These are exemplified by the impact of CD40L-mediated stimulation, and the provision of growth and differentiation factors such as IL-2 and IL-21.

It is thought that the variations in timing and interactivity of Signal 3 stimuli during primary $CD8^+$ T cell responses lead to diverse intrinsic signals, including induction of transcriptional factors such as T-bet and Eomesodermin (Eomes), which contribute to the heterogeneity of effector $CD8^+$ cell responses and thus 'program' cell differentiation and fate decision. However, our knowledge of the interactivity of distinct Signal 3 molecules and their regulation of $CD8^+$ T cell responses to date is relatively limited.

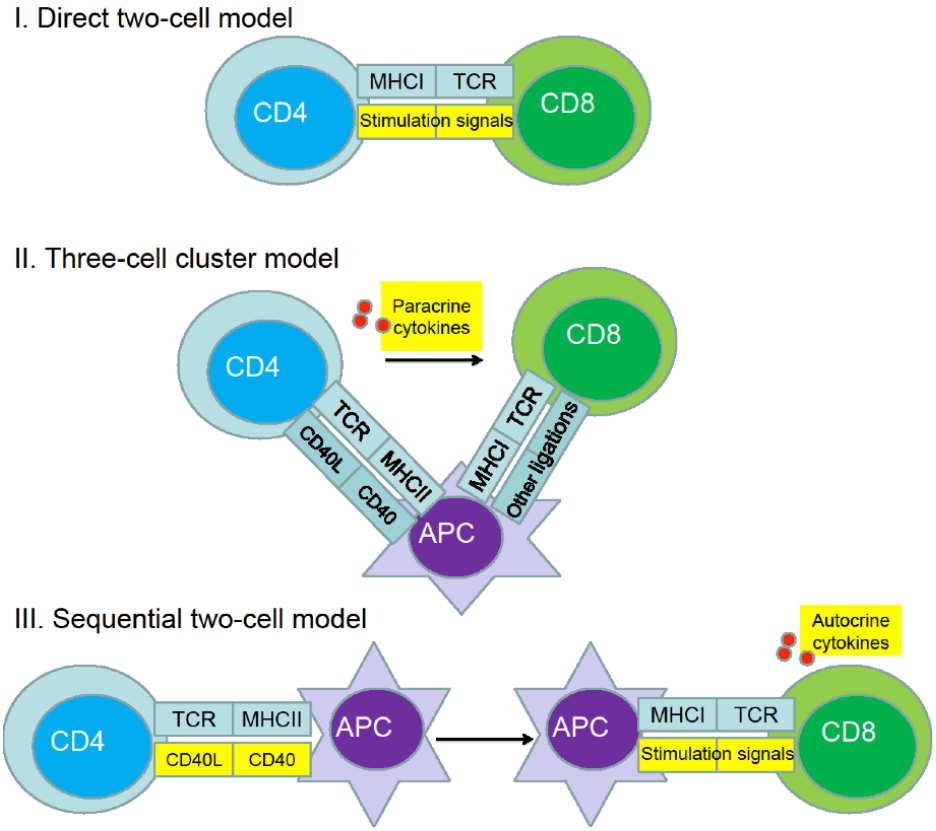
1.4 $CD4^+$ T cell help for $CD8^+$ T cell responses

$CD4^+$ helper T cells have been shown to play an important role in eliciting many primary $CD8^+$ T cell responses[3-6]. Despite quite intensive studies at this point in time, the mechanisms by which $CD4^+$ helper T cell promotes $CD8^+$ T cell responses are not fully understood, and a long-standing controversy in this process has focused on the sequence and nature of the cellular interactions through which help is provided.

Some studies[7, 8] indicate that direct stimulation of $CD8^+$ T cells by $CD4^+$ T cells can enhance $CD8^+$ T cell activation and survival, supporting the **direct two-cell model** (Figure 1.1 Model I). Alternatively, some other studies[9, 10] suggest a **three-cell cluster model** wherein a $CD4^+$ T cell helps a $CD8^+$ T cell via paracrine secretion of growth and differentiation factors, such as IL-2[5, 11-14]and IL-21[15-17], in a three-cell cluster with an APC

(Figure 1.1 Model II). Of note, $CD4^+$ T cells also play a key role in up-regulating the activation state ('licensing') of dendritic cells (DC, the professional APC) to support $CD8^+$ T cell responses, predominantly via CD40L-CD40 interactions. Evidence from our lab[18] and others[7, 9, 19] demonstrates that direct stimulation of CD40 on DC overcomes the necessity of $CD4^+$ T cells for the generation of primary $CD8^+$ T cell responses, thereby supporting the **sequential two-cell model** (Figure 1.1 Model III). Nevertheless, it is of particular significance to note that these models are not mutually exclusive, and in fact one would anticipate a mixture of these mechanisms occurs based on the versatile role and longitudinal requirement[20] of $CD4^+$ helper T cells in $CD8^+$ T cell activation and fate decisions.

Figure 1.1 Models for $CD4^+$ helper T cell-dependent $CD8^+$ T cell responses.



1.5 Do $CD4^+$ T cells send 'help' signal via induction of CD70 on DC?

What are the help signals transmitted by $CD4^+$ T cell-'licensed' DC to $CD8^+$ T cells? Studies from our lab[18] and others[21-24] have demonstrate that CD40-stimulated DC up-regulate the expression of the TNF-superfamily member CD70, the ligand for CD27. CD40-stimulated bone marrow-derived DC (BMDC) or subunit vaccines that utilize agonistic CD40 antibodies can bypass the necessity of $CD4^+$ helper T cells to elicit competent primary $CD8^+$ T cell responses, and these responses are potently reduced by blockade of CD70. These data support a prominent role for CD70 induction in a 'licensed' DC. Furthermore, by utilizing antagonistic CD70 antibodies in adenovirus- or DC-immunized CD40-deficient mice, our recent study[25] has revealed a critical role of CD70 expression by DC in the immunogenicity of $CD4^+$ T cell-dependent, CD40-independent $CD8^+$ T cell responses. Taken together, these data indicate that a main consequence of $CD4^+$ T cell-mediated 'licensing' of DC is the induction of CD70, and support the hypothesis that the $CD4^+$ T cell help signal is transmitted from DC to $CD8^+$ T cells via CD27-CD70 interactions.

1.6 Role of CD27 costimulation in primary $CD8^+$ T cell responses

CD27 is expressed on naive T cells and early thymocytes, as well as subsets of NK cells and B cells. Activity of CD27 is primarily governed by the transient availability of its ligand CD70 on DC or lymphocytes. CD27, with its closely related TNF receptor superfamily members like 4-1BB (CD137), CD30, herpes virus entry mediator (HVEM), OX40 (CD134), and glucocorticoid-induced TNFR family-related gene (GITR), accounts for an important costimulatory molecule besides the best characterized costimulatory receptor CD28[26, 27].

However distinct from CD28 signal that mainly impacts cell proliferation, CD27 costimulation has been reported to primarily promote CTL survival throughout clonal expansion – mechanisms of which include up-regulation of anti-apoptotic Bcl-2 family m[28]embers[28], induction of Pim-1 kinase-mediated survival pathway[29], up-regulation of CD25 and induction of sustained IL-2[3031], and preventing activation-induced and Fas-dependent apoptosis[32].

By conferring pro-survival effects, CD27 costimulation by CD70 supports effector cell generation against multiple viral and bacterial infections. For instance, by abrogating CD27 signals by utilizing antagonistic CD70 antibodies or genetic models with CD70 or CD27 deficiency, many studies have revealed key contributions of CD27-CD70 costimulatory axis to primary $CD8^+$ T cell responses during recombinant adenovirus, vaccinia virus, lymphocytic choriomeningitis virus, influenza virus, vesicular stomatitis virus and Listeria monocytogenes infections[25, 33-36]. Reciprocally, the enforcement of CD27 signals by transgenic expression of CD70 on immature DC or by utilizing soluble recombinant CD70 has been shown to break $CD8^+$ T cell tolerance or allow strong induction of effector $CD8^+$ T cells to peptide immunization [37-39]. These studies in fact suggest that CD27 costimualtion may suffice to drive primary $CD8^+$ T cell responses along with Ag signal.

1.7 CD70 versus inflammation

Most primary $CD8^+$ T cell responses to viral infections are not only $CD4^+$ helper T cell-dependent, but also require soluble inflammatory cytokines downstream of innate receptors. Co-targeting CD40 and TLR has been identified as a vigorous strategy for subunit vaccination, where the strong primary $CD8^+$ T cell responses are promoted by CD70[24, 40] and IFN-1[23]. Intriguingly, while the expression of CD70 is primarily induced by

stimulation of CD40, it can be strongly enhanced by concurrent stimulation of TLRs and signaling via IFNαβ receptors[18, 24, 25, 41], raising the question of whether the contribution of TLR/IFN-1 to $CD8^+$ T cell responses could be manifested mainly via optimized expression of CD70 on the DC. Furthermore, if CD70 is a nexus for Signal 3 pathways derived from both $CD4^+$ helper T cells and innate immunity, the finding would suggest that strong stimulation of CD27 may be capable to bypass the necessity for $CD4^+$ T cell help and inflammatory signals in the generation of primary $CD8^+$ T cell responses.

In contrast, there is considerable evidence that supports key contributions of inflammation to CTL expansion and differentiation beyond up-regulation of CD70 on DC. TLR stimulation induces the production of inflammatory cytokines including the classic Signal 3 molecules IFN-1 and IL-12. IFN-1 was found to act directly on $CD8^+$ T cells to allow clonal expansion and memory differentiation[42, 43], while IL-12 has been shown by multiple infection models to support $CD8^+$ T cell expansion and direct their differentiation into KLRG1-expressing short-lived effector cells via the induction of transcription factor T-bet [44-48]. Of note, distinct from CD28 costimulation (Signal 2), the requirement of CD27 costimulation in $CD8^+$ T cell primary responses was found to vary depending on the nature of the immunogen[34]. Our preliminary studies suggested that in response to non-replicating, less inflammatory immunogens (e.g. recombinant adenovirus), CD27 costimulation was required to drive a robust primary $CD8^+$ T cell response, whereas in models of infection with substantial Ag strength/duration and inflammation (e.g. vaccinia infection), lack of CD27 costimulation during priming and effector clonal expansion did not necessarily lead to a severe impairment of primary $CD8^+$ T cell response. These findings suggest that there is some potential redundancy between inflammation and CD27 costimulation in the generation of primary $CD8^+$ T cell responses. Moreover,

regardless of their potential overlapping role in regulating the quantity of the primary $CD8^+$ T cell response, it is of particular interest to identify whether CD27 costimulatory and inflammatory cytokine pathways impact diversely on the functional quality of those $CD8^+$ T cells.

1.8 Rationale and proposal for the thesis project in Chapter 2

Although the necessity of CD27 costimulatory signal in $CD4^+$ T cell help for primary $CD8^+$ T cell responses has been demonstrated by extensive studies, whether it is sufficient to drive effective induction of CTLs has not been well established. Furthermore, despite reasonable evidence interpreting the primary $CD8^+$ T cell responses as an integrated outcome from CD27 costimulatory and soluble inflammatory signals, to date the relative contributions and their interplay in the generation of primary $CD8^+$ T cell responses has not been addressed. In addition, distinct from inflammatory signals, little is known about the contributions of CD27 costimulation to $CD8^+$ T cell effector function and differentiation besides its relatively well-studied pro-survival role. We sought to address these questions in Chapter 2, where we restricted $CD4^+$ T cell help (thus limiting natural CD70 expression) and inflammation in the protein immunization model, and assessed the differential impact of CD27 costimulation and inflammation on primary $CD8^+$ T cell responses by applying agonistic CD27 antibodies and recombinant cytokine treatment alone and in combination. Our findings have revealed complex interactions between inflammatory and costimulatory pathways, and highlighted opportunities to influence the differentiation of $CD8^+$ T cell responses.

1.9 $CD8^+$ T cell memory differentiation

The concepts of the induction and maintenance of immunologic memory have governed the design of highly effective preventative vaccines, although the underlying

mechanisms by which memory develops and is sustained remain poorly defined. The formation of immunological memory is a cardinal character of adaptive immunity, by which an organism recognizes an original pathogen that it encountered before, and exerts more robust humoral and cellular responses to control the infection[2]. A potent primary $CD8^+$ T cell response is often a prerequisite yet not a guarantee for the generation of competent memory and prominent secondary response. Primary CTLs go through memory differentiation – a program which effector $CD8^+$ T cells die after clearance of pathogens, and which ones are sustained during the contraction stage and develop into long-term memory cells. During the differentiation, memory $CD8^+$ T cells attain not only quantitative but also qualitative differences from naïve populations.

Three possible models account for $CD8^+$ T cell memory differentiation[49] (Figure 2.1). The **linear differentiation model** proposes that stimulation of naïve $CD8^+$ T cells with Ag results in the generation of effector T cells, which then either become short-lived effector cells (SLECs) with little capacity to survive long-term, or differentiate into memory precursor effector cells (MPECs) with a greater capacity for survival. In the **bifurcative differentiation model**, the memory precursors split from primary effectors at early stages of primary responses and develop as a parallel population. Further, the so-called "**self-renewing effector model**" suggests that a naïve T cell can develop into a memory cell that can self-renew upon Ag ligation, and that the effectors are derived from these stem cell-like cells. Recent data indicate that naïve $CD8^+$ T cells have the capacity to form either effector or memory cells[50], and that at least some memory cells show evidence of previous effector activity[51], supporting the linear differentiation model.

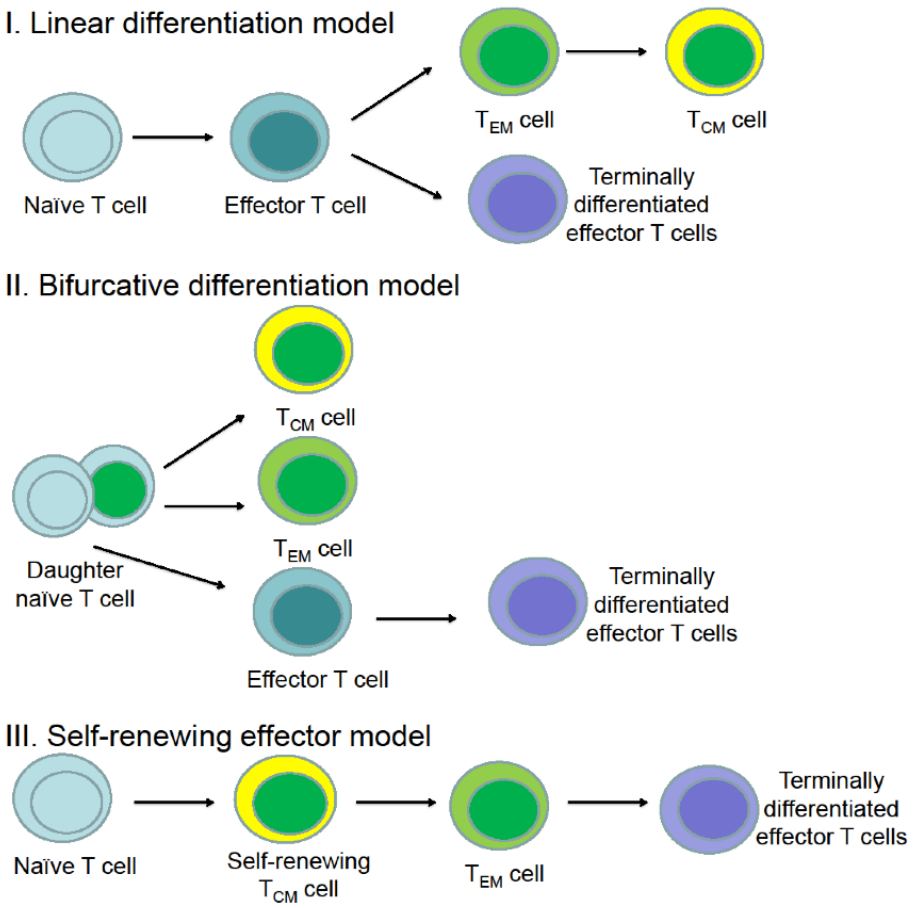
The development of more effective vaccine regimens requires the knowledge on the factors that control memory differentiation of primary CTLs are of particular interest.

Multiple layers of extrinsic and intrinsic signals have been proposed in this cell fate-determining process, including **inflammatory cytokines**, **CD4$^+$ T cell help**, transcriptional regulations[52, 53], metabolic switches, and uneven segregation of lineage-determining factors[2].

1.10 IL-7Rα and MPEC differentiation

IL-7/IL-7R-mediated signal is particularly critical for the long-term survival of primary CD8$^+$ T cells during the contraction phase[1]. IL-7 is a member of the common γ-chain (γc) family of type I cytokines, and signaling through the receptor occurs via heterodimerization of the γc and IL-7Rα[54]. Naïve CD8$^+$ T cells constitutively express IL-7Rα, and the down-regulation occurs upon TCR-mediated activation[55]. MPECs are enriched in a small subset of effector cells that regains expression of IL-7Rα (referred to IL-7Rα^{hi}) at the peak of primary CD8$^+$ T cell responses, while most IL-7Rα^{lo} effector CD8$^+$ T cells express KLRG1, are frequently terminally differentiated and disappear during the contraction phase[2, 44, 56]. IL-7Rα has been demonstrated as necessary yet not sufficient for the long-term survival of primary CD8$^+$ T cells and is regarded as a defining marker of MPECs, however the factors that regulate its re-expression on MPECs are not yet clear.

Figure 1.2[49] Models of T cell memory differentiation, *revised from Ahmed et al, Nature 2009*. $T_{CM,}$ central memory T cells; $T_{EM,}$ effector memory T cells.



1.11 Inflammation and SLEC differentiation

Along with Ag exposure (Signal 1) and CD28 constimulation (Signal 2), soluble inflammatory cytokines during priming, typically IL-12 and IFN-1, provide a Signal 3 to support $CD8^+$ T cell clonal expansion and development of effector function[2]. The amount of inflammation is also a critical determinant in the extent to which $CD8^+$ T cells differentiate into memory cells[57, 58]. For instance, IL-12 has been highlighted to direct SLECs and MPECs fate decision via alterations to transcriptional programming for $CD8^+$ T cells. IL-12 has been described as a strong promoter of $CD8^+$ T cell expansion, and promotes differentiation into KLRG1-expressing SLECs after LCMV, Listeria monocytogenes and Toxoplasma gondii infection[44, 46-48, 58]. However, the influence of IL-12 on $CD8^+$ T cell memory has remained controversial, with both positive and negative effects been observed. IL-12 was identified as being required for the programming of $CD8^+$ T cell memory in response to vaccinia infection[43]. Studies using IL-12 as vaccine adjuvant, however, reveal no effect of IL-12 on number of MPECs or memory cells despite a robust increase in the total number of $CD8^+$ effector T cells[47]. In fact, some studies even suggest that IL-12 may have a detrimental outcome on memory $CD8^+$ T cell differentiation[45, 46]. Consistent with this, differentiation into KLRG1-expressing SLECs is strongly enhanced by IL-12-driven induction of T-bet[44, 46], and genetically limiting T-bet induction can enhance $CD8^+$ T cell memory[44]. Besides up-regulation of T-bet, IL-12 in contrast down-regulates Eomesodermin (Eomes) [59], a transcription factor that acts to reciprocally promote self-renewal of Ag-specific memory cells versus terminal differentiation[60]. These findings have led to the hypothesis that IL-12-driven SLEC differentiation may come at the expense of MPECs and memory $CD8^+$ T cells.

1.12 $CD4^+$ T cell help for $CD8^+$ T cell memory

Not only do $CD4^+$ helper T cells play an important role in eliciting most primary $CD8^+$ T cell responses, but in many cases they also dictate the generation of memory $CD8^+$ T cells and secondary responses[61-65]. How $CD4^+$ T cells 'help' $CD8^+$ T cell memory is not well understood. $CD4^+$ T cells can direct stimulate $CD8^+$ T cells via CD40L-CD40 interactions and reduce activation-induced cell death in some studies[8] (**direct two-cell model,** Figure 1.1 Model I). In other studies, the help effects to $CD8^+$ T cell memory are dependent on IL-2 and IL-21, suggesting that $CD4^+$ T cells contribute to $CD8^+$ T cell memory via induction of paracrine cytokines (**three-cell cluster,** Figure 1.1 Model II). Alternatively, as a main consequence of $CD4^+$ T cell-mediated 'licensing' of DC is the induction of CD70, and we have shown that competent $CD8^+$ T cell memory can develop after immunization with CD40L activated DC in the absence of $CD4^+$ T cells[18]. Together, these observations support the concept that the CD27-CD70 interactions elicited by $CD4^+$ T cell-'licensed' DC induce a programming process of $CD8^+$ T cells that is sufficient to drive memory formation[18, 24, 25] (**sequential two-cell model,** Figure 1.2 Model III), and in this model, paracrine cytokines secreted by $CD4^+$ T cells may support, but are not indispensable for, $CD8^+$ T cell memory.

1.13 CD70 on DC = help signal from $CD4^+$ T cell for $CD8^+$ T cell memory?

To extend our previous hypothesis that the $CD4^+$ T cell help signal for primary $CD8^+$ T cell responses is transmitted from DC to $CD8^+$ T cells via CD27-CD70 interactions, secondary $CD8^+$ T cell responses to influenza and LCMV infection have been reported to be impaired in CD27-knockout mice[33, 66, 67] and by blocking CD70-CD27 interactions [32, 40]. Mechanistically, late signals from CD27 have been shown to prevent FasL-mediated cell death, suggesting CD27 costimulation may promote long-term survival of primary $CD8^+$ T cells through the contraction phase[32]. Besides, tonic CD27 stimulation

has recently been reported to regulate the self-renewal process of quiescent memory[68], implying CD27 costimulation may be involved in the regulation of memory $CD8^+$ T cells homeostatic proliferation. Furthermore, CD27 signaling has been demonstrated to induce autocrine IL-2 production of CTLs[30], while during priming IL-2 signals[11] as well as $CD4^+$ T cell help[63] are found to be crucial in driving secondary expansion of memory $CD8^+$ T cells. Importantly, in a recent study the contribution of $CD4^+$ T cells to $CD8^+$ T cell memory in response to vaccinia infection was shown to be mediated through their ability to activate APCs to a state in which they become able to endow the $CD8^+$ T cells they primed with the ability to produce autocrine IL-2[69]. This suggests that CD27 may be one of the APC-derived signals that 'imprint' $CD8^+$ T cells with the ability to produce autocrine IL-2 and therefore enhances the secondary population expansion of $CD8^+$ memory T cells. These data together reveal a significant role of CD27 costimulation for the generation of $CD8^+$ T cell memory and secondary responses, which is highly consistent with contributions of $CD4^+$ helper T cells, leading us to hypothesize that the defects in $CD8^+$ T cell memory that occur in the absence of $CD4^+$ helper T cells are a consequence of inadequate CD27 stimulation.

1.14 CD70 versus inflammation for $CD8^+$ T cell memory

A better understanding is needed for the underlying mechanisms by which CD27-CD70 costimulation promotes $CD8^+$ T cell memory. We believe this critical question is currently blurred by the variation in results from studies on the requirement of CD27 costimulation depending on the nature of the immunogen[34]. Suggested by studies from others[33, 66, 70] and our preliminary data, CD27 costimulaiton establishes key contributions to the programming and/or maintenance of memory $CD8^+$ T cells especially in cases with concurrent presence of high inflammation: in response to non-replicating, less

inflammatory immunogens (e.g. BMDC-based immunization), CD27 costimulation was required to drive a profound primary $CD8^+$ T cell response, yet was found dispensable for $CD8^+$ T cell memory; whereas in models of infection with substantial inflammation (e.g. vaccinia infection), while lack of CD27 costimulation during priming and effector clonal expansion did not necessarily lead to a severe impairment of primary $CD8^+$ T cell response, its absence could completely abolish $CD8^+$ T cell memory. Therefore, despite the potential redundancy/cooperation between CD70 and inflammatory cytokines in driving primary effector expansion, these two factors may govern antagonistic roles in memory differentiation.

1.15 Rationale and proposal for the thesis project in Chapter 3&4

A variety of Signal 3 stimuli interact and integrate in $CD8^+$ T cells, influencing multiple pathways that regulate the memory differentiation process. We hypothesize that $CD4^+$ helper T cells contribute to $CD8^+$ T cell memory mainly via up-regulation of CD70 on 'licensed' DC during priming, as the deficient $CD8^+$ T cell memory that occurs in the absence of CD27-CD70 interactions phenocopies the 'helpless' memory. If true, exogenous CD27 stimulation should bypass the necessity of $CD4^+$ helper T cells in the generation of $CD8^+$ T cell memory and secondary responses. Furthermore, despite its well-known pro-survival effect that may help sustain long-lasting memory, our knowledge on the contributions of CD27 costimulation to provide instructive signals for memory differentiation is limited. As IL-12 has been highlighted to direct SLECs and MPECs fate decision via altering the transcriptional programming for $CD8^+$ T cells, and IL-12 is dominant during vaccinia infection where $CD4^+$ T cell help is critical for $CD8^+$ T cell memory, it's of particular interest for us to determine whether and how this classic Signal 3 could interact with or be modulated by CD27 costimulation. For instance, whether

CD27 costimulation promotes IL-7Rα-expressing MPEC differentiation remains to be an open question. We sought to address these questions and test the corresponding hypothesis in Chapter 3. We have used a combination of agonistic or blocking antibodies and genetically deficient mice, in both high- and low-inflammatory immunization models, to determine the extent to which CD27 costimulation influences the fate decisions made by primary $CD8^+$ T cells. Because our results indicate that CD27 costimulation supports IL-7Rα-expressing MPECs, we proceeded in Chapter 4 the underlying cellular and molecular mechanisms of this regulation. These studies together provide insights into the mechanistic basis by which CD27 costimulation influences $CD8^+$ T cell memory differentiation, and highlight the potential of targeting CD27-CD70 axis for antiviral/antitumor immunotherapy.

Chapter 2: CD70 and IFN-1 selectively induce Eomesodermin or T-bet and synergize to promote CD8$^+$ T cell responses

2.1 Abstract

CD70-mediated stimulation of CD27 is an important cofactor of CD4$^+$ T cell licensed dendritic cells. However, it is unclear how CD70-mediated stimulation of T cells is integrated with signals that emanate from Signal 3 pathways, such as type-1 interferon (IFN-1) and IL-12. We find that while stimulation of CD27 in isolation drives weak Eomesodermin^{hi}T-bet^{lo} CD8$^+$ T cell responses to OVA immunization, profound synergistic expansion is achieved by co-targeting TLR. This co-operativity can substantially boost anti-viral CD8$^+$ T cell responses during acute infection. Concomitant stimulation of TLR significantly increases per-cell IFNγ-production and the proportion of the population with characteristics of short-lived effector cells, yet also promotes the ability to form long-lived memory. Notably, while IFN-1 contributes to the expression of CD70 on dendritic cells, the synergy between CD27 and TLR stimulation is instead dependent upon IFN-1's effect directly on CD8$^+$ T cells, and is associated with the increased expression of T-bet. Surprisingly, we find that IL-12 fails to synergize with CD27 stimulation to promote CD8$^+$ T cell expansion, despite its capacity in driving effector differentiation. Together these data identify complex interactions between Signal 3 and costimulatory pathways, and identify new opportunities to therapeutically target the differentiation of CD8$^+$ T cell responses.

2.2 Introduction

Effective therapeutic vaccination strategies against viral infections and tumors will likely require the development of potent CD8$^+$ T cells. By defining the cellular and

molecular determinants of naïve $CD8^+$ T cell differentiation into either effectors or long-lived poly-functional memory cells, it can be anticipated that vaccine efficacy will be improved, and that vaccines may be tailored according to the type of protection that is needed.

Initial $CD8^+$ T cell activation by TCR engagement (Signal 1) and CD28 stimulation (Signal 2) is followed by a program of proliferation[71, 72] and differentiation into short-lived effector cells (SLECS; characterized by $CD127^{lo}$ $KLRG1^{hi}$) or memory precursor effector cells (MPECs; $CD127^{hi}KLRG1^{lo}$) [44, 52, 73, 74]. During and after initial activation, $CD8^+$ T cells receive a variety of stimuli that influence fate decisions, dictating the expression of transcription factors such as T-bet, Eomesodermin (Eomes), and B lymphocyte-induced maturation protein-1 (BLIMP1) that regulate $CD8^+$ T cell proliferation, differentiation and survival (Signal 3), and subsequent differentiation into effector and memory cells[53]. Some Signal 3 stimuli are normally elicited by the response to pathogenic infection, sensed by TLR and other PRR, and are manifest in the form of inflammatory cytokines such as IL-12, type I IFN (IFN-1), IFN-γ and IL-6. IL-12 has been shown to direct $CD8^+$ T cells to take the characteristics of SLECs by the induction of T-bet[44-46]. IFN-1 has been shown to elicit DC activation[75] and also contribute to both the expansion and differentiation of T cell responses[42, 43]. Its contribution to the expression of the transcription factors that regulate SLEC/MPEC differentiation *in vivo* is less well characterized. Other stimuli that influence T cell fate are derived from components of adaptive immunity, and are primarily orchestrated by helper $CD4^+$ T cells.

Evidence from our lab and others has demonstrated that a major consequence of $CD4^+$ T cell-mediated licensing of DC via CD40 is the induction of the TNF-superfamily member CD70[18, 21, 22, 25, 34, 76]. CD70 stimulates CD27, which, among other functions,

reduces activation-induced[31] and Fas-L mediated cell death[32]. Prolonged survival is in part by inducing sustained expression of IL-2[30] in peripheral $CD8^+$ T cells, and CD27 stimulation supports effector cell generation against viral infections[33, 35, 77, 78], and subsequent differentiation to memory cells[33, 66, 79-81]. While the expression of CD70 on DC is primarily induced by stimulation of CD40, it is strongly enhanced by concurrent stimulation of TLR and signaling via IFNαβ receptors, leading to the potent activation of $CD8^+$ T cell responses and a strategy for subunit vaccination[18, 22, 24, 25, 41]. However, IFNαβR-independent, IL-12R-dependent, activation of $CD8^+$ T cells can occur, particularly to IL-12-inducing TLR agonists[23, 82]. Whether IL-12 can synergize with CD40 to induce CD70 is not known. Thus, on the one hand, the contribution of TLR/IL-12/IFN-1 to $CD8^+$ T cell expansion and differentiation could be to sensitize DC to enhance CD70 expression[82]. On the other hand, as recent studies have implicated a role for IL-12 and IFN-1 in the direct stimulation of $CD8^+$ T cells[42, 43, 48, 58, 83-86], stimulation by CD27 and IFN-1/IL-12 may co-operate to induce transcription factors that regulate the expansion, survival and differentiation of $CD8^+$ T cells. This raises the question whether CD27 stimulation can drive $CD8^+$ T cell proliferation and differentiation alone, as suggested by studies using transgenic expression of CD70 by DC and recombinant CD70[38-40] , or whether concomitant IFN-1 or other Signal 3 co-factors are also required[24, 87].

2.3 Materials and Methods

2.3.1 Animals. C57Bl/6 mice were obtained from National Cancer Institute (Frederick, MD). MHC class II-deficient mice were obtained from Taconic (B6.129-*H2-Ab1*^{tm1Gru} N12, model # ABBN12). IL-12p40KO mice (B6.129S1-*Il12b*^{tm1Jm}/J, stock # 002693) were purchased from the Jackson Laboratory (Bar Habor, ME). IFNαβRKO mice were provided by Dr. Ross Kedl (University of Colorado). OT-I mice, expressing TCRs specific

for $OVA_{257-264}$ peptide in complex with H-$2K^b$, were purchased from Taconic (B6.129S7-*Rag1*^{tm1Mom} Tg(TcraTcrb)1100Mjb N9+N1, model # 4175), and crossed on Thy1.1$^+$ (B6.PL-*Thy1*^{a}/CyJ stock # 000406) mice obtained from the Jackson Laboratory. CD27KO mice were provided by Dr. Steven Schoenberger (La Jolla Institute of Immunology and Allergy), with the permission of Dr. Jannie Borst (Netherlands Cancer Institute). IL-12p40KO mice (B6.129S1-*Il12b*^{tm1Jm}/J, stock # 002693) were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were maintained in specific pathogen-free facilities and were treated in accordance with the guidelines established by the Animal Care and Use Committee at the University of Virginia.

2.3.2 Peptide and protein. Synthetic peptide $OVA_{257-264}$ (SIINFEKL) was purchased from GenScript (Piscataway, NJ). Endotoxin was removed by Detoxi-Gel endotoxin-removal kit (Pierce, Rockford, IL). OVA protein was purchased from Sigma (St Louis, MO) and endotoxin was removed as above.

2.3.3 Virus and viral titer assay. OVA-adeno was either purchased from Gene Transfer Vector Core (University of Iowa) or provided by Dr. Young Hahn (University of Virginia), and was propagated on 293A fibroblasts. Recombinant vaccinia virus expressing OVA (OVA-vac) was provided by J. Yewdell (National Institute of Allergy and Infectious Diseases), and was propagated on HuTK$^-$ cells. Virus titers from infected mice were determined 7 d after i.p. priming with 1×10^8 PFU OVA-vac. Spleens were excised and then homogenized. Homogenate was subject to three cycles of freezing and thawing and then sonicated. Sonicate was cleared of particulate matter by a centrifugation, and the supernatant was used to infect HuTK$^-$ cells. Virus plaques were revealed 48 h later by crystal violet staining.

2.3.4 Antibodies and other reagents. GK1.5-depleting anti-CD4 was obtained from ATCC. NK-depleting PK136 was provided by Dr. Michael Brown (University of Virginia). C17.8-neutralizing anti-IL-12/23 was purchased from Bio-X-Cell (West Lebanon, NH). Agonistic FGK45 anti-CD40 was provided by Dr. Steven Schoenberger. Agonistic AT124.1 anti-CD27 has been described[88] and endotoxin was removed as above. FR70-blocking anti-CD70 has been described before[18]. TLR3 agonist PolyI:C was purchased from Invivogen (San Diego, CA). Mouse IL-12 p70 recombinant protein was purchased from eBioscience (San Diego, CA). Recombinant IFNα was provided by Dr. Ross Kedl (University of Colorado). Control Ig was purchased from Sigma.

2.3.5 Immunization. Unless indicated otherwise, mice were depleted of peripheral $CD4^+$ T cells by i.p. injection of 200 µg GK1.5 7 and 3 d before primary immunization and confirmed by tail vain bleed. NK cell depletion was performed by injecting 200 µg PK136 i.p. 3 d before immunization. For the generation of primary $CD8^+$ T cell responses, 500 µg OVA protein was injected i.p. alone or in combination with the indicated adjuvants in 200 µL total volume. CD40 stimulation was performed by injecting 50 µg FGK45 i.p. on d0. CD27 stimulation was performed by injecting 50 µg AT124.1 i.p. on d0, 3 and 6. CD70 blockade was performed by injecting 500 µg FR70 i.p. on d0, 2, 4 and 6. IL-12 blockade was performed by injecting 500 µg C17.8 i.p. on d 0, 2, 4 and 6. TLR stimulation was performed by injecting 50 µg the indicated agonist i.p. on d0. For recombinant cytokine administration, approximately $1X10^7$ units of IFNα was given i.p. on d0, or 1ug IL-12 was given i.p. on d0, 1 and 2[89]. For the generation of secondary $CD8^+$ T cell responses, $2x10^8$ p.f.u. OVA-adeno was injected i.p.

2.3.6 In vivo cytotoxicity assay. Splenocytes from CD45.1 B6 mice were either pulsed with $OVA_{257-264}$ peptide and labeled with high levels of CFSE (5 µM) as targeting cells, or

labeled with low levels of CFSE (0.5 μM) without peptide pulse as control cells. 3 X 10^6 targeting cells and 3 X 10^6 control cells were co-transferred into CD45.2 pre-immunized mice at d6, and the cytotoxicity was assessed 16 hr later.

2.3.7 Flow cytometry. Lymphocytes were isolated from blood or homogenized spleens 7d after primary immunization or 5d after secondary challenge. Cells were first stained with Aqua vital dye (life technologies; Carlsbad, CA) for 20 min at 4°C, and pre-incubated with $OVA_{257-264}$-specific H2-K^b dextramer-APC (Immudex, Denmark) for 20 min at 4°C before other antibodies were applied. After Fc blockade, surface markers were stained for 30 min at 4°C, with antibodies anti-CD8-ef450 (53-6.7), anti-CD44-FITC (IM7), anti-KLRG1-PE (2F1), anti-CD127-PerCP-Cy5.5 (A7R34) from eBioscience. In some cases, anti-Thy1.1-FITC (HIS51) was applied to identify adoptively transferred OT-I population. In some early experiments, $OVA_{257-264}$-specific H2-K^b tetramer-APC (Dr. Vic Engelhard, University of Virginia) was co-stained with surface markers as an alternative for the dextramer. For transcriptional factor staining, lymphocytes were then permeabilized (eBioscience Fixation/Permeabilization) and stained with T-bet-PE (4B10) and Eomes-PercP-eFlour710 (Dan11mag) from eBioscience for 30 min at 4°C. Functional assays for the production of intracellular IFNγ, IL-2 and expression of CD107a were performed as previously described with $OVA_{257-264}$-peptide-pulsed or unpulsed LB15.13 hybridoma [14].Staining was assessed by flow cytometry on a FACS Canto II (Becton Dickinson; Franklin Lakes, NJ) and analyzed using FlowJo 7.6.5 Software (Treestar, OR).

2.3.8 Statistical analysis. Statistical analysis was performed with Prism 5 (GraphPad Software, Inc., La Jolla, CA) and data were presented as the Mean ± SD. Comparisons between groups were performed by unpaired two-tailed Student's *t* test or one-way

ANOVA. Statistical significance was determined for 95% confidence interval. Synergism of two treatment arms was identified by a significant interaction determined in two-way ANOVA as previously described[90].

2.4 Results

2.4.1 Co-targeting CD40 and TLR results in CD70-dependent, helper CD4$^+$ T cell independent primary and memory CD8$^+$ T cell responses.

Concurrent stimulation of CD40 and TLR has been shown to bypass the necessity for CD4$^+$ T cell help in the generation of primary CD8$^+$ T cell responses to OVA protein immunization[23]. It is not certain whether this combination of stimulations is sufficient for the formation of functional memory CD8$^+$ T cells in the absence of CD4$^+$ T cell help and if so, whether memory formation is dependent upon CD70. In agreement with the previous studies[24, 37], we found that mice deficient of CD4$^+$ T cells (MHC class II-knockout) generated substantial primary CD8$^+$ T cell responses to OVA protein when given both agonistic αCD40 and polyI:C (pIC, TLR3 agonist) (Figure 2.1A). These responses were equivalent in magnitude to those generated in mice with an intact CD4$^+$ T cell population (Figure 2.2A). In the absence of either αCD40 or pIC, minimal primary CD8$^+$ T cell responses were detected in either WT or MHC class II-knockout animals (data not shown). Consistent with previous findings in CD4-intact animals[24], the primary CD8$^+$ T cell response in CD4-deficient mice, or mice depleted of CD4$^+$ T cells (not shown) elicited by immunization of OVA and combined αCD40/pIC was ~75% dependent upon CD70 (Figure 2.1A, B). Notably, CD70 blockade resulted in a greater loss of KLRG1-

Figure 2.1 **Impact of CD70 induction on primary and secondary CD8$^+$ T cell responses**. Cohorts of MHC class II-deficient mice (n=3/cohort) were immunized with OVA alone, or concurrently with combined αCD40/pIC and treated with either αCD70 or

clg. Secondary responses were initiated by challenging with OVA-adeno >35 d post primary immunization. Data are representative of two-three similar, independent experiments. **(A)** Frequency of primary $OVA_{257-264}$-specific $CD8^+$ T cells 7d after immunization. One-way ANOVA with Tukey's post test, **p<0.01 compared to the first column, ##p<0.01 compared to the second column. **(B)** Top plots: representative data showing CD127/KLRG1 expression on $OVA_{257-264}$-specific $CD8^+$ T cells; regions are based on FMO stains. Top histogram: enumeration of $OVA_{257-264}$-specific $CD8^+$ T cells. Bottom histograms: enumeration of SLECs and MPECs. Student t test, ***p<0.001. **(C)** Frequency of secondary $OVA_{257-264}$-specific $CD8^+$ T cells 5d after OVA-adeno challenge. One-way ANOVA with Tukey's post test, ***p<0.001 compared to the first column, ###p<0.001 compared to the second column.

Figure 2.1

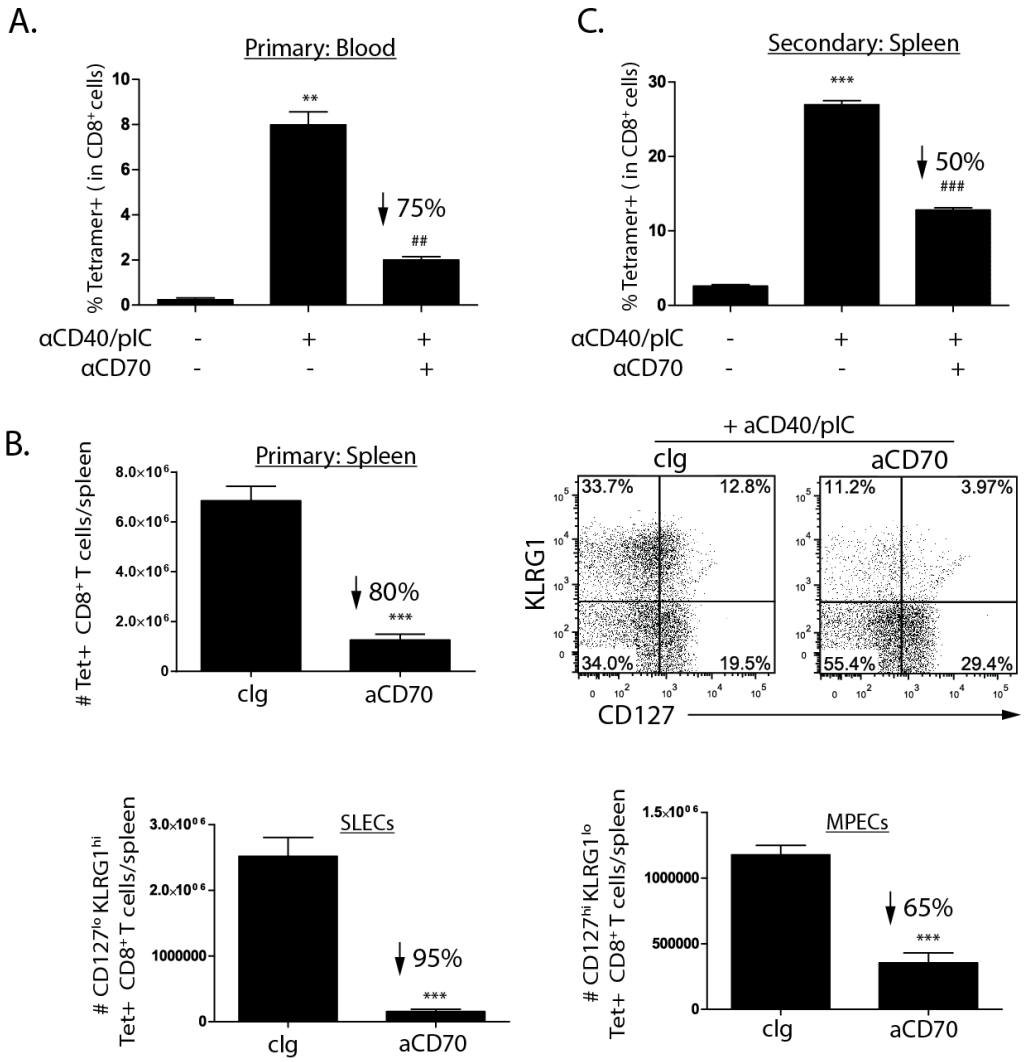
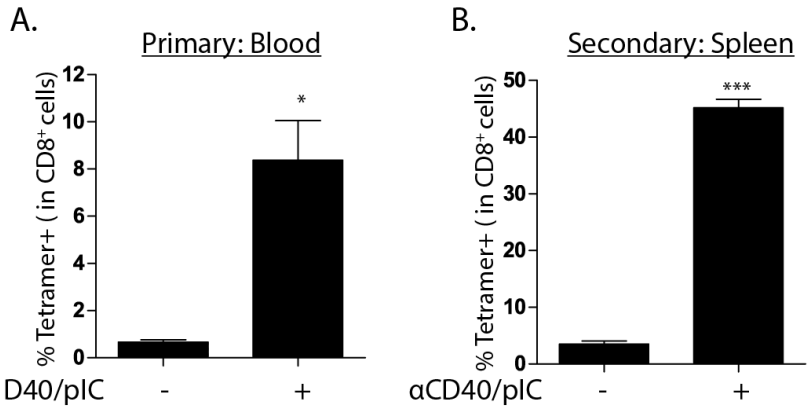

A.
Primary: Blood
% Tetramer+ (in CD8+ cells)
**
75%
##
αCD40/pIC - + +
αCD70 - - +
C.
Secondary: Spleen
% Tetramer+ (in CD8+ cells)
***
50%
###
αCD40/pIC - + +
αCD70 - - +
B.
Primary: Spleen
Tet+ CD8+ T cells/spleen
80%
***
cIg
aCD70
+ aCD40/pIC
cIg
aCD70
KLRG1
CD127
33.7%
12.8%
34.0%
19.5%
11.2%
3.97%
55.4%
29.4%
SLECs
CD127lo KLRG1hi Tet+ CD8+ T cells/spleen
95%
***
cIg
aCD70
MPECs
CD127hi KLRG1lo Tet+ CD8+ T cells/spleen
65%
***
cIg
aCD70

Figure 2.2 **Potent primary and secondary $CD8^+$ T cell responses by co-targeting CD40 and TLR.** WT (CD4-competent) mice were immunized with OVA alone, or concurrently with combined αCD40/pIC and treated with either αCD70 or cIg. Secondary responses were initiated by challenging with OVA-adeno >35 d post primary immunization. Data are representative of two-three similar, independent experiments with 3 mice per cohort. **(A)** Frequency of primary $OVA_{257-264}$-specific $CD8^+$ T cells. Student t test, *$p<0.05$. (B) Frequency of secondary $OVA_{257-264}$-specific $CD8^+$ T cells. Student t test, ***$p<0.001$. Data are representative of two similar, independent experiments.

Figure 2.2


A.
Primary: Blood
% Tetramer+ (in CD8+ cells)
12
10
8
6
4
2
0
*
D40/pIC
-
+
B.
Secondary: Spleen
% Tetramer+ (in CD8+ cells)
50
40
30
20
10
0
***
αCD40/pIC
-
+

expressing SLECs (95% reduction) than of CD127-expressing MPECs (65% reduction) (Figure 2.1B).

We next assessed whether immunization with combined αCD40/pIC resulted in the generation of functional memory in the absence of $CD4^+$ T cells. Mice that had been rested for 35d after protein immunization were challenged with recombinant adenovirus expressing OVA (OVA-adeno). MHC-tetramer staining revealed that the secondary $CD8^+$ T cell response elicited in CD4-deficient mice approached ~60% of the level achieved in WT mice (Figure 2.1C and Figure 2.2B). Further, short term *in vitro* culture of secondary effector cells with $OVA_{257-264}$ peptide-pulsed APC demonstrated an equivalent degree of both CD107a expression and IFNγ production (data not shown). Notably, as predicted by the reduction in MPECs after CD70 blockade, CD27 stimulation during the primary response also substantially contributes to the magnitude of $OVA_{257-264}$-specific secondary $CD8^+$ T cell response, though to a lesser extent (50%, Figure 2.1C) than seen in the primary response (75%, Figure 2.1A).

2.4.2 Synergy between CD27 and TLR stimulation to generate primary $CD8^+$ T cell response.

Previous studies have indicated that the synergy between CD40 and TLR stimulation in the generation of primary $CD8^+$ T cell responses is dependent upon CD70 and IFN-1[22, 23]. Further, IFN-1 has been shown to synergize with CD40 stimulation to induce CD70 expression[82], leading us to hypothesize that the major contribution of pIC in the primary $CD8^+$ T cell response is to support CD40-mediated up-regulation of CD70 expression via IFN-1 induction. Indeed, in mice lacking the IFNαβ receptor, the ability to elicit CD70 expression on DC after infusion with combined αCD40/pIC is significantly

reduced. In contrast, IL-12 deficiency has negligible impact on CD70 expression (Figure 2.3A).

However, IFN-1 has also been shown to directly support $CD8^+$ T cell expansion and differentiation[42, 43, 84, 85, 91]. Therefore, to begin to dissect how αCD40 and pIC drive $CD8^+$ T cell responses, we examined the effect of directly stimulating CD27. If the role of IFN-1 is primarily to synergize with αCD40 in inducing CD70 expression, then CD27 stimulation should be sufficient to elicit primary $CD8^+$ T cell responses to OVA protein in the absence of $CD4^+$ helper T cells. Surprisingly, when applied as a sole adjuvant, endotoxin-free αCD27 only drove weak $OVA_{257-264}$-specific $CD8^+$ T cell responses (Figure 2.4). Thus, while CD27-mediated costimulation is necessary for generation of $CD8^+$ T cell responses to OVA with combined αCD40/pIC, alone it is inefficient at stimulating a robust primary response, at least not from an endogenous population where the precursor frequency is low.

As the combination of pIC and αCD40, but neither alone, had driven a CD70-dependent $CD8^+$ T cell response to OVA immunization, we then sought to determine whether either of these adjuvants would synergize with CD27 stimulation. We found that co-treatment with αCD40 did not increase the αCD27-driven response (Figure 2.3B). In contrast, co-targeting CD27 and TLR3 synergized, substantially enhancing the $OVA_{257-264}$-specific endogenous primary $CD8^+$ T cell response in the absence of $CD4^+$ T cell-mediated help (Figure 2.4). Thus, together these data indicate that the inclusion of TLR stimulation promotes primary $CD8^+$ T cell responses independently from their effects on inducing CD70 expression.

2.4.3 IFN-1 but not IL-12 supports the ability of CD27 stimulation to promote $CD8^+$ T cell responses.

Figure 2.3 **(A) Regulation of CD70 expression by IFN-1.** Expression of CD70 on splenic DC 48 h after treatment with aCD40 and plC. Plots are gated on $CD11c^{hi}$ $CD3^{-}$ $CD19^{-}$ cells. Numbers in quadrants show percent of DC expressing CD86 and/or CD70. **(B) No synergy between CD27 and CD40 stimulation in driving $CD8^{+}$ T cell responses.** Frequency of $OVA_{257-264}$-specific $CD8^{+}$ T cells in spleens of CD4-deficient mice 7d after immunization with OVA and indicated adjuvant(s). Data represent of two-three similar, independent experiments.

Figure 2.3

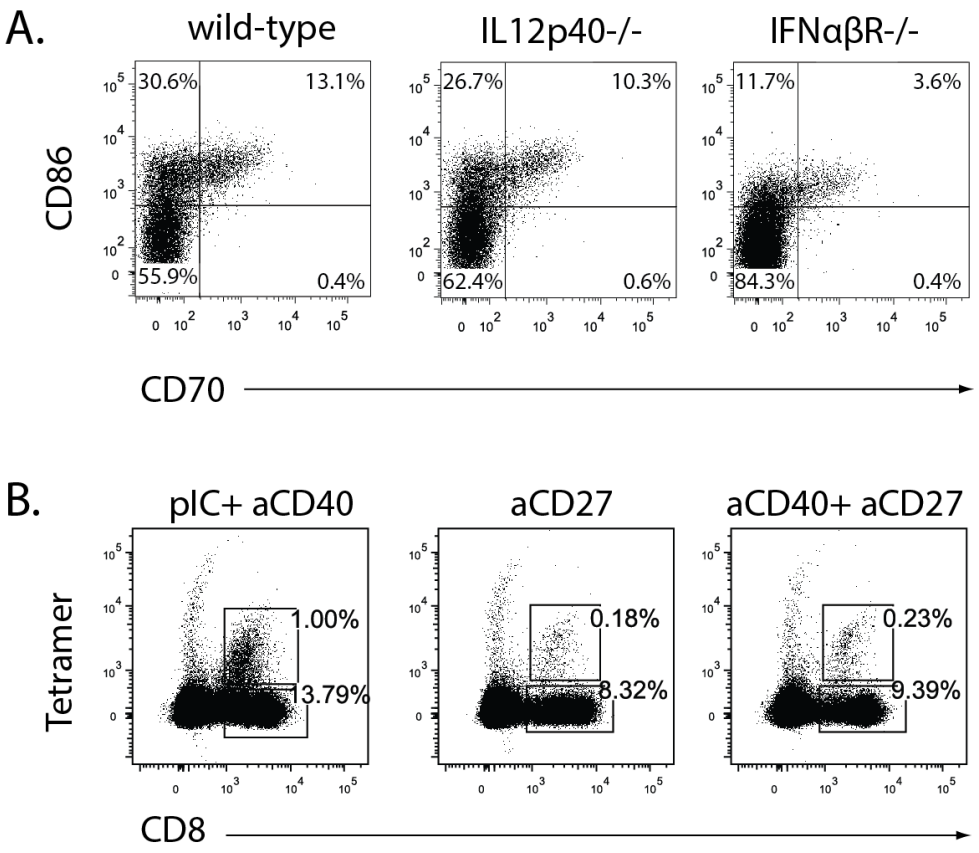
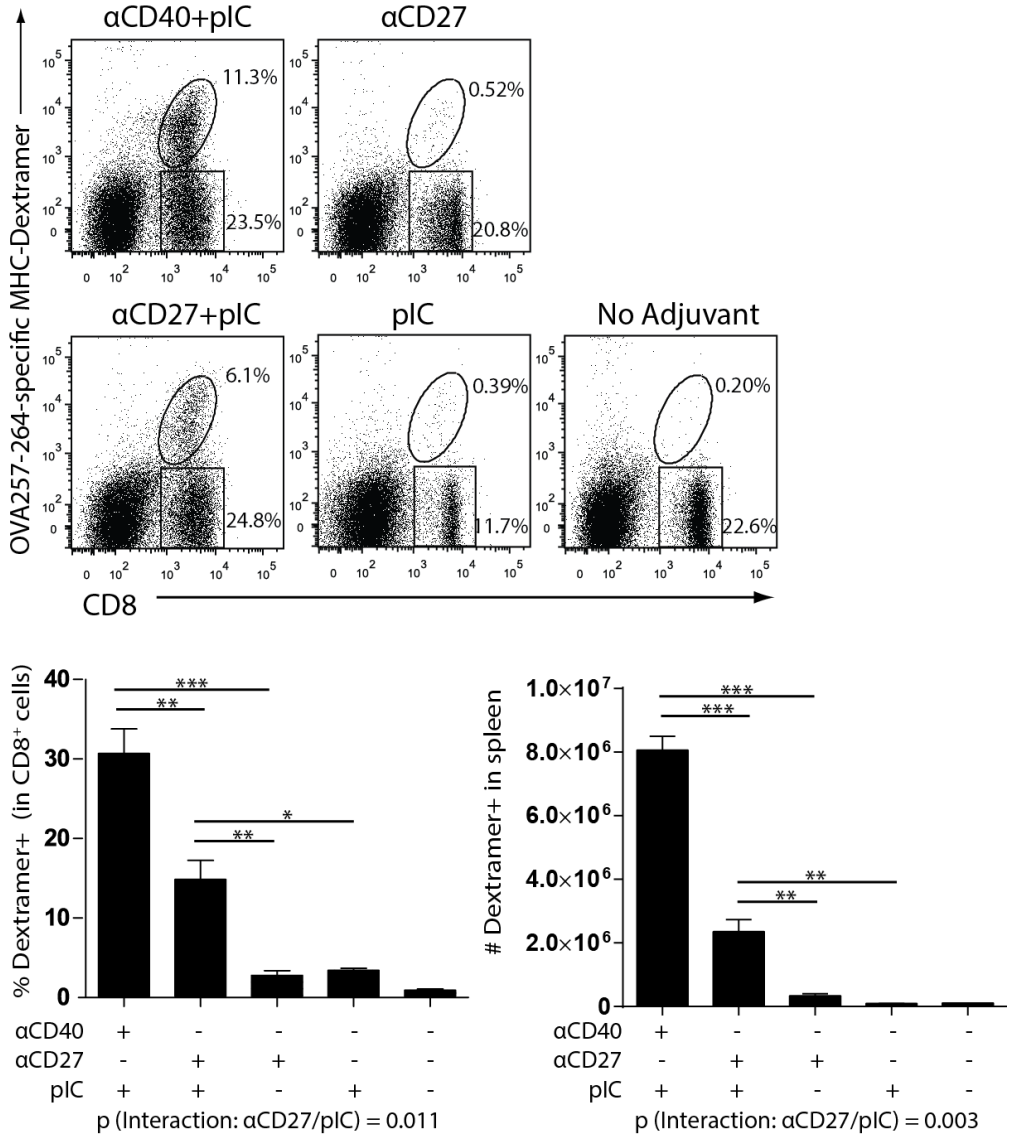

A.
wild-type
IL12p40-/-
IFNαβR-/-
CD86
30.6%
13.1%
55.9%
0.4%
26.7%
10.3%
62.4%
0.6%
11.7%
3.6%
84.3%
0.4%
CD70
B.
pIC+ aCD40
aCD27
aCD40+ aCD27
Tetramer
1.00%
13.79%
0.18%
8.32%
0.23%
9.39%
CD8

Figure 2.4 **Synergy between CD27 and TLR stimulation to promote $CD8^+$ T cell responses**. Frequency and absolute number of $OVA_{257-264}$-specific $CD8^+$ T cells in spleens of CD4-depleted mice 7d after immunization with OVA and indicated adjuvant(s). Dot plots show representative mice from each cohort. Histogram shows quantification of a representative experiment from at least four independent experiments with three mice per group. One-way ANOVA with Tukey's post test, *p<0.05, **p<0.01, ***p<0.001. p(Interaction) was determined by two-way ANOVA of the last four columns.

Figure 2.4


αCD40+pIC
αCD27
αCD27+pIC
pIC
No Adjuvant
11.3%
23.5%
0.52%
20.8%
6.1%
24.8%
0.39%
11.7%
0.20%
22.6%
OVA257-264-specific MHC-Dextramer
CD8
% Dextramer+ (in CD8+ cells)
αCD40 + - - - -
αCD27 - + + - -
pIC + + - + -
p (Interaction: αCD27/pIC) = 0.011
Dextramer+ in spleen
p (Interaction: αCD27/pIC) = 0.003

The preceding data indicated that additional stimulatory cofactor(s) cooperate with CD27 stimulation to elicit primary $CD8^+$ T cell responses to OVA protein in the absence of helper $CD4^+$ T cells. Both IFN-1 and IL-12 have been implicated as additional Signal 3 molecules that are capable of driving the expansion of primary $CD8^+$ T cell responses[92] and are produced by pIC stimulation[93]. To address the role of these cytokines in supporting CD27-mediated $CD8^+$ T cell expansion, we immunized $CD4^+$ T cell-depleted mice with OVA and αCD27, with or without endotoxin-free recombinant IFNα or IL-12. We found that IFNα synergized with CD27 stimulation, resulting in a >3-fold greater $CD8^+$ T cell response compared to either IFNα or CD27 stimulation alone (Figure 2.5A). In contrast, IL-12 not only failed to synergize with CD27 stimulation, but reduced the limited response driven by CD27 stimulation alone (Figure 2.5B). Despite its failure to drive effector expansion, inclusion of IL-12 successfully promoted KLRG1 expression and SLEC differentiation (Figure 2.5B inset). Minimal $OVA_{257-264}$-specific $CD8^+$ T cell responses were achieved when either recombinant cytokine was applied as a sole adjuvant (Figure 2.5A, B), consistent with the observation of limited responses driven by pIC alone (Figure 2.4). Thus, the capacity of CD27 to synergize with pIC to augment $CD8^+$ T cell responses is dictated by the elaboration of IFN-1 by pIC. The synergy between IFN-1 and CD27 stimulation was further confirmed by immunizing $CD4^+$ T cell-depleted IFNαβRKO mice with OVA and combined αCD27/pIC. We found that the primary $CD8^+$ T cell response to OVA was substantially reduced (~75%) in the absence of IFNαβR signaling (Figure 2.6A). In contrast to the results with the IFNαβRKO mice, the $OVA_{257-264}$-specific primary $CD8^+$ T cell response was unimpeded if not enhanced after IL-12 blockade or in IL-12p40KO mice (Figure 2.6B-D), and that the requirement for IFNαβR signaling was not overcome by blocking IL-12 (Figure 2.6D).

Figure 2.5 **IFNα but not IL-12 synergizes with CD27 stimulation.** Analysis of spleens from CD4-depleted mice 7d after immunization with OVA and indicated adjuvant(s). **(A)** Top dot plots show $OVA_{257-264}$-specific $CD8^+$ T cells from representative mice in each cohort where αCD27 and/or rIFNα were applied as adjuvant(s); bottom plots show target cell killing in representative mice. Histogram shows quantification of a representative experiment from four independent experiments, with three-four mice in each cohort. One-way ANOVA with Tukey's post test, ***p<0.001. p(Interaction) was determined by two-way ANOVA. **(B)** A representative experiment from at least three independent experiments where αCD27 and/or rIL-12 were applied; inset dot plots show representative data of CD127/KLRG1 expression on $OVA_{257-264}$-specific $CD8^+$ T cells. One-way ANOVA with Tukey's post test, *p<0.05. (C) Degradulation and cytokine production induced by $OVA_{257-264}$-peptide pulse *in vitro*. Left histogram shows population frequency while right histrogram shows per-cell expression levels compared to no adjuvant control. Data are derived from a representative experiment with three mice per group, which independently recapitulated three times. One-way ANOVA with Tukey's post test, *p<0.05, **p<0.01, ***p<0.001.

Figure 2.5

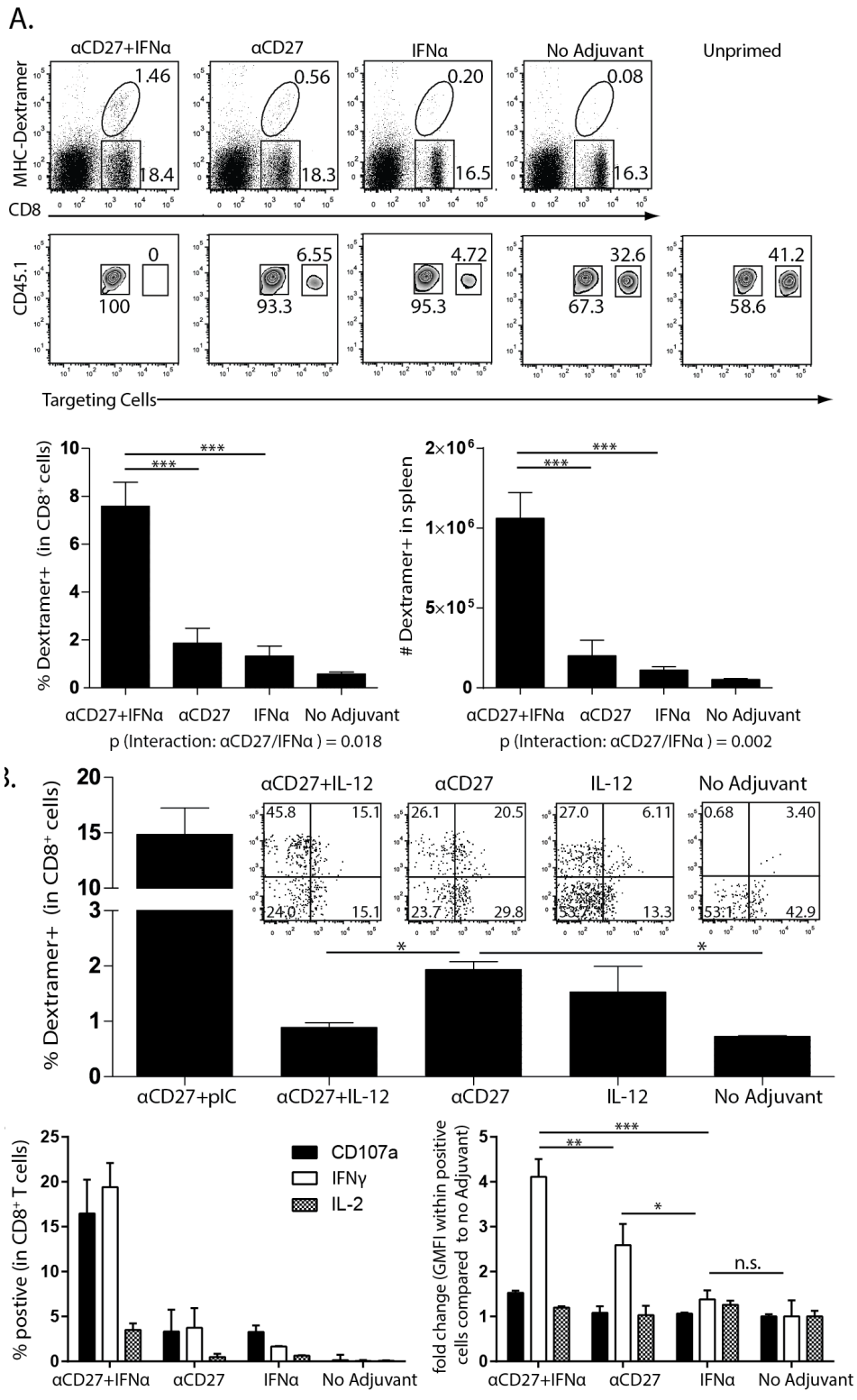
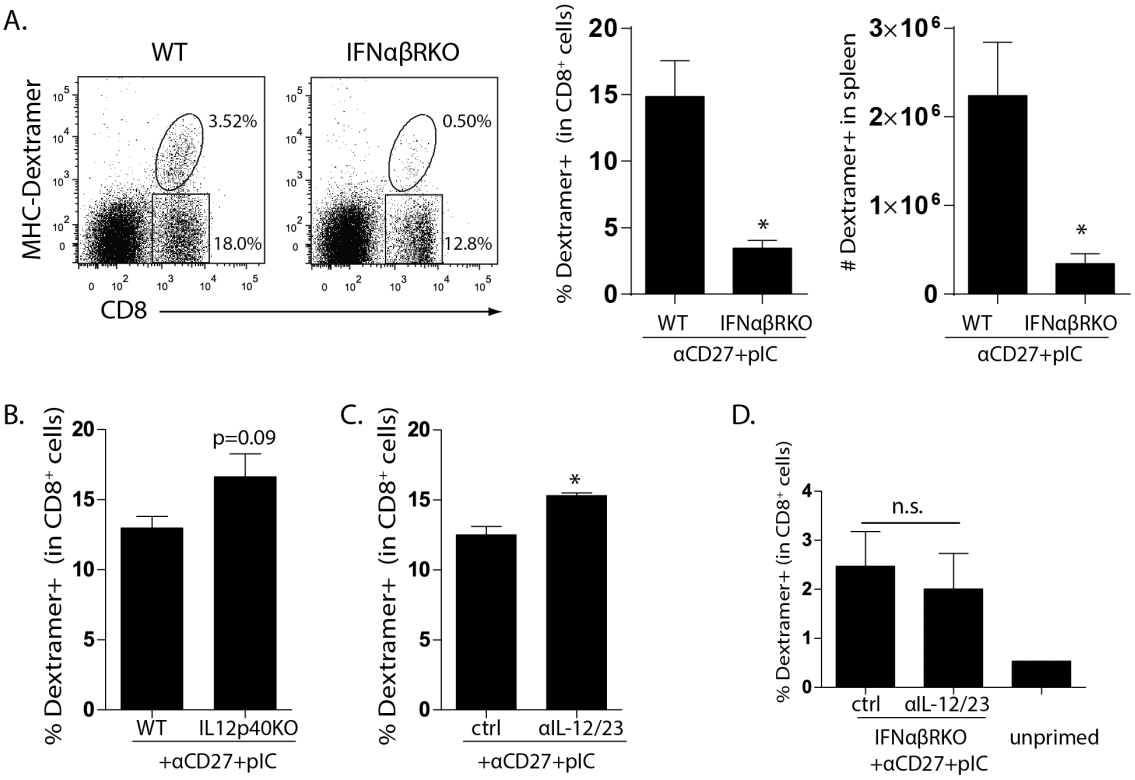

A.
αCD27+IFNα
αCD27
IFNα
No Adjuvant
Unprimed
MHC-Dextramer
CD8
1.46
18.4
0.56
18.3
0.20
16.5
0.08
16.3
CD45.1
0
100
6.55
93.3
4.72
95.3
32.6
67.3
41.2
58.6
Targeting Cells
% Dextramer+ (in CD8+ cells)
# Dextramer+ in spleen
***
***
αCD27+IFNα αCD27 IFNα No Adjuvant
p (Interaction: αCD27/IFNα) = 0.018
p (Interaction: αCD27/IFNα) = 0.002
B.
αCD27+IL-12
αCD27
IL-12
No Adjuvant
45.8
15.1
24.0
15.1
26.1
20.5
23.7
29.8
27.0
6.11
53.7
13.3
0.68
3.40
53.1
42.9
*
*
αCD27+pIC
αCD27+IL-12
αCD27
IL-12
No Adjuvant
% postive (in CD8+ T cells)
CD107a
IFNγ
IL-2
fold change (GMFI within positive cells compared to no Adjuvant)
**
*
n.s.

Figure 2.6 **Requirement for IFN-1 but not IL-12 in αCD27/TLR synergy**. **(A)** Frequency of $OVA_{257-264}$-specific $CD8^+$ T cells in spleens from CD4-depleted WT or IFNαβRKO mice 7d after immunization. Data are derived from a representative experiment with three mice per cohort, which independently recapitulated three times. **(B)** Frequency of $OVA_{257-264}$-specific $CD8^+$ T cells in spleens from CD4-depleted WT or IL12p40KO mice. Independent repeats were performed at least three times. IL-12 was blocked by applying αIL12/23 in CD4-depleted WT **(C)** or IFNαβRKO mice **(D)**; histograms show combined data for three mice per group from representative experiments with two independent repeats. All Student t test except (D) where one-way ANOVA with Tukey's post test was performed. *$p<0.05$, n.s.= not significant.

Figure 2.6


A.
WT
IFNαβRKO
MHC-Dextramer
CD8
3.52%
18.0%
0.50%
12.8%
% Dextramer+ (in CD8+ cells)
# Dextramer+ in spleen
WT
IFNαβRKO
αCD27+pIC
B.
p=0.09
WT
IL12p40KO
+αCD27+pIC
C.
ctrl
αIL-12/23
+αCD27+pIC
D.
n.s.
ctrl
αIL-12/23
IFNαβRKO
+αCD27+pIC
unprimed

These data together demonstrate that the synergy achieved by co-targeting CD27 and TLR3 is dependent on IFN-1, and IFNα is sufficient to replicate the majority of the synergy with αCD27, while IL-12 surprisingly does not promote CD27-driven responses.

2.4.4 CD27 and IFNα augment the functional activity of primary CD8$^+$ T cell responses.

We next sought to understand whether the synergy between CD27 and IFNα at driving the expansion of CD8$^+$ T cells had an impact on their functional activity. We found that mice immunized with the combination of αCD27 and IFNα had the greatest ability to clear antigen loaded target cells *in vivo* (Figure 2.5A, bottom plots). As this enhanced *in vivo* cytotoxicity could simply reflect the increase in magnitude of the cytotoxic T cell response, we examined effector functions on a per-cell basis. In general the magnitude of the CD8$^+$ T cell populations that could de-granulate or produce IFNγ or IL-2 followed the response size as gauged by MHC-dextramer staining (Figure 2.5C, left). However, CD8$^+$ T cells that respond to αCD27+IFNα produce IFNγ at a greater amount per cell (3-fold increase compared to IFNα and 60% increase compared to CD27 alone; Figure 2.5C, right). Interestingly, this increased functional activity per cell was not evident in degranulation or IL-2 production, suggesting the combination specifically targets IFNγ production. Thus, not only does the combination of CD27 and IFNα synergize in the expansion of CD8$^+$ T cells, the responding CD8$^+$ T cells are poly-functional and have enhanced ability to secrete IFNγ relative to controls.

The physiological relevance of this immunization strategy is demonstrated by an increase in the magnitude of the $OVA_{257-264}$-specific CD8$^+$ T cell population achieved by OVA+ αCD27+pIC super-imposed upon the vaccinia infection that was not achieved by

immunizations that used OVA+αCD27, OVA+pIC or OVA alone (Figure 2.7A). The magnitude of the $CD8^+$ T cell response to $OVA_{257-264}$ correlates closely with the viral titer in the spleen at day 7 (Figure 2.7B). Therefore, these data highlight the potential of co-targeting CD27 and IFNα along with antigen delivery to augment a functional $CD8^+$ T cell response that constrains acute viral infections.

2.4.5 $CD8^+$ T cell expression of IFNαβR and CD27 is necessary for TLR synergy with CD27 stimulation.

IFN-1 can influence both DC and T cell function, while CD27 can stimulate multiple subsets of lymphocytes. To determine the cellular target of IFN-1, we transferred ~1000 IFNαβRKO or WT OT-I into CD4-depleted, IFNαβR-competent mice and challenged with OVA and combined αCD27/pIC. The magnitude of the primary OT-I response at day 7 after immunization was ~3-fold lower when the responding OT-I cells did not express the IFNαβR, indicating that the ability of IFN-1 to synergize with CD27 stimulation is highly dependent on the expression of IFNαβR on $CD8^+$ T cells (Figure 2.8A). Conversely, the magnitude of a WT OT-I response was not impeded in IFNαβRKO hosts compared to WT hosts (Figure 2.8B), indicating that the expression of IFNαβR on host population (e.g. DC) is dispensable. Likewise, the expansion of WT OT-1 cells is unimpeded in CD27-deficient hosts (Figure 2.9A), and the absence of either $CD4^+$ T cells or NK cells (Figure 2.9B) did not reduce the response to the vaccine. Thus, CD27 and IFN-1 synergize directly on $CD8^+$ T cells to generate primary $CD8^+$ T cell responses to protein immunization.

2.4.6 Induction of transcription factors T-bet and Eomesodermin by stimulation of IFN-1 and CD27.

Figure 2.7 **The physiological relevance of αCD27+pIC immunization strategy.** Mice were i.p. primed with $1*10^8$ OVA-vac and then therapeutically treated with the indicated treatments initiated 24 h later. Splenocytes were assessed by flow cytometry on d 7. **(A)** Magnitude of $OVA_{257-264}$-specific $CD8^+$ T cells. One-way ANOVA with Tukey's post test. *$p<0.05$. **(B)** Correlation between the magnitude of $OVA_{257-264}$-specific $CD8^+$ T cells and the viral titer.

Figure 2.7

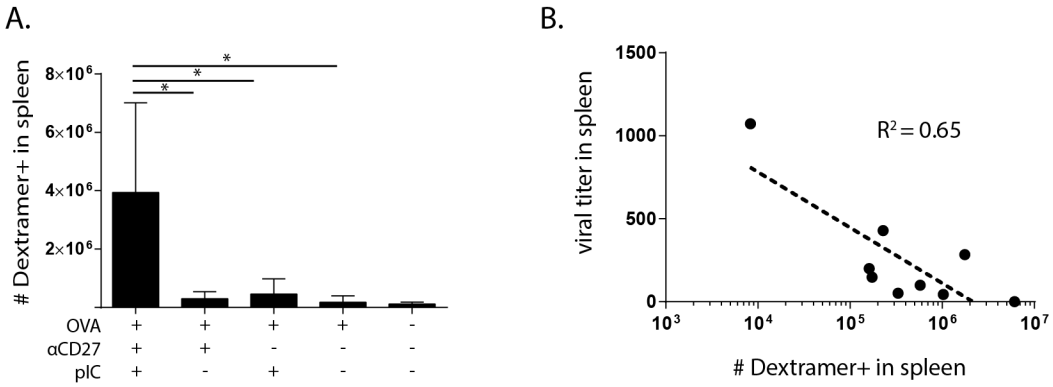

A.
Dextramer+ in spleen
8×10⁶
6×10⁶
4×10⁶
2×10⁶
*
*
*
OVA + + + + -
αCD27 + + - - -
pIC + - + - -
B.
viral titer in spleen
1500
1000
500
0
R² = 0.65
10³
10⁴
10⁵
10⁶
10⁷
Dextramer+ in spleen

Figure 2.8 **$CD8^+$ T cell expression of IFNαβR is necessary for IFN-1 synergy with CD27 stimulation**. **(A)** ~1000 $CD45.2^+$ OT-I (WT or IFNαβRKO) $CD8^+$ T cells were transferred into CD4-depleted $CD45.1^+$ C57Bl/6 (WT) and challenged with OVA and combined αCD27/pIC. The frequency and absolute number of $CD8^+CD45.2^+OVA_{257-264}$-specific $tetramer^+$ cells were measured in spleens 7d later. **(B)** ~1000 $Thy1.1^+$OT-I (WT) $CD8^+$ T cells were transferred into CD4-depleted $Thy1.2^+$C57Bl/6 (WT or IFNαβRKO) mice, and challenged with OVA and combined αCD27/pIC. The frequency and absolute number of $CD8^+Thy1.1^+OVA_{257-264}$-specific $tetramer^+$ cells were measured in spleens 7d later. Bar graphs show combined data with three mice per cohort, which are derived from one of two identical, independent experiments. Student t test, *$p<0.05$, **$p<0.01$.

Figure 2.8

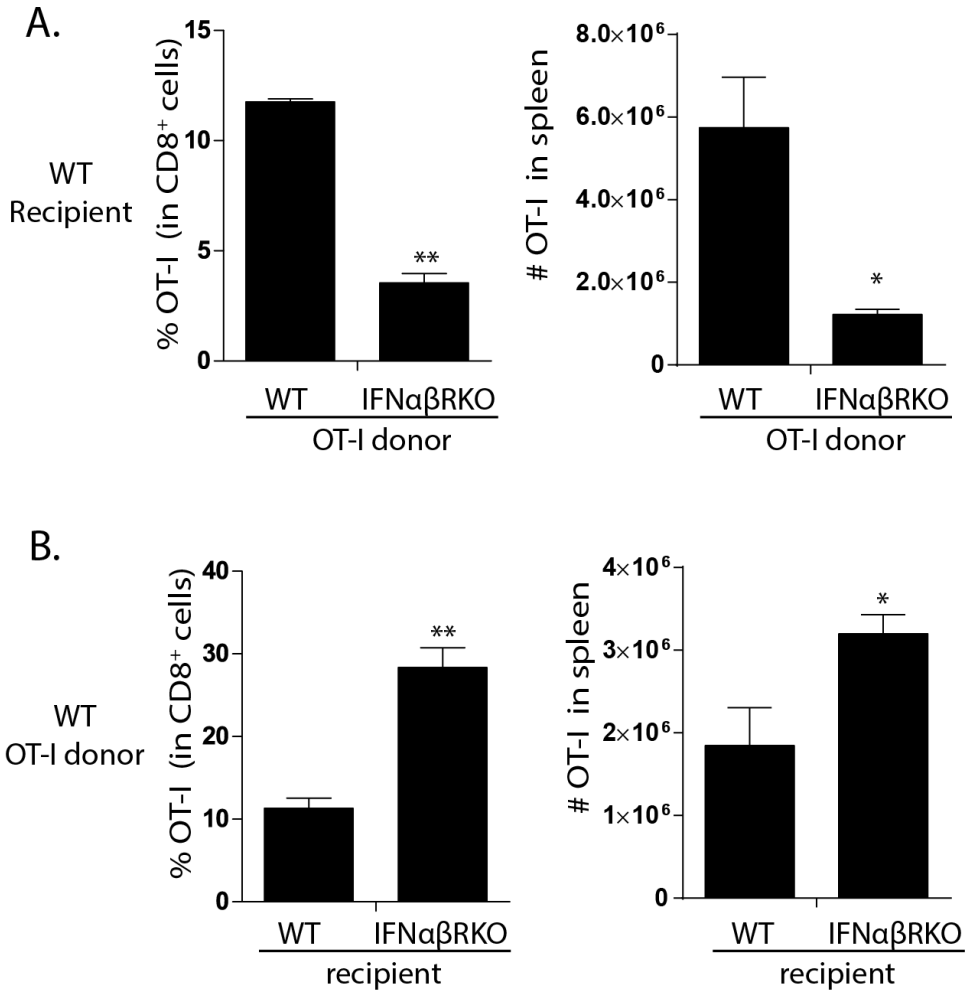
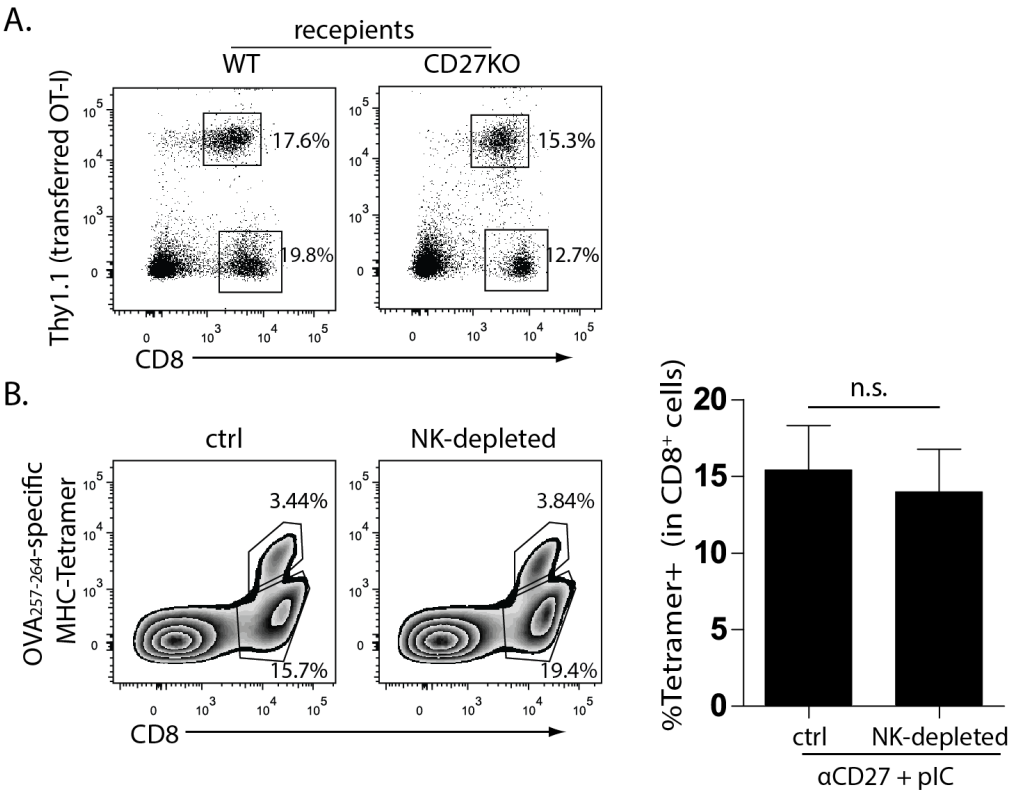

A.
WT
Recipient
% OT-I (in CD8+ cells)
15
10
5
0
**
WT
IFNαβRKO
OT-I donor
OT-I in spleen
8.0×10^6
6.0×10^6
4.0×10^6
2.0×10^6
0
*
WT
IFNαβRKO
OT-I donor
B.
WT
OT-I donor
% OT-I (in CD8+ cells)
40
30
20
10
0
**
WT
IFNαβRKO
recipient
OT-I in spleen
4×10^6
3×10^6
2×10^6
1×10^6
0
*
WT
IFNαβRKO
recipient

Figure 2.9 **$CD8^+$ T cell expression of CD27 is necessary for CD27 stimulation synergy with pIC**. **(A)** 10,000 $Thy1.1^+$ OT-I cells were adoptively transferred to $Thy1.2^+$ WT and CD27KO recipients a day before immunization with OVA and combined αCD27/pIC. Representative plots showed the presence of OT-I cells in spleens 7d later. **(B)** Frequency of $OVA_{257-264}$-specific $CD8^+$ T cells in spleens of NK-intact versus NK-depleted mice 7d after immunization. Student t test, n.s.= not significant. Assays were repeated at least twice independently.

Figure 2.9


A.
recepients
WT
CD27KO
Thy1.1 (transferred OT-I)
17.6%
19.8%
15.3%
12.7%
CD8
B.
ctrl
NK-depleted
OVA257-264-specific MHC-Tetramer
3.44%
15.7%
3.84%
19.4%
CD8
%Tetramer+ (in CD8+ cells)
n.s.
20
15
10
5
0
ctrl
NK-depleted
αCD27 + plC

The T-box transcription factors T-bet and Eomes have been well defined as two key drivers governing $CD8^+$ T cell differentiation and cytolytic function. We previously reported CD27-mediated Eomes up-regulation during acute viral infection[80]. Consistent with this, here we found that the few $CD8^+$ T cells that respond to OVA alone has little expression of T-bet or Eomes, while those responding to pIC+OVA mostly induced T-bet expression with a subset also expressing Eomes (Figure 2.10A). Stimulation of CD27 during priming with OVA resulted in both a ~2-fold increase in frequency of Eomes-expressing $CD8^+$ T cells and a >1.5-fold increase in the level of Eomes expression (within Eomes-expressing $CD8^+$ T cells) when compared to mice primed with OVA and either IFNα or IL-12 (Figure 2.10B). In contrast, αCD27 only moderately induces T-bet (Figure 2.10B), which is commonly required for effector cell expansion[94]. Notably, while IL-12 and IFNα have both been described as good inducers of T-bet in other settings[44, 95], in this protein-based vaccination system we observed higher levels of T-bet with IFNα compared to IL-12 treatment (Figure 2.10B).

We then sought to determine how CD27 and the cytokine (IFN-1/IL-12) stimulation, when applied in combination, would impact Eomes and T-bet expression in effector $CD8^+$ T cells. We found that the αCD27-dependent Eomes up-regulation was not impeded by inclusion of either IFNα or IL-12 (or pIC) (Figure 2.10C, left). Interestingly, while adding IFNα (or pIC) with αCD27 led to a 2-fold increase of T-bet in $OVA_{257-264}$-specific $CD8^+$ T cells, no significant increase in T-bet was identified by adding IL-12 with αCD27 (Figure 2.10C, middle). On a per cell basis, the inclusion of pIC or IFNα doubled the ratio of T-bet:Eomes (Figure 2.10C, right). Conversely, T-bet levels were 25% lower in response to combined αCD27/pIC in IFNαβRKO mice, yet remained stable in IL-12p40KO (Figure 2.10D). These data correlate with the failure of IL-12 to

synergize with αCD27 in promoting primary expansion of $CD8^+$ T cells. Consistent with the notion that T-bet levels are a critical regulator of the expansion of $CD8^+$ T cells in response to combined αCD27/pIC immunization, the magnitude of the $OVA_{257-264}$-specific $CD8^+$ T cell response positively correlated with their T-bet levels (Figure 2.10E). Together our data suggested that the synergy between αCD27 and IFN-1 (or pIC) is a product of optimized regulation of transcription factors, including αCD27-mediated induction of Eomes and IFN-1-mediated induction of T-bet.

2.4.7 Co-targeting CD27 and TLR stimulation generates functional memory $CD8^+$ T cells.

CD70 blockade had a more profound impact on effector $CD8^+$ T cell expansion than the generation of memory (Figure 2.1B). This result, together with the ability of CD27 to promote Eomes expression, which is known to support memory cell development, led us to question whether CD27 stimulation is sufficient to promote memory $CD8^+$ T cells in the context of protein immunization. We found that although CD27 stimulation increased the proportion of SLECs compared to OVA immunization alone (despite modestly reducing the T-bet:Eomes ratio), there was nonetheless a clearly discernable MPEC population (Figure 2.11A). Remarkably, however, mice primed with OVA+αCD27 made very limited secondary responses after rechallenge (Figure 2.11B). The addition of pIC to OVA+αCD27 further increased the proportion of SLECs in the primary response (in accordance with the increase with the T-bet:Eomes ratio) yet did not increase the number of MPECs (Figure 2.11A). Despite this, mice primed with OVA+combined αCD27/pIC generated a ~10-fold greater secondary response than those primed with αCD27 (Figure 2.11B). These data indicate that TLR and CD27 stimulation not only synergize to generate primary $CD8^+$ T cell responses to

Figure 2.10 **CD27 stimulation induces Eomes while IFN-1 induces T-bet.** Eomes/T-bet expression in $OVA_{257-264}$-specific dextramer$^+$ $CD8^+$ T cells from spleens 7d after immunization with OVA and indicated adjuvant(s). Representative plots from two seperate experiments where mice were immunized with either OVA alone or in combination with pIC **(A)**; or IL-12, IFNα or αCD27, **(B)**. Regions are based on FMO stains. Percentage of positive and geometric mean of fluorescence intensity (GMFI) for the positive subsets are listed below. **(C)** Expression of Eomes and T-bet (gated on positively expressing cells), and T-bet/Eomes ratio from mice treated with αCD27 alone, or in combination with pIC, IFNα or IL-12, respectively. Histograms show combined data for three mice per group from representative experiments, which were repeated twice independently. **(D)** T-bet expression from WT, IL12p40KO and IFNαβRKO mice. Data are representative of two independent repeats. **(E)** Correlation between T-bet protein level and the magnitude of $CD8^+$ T cells responses. Each dot presents an individual mouse from three independent experiments. For **(C) (D)**, one-way ANOVA with Tukey's post test, *p<0.05, **p<0.01, ***p<0.001, n.s.= not significant.

Figure 2.10

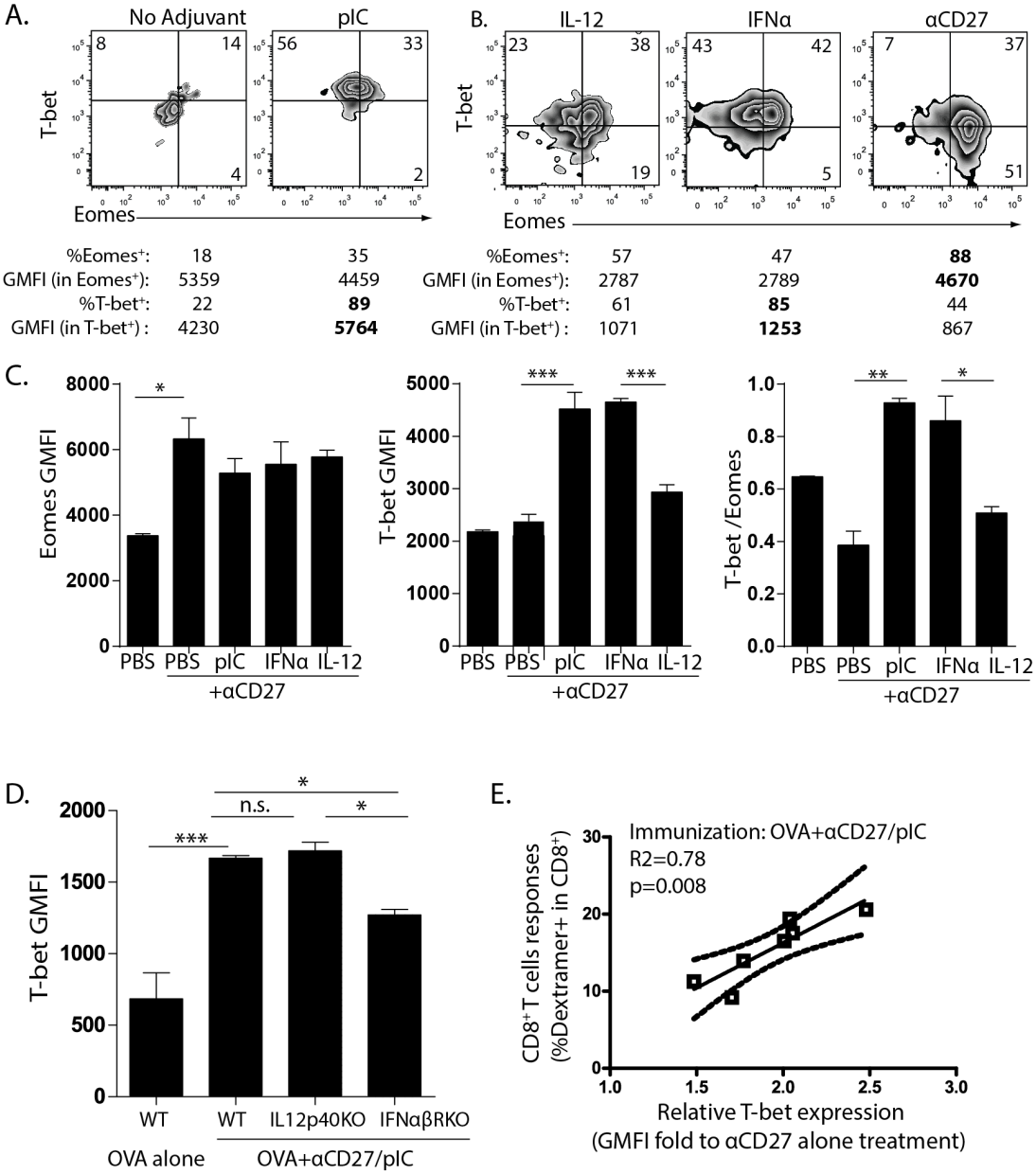
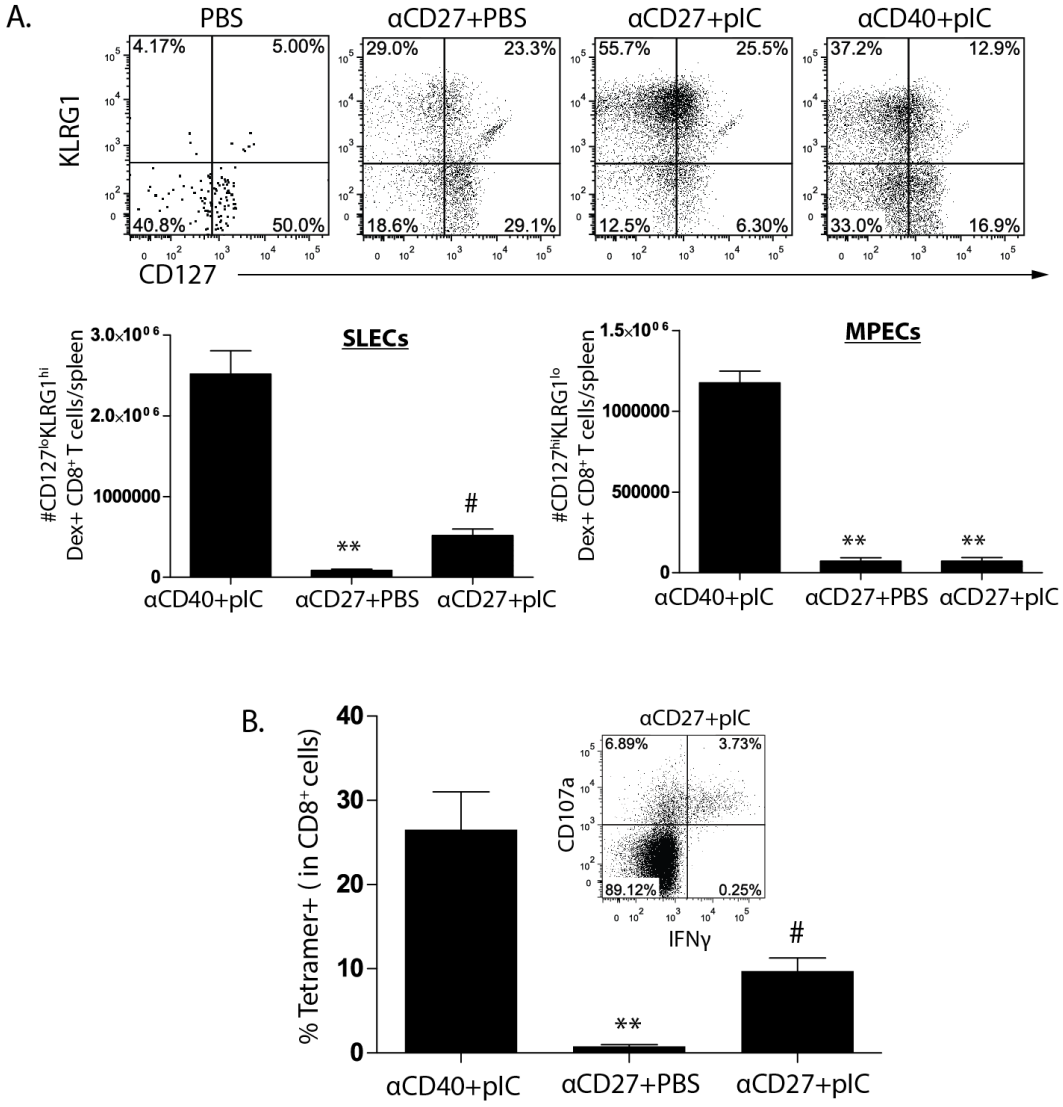

A.
No Adjuvant
pIC
T-bet
Eomes
8
14
4
56
33
2
B.
IL-12
IFNα
αCD27
23
38
19
43
42
5
7
37
51
%Eomes+: 18 35
GMFI (in Eomes+): 5359 4459
%T-bet+: 22 89
GMFI (in T-bet+) : 4230 5764
%Eomes+: 57 47 88
GMFI (in Eomes+): 2787 2789 4670
%T-bet+: 61 85 44
GMFI (in T-bet+) : 1071 1253 867
C.
Eomes GMFI
T-bet GMFI
T-bet /Eomes
PBS PBS pIC IFNα IL-12
+αCD27
D.
T-bet GMFI
n.s.
WT WT IL12p40KO IFNαβRKO
OVA alone OVA+αCD27/pIC
E.
Immunization: OVA+αCD27/pIC
R2=0.78
p=0.008
CD8+ T cells responses
(%Dextramer+ in CD8+)
Relative T-bet expression
(GMFI fold to αCD27 alone treatment)

Figure 2.11 **Functional secondary $CD8^+$ T cell responses from αCD27/TLR-primed mice**. **(A)** Top plots: representative data showing CD127/KLRG1 expression on $OVA_{257-264}$-specific dextramer$^+$ $CD8^+$ T cells from spleens 7d after immunization with OVA and indicated adjuvant(s); regions are based on FMO stains. Bottom histograms: enumeration of SLECs and MPECs (n=3/cohorts) in a representative experiment, which were repeated at least twice independently. **(B)** Frequency of secondary $OVA_{257-264}$-specific $CD8^+$ T cells in spleens of mice with indicated priming was measured 5d after OVA-adeno challenge. Data shown are from one of two similar experiments with three mice per group. Inset: representative data showing CD107a/IFNγ expression after 5h in *vitro* $OVA_{257-264}$ peptide stimulation of secondary effectors (identified by $CD44^{hi}CD8^+$ live cells) from spleen of mice primed with OVA+αCD27/pIC; gatings are based on unstimulated controls. One-way ANOVA with Tukey's post test for all histograms, **p<0.01 compared to the first column, #p<0.05 compared to the second column.

Figure 2.11


A.
PBS
αCD27+PBS
αCD27+plC
αCD40+plC
KLRG1
CD127
4.17%
5.00%
40.8%
50.0%
29.0%
23.3%
18.6%
29.1%
55.7%
25.5%
12.5%
6.30%
37.2%
12.9%
33.0%
16.9%
SLECs
MPECs
#CD127loKLRG1hi Dex+ CD8+ T cells/spleen
#CD127hiKLRG1lo Dex+ CD8+ T cells/spleen
3.0x10^06
2.0x10^06
1000000
1.5x10^06
500000
0
**
#
αCD40+plC
αCD27+PBS
αCD27+plC
B.
% Tetramer+ (in CD8+ cells)
40
30
20
10
0
αCD27+plC
CD107a
IFNγ
6.89%
3.73%
89.12%
0.25%

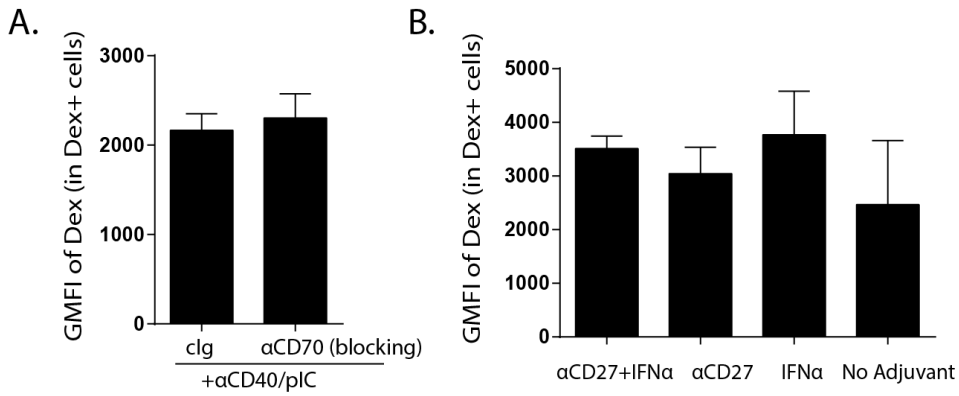
protein immunization, but also that the addition of TLR stimulation provides a critical co-factor that promotes the development of memory $CD8^+$ T cells.

2.5 Discussion

We demonstrate that direct stimulation of CD27 in conjunction with TLR stimulation can drive the expansion and differentiation of the $CD8^+$ T cell response and that CD27 stimulation is an obligatory component of the activity of CD40 stimulation. Blocking CD70 dramatically reduced the frequency of KLRG1-expressing SLECs, a phenotype that also occurs in the absence of $CD4^+$ T cells[80]. Yet in contrast, we find that CD27 stimulation by itself is a weak agonist for $CD8^+$ T cell responses to protein immunization, and the majority of the response is equally distributed between SLECs ($CD127^{lo}KLRG1^{hi}$), MPECs ($CD127^{hi}KLRG1^{lo}$) and $CD127^{hi}KLRG1^{hi}$ cells. Thus, CD27 promotes the expansion of primary effectors, but is insufficient to drive SLEC expansion in isolation. Profound synergistic expansion of the primary KLRG1-expressing SLEC component of the $CD8^+$ T cell response after CD27 stimulation was achieved by the inclusion of TLR stimulation. Notably, therapeutic vaccination substantially increased the magnitude of the $CD8^+$ T cell response during an acute viral infection, and this correlated strongly with reduced viral titer, suggesting an approach for curtailing viral infection prior to the establishment of protective antibodies. Pointedly, we don't find that CD27 stimulation leads to reduced staining with MHC-multimers (Figure 2.12), arguing against the notion that CD27 stimulation increases the magnitude of the primary response by recruiting low affinity $CD8^+$ T cells[96]. Underscoring the interaction between CD27 and TLR stimulation, we have found that if endotoxin is not removed from the agonistic αCD27, then it can initiate substantial $CD8^+$ T cell responses independently from further TLR stimulation. This could potentially explain the difference in CD27-driven

Figure 2.12 **CD27 costimulation has minimal impact on the TCR affinity of primary CD8$^+$ T cells.** **(A)** CD4-depleted mice were immunized with OVA+combined αCD40/pIC, and either control IgG (cIg) or blocking Ab of CD70. **(B)** CD4-depleted mice were immunized with OVA, and αCD27 and/or IFNα as adjuvant(s). Histograms show the geometric mean of florescence intensity (GMFI) of MHC-dextramer staining of $OVA_{257-264}$-specific CD8$^+$ T cells in spleen at d7. Assays were independently repeated at least twice, with three mice in each cohort.

Figure 2.12


A.
GMFI of Dex (in Dex+ cells)
3000
2000
1000
0
cIg
αCD70 (blocking)
+αCD40/pIC
B.
GMFI of Dex (in Dex+ cells)
5000
4000
3000
2000
1000
0
αCD27+IFNα
αCD27
IFNα
No Adjuvant

response sizes in this study compared to those in a recent publication describing differences in the influence of CD27 and 4-1BB driven on $CD8^+$ T cell differentiation[97]. Additionally, previous studies using soluble recombinant CD70[37] did not require TLR stimulation to support $CD8^+$ T cell responses to $OVA_{257-264}$ peptide; however, these studies were performed in chimeric mice containing large numbers of OT-I TCR transgenic T cells, a situation which we find to be independent of either CD27 or TLR stimulation (not shown).

Other studies have indicated the synergy achieved between αCD40 and TLR agonists[23, 82, 98] is dependent upon IFN-1 and associated with its ability to upregulate CD70 expression on DC. However, by using agonist antibodies to CD27, consistent with previous reports[42, 43, 84-86, 91, 99], we reveal an additional critical role for IFN-1 as a major cofactor with CD27 stimulation at the level of the T cell. This is demonstrated by the reduced expansion of $CD8^+$ T cells deficient in IFNαβR expression in response to OVA with combined αCD27/pIC, and reciprocally the unimpeded expansion of WT OT-I in IFNαβRKO mice in which only the OT-I cells are responsive to IFN-1. IFN-1-mediated signaling in host populations is dispensable in this priming system – indeed, the magnitude of OT-I responses were even elevated in IFNαβRKO hosts, probably due to a reduced competition from the deficient endogenous $CD8^+$ T cell responses. The competitive advantage of IFNabR-expressing OT1 could also be as a consequence of increased IFN-1 availability to OT-I cells due to the reduced consumption by the host, or the ability of IFN-1 to impart resistance of $CD8^+$ T cells to NK-mediated lysis via the expression of NRC-1[100, 101]. Although we have determined that NK cells are not required for αCD27/pIC-mediated expansion of primary $CD8^+$ T cell responses, we are currently assessing whether NK cells are deleterious in the absence of either CD27 or IFN-1.

Our data argue that the varied ability of costimulatory/Signal 3 molecules to induce T-bet or Eomes dictates their contribution to the primary $CD8^+$ T cell response after protein immunization. CD27 stimulation weakly induces T-bet expression in primary effectors. Rather, T-bet is more strongly promoted by either TLR or IFN-1, and the level of T-bet, and the ratio of T-bet:Eomes, positively correlates with the magnitude of $CD8^+$ T cell response, providing a probable explanation for the ability of TLR to augment CD27-stimulated responses. If T-bet induction is sufficient to drive primary $CD8^+$ T cell responses, it raises the question why CD27 stimulation is necessary to support TLR agonists and IFN-1. Consistent with our previous studies with vaccinia virus, and recent reports studying 4-1BB[97, 102] (a related TNF-superfamily member) we find that CD27 stimulation significantly increases the expression of Eomes compared to either TLR or cytokine stimulation. In viral infection models, Eomes has been demonstrated to play a critical role in effector $CD8^+$ T cell differentiation and function, yet is not thought to play a significant role in promoting the expansion or survival of well-differentiated primary effectors, perhaps due to some redundancy between Eomes and T-bet[94, 103]. We hypothesize that the $CD8^+$ T cell response to protein immunization might be enhanced by CD27-derived Eomes expression as the level of T-bet expression induced by soluble TLR agonists/IFN-1 may be low compared to that achieved by viral infections, reducing the potential redundancy between these molecules[94]. We are currently generating the appropriate knockouts to directly test this hypothesis.

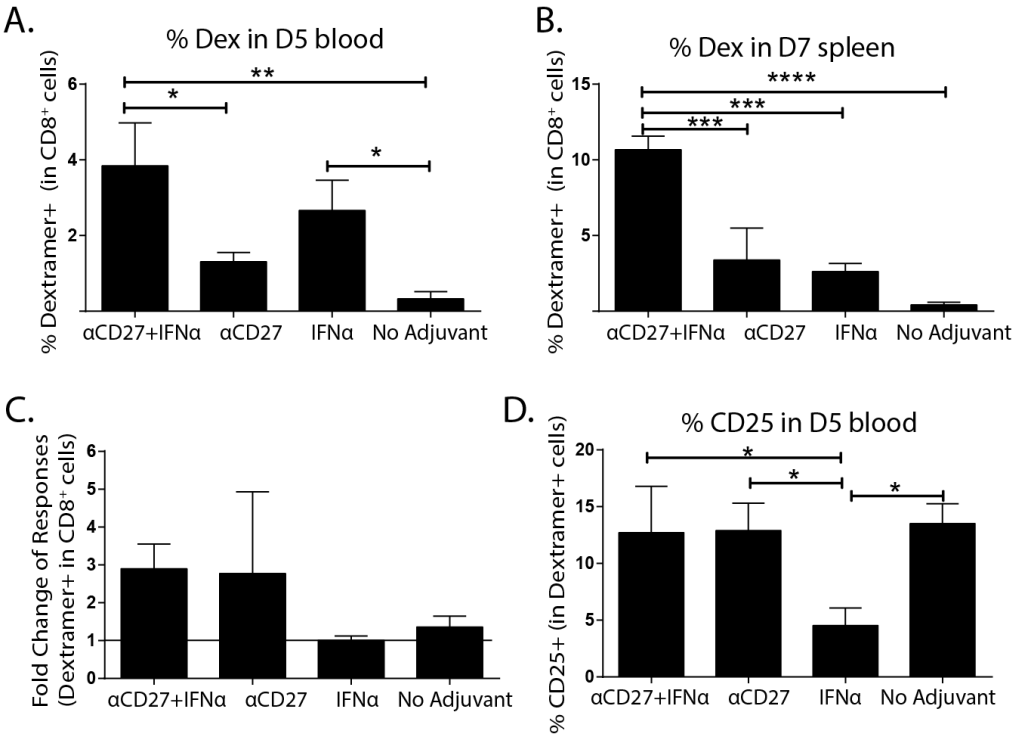
How CD27 increases Eomes expression is not currently clear. CD27 is known to support the expression of CD25, the high affinity receptor for IL-2 ([30]; and our data not shown), and STAT5 augments Eomes expression[13]. Thus, the enhanced expansion achieved with CD27 stimulation may be a consequence of its ability to regulate IL-2

signals. Previous studies have demonstrated that receptivity to IL-2 is critical for sustained $CD8^+$ T cell expansion[12, 14], but sustained IL-2R expression has been attributed to IFN-1[104]. Preliminary studies (Figure 2.13) suggest that, in the current system, IFNα (in contrast to CD27) is not able to support CD25 expression at d5, and that CD27 stimulated cells continue to expand from d5 to d7, while IFNα stimulated cells contract. Thus, our current hypothesis is that IFN-1 is needed for the initial expansion of primary $CD8^+$ T cells, perhaps by inducing T-bet or preserving them from NK-mediated lysis[100, 101], while signals emanating from CD27 support the sustained expansion, perhaps by activating an IL-2-Eomes node. Aside from influencing the magnitude of the $CD8^+$ T cell response, CD27 augmented IFNγ production on a per cell basis. CD27-mediated potentiation of $CD8^+$ T cell function was not found for degranulation (CD107a expression) or IL-2 production. The capacity for CD27 stimulation to augment IFNγ production in $CD8^+$ T cells is under-appreciated[97, 105], and important for potential therapeutic interventions based on CD27 stimulation (tumor control[106, 107]; viral infection[108, 109]), and may be a function of the increased Eomes expression[103] achieved by CD27 stimulation.

It is currently unclear why IL-12 does not support the effects of CD27 stimulation in a manner similar to IFN-1. In the context of protein immunization used in the current study we found that IFN-1 is a stronger inducer of T-bet than IL-12, and that IL-12 is in fact dispensable for T-bet induction in the presence of pIC. We have confidence that recombinant IL-12 is bio-active in our system as it induced higher KLRG1 expression in conjunction with CD27 stimulation. It is noteworthy that IL-12 has been reported to be a negative regulator of Eomes, and IFN-1 an inducer of T-bet[59, 86]. However, we found no detrimental effect of IL-12 on Eomes expression in the context of CD27 immunization,

Figure 2.13 **CD27 stimulation but not IFNα induces CD25, which correlates with an enhanced expansion during late stages of primary responses.** CD4-depleted mice were immunized with OVA, and αCD27 and/or IFNα as adjuvant(s). Frequency of $OVA_{257-264}$-specific $CD8^+$ T cells in **(A)** d5 blood and in **(B)** d7 spleen. **(C)** Fold of expansion between d 5~7. **(D)** CD25 expression on $OVA_{257-264}$-specific $CD8^+$ T cells in blood at d5. Histograms show quantification of a single experiment with three mice in each cohort. One-way ANOVA with Tukey's posttest, *p<0.05, **p<0.01, ***p<0.001.

Figure 2.13


A.
% Dex in D5 blood
% Dextramer+ (in CD8+ cells)
αCD27+IFNα
αCD27
IFNα
No Adjuvant
B.
% Dex in D7 spleen
% Dextramer+ (in CD8+ cells)
αCD27+IFNα
αCD27
IFNα
No Adjuvant
C.
Fold Change of Responses (Dextramer+ in CD8+ cells)
αCD27+IFNα
αCD27
IFNα
No Adjuvant
D.
% CD25 in D5 blood
% CD25+ (in Dextramer+ cells)
αCD27+IFNα
αCD27
IFNα
No Adjuvant

suggesting that this is not the mechanism in play here. Of interest, we previously reported that CD27 stimulation can antagonize the polarizing effects of IL-12 on $CD8^+$ T cells[80]. While we do not know the mechanism by which this occurs, an antagonistic effect of CD27 stimulation on IL-12 signaling could explain their inability to cooperate in the context of protein immunization.

Surprisingly, mice stimulated by αCD27 alone did not mount strong secondary responses upon antigen re-challenge, despite the expansion of CD127-expressing primary effector $CD8^+$ T cells, indicating that an additional signal is necessary for the programming of fully-functional memory precursors. Given the synergy between IFN-1 and CD27 stimulation during primary response we hypothesize that IFN-1 is also promoting memory cell differentiation or capacity to re-expand as the addition of IFN-1 to CD27 stimulation did not increase the absolute number of MPECs. This may be related to the increase in T-bet expression in response to IFN-1, as T-bet is known to support memory $CD8^+$ T cell development ([110], and our unpublished results). Thus, there may be a "Goldilocks" level of T-bet that is necessary for effector cell expansion and differentiation yet also engenders memory cell survival, without driving to terminally differentiated effector cells[44]. Interestingly, the ration of T-bet:Eomes is equivalent in both KLRG1-expressing SLECs and IL-7R-expressing MPECs (data not shown), suggesting that the ratio of these transcription factors is not decisive in their fate decisions. The necessity of IFN-1 during the primary response to protein immunization complicates directly dissecting its role in memory cell differentiation. However, preliminary data (not shown) using adenoviral immunization indicates a critical role for IFN-1 in CD27-dependent memory cell generation, independent from its role in supporting primary $CD8^+$ T cell expansion described here. It should also be noted that

although priming with αCD27/pIC produced 30-fold less $CD127^{hi}KLRG1^{lo}$ MPECs than achieved with αCD40/pIC, there was only a 3-fold difference in the magnitude of the secondary response between these cohorts. Thus, αCD27/pIC immunization may generate either more memory $CD8^+$ T cells or memory $CD8^+$ T cells with greater expansion potential compared to those that are generated with αCD40/pIC. Pertaining to this, we note that αCD27/pIC results in a large increase in $CD127^{hi}KLRG1^{hi}$ cells. As these cells have previously been characterized as having a better ability to become long-term memory cells than $CD127^{lo}KLRG1^{hi}$ SLECs[44], it is possible that the expansion of this population contributes to the increased secondary responses. Future studies that focus on these subpopulations (dissected by CD127/KLRG1 expression) will help define how the addition of IFN-1 to stimulation augments memory cell development.

Together, these data indicate that the combinatorial targeting of CD27 with TLR stimulation can be a potent mechanism for eliciting the expansion of effector and memory $CD8^+$ T cells to helper-dependent antigens. IFN-1 is critical for the efficacy of this approach. Thus, coordinate targeting of CD27 and IFNαβR defines a potentially effective therapeutic avenue, circumventing the need for potentially dangerous pathogen-derived vectors.

Chapter 3: CD27 stimulation promotes the frequency of IL-7 receptor-expressing memory precursors and prevents IL-12-mediated loss of memory in the absence of $CD4^+$ T cell help

3.1 Abstract

Fully functional $CD8^+$ T cell memory is highly dependent upon $CD4^+$ T cell support. $CD4^+$ T cells play a critical role in inducing the expression of CD70, the ligand for CD27, on dendritic cells. In this study, we demonstrate that CD27 stimulation during primary $CD8^+$ T cell responses regulates the ability to mount secondary $CD8^+$ T cell responses. CD27 stimulation during vaccinia and dendritic cell immunization controls the expression of the IL-7R (CD127), which has been shown to be necessary for memory $CD8^+$ T cell survival. Furthermore, CD27 stimulation during primary $CD8^+$ T cell responses to vaccinia virus restrained the late expression on memory precursor cells of cytokine receptors that support terminal differentiation. The formation of $CD8^+$ T cell memory precursors and secondary $CD8^+$ T cell responses was restored in the absence of CD27 costimulation when endogenous IL-12 was not available. Similarly, the lesion in $CD8^+$ T cell memory that occurs in the absence of $CD4^+$ T cells did not occur in mice lacking IL-12. These data indicate that $CD4^+$ T cell help and, by extension, CD27 stimulation support $CD8^+$ T cell memory by modulating the expression of cytokine receptors that influence the differentiation and survival of memory $CD8^+$ T cells.

3.2 Introduction

In the search for more effective vaccine regimens, there is a continuing need to understand the basis by which $CD8^+$ T cell memory develops and is sustained. Two competing hypotheses account for $CD8^+$ T cell memory, as follows: first, that a subset of

less differentiated primary $CD8^+$ T cells survives at the end of the response[111]; second, that memory precursors split from primary effectors at early stages of the primary response and develop as parallel population[112]. Recent data indicate that naive $CD8^+$ T cells have the capacity to form either effector or memory $CD8^+$ T cells[50], and that at least some memory cells show evidence of previous effector activity[51], supporting a linear differentiation model. From the pool of $CD8^+$ T cells that expand in response to immunization, those with a greater capacity for survival (termed memory precursor effector cells [MPECs]) are enriched within a population of cells that re-express the IL-7R[56, 113], whereas terminally differentiated effector cells with little capacity to survive long-term (termed short-lived effector cells [SLECs]) frequently express KLRG1[44]. Loss of IL-7R expression has been shown to be influenced by TCR engagement and the binding of IL-7, but the factors that influence its re-expression on MPECs are not known[55].

The factors that control the fate decisions of primary $CD8^+$ T cells are therefore of considerable interest. Recent studies have elucidated that the extent of inflammation that accompanies exposure to Ag is a critical determinant in the differentiation of primary $CD8^+$ T cells into SLECs. $CD8^+$ T cell responses to dendritic cell (DC) immunization are dominated by cells with MPEC phenotype, and the addition of proinflammatory TLR agonists increases the proportion of KLRG1-expressing SLECs in the response[57]. Differentiation into KLRG1-expressing SLECs is strongly enhanced by IL-12–driven induction of T-bet and BLIMP-1[44, 46, 114, 115]. Genetically limiting T-bet expression enhances CD8 T cell memory in some but not all cases[44, 116], suggesting that SLECs arise from the same common precursor as MPECs, and that inflammation-driven differentiation might come at the expense of MPECs and memory $CD8^+$ T cells.

This leads to the hypothesis that differentiation into memory precursors is the default pathway for activated $CD8^+$ T cells that have not received effector cell differentiation signals. However, $CD4^+$ T cells have also been shown to provide important contributions to memory $CD8^+$ T cell development and function in many[61-63], but not all responses to pathogens[64]. Our understanding of the mechanistic basis behind $CD4^+$ T cell-mediated promotion of $CD8^+$ T cell memory is incomplete. In some studies, expression of IL-2R or IL-21R is necessary for $CD8^+$ T cell memory[11, 15, 16], suggesting that $CD4^+$ T cells support $CD8^+$ T cell memory via the provision of paracrine cytokines. Alternatively, direct stimulation of CD40 on $CD8^+$ T cells by $CD4^+$ T cells can enhance $CD8^+$ T cell activation[7] and survival[8].

$CD4^+$ T cell-mediated stimulation of CD40 also plays an important role in upregulating the activation state of DC to support $CD8^+$ T cell responses. Direct stimulation of CD40 on DC has been shown to overcome the necessity of $CD4^+$ T cells for the generation of primary $CD8^+$ T cell responses[18, 23, 117] and subsequent development into fully functioning memory $CD8^+$ T cells[18], indicating that paracrine cytokines provided by $CD4^+$ T cells may support but are not required for $CD8^+$ T cell memory. These studies indicate that DC that have been activated by $CD4^+$ T cells induce a program of proliferation and differentiation in $CD8^+$ T cells that is sufficient for long-term survival and homeostatic proliferation. However, our understanding of the mechanistic basis by which $CD4^+$ T cell-stimulated DC regulate $CD8^+$ T cell memory programming is limited.

CD40-stimulated DC upregulate the expression of CD70, the ligand for CD27, and blockade of CD70 potently reduces primary $CD8^+$ T cell responses [18, 22, 24], demonstrating a prominent role for CD70 expression in a licensed DC. Importantly,

memory $CD8^+$ T cell responses to influenza infection and lymphocytic choriomeningitis virus (LCMV) infection have been reported to be curtailed in CD27 knockout mice[27], and blocking CD70–CD27 interactions results in diminished $CD8^+$ T cell memory [32, 40]. Therefore, we hypothesized that the defects in the quantity and quality of $CD8^+$ T cell memory that occur in the absence of $CD4^+$ T cell help are a consequence of inadequate CD27 stimulation. To test this hypothesis, we used combinations of blocking Abs to CD70 and agonistic Abs to CD27, either in the context of highly inflammatory infections with recombinant vaccinia virus or weakly inflammatory DC-based immunizations, to determine the extent to which CD27 stimulation during primary $CD8^+$ T cell responses influences the fate decisions made by primary $CD8^+$ T cells. Our results indicate that CD27-mediated stimulation strongly supports $CD8^+$ T cell differentiation to MPECs, and protects against IL-12–mediated terminal differentiation.

3.3 Materials and Methods

3.3.1 Animals C57BL/6Y (B6) mice were obtained from National Cancer Institute (Frederick, MD). IL-12 p35 (B6.129S1-Il12a^{tm1Jm}/J stock 002692) and IL- 12p40 knockout mice (B6.129S1-Il12b^{tm1Jm}/J stock 002693) were pur- chased from The Jackson Laboratory (Bar Habor, ME). OT-I transgenic mice, expressing TCRs specific for OVA257 peptide in complex with H-$2K^b$, were purchased from The Jackson Laboratory (C57BL/6-Tg [TcraTcrb] 1100Mjb/J, stock 003831) and crossed onto $Thy1.1^+$ (B6.PL-$Thy1^a$/CyJ stock 000406) mice obtained from The Jackson Laboratory. CD27 knockout mice[33] were provided by S.Schoenberger (La Jolla Institute for Allergy and Immunology), with the permission of J. Borst (Netherlands Cancer Institute). Mice were maintained in specific pathogen- free facilities and were treated in accordance with the guidelines established by the Animal Care and Use Committee at the University of Virginia.

3.3.2 Cell lines and viruses Recombinant vaccinia expressing OVA (OVA-vac) was provided by J. Yewdell (National Institute of Allergy and Infectious Diseases), and was propagated on HuTK^2 cells. Recombinant adenovirus expressing OVA (OVA-adeno) was provided by Y. Hahn (University of Virginia), and was propagated on 293A fibroblasts. LB15.13 hybridoma was obtained from American Type Culture Collection (Frederick, MD) and maintained in RPMI 1640 with 5% FBS (Hyclone, Logan, UT).

3.3.3 Antibodies Agonistic AT124.1 anti-mouse CD27 has been described[88]. FR70-blocking anti-mouse CD70 has been described[118]. Control Ig (cIg) was purchased from Sigma-Aldrich (St. Louis, MO).

3.3.4 Bone marrow DC generation Bone marrow DC (BMDC) were expanded from mouse bone marrow in the presence of GM-CSF and IL-4, as previously described[119]. D7 BMDCs were isolated by negative selection on magnetic columns (Stemcell, Vancouver, BC), incubated overnight in culture with CD40L-expressing 3T3 cells and media containing 10 mg/ml OVA257 peptide.

3.3.5 Peptides and protein Synthetic peptides were purchased from Genscript (Piscataway, NJ). OVA was purchased from Sigma-Aldrich. Endotoxin was removed by Detoxi-Gel endotoxin-removal kit (Pierce, Rockford, IL).

3.3.6 Immunization For the generation of chimeric mice, 1000–5000 Thy1.1$^+$ CD45.2$^+$ OT-I cells were transferred into recipient mice. Depletion of CD4$^+$ T cells was achieved by i.p. injection of 200 mg GK1.5 (American Type Culture Collection) 7 and 3 d prior to generation of chimeric mice, and confirmed by tail vein bleed. CD27 stimulation was performed by injecting 50 mg AT124-1 i.p. on days 0, 3, and 6. CD70 blockade was performed by injecting 500 mg FR70 i.p. on days 0, 2, 4, and 6. IL-12 blockade was performed by injecting 500 mg C17.8 (BioXCell, Hanover, NH) on days 0, 2, 4, and 6

after immunization. rIL-12 (eBioscience) was delivered by i.p. injection of 500 mg 24 and 48 h after immunization. Primary $CD8^+$ T cell responses were generated by injecting mice i.v. with 10^7 PFU OVA-vac, or 10^5 CD40L-activated OVA257-pulsed BMDC. Secondary responses were initiated in primed mice by i.p. challenge with 2 x 10^8 PFU OVA-adeno.

3.3.7 Viral titers Virus titers from infected mice were determined 4 d after i.p. challenge with 1 x 10^8 PFU naive or previously immunized mice. Ovaries were excised and digested with collagenase/DNase/hyaluronidase and then homogenized. Homogenate was subject to three cycles of freezing and thawing and then sonicated. Sonicate was cleared of particulate matter by a centrifu- gation, and the supernatant was used to infect $HuTK^2$ cells. Virus plaques were revealed 48 h later by crystal violet staining.

3.3.8 Tetramer staining H2-K^b tetramers that had been folded around OVA_{257} were provided by V. Engelhard (University of Virginia). Lymphocytes were isolated from blood or homogenized spleens and were coincubated for 30 min at 4℃ with tetramer-allophycocyanin. Abs described were purchased from eBioscience, with the exception of anti-CD44 Pacific Blue, anti–IL-21R PE, and anti–IFN$\alpha\beta$R-PE, which were purchased from BioLegend. Anti- CD212 (IL-12Rβ1) PE and anti-CD107a PE-Cy7 were purchased from BD Biosciences (Franklin Lakes, NJ). Anti-KLRG1 PE was purchased from Abcam (Cambridge, MA). Staining was assessed by flow cytometry on a FACS Canto II (BD Biosciences) and analyzed using FlowJo software (Tree Star).

3.3.9 Memory adoptive transfers $CD8^+$ T cells were enriched from $CD45.1^+$ mice by magnetic bead-based negative selection (Stemcell) from the spleens and lymph nodes of day 90 mice. OT-1 cells were enumerated by staining for Thy1.1, and ~1000 OT-1 cells were transferred into recipient $CD45.2^+$ mice.

3.3.10 Statistics Statistical significance of differences between comparison groups was determined by performing unpaired two-tailed Student t tests for 95% confidence limits using GraphPad Prism software (San Diego, CA).

3.4 Results

3.4.1 Stimulation of CD27 on $CD8^+$ T cells promotes $CD8^+$ T cell memory in the absence of $CD4^+$ T cell help

To begin to test whether the defect in $CD8^+$ T cell memory that occurs in the absence of CD4 induction of CD27 costimulation, we first asked whether direct stimulation of CD27 promotes $CD8^+$ T cell memory in the absence of $CD4^+$ T cells. To facilitate tracking $CD8^+$ T cell responses, and to allow genetic manipulation of responding $CD8^+$ T cell populations, we generated chimeric mice by transferring ~1000 $Thy1.1^+$ OT-I TCR transgenic cells into $Thy1.2^+$ recipient mice. At this frequency, ~70% of the OVA257-specific response is composed of OT-I cells, whereas 30% is derived from endogenous sources. This relationship is maintained through memory and subsequent secondary expansion (Figure 3.1). $CD4^+$ T cell-depleted OT-1 chimeric mice were immunized with OVA-vac, and treated with an agonistic Ab to CD27 or cIg during the primary response. Compared with nondepleted control mice, the frequency of the OVA257-specific primary $CD8^+$ T cells in CD4-depleted mice was ~2-fold lower. CD27 stimulation modestly increased the magnitude of the OVA_{257}-specific primary response in CD4-depleted mice (Figure 3.2A). After 90 days of rest, we assessed the ability of these mice to mount secondary $CD8^+$ T cell responses to a heterologous challenge with OVA-adeno. As anticipated, secondary OVA257-specific $CD8^+$ T cell responses were highly compromised in cIg-treated, $CD4^+$ T cell- depleted mice as compared with nondepleted

FIGURE 3.1. Both endogenous and OT-I $CD8^+$ T cells contribute to primary, memory and secondary CD8+ T cell responses. ~1000 OT-I $Thy1.1^+$ $CD8^+$ T cells were transferred into $Thy1.2^+$ mice which were subsequently challenged with OVA-vac. Mice were bled on d7, and cohorts were either sacrificed on d60 for memory responses, or rechallenged with OVA-adeno and sacrificed 5d later for secondary responses. In each case, cells were stained with anti-CD8, MHC-tetramer and anti-Thy1.1. The proportion of $Thy1.1^+$ cells in the primary response at d7 is shown in the histogram, and summarized in the chart along with the proportion of the memory and secondary $CD8^+$ T cell populations that are $Thy1.1^+$.

FIGURE 3.1.

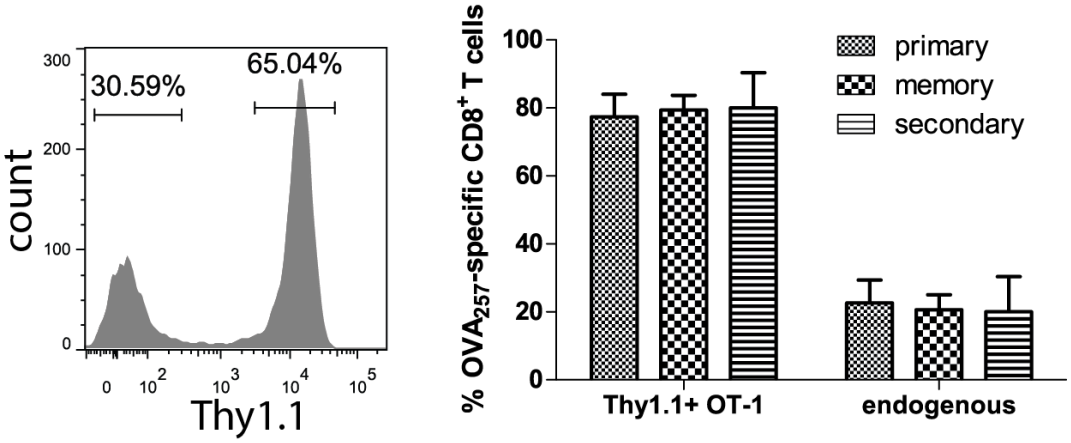
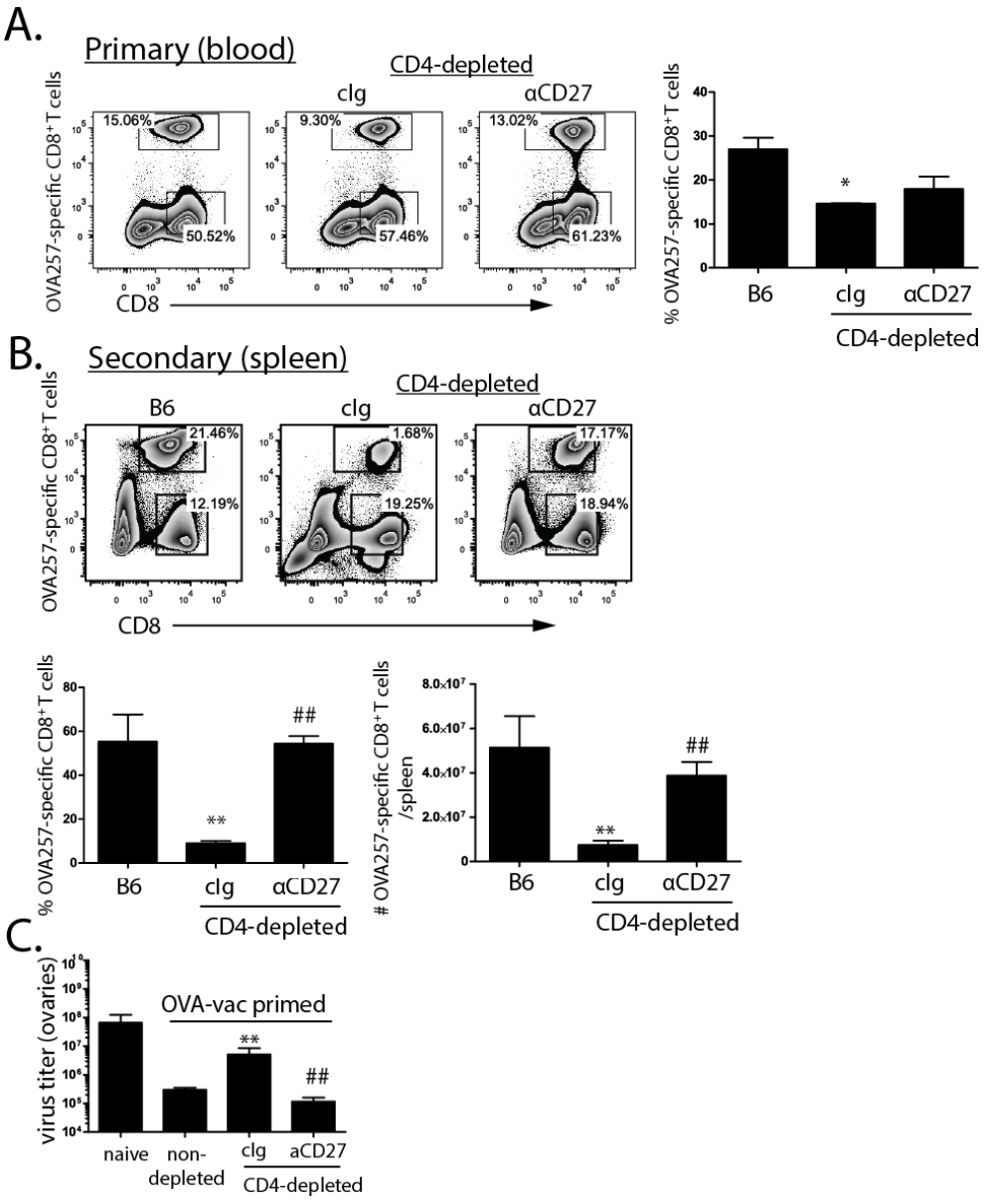

30.59%
65.04%
count
Thy1.1
% OVA257-specific CD8+ T cells
primary
memory
secondary
Thy1.1+ OT-1
endogenous

FIGURE 3.2. CD27 stimulation promotes $CD8^+$ T cell memory in the absence of $CD4^+$ T cells. OT-1 chimeric mice (n = 3 mice per cohort) were depleted of $CD4^+$ T cells, and then immunized with OVA-vac and treated with cIg or anti-CD27. (A) Primary OT-1 responses in blood 7 d after OVA-vac immunization. (B) Secondary OT-1 responses in spleens 5 d after OVA-adeno challenge of mice from (A) rested for 35 d. (C) Vaccinia titers from the ovaries of naive mice or mice primed 60 d previously with OVA-vac under the indicated conditions. Plots are derived from representative individual mice within an experimental cohort. Histograms contain compiled cohort data, showing median responses +/- SEM. Data from one of five similar experiments. *$p<0.05$, **$p<0.01$ compared with B6. $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared with cIg-treated mice.

FIGURE 3.2


A.
Primary (blood)
CD4-depleted
cIg
αCD27
15.06%
50.52%
9.30%
57.46%
13.02%
61.23%
OVA257-specific CD8+ T cells
CD8
% OVA257-specific CD8+ T cells
B6
cIg
αCD27
CD4-depleted
B.
Secondary (spleen)
B6
21.46%
12.19%
1.68%
19.25%
17.17%
18.94%
% OVA257-specific CD8+ T cells
# OVA257-specific CD8+ T cells /spleen
##
**
C.
virus titer (ovaries)
OVA-vac primed
naive
non-depleted
cIg
aCD27

mice. In contrast, $CD4^+$ T cell-depleted mice treated with anti-CD27 mounted secondary OVA_{257}-specific $CD8^+$ T cell responses that were equivalent in magnitude to those found in nondepleted mice (Figure 3.2B). Similar results were achieved using MHC II-deficient mice (data not shown). The ability of anti-CD27 treatment to rescue secondary $CD8^+$ T cell responses corresponded to a significant reduction in the amount of virus found in the ovaries of infected mice compared with control-treated counterparts (Figure 3.2C). CD27 stimulation worked directly on $CD8^+$ T cells, as anti-CD27 treatment restored $CD8^+$ T cell memory in CD4-depleted, $CD27^{-/-}$ mice containing wild-type OT-1 (Figure 3.3). Therefore, the deficiency in $CD8^+$ T cell memory that develops in the absence of $CD4^+$ T cells can be overcome by direct stimulation of CD27 on primary $CD8^+$ T cells.

3.4.2 CD70 blockade during the primary CD8 abrogates $CD8^+$ T cell memory T cell response

We reasoned that if insufficient CD27 stimulation was responsible for the defect in $CD8^+$ T cell memory in the absence of $CD4^+$ T cell help, then blocking CD27 stimulation in mice replete for $CD4^+$ T cells should abrogate memory. We prevented CD27 stimulation by infusing a CD70-blocking Ab after OVA-vac infection of OT-1 chimeric mice, and the magnitude of the primary $CD8^+$ T cell response was determined in the blood or the spleen. We found statistically significant, yet small reduction in the magnitude of the OVA_{257}-specific response compared with control-treated mice (Figure 3.4A). In contrast to the primary $CD8^+$ T cell response, the secondary response from mice treated with anti-CD70 was almost completely absent from both lymphoid (spleen, lymph nodes) and peripheral tissues (lungs) (Figure 3.4B). Thus, as with $CD8^+$ T cell responses to vaccinia virus in the absence of $CD4^+$ T cells[62, 65, 120], the primary $CD8^+$ T cell responses to recombinant vaccinia virus are not strongly dependent upon CD70

FIGURE 3.3. Anti-CD27 promotes $CD8^+$ T cell memory by direct stimulation of $CD8^+$ T cells. Secondary OT-1 response in B6 control or CD4-depleted $CD27^{-/-}$ OT-1 chimeric mice that were treated with cIg or aCD27 during the primary response to OVA-vac. Plots are derived from representative individual mice within an experimental cohort. Histograms contain compiled cohort data, showing median responses +/- SEM. *$p<0.05$; ** $p<0.01$ compared to B6. $^{\#}p<0.05$; $^{\#\#}p<0.01$ compared to cIg- treated mice. Data are from one of 3 similar experiments.

FIGURE 3.3.

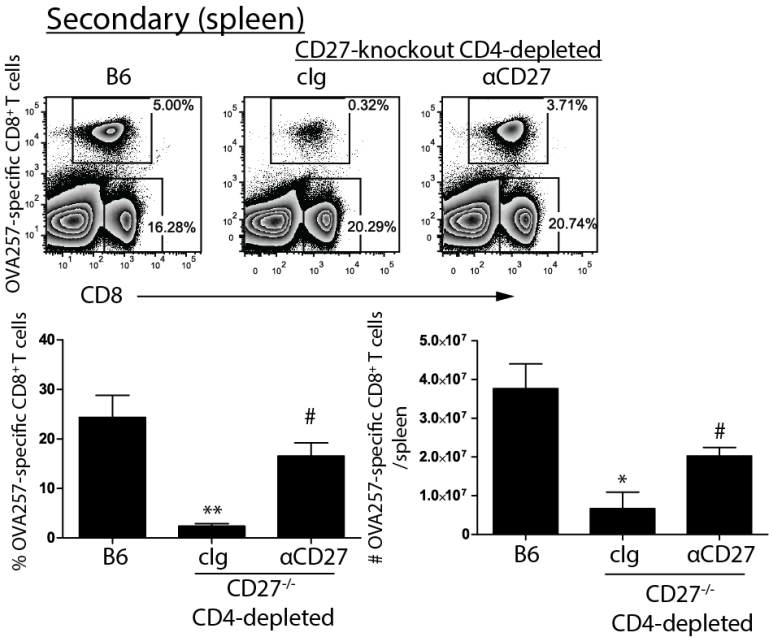
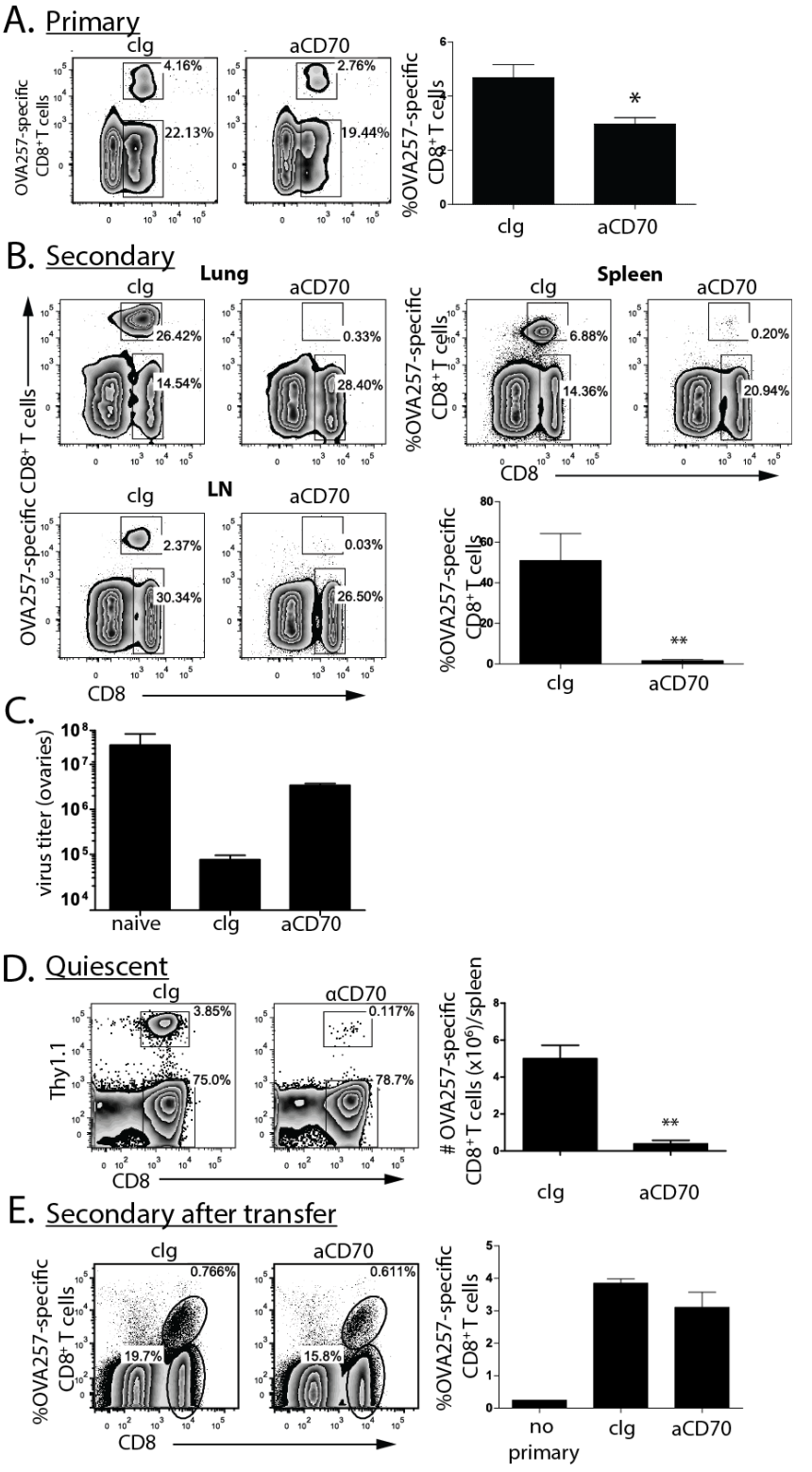

Secondary (spleen)
CD27-knockout CD4-depleted
B6
cIg
αCD27
5.00%
16.28%
0.32%
20.29%
3.71%
20.74%
OVA257-specific CD8+ T cells
CD8
% OVA257-specific CD8+ T cells
# OVA257-specific CD8+ T cells /spleen
**
*
#
B6
cIg
αCD27
CD27-/-
CD4-depleted

FIGURE 3.4. CD70 stimulation during the primary $CD8^+$ T cell response to OVA-vac is required for secondary $CD8^+$ T cell expansion. OT-I chimeric mice (n = 3 mice per cohort) were challenged with OVA-vac and were treated with anti-CD70 or cIg during the expansion of the primary response. (A) Magnitude of the primary OT-1 response in spleens on day 7 in cIg- or anti-CD70–treated mice. (B) Magnitude of secondary responses in lung, spleen, and lymph nodes (LN) 5 d after challenge with OVA-adeno, 35 d after initial priming with OVA-vac. Dot plots are from individual mice within representative experiments. Histograms contain data from spleens of each cohort. (C) Vaccinia titers from the ovaries of naive or mice primed 60 d previously with OVA-vac under the indicated conditions. (D) Frequency and number of quiescent memory $CD8^+$ T cells enriched from the spleens of OVA-vac–immunized OT-1 chimeric mice 90 d after priming. (E) Magnitude of secondary OT-1 response from 1000 transferred quiescent memory OT-1 5 d after OVA-adeno challenge. Numbers in plots indicate percentage of cells within plot within the indicated region. Each histogram shows the median value of the cohort 6 SEM. Data from experiments performed three to five times. *$p<0.05$, **$p<0.01$ compared with cIg-treated mice.

FIGURE 3.4.


A. Primary
clg
aCD70
4.16%
22.13%
2.76%
19.44%
OVA257-specific CD8+T cells
%OVA257-specific CD8+T cells
*
clg
aCD70
B. Secondary
Lung
Spleen
clg
aCD70
26.42%
14.54%
0.33%
28.40%
6.88%
14.36%
0.20%
20.94%
OVA257-specific CD8+ T cells
%OVA257-specific CD8+T cells
CD8
LN
2.37%
30.34%
0.03%
26.50%
**
C.
virus titer (ovaries)
naive
clg
aCD70
D. Quiescent
αCD70
3.85%
75.0%
0.117%
78.7%
Thy1.1
CD8
OVA257-specific CD8+T cells (x10^6)/spleen
E. Secondary after transfer
0.766%
19.7%
0.611%
15.8%
%OVA257-specific CD8+T cells
no primary

stimulation. However, the secondary $CD8^+$ T cell response after re-exposure to Ag was highly compromised, indicating a critical role for CD70-mediated stimulation in promoting $CD8^+$ memory T cell formation or function. This fact is further emphasized by the significantly higher vaccinia virus titer found in the ovaries of aCD70-treated mice rechallenged with vaccinia compared with control-treated mice (Figure 3.4C).

3.4.3 Reduction in the frequency of memory $CD8^+$ T cells in the absence of CD70 costimulation

The critical role of CD70 in establishing the ability to mount a secondary $CD8^+$ T cell response implicated that CD70 stimulation might be needed in the proper formation or survival of memory $CD8^+$ T cells, or their ability to expand upon challenge. To address this question, we next determined whether CD27 costimulation during the primary response altered the number of quiescent memory $CD8^+$ T cells. At 90 days postinfection, very few OVA_{257}-specific memory cells were found in mice that were blocked from CD70 costimulation during the primary response (Figure 3.4D). Those that remained expressed similar surface molecules as the $CD8^+$ T cells found in control-treated mice. The majority (55–60%) expressed IL-7R and CD27, but only 10–20% expressed CD62L and CCR7 (data not shown). Therefore, effector memory phenotype cells dominated in the spleen of both cIg and anti-CD70–treated mice that have been infected with OVA-vac, but no significant differences were apparent between control and anti-CD70–treated mice. Thus, CD70 costimulation during the primary $CD8^+$ T cell response enhances the number of memory $CD8^+$ T cells.

We next determined whether memory $CD8^+$ T cells that developed in the absence of CD70 costimulation have defects in their ability to proliferate in response to Ag challenge. Approximately 1000 memory OT-1 $CD45.2^+$ cells were transferred into

recipient CD45.1 mice and challenged with OVA-adeno. Memory $CD8^+$ T cells from either control or CD70-blocked mice were able to expand equivalently (Figure 3.4E), and the subsequent secondary effectors had the same ability to expose CD107a (a marker of cellular degranulation) and produce IFNγ (data not shown). Therefore, the absence of CD70 stimulation during priming with OVA-vac abrogates the ability of mice to mount secondary $CD8^+$ T cell responses, and this is predominantly a consequence of diminished memory $CD8^+$ T cell numbers, rather than the type or function of the memory $CD8^+$ T cells that do form.

3.4.4 CD70 stimulation during primary OVA-vac infection supports IL-7R–expressing memory precursor $CD8^+$ T cells

To establish how CD70 stimulation supported memory $CD8^+$ T cells, we next asked whether it played a role in the formation of $CD8^+$ T cell memory precursors, which are identified by the expression of the IL-7R (CD127) and the absence of KLRG1 expression [44, 113]. First, we determined the frequency and number of MPECs in the spleens of control or CD70-blocked mice 7 days after priming, and found a 55% reduction in the number of primary OT-1 cells that express IL-7R (Figure 3.5A). A similar outcome was found for the endogenous component of the primary $CD8^+$ T cell response (Figure 3.6). We found no significant increase in KLRG1 expression by OT-1 cells, indicating that CD70 blockade does not enhance terminal differentiation of effector $CD8^+$ T cells. Rather, an increase in the frequency of OT-1 cells that express neither IL-7R nor KLRG1 (termed early effector cells) [121] was noted (Figure 3.5A), suggesting that CD27 stimulation promotes either the formation or persistence of IL-7R–expressing $CD8^+$ T cells.

We next assessed whether augmented CD27 stimulation enhanced the frequency

FIGURE 3.5. CD70-CD27 stimulation modulates the frequency of CD127-expressing primary effector $CD8^+$ T cells. (A) KLRG1 and CD127 expression by day 7 OT-1 (top left plot; $Thy1.1^+$) cells from chimeric mice challenged with OVA-vac and treated with cIg (top plots) or anti-CD70 (bottom plots). cIg and anti-CD70 plots are gated on Thy1.1, whereas bottom left-hand plot shows KLRG1 and CD127 expression on total CD8 for comparative purposes. (B) Expression of CD127 and KLRG1 on day 7 OT-1 cells from spleens of nondepleted or CD4-depleted mice, treated with cIg or anti-CD27, that were primed with OVA-vac. (C) Intracellular expression of T-bet and Eomes in day 7 OT-1 MPEC that expanded in nondepleted (solid lines) or CD4- depleted mice treated with cIg (dashed lines) or anti-CD27 (dotted lines), gated on $Thy1.1^+$ $CD127^+$ $CD8^+$ T cells. Dot plots and overlays are from representative mice. Numbers in dot plots indicated the percentage of cells within the indicated regions or quadrants. Histograms show the number of OT-1–expressing KLRG1 and/or CD127 in the spleens of B6 or CD4-depleted mice treated with cIg- or anti-CD27– treated mice on day 7 and the geometric mean fluorescence (GMF) expression of T-bet and Eomes. Each histogram shows the median value of the cohort (n = 3) +/- SEM. *$p<0.05$; compared with cIg-treated or B6 mice. Data represent experiments repeated at least twice. $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared with cIg-treated CD4-depleted mice.

FIGURE 3.5.

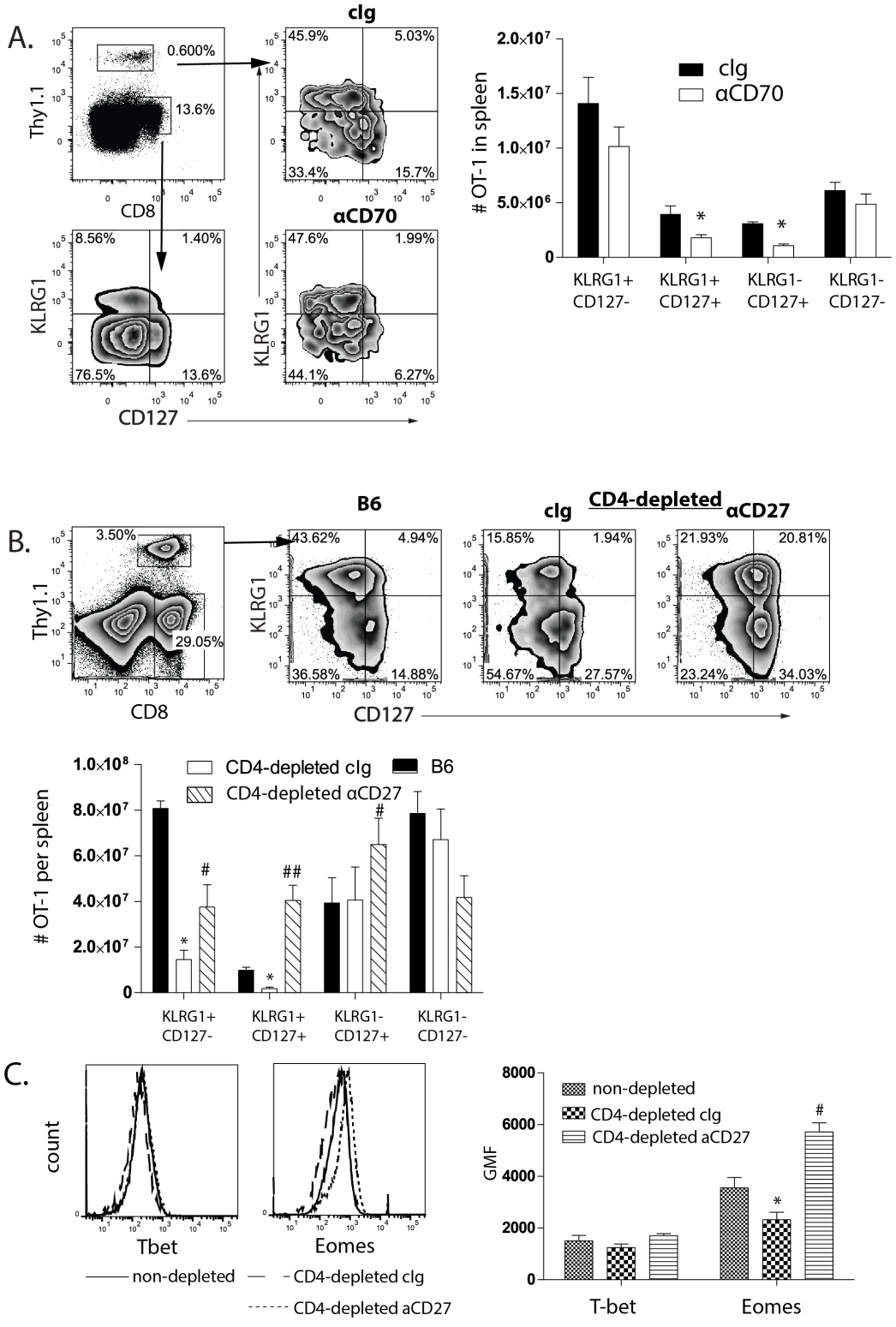
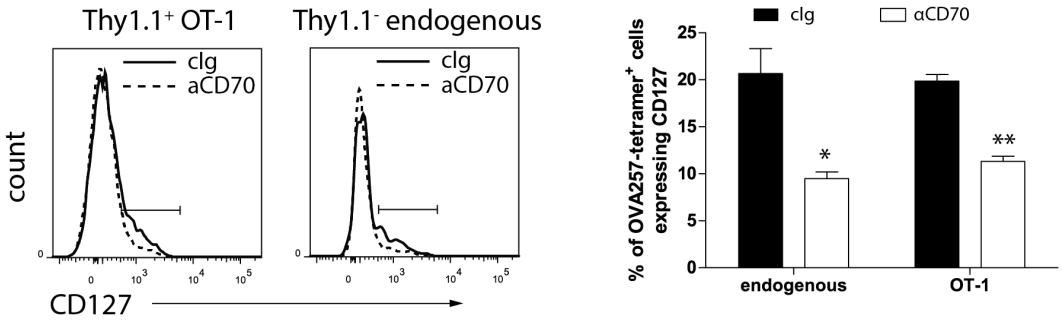

A.
cIg
αCD70
Thy1.1
CD8
0.600%
13.6%
45.9%
5.03%
33.4%
15.7%
KLRG1
CD127
8.56%
1.40%
76.5%
13.6%
47.6%
1.99%
44.1%
6.27%
OT-1 in spleen
cIg
αCD70
KLRG1+ CD127-
KLRG1+ CD127+
KLRG1- CD127+
KLRG1- CD127-
B.
B6
cIg
CD4-depleted
αCD27
3.50%
29.05%
43.62%
4.94%
36.58%
14.88%
15.85%
1.94%
54.67%
27.57%
21.93%
20.81%
23.24%
34.03%
OT-1 per spleen
CD4-depleted cIg
B6
CD4-depleted αCD27
C.
count
Tbet
Eomes
non-depleted
CD4-depleted cIg
CD4-depleted aCD27
GMF
T-bet
Eomes

FIGURE 3.6. CD70-blockade reduced the frequency of CD127-expressing cells within the endogenous response. 1000 OT-I Thy1.1^{+} CD8^{+} T cells were transferred into Thy1.2^{+} mice which were subsequently challenged with OVA-vac in the presence of cIg or anti-CD70, as described in the Methods. On d7, spleens were stained for the expression of CD8, Thy1.1, CD127 and MHC-tetramer. Histograms show overlaid CD127-expression on MHC-tetramer^{+} CD8^{+} T cells gated into either Thy1.1^{+} OT-I or Thy1.1- endogenous responders. Solid lines are from cIg treated mice, and dotted lines from anti-CD70 treated. Region gate is based on staining from FMO. Compiled data from cohorts of 3 mice are presented in the chart. *$p<0.05$; **$p<0.01$ compared to cIg treated mice.

FIGURE 3.6.


Thy1.1+ OT-1
Thy1.1- endogenous
clg
aCD70
count
CD127
clg
αCD70
% of OVA257-tetramer+ cells expressing CD127
endogenous
OT-1
*
**

and number of IL-7R–expressing memory precursors. Somewhat unexpectedly[122], we found that CD4-depleted mice had a dramatically reduced frequency and number of KLRG1^{high} SLECS, yet did not have a lower frequency of IL- 7R^{+} MPEC CD8^{+} T cells (Figure 3.5B). However, CD4^{+} T cell-depleted mice treated with anti-CD27 had a significantly greater frequency of IL-7R–expressing cells than either nondepleted mice, or CD4^{+} T cell-depleted mice treated with cIg (Figure 3.5B). Notably, we found increases in both the frequency of KLRG1^{-} IL-7R^{+} and IL-7R^{+} KLRG1^{+} CD8^{+} T cells (Figure 3.5B). Again, these data were recapitulated in the smaller endogenous response to OVA-vac (Figure 3.7A, B). Thus, stimulation of CD27 during primary CD8^{+} T cell responses to OVA-vac results in a far greater proportion of CD8^{+} T cells with characteristics of cells with potential to survive into memory. Together, these data indicate that CD70/CD27 costimulation either regulates the expression of IL- 7R by primary effector cells, or is integral to either the proliferation or survival of IL-7R–expressing MPECs.

A deficiency in CD4^{+} T cells during LCMV infection has been previously shown to result in excessive expression of the transcription factor T-bet and reduced expression of Eomesodermin (Eomes), resulting in repression of the IL-7R and a corresponding loss of central memory CD8^{+} T cells[115, 116]. Therefore, we determined whether CD27 stimulation modulated the expression of T-bet and Eomes. Whereas both transcription factors were induced in effector CD8^{+} T cells compared with naive CD8^{+} T cells (data not shown), we found little difference in T-bet expression in CD8^{+} T cells that expand in response to OVA-vac nondepleted and CD4-depleted mice (Figure 3.5C). In contrast, the expression of Eomes was significantly reduced in CD8^{+} T cells that expanded in CD4-depleted mice compared with nondepleted. Treatment with anti-CD27 restored Eomes expression to a level even higher than found in nondepleted mice (Figure 3.5C). Thus, ability of CD27

stimulation to promote secondary $CD8^+$ T cell responses after vaccinia immunization closely correlates with the induction of Eomes expression, but not T-bet expression.

3.4.5 Promotion of $CD8^+$ T cell memory by CD27 costimulation correlates with decreased expression of IL-12R and IL-2R

The preceding data indicate that CD27 costimulation strongly influences $CD8^+$ T cell memory potential. However, IL-7R expression is not sufficient for memory $CD8^+$ T cell function[123, 124]. Furthermore, whereas CD27 stimulation augmented the frequency of IL-7R–expressing cells, the absence of $CD4^+$ T cells did not substantially reduce the frequency of IL-7R–expressing cells compared with control mice. These data suggested that CD27 stimulation modulates additional factors that influence memory $CD8^+$ T cell differentiation or survival. Neither the expression of IL-21R or IFNabR (Figure 3.8A), nor autocrine IL-2[30] (which was strongly induced by CD27stimulation; Figure 3.8B) correlated with the ability to generate $CD8^+$ T cell memory in CD4-depleted mice. In contrast, we found that in $CD4^+$ T cell-depleted mice, IL-$7R^+$ OT- 1 retained expression of CD25, the high-affinity IL-2R that marks terminally differentiated cells[12, 14]. Stimulation with CD27 significantly reduced the frequency of IL-$7R^+$ cells that expressed CD25 (Figure 3.7C, Figure 3.8C). This suggested that in the absence of $CD4^+$ T cells, MPECs retain the expression of cytokine receptors that could lead to terminal differentiation. Supporting this, the expression of IL-12Rβ1 on IL-$7R^+$ OT-1 inversely correlated with the capacity to form $CD8^+$ T cell memory (Figure 3.8D). Of particular interest, IL-7R–expressing OT-1 that coexpressed CD25 also expressed IL-12Rβ1, and this subpopulation was 3 times more prevalent in CD4-depleted mice compared with non-depleted, yet was absent from CD4-depleted mice treated with anti-CD27 (Figure 3.8E). Therefore, the absence of $CD4^+$ T cells during primary $CD8^+$ T cell responses is

FIGURE 3.7. Effect of anti-CD27 on the frequency of CD127- and KLRG1- or CD25-expressing endogenous $CD8^+$ T cells in CD4-depleted mice. Control or CD4-depleted mice were primed with OVA-vac and treated with cIg or anti-CD27, as described in the Methods. On d7, mice were bled and stained with anti-CD8, MHC-tetramer, anti-KLRG1, anti-CD127 and anti-CD25. (A) Plots show co-expression of KLRG1 and CD127 on $CD8^+$ MHC-tetramer$^+$ cells from bleeds of individual mice. (B) Compiled data from cohorts of 3 mice are shown. *$p<0.05$; **$p<0.01$ compared to non-depleted mice. (C) Representative plots from individual mice showing CD25 expression on OVA_{257}-specific primary $CD8^+$ T cells responding to OVA-vac immunization.

FIGURE 3.7.

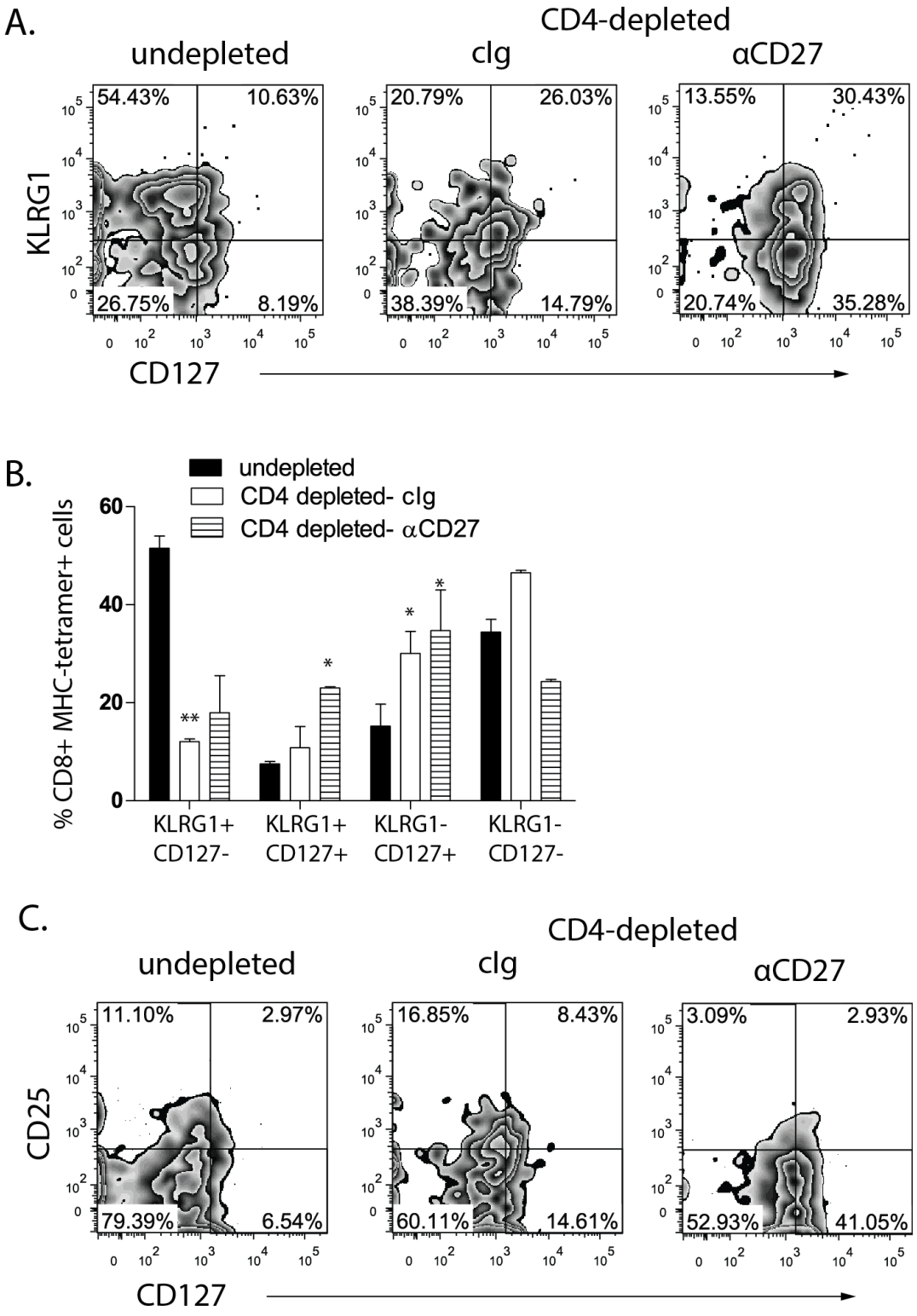
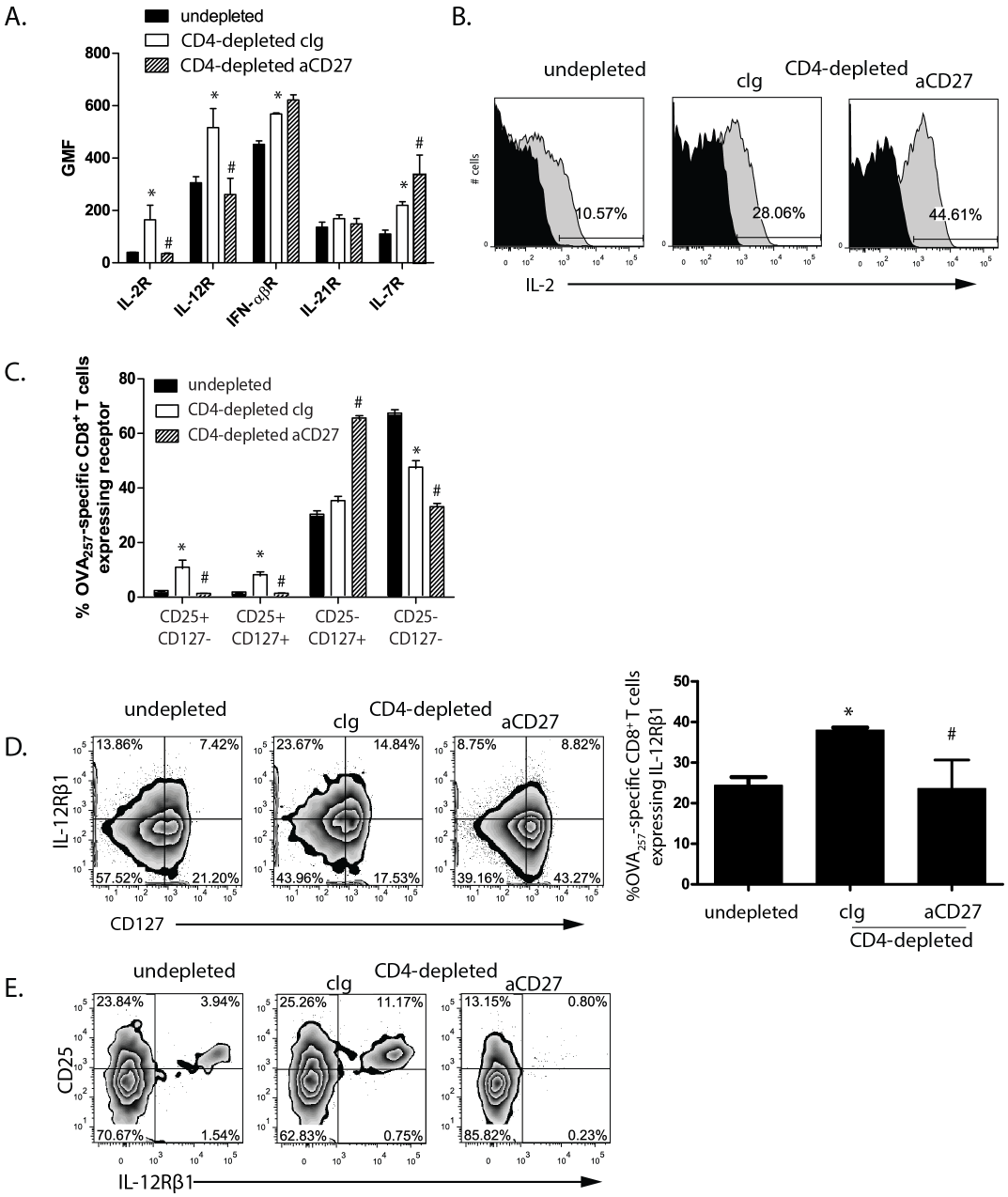

A.
undepleted
CD4-depleted
cIg
αCD27
54.43%
10.63%
26.75%
8.19%
20.79%
26.03%
38.39%
14.79%
13.55%
30.43%
20.74%
35.28%
KLRG1
CD127
B.
undepleted
CD4 depleted- cIg
CD4 depleted- αCD27
% CD8+ MHC-tetramer+ cells
KLRG1+ CD127-
KLRG1+ CD127+
KLRG1- CD127+
KLRG1- CD127-
C.
CD4-depleted
undepleted
cIg
αCD27
11.10%
2.97%
79.39%
6.54%
16.85%
8.43%
60.11%
14.61%
3.09%
2.93%
52.93%
41.05%
CD25
CD127

FIGURE 3.8. The ability of CD27 stimulation to promote $CD8^+$ T cell memory inversely correlates with CD25 and IL-12R expression. $Thy1.1^+$ OT-1 were transferred into nondepleted or CD4-depleted mice that were infected with OVA-vac and treated with cIg or anti-CD27. Seven days after OVA-vac infection, spleens and/or lymph nodes were excised and stained for the presence of $CD44^{high}$ $CD8^+$ $Thy1.1^+$ OT-1 cells. (A) Expression of cytokine receptors on $Thy1.1^+$ OT-1–gated cells. Histogram shows the geometric mean fluorescence (GMF) of the respective cytokine receptors. (B) Expression of IL-2 by day 7 OT-1 cells after short-term in vitro stimulation of splenocytes (gray plots). Black areas are from nonstimulated controls. Numbers in plots show percentage of OT-1–expressing IL-2. (C) Co-expression of CD127 and CD25 on d7 OT-1 cells. (D) Coexpression of IL-12Rβ1 and CD127 on OT-1. Dot plots show expression in individual mice. Histogram shows percentage of OT-1–expressing IL-12Rβ1 in each cohort. (E) Coexpression of CD25 and IL-12Rβ1 gated on CD127- expressing OT-1 cells. Data represent one of three similar experiments. *$p<0.05$ compared with nondepleted. Each histogram shows the median value of the cohort ($n = 3$) +/- SEM. $^{\#}p<0.05$ compared with cIg treated.

FIGURE 3.8.


A.
undepleted
CD4-depleted clg
CD4-depleted aCD27
GMF
IL-2R
IL-12R
IFN-αβR
IL-21R
IL-7R
B.
undepleted
clg
CD4-depleted
aCD27
cells
10.57%
28.06%
44.61%
IL-2
C.
undepleted
CD4-depleted clg
CD4-depleted aCD27
% OVA257-specific CD8+ T cells expressing receptor
CD25+ CD127-
CD25+ CD127+
CD25- CD127+
CD25- CD127-
D.
undepleted
clg
CD4-depleted
aCD27
IL-12Rβ1
13.86%
7.42%
57.52%
21.20%
23.67%
14.84%
43.96%
17.53%
8.75%
8.82%
39.16%
43.27%
CD127
%OVA257-specific CD8+ T cells expressing IL-12Rβ1
undepleted
clg
aCD27
CD4-depleted
E.
undepleted
clg
CD4-depleted
aCD27
CD25
23.84%
3.94%
70.67%
1.54%
25.26%
11.17%
62.83%
0.75%
13.15%
0.80%
85.82%
0.23%
IL-12Rβ1

associated with increased expression of receptors for cytokines associated with the differentiation and survival of effector cells on IL-7R–expressing MPECs. Stimulation of CD27 suppresses the generation of cells with this phenotype.

3.4.6 CD70 costimulation moderates the influence of IL-12 on MPEC formation and CD8$^+$ T cell memory

Recent studies have implicated IL-12 as a critical mediator of CD8$^+$ T cell fate decisions[44-46, 89], and as CD27 costimulation modulated the expression of the IL-12Rβ1 chain, we assessed whether IL-12 is responsible for the loss of memory cell formation in the absence of CD27 costimulation. IL-12 only moderately contributed to the development of OT-1 primary effector CD8$^+$ T cell responses to recombinant vaccinia, but concomitantly blocking CD70 had an additive effect, leading to a significant reduction in the primary OT-1 CD8$^+$ T cell response (Figure 3.9A). In contrast, we found that the magnitude of the OT-1 secondary CD8$^+$ T cell response IL-12–deficient mice treated with anti-CD70 was equivalent to that of either IL-12–deficient or wild-type mice treated with cIg (Figure 3.9A). Similar results were found with IL-12p40 knockout mice (data not shown). To define the basis for this difference, we examined the impact of CD70 blockade on memory precursors. We found that in the absence of IL-12, the proportion of the responding CD8$^+$ T cells that expressed KLRG1 was reduced. Furthermore, in the absence of IL-12, CD70 blockade did not significantly reduce the frequency and number of IL-7R–expressing memory precursors (Figure 3.9B). Therefore, we conclude that CD27 stimulation plays a critical role in controlling the influence of IL-12 on the differentiation and survival of MPECs.

3.4.7 CD70 blockade does not abrogate CD8$^+$ T cell memory in BMDC-primed mice

We next investigated whether CD70 costimulation is generally necessary for the

development of IL-7R–expressing MPECs, even under noninflammatory conditions. Cohorts of mice were immunized with OVA257-pulsed, CD40L-activated BMDC and treated with anti-CD70 or cIg. Analysis of the primary $CD8^+$ T cell response revealed a significant reduction (~75%) in mice treated with anti-CD70, consistent with our previous results using BMDC preincubated with anti-CD70 (Figure 3.10A). Strikingly, the magnitude of the secondary $CD8^+$ T cell response elicited by OVA-adeno challenge was equivalent between the two cohorts (Figure 3.10B). Therefore, under immunization conditions that induce weak inflammation, CD70 blockade had a large impact on the development of the primary $CD8^+$ T cell response, but no apparent impact on the survival or differentiation of $CD8^+$ T cell memory.

We reasoned that CD70 blockade during BMDC immunization had little impact on $CD8^+$ T cell memory due to the minimal IL-12 produced by this immunization system. We therefore immunized cohorts of OT-1 chimeras and treated with anti-CD70 or cIg with supplemental IL-12 or PBS. Inclusion of IL-12 had little impact on the overall magnitude of the OT-1 response generated in cIg- treated mice. However, IL-12 significantly increased the size of the primary $CD8^+$ T cell response in anti-CD70–treated mice (Figure 3.10A), indicating that IL-12 and CD70 stimulation nonredundantly support the expansion of primary effector $CD8^+$ T cells. However, mice that had received IL-12 and anti-CD70 during the primary response to BMDC immunization made very poor secondary $CD8^+$ T cell responses (Figure 3.10B). Therefore, $CD8^+$ T cells that respond to BMDC immunization require stimulation by CD70 to form functional $CD8^+$ T cell memory in the presence of IL-12.

To understand the basis of the difference between the effect of CD70 blockade on the development of $CD8^+$ T cell memory after OVA-vac and BMDC immunization, we

FIGURE 3.9. IL-12 prevents secondary $CD8^+$ T cell responses in the absence of CD70 stimulation. (A) OT-1 chimeric B6 or IL-12p35 knockout mice (n = 3) were primed with OVA-vac in the presence of cIg or anti- CD70. Primary OT-1 responses (top plots) were determined by Thy1.1 staining in blood 7 d postinfection. Secondary OT-1 responses (bottom plots) were initiated by challenge with OVA-adeno 35 d after initial priming with OVA-vac, and determined by staining spleens 5 d later for Thy1.1-expressing $CD8^+$ T cells. Naive indicates the primary response generated 5 d after OVA-adeno. (B) Expression of KLRG1 and CD127 (dot plots) by day 7, gated on $Thy1.1^+$ OT-1, in the spleens of mice described in (A), and enumeration of the number of OT-1 with each phenotype per spleen (histogram). Numbers in dot plots indicate the percentage of cells within each region. Plots are derived from representative individual mice within an experimental cohort. Histograms contain compiled cohort data, showing median responses +/- SEM. Data from one of five similar experiments. *$p<0.05$, **$p<0.01$ compared with cIg-treated B6. $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared with anti-CD70–treated mice.

FIGURE 3.9.

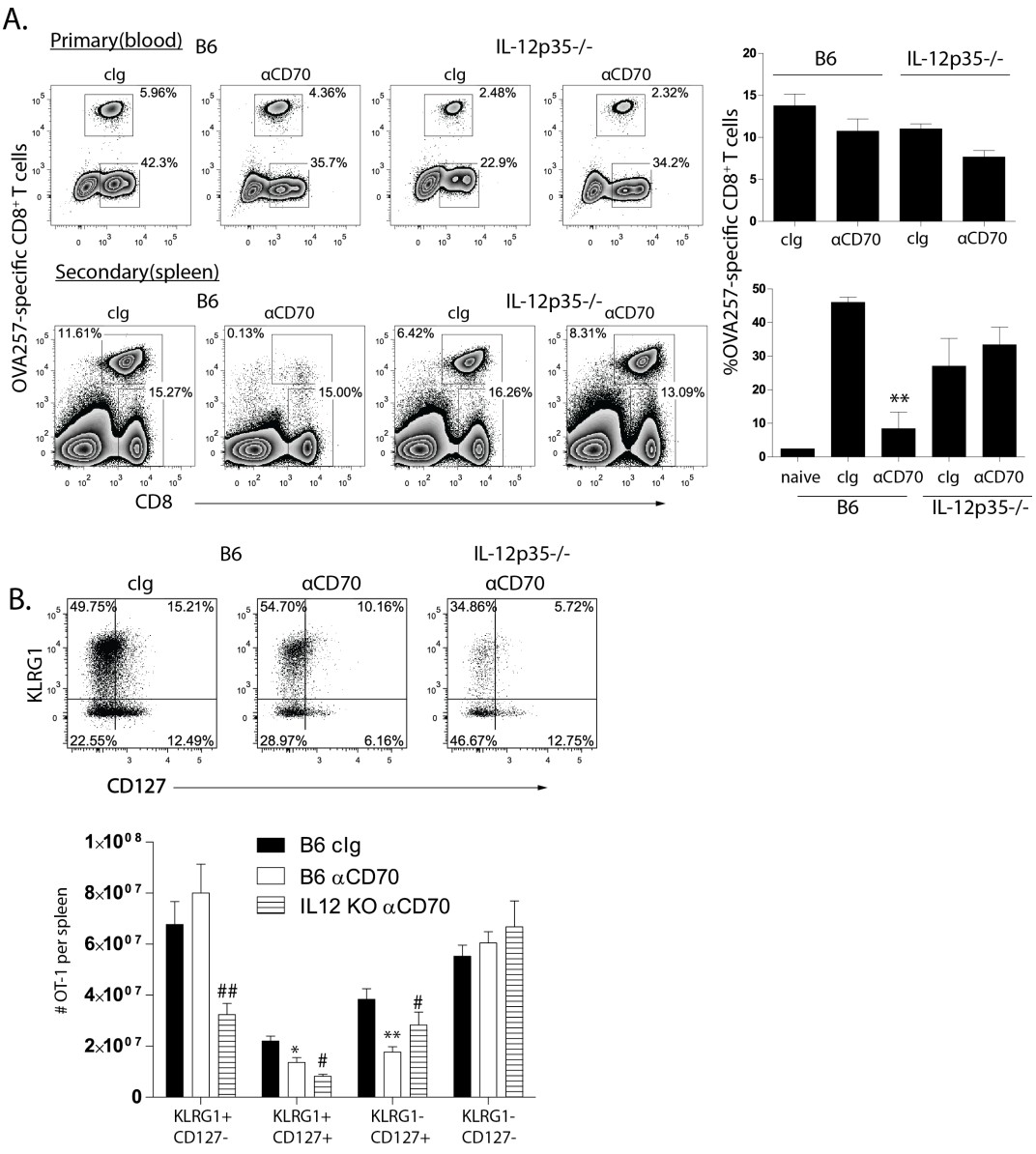
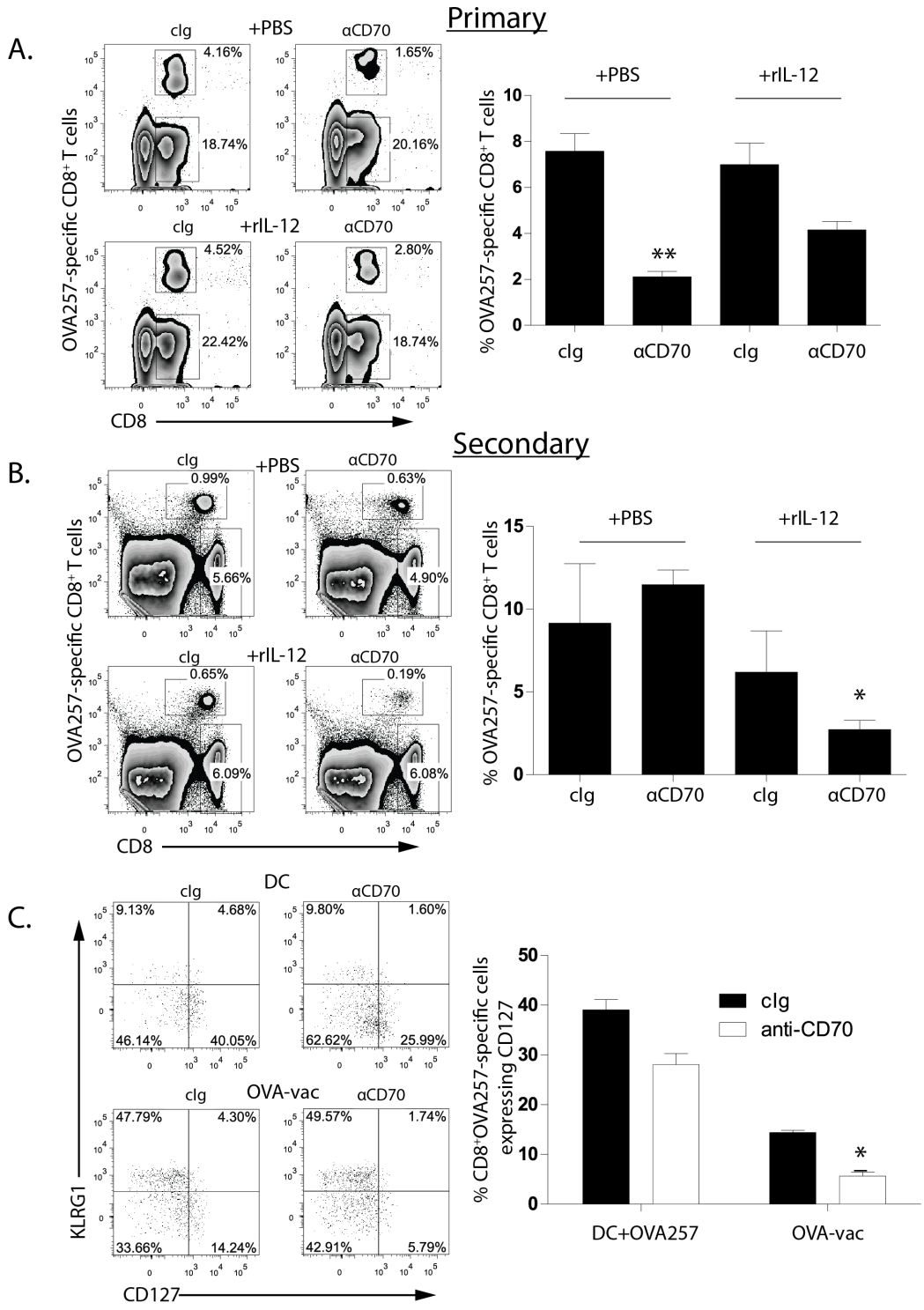

A.
Primary(blood)
B6
IL-12p35-/-
clg
αCD70
5.96%
42.3%
4.36%
35.7%
2.48%
22.9%
2.32%
34.2%
OVA257-specific CD8+ T cells
Secondary(spleen)
11.61%
15.27%
0.13%
15.00%
6.42%
16.26%
8.31%
13.09%
CD8
%OVA257-specific CD8+ T cells
naive
**
B.
KLRG1
CD127
49.75%
15.21%
22.55%
12.49%
54.70%
10.16%
28.97%
6.16%
34.86%
5.72%
46.67%
12.75%
OT-1 per spleen
B6 clg
B6 αCD70
IL12 KO αCD70
KLRG1+ CD127-
KLRG1+ CD127+
KLRG1- CD127+
KLRG1- CD127-

FIGURE 3.10. IL-12 induces memory $CD8^+$ T cell susceptibility to CD70 blockade after immunization with BMDC. Cohorts (n = 3) of OT-1 chimeric mice were primed with OVA257-pulsed, CD40L-activated BMDC in the presence of cIg (left panels) or anti-CD70 (right panels). Half the cohorts received PBS (top panels), and the other half rIL-12 (bottom panels) on days 0 and 2 after BMDC. Primary OT-1 responses (A) and secondary OT-1 responses (B) were assessed 7 and 5 d after immunization with BMDC or challenge with OVA-vac, respectively. Histograms contain compiled cohort data, showing median responses +/- SEM. (C) SLEC/ MPEC phenotype of day 7 primary OT-1 cells in spleens elicited by either BMDC (top panels) or OVA-vac (bottom plots) in mice treated with cIg or anti-CD70. Plots are derived from representative individual mice within an experimental cohort. Histograms contain compiled cohort data, showing median responses 6 SEM. Data are from one of two similar experiments. *$p<0.05$, **$p< 0.01$ compared with cIg treated.

FIGURE 3.10.


A.
Primary
cIg
+PBS
αCD70
4.16%
18.74%
1.65%
20.16%
+rIL-12
4.52%
22.42%
2.80%
18.74%
OVA257-specific CD8+ T cells
CD8
+PBS
+rIL-12
% OVA257-specific CD8+ T cells
**
cIg
αCD70
B.
Secondary
0.99%
5.66%
0.63%
4.90%
0.65%
6.09%
0.19%
6.08%
*
C.
DC
OVA-vac
9.13%
4.68%
46.14%
40.05%
9.80%
1.60%
62.62%
25.99%
47.79%
4.30%
33.66%
14.24%
49.57%
1.74%
42.91%
5.79%
KLRG1
CD127
% CD8+OVA257-specific cells expressing CD127
anti-CD70
DC+OVA257
OVA-vac

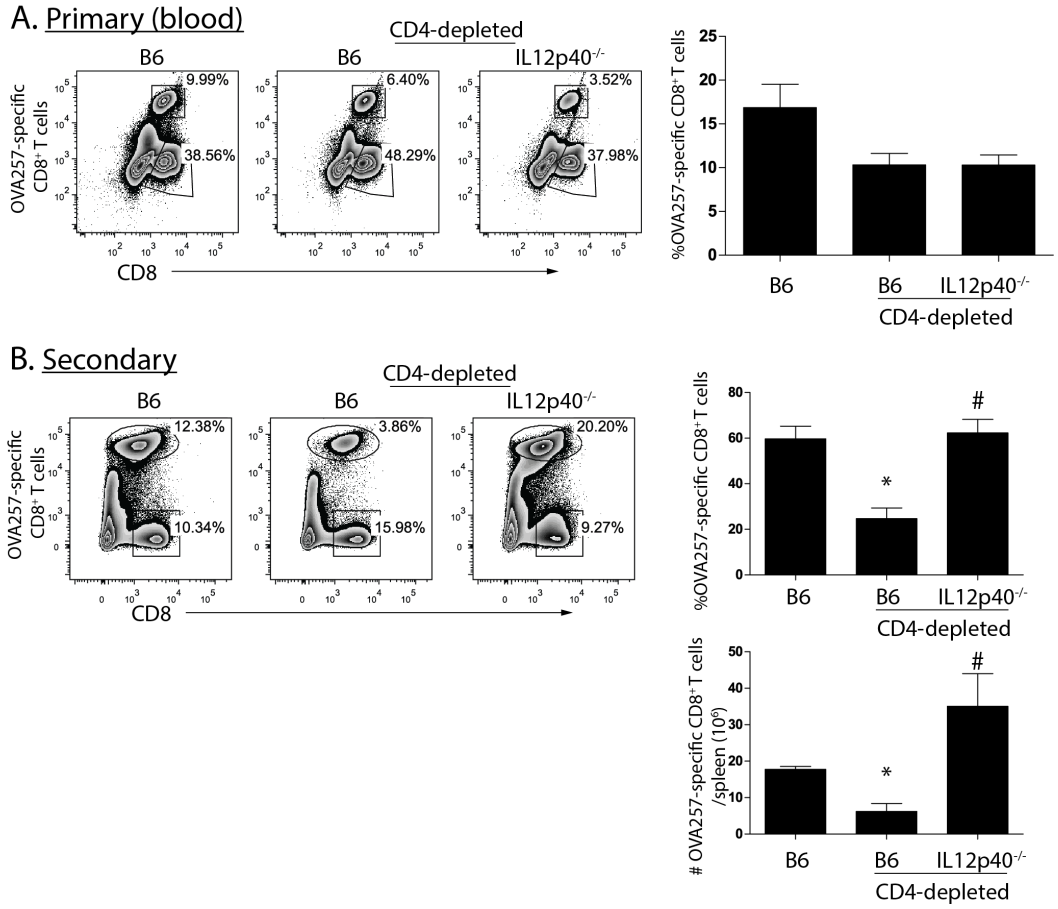
examined the impact of CD70 blockade on MPEC generation in response to BMDC. Notably, considerably fewer OT-1 that responded to BMDC immunization expressed KLRG1, whereas the proportion that expressed IL- 7R was significantly higher (Figure 3.10C). Surprisingly, we found that pothesized that the loss of $CD8^+$ T cell memory in the absence of $CD4^+$ T cells might be a consequence of weak CD70-mediated protection against terminal differentiation induced by IL-12. We depleted wild-type or IL-12–deficient OT-I chimeric mice of $CD4^+$ T cells, and challenged with OVA-vac. As previously observed, the OVA257-specific primary $CD8^+$ T cell response was ~2-fold lower in CD4-depleted mice (Figure 3.11A). However, com- pared with CD4-depleted wild-type mice, secondary $CD8^+$ T cell responses elicited by OVA-adeno in CD4-depleted IL-12–deficient mice were not compromised (Figure 3.11B). Thus, we conclude that the inability to mount secondary $CD8^+$ T cell responses from memory $CD8^+$ T cells primed in the absence of $CD4^+$ Th cells can be attributed to IL-12 and can be overcome by direct stimulation of CD27 on $CD8^+$ T cells.

3.5 Discussion

The studies presented in this work demonstrate that CD70-mediated costimulation has a major influence on $CD8^+$ T cell memory, particularly in situations in which IL-12 expression is strongly induced. Our data indicate that CD70–CD27 interaction plays either a significant role in the expansion/survival of IL-7R–expressing cells, or directly modulates the expression of the IL-7R. We find that CD70 stimulation dictates the frequency and number of IL-7R–expressing putative memory precursor $CD8^+$ T cells found at the peak of the primary response, which is significant, as little is known about the stimuli that promote MPECs. Together these data illuminate an unappreciated role for CD27-mediated costimulation in promoting the frequency of IL-7R–expressing cells

FIGURE 3.11. IL-12 is responsible for the loss of $CD8^+$ T cell memory to OVA-vac in absence of $CD4^+$ T cells. Cohorts of nondepleted, or CD4- depleted B6 or IL-$12p40^{-/-}$ OT-1 chimeric mice (n = 3) were primed with OVA-vac. (A) Frequency of primary OT-1 response in blood 7 d after priming. (B) Frequency and total number of secondary OT-1 responses in spleen 5 d after challenging mice from (A) with OVA-adeno. Histograms contain compiled cohort data, showing median responses +/- SEM. Data from one of two similar experiments. *$p<0.05$ compared with non-depleted, $^{\#}p<0.05$ compared with CD4-depleted B6.

FIGURE 3.11.


A. Primary (blood)
CD4-depleted
B6
B6
IL12p40-/-
9.99%
38.56%
6.40%
48.29%
3.52%
37.98%
OVA257-specific CD8+ T cells
CD8
%OVA257-specific CD8+ T cells
B6
B6
IL12p40-/-
CD4-depleted
B. Secondary
CD4-depleted
B6
B6
IL12p40-/-
12.38%
10.34%
3.86%
15.98%
20.20%
9.27%
OVA257-specific CD8+ T cells
CD8
%OVA257-specific CD8+ T cells
*
#
B6
B6
IL12p40-/-
CD4-depleted
OVA257-specific CD8+ T cells /spleen (10^6)
*
#
B6
B6
IL12p40-/-
CD4-depleted

and constraining the influence of IL-12 on $CD8^+$ T cell differentiation, and mechanistically link the induction of CD70 expression on DC by $CD4^+$ T cells with the programming of IL-12 resistance in memory $CD8^+$ T cell differentiation.

As the expression of CD70 on DC generally requires ligation of CD40, we hypothesized that the dysfunctional nature of memory $CD8^+$ T cells that develops in the absence of $CD4^+$ T cell help can be attributed to a failure to induce CD70 expression. Supporting the hypothesis, direct stimulation of CD27 bypassed the requirement for $CD4^+$ T cell help in the promotion of $CD8^+$ T cell memory, whereas CD70 blockade abrogated $CD8^+$ T cell memory; in both cases, loss of $CD8^+$ T cell memory was dependent upon IL-12. However, there is some divergence in the impact of CD70 blockade and $CD4^+$ T cell depletion on the subsets of $CD8^+$ T cells at the peak of the primary response. The absence of $CD4^+$ T cells had a more profound effect on $KLRG1^+$ SLEC numbers than on either the frequency or number of IL-7R–expressing MPECs, highlighting the role for $CD4^+$ T cells in the support of SLEC survival. However, autocrine IL-2 expression in primary effector $CD8^+$ T cells, which has recently been implicated in dictating the ability of $CD8^+$ T cells to become memory cells[69], was not reduced in the absence of $CD4^+$ T cell help. Conversely, CD27 stimulation strongly induced both IL-7R and autocrine IL-2 expression. These data indicate that CD27 stimulation and $CD4^+$ T cell help have some overlapping effects on $CD8^+$ T cell phenotype and function; however, they are not surprisingly incompletely synonymous. Alternatively, other mechanisms for inducing low levels of CD70 expression independent of $CD4^+$ T cells, perhaps NK or NKT cells, may be at play during viral infections. Thus, some limited CD70-mediated stimulation available in the absence of $CD4^+$ T cells may allow the development of cells with MPEC characteristics, but not the ability to fully differentiate

into long-lived memory $CD8^+$ T cells.

The contribution of CD27 stimulation to $CD8^+$ T cell fate decisions appears to be highly associated with the extent of inflammation associated with the type of immunization. We identified IL-12 as the critical mediator that regulates memory $CD8^+$ T cell loss in the absence of CD27 stimulation or $CD4^+$ T cell help during the primary $CD8^+$ T cell response. IL-12 has been described as a strong promoter of $CD8^+$ T cell expansion, and promotes differentiation into KLRG1-expressing effector cells after LCMV[44, 47], Listeria monocytogenes[48], and Toxoplasma gondii infection[46]. Furthermore, it has been suggested that IL-12 may have a detrimental outcome on memory $CD8^+$ T cell differentiation[45, 46], although it is unclear whether this is due to forced differentiation into KLRG1-expressing terminally differentiated cells, or activation-induced death of memory precursors. The studies presented in this work demonstrate that in the absence of balancing stimulation by CD27, IL-12 can result in the failure to generate long-lived $CD8^+$ T cell memory. We found that $CD8^+$ T cells that expand in the absence of $CD4^+$ Th cells overexpress IL-12Rβ1 compared with controls, whereas those that received supplemental CD27 stimulation had normal levels of IL-12Rβ1 expression. This suggests that CD27 stimulation and $CD4^+$ T cell help regulate the extent to which expanding $CD8^+$ T cells are responsive to IL-12. IL-12Rβ1 expression on CD4 part controlled by a positive feedback loop activated by IFNγ –mediated induction of T-bet[125] and IL-2[126], but less is known about its regulation on $CD8^+$ T cells. The reduced expression of IL-12R on $CD8^+$ T cells after CD27 stimulation is an unexpected result given that stimulation of CD27 has been reported to enhance IL-12Rβ expression on human $CD4^+$ T cells[28], but provides a rationale for how CD27 engagement regulates the sensitivity of $CD8^+$ T cells to IL-12–mediated differentiation. Pertaining to this, we found

that Eomes expression, but not T-bet expression, correlated with $CD4^+$ T cell help and CD27 stimulation. This is consistent with the notion that T-bet is initially induced by IFNγ, rather than IL-12, and suggests that Eomes is either a novel downstream target of CD27 signaling, or that the reduction in IL-12R expression abrogates IL-12–mediated suppression of Eomes[59]. In either case, the data presented in this work argue that the importance of CD27 stimulation in promoting $CD8^+$ T cell memory can be amplified by the extent of accompanying inflammatory cytokines. Interestingly, the contribution of CD27 stimulation for $CD8^+$ T cell memory was first noted in studies using influenza virus[32, 33], yet a recent study utilizing a different strain of influenza found no role for CD27 stimulation in $CD8^+$ T cell memory[96]. This, together with DC immunization data presented in this work, indicates that the requirement for CD27 and potentially other TNF-superfamily members such as 4-1BB (CD137) [127] for $CD8^+$ T cell memory development may be exacerbated by the inflammatory context present during the initial expansion of the primary $CD8^+$ T cell response.

We found that CD27 stimulation strongly influenced the frequency and number of IL-7R–expressing primary $CD8^+$ T cells, which are putative memory precursors. To date, the control of IL- 7R expression has generally been attributed to either TCR engagement or IL-7 binding. Currently, we do not know whether the change in frequency of IL-7R–expressing cells induced by CD27 stimulation is a consequence of augmented proliferation, differentiation, or survival, and/or by direct regulation of IL-7R expression. However, CD27 stimulation profoundly upregulated the expression of IL-7R expression in differentiated KLRG1-expressing cells. Furthermore, the increase in IL-7R–expressing OT-1 obtained after CD27 stimulation occurred without a concomitant increase in total OT-1 numbers. Together, these data argue that CD27 stimulation controls IL-7R

expression, rather than supporting the survival of IL-7R–expressing cells. IL-7R expression is controlled by the opposing actions of the transcription factors GABPα1 and Gfi-1[128], Foxo1 and Foxp1[129, 130], and potentially ETS-1[131], suggesting a role for CD27 signaling in the mobilization of these factors. Future studies will elucidate whether there is a transcriptional cassette that is elicited by CD27 stimulation that directly accounts for IL-7R expression, or whether CD27 stimulation impacts on the ability of other cytokines (such as IL-12) to induce epigenetic silencing or transcriptional repression of IL-7R. Furthermore, as both IL-7R stimulation and CD27 stimulation induce anti-apoptotic molecules, it is interesting to speculate whether CD27 stimulation operates through IL-7[68], and whether IL-7R–expressing KLRG1$^+$ cells can survive to become memory CD8$^+$ T cells.

Together the data presented in this work define a mechanism by which CD70-CD27 stimulation regulates CD8$^+$ T cell memory, and provide a link between CD4$^+$ T cell-mediated licensing of DC and CD8$^+$ T cell memory. Importantly, these data argue that targeting CD70 expression, or CD27 stimulation, will provide a mechanism for generating long-term CD8$^+$ T cell memory in the absence of CD4$^+$ T cell help. This has significant ramifications in the design of vaccines for individuals with degraded CD4$^+$ T cell populations. Furthermore, the capability of CD27 stimulation to induce IL-7R expression may provide opportunities to augment IL-7–based immunotherapies for cancer and chronic viral infections[132, 133]. Furthermore, these data indicate that vaccine adjuvants, such as TLR agonists, that elicit high or sustained levels of IL-12 in the absence of concomitant induction of CD70 expression will possibly lead to poor memory populations. Thus, for vaccines based upon minimal MHC class I-restricted peptides, combination of TLR agonists with CD40 stimulation to induce CD70 expression, or the

inclusion of peptide epitopes that elicit $CD4^+$ T cell responses is likely to be significantly more effective at generating $CD8^+$ T cell memory.

Chapter 4. CD27 costimulation promotes IL-7 receptor α re-expression in effector CD8$^+$ T cells by mRNA regulation during acute viral infection

4.1 Abstract

IL-7 plays a critical role in the generation and maintenance of memory CD8$^+$ T cells, and IL-7Rα has been regarded as a functional marker of long-lived memory precursor effector cells. Using vaccinia virus, we previously demonstrated that CD27 costimulation during primary response is critical for the generation of IL-7Rα-expressing effectors and promotes CD8$^+$ T cell memory. While IL-7Rα is down-regulated acutely upon TCR stimulation, its re-expression around the peak of primary CD8$^+$ responses is generally thought to be a default outcome from withdrawal of TCR stimulation. An alternative hypothesis is that specific stimuli actively antagonize the down-regulation or promote the recovery of IL-7Rα in Ag-activated CD8$^+$ T cells. By utilizing agonistic mAb and transgenic models, here we show 1) CD27 stimulation acts directly on CD8$^+$ T cells to enhance IL-7Rα-expressing effectors; 2) CD27 stimulation neither alleviates the down-regulation of IL-7Rα upon TCR signaling nor promotes the expansion/survival of IL-7Rα-expressing effectors, but facilitates IL-7Rα re-expression; 3) CD27 stimulation regulates *Il7ra* mRNA abundance but not protein distribution. Importantly, CD27 stimulation promotes not only IL-7Rα, but also the common γ chain of the receptor and the downstream signaling mediated by pSTAT5. Intriguingly, stimulation of other TNFSF members OX-40 and 4-1BB showed differing ability to promote IL-7Rα. Our results demonstrate a previously unappreciated role of CD27 costimulation as a positive regulator of IL-7Rα during CD8 T cell responses, provide insights into the mechanistic

basis by which CD27 costimulation influences $CD8^+$ T cell memory differentiation, and highlight the potential of targeting CD27-CD70 axis to enhance IL-7 signaling for antiviral/antitumor immunotherapy.

4.2 Introduction

After encountering pathogens, naïve $CD8^+$ T cells are activated and start rapid proliferation and differentiation into functional CTLs, secret cytokines and granzymes and kill target cells. Following pathogen clearance, the majority of CTLs die via apoptosis during contraction stage, while a small subset of them is maintained stably and becomes long-lived memory cells. Interleukin-7 (IL-7) signal is critical for the long-term maintenance of antigen (Ag)-specific $CD8^+$ T cells during the contraction phase[56, 134]. IL-7 is constitutively produced by stromal cells, and T cell responsiveness to IL-7 is primarily regulated by expression of the IL-7 receptor (IL-7R). IL-7R on the cell surface consists of two transmembrane subunits: an IL-7 specific α chain – IL-7Rα, and a common cytokine receptor γ chain (γc) that is shared with the receptors for cytokines IL-2, IL-4, IL-9, IL-15, and IL-21[55, 135]. Increased expression of IL-7Rα directly correlates with Ag-specific $CD8^+$ T cells survival after resolution of infection during contraction, and thus IL-7Rα has been regarded as a functional marker of long-lived memory precursor effector cells (MPECs)[56]. Using vaccinia virus, we previously demonstrated that CD27 costimulation promotes $CD8^+$ T cell memory formation, which correlates with an increase in the frequency of IL-7Rα-expressing effector cells at the peak of primary responses[80]. However it was not clear whether CD27 costimulation selectively expands or drives the development of IL-7Rα-expressing effector cells.

Much work has been done to study how IL-7Rα expression is regulated at transcriptional level in steady-state $CD8^+$ T cells. IL-7Rα expression during T cell

development in thymus is dynamically regulated by transcriptional activator GABPα[136] and transcriptional suppressor Gfi-1[137, 138]. Forkhead family transcription factor Foxo1 induces *Il7ra* gene transcription in mature T cells in the periphery[129, 139], while Foxp1 from the same family represses *Il7ra* transcription by antagonizing Foxo1 binding and maintains the quiescence of naïve T cells[130, 140]. Only several extrinsic regulators of IL-7Rα have been reported, including IL-7, TCR stimulation and TGF-β. IL-7[138] and TCR stimulation inhibit *Il7ra* transcription via Gfi-1 during $CD8^+$ T cell development and peripheral homeostasis, while TGF-β induces IL-7Rα during thymic positive selection by suppression of Gfi-1[141]. Recent studies indicate that IL-7Rα also undergoes posttranslational regulation. IL-7 has been reported to acutely induce clathrin-mediated internalization of surface IL-7Rα proteins, decrease the proportion of intracellular IL-7Rα proteins that recycle back onto cell surface by class III PI3K-dependent intracellular trafficking, while increase the proportion targeted for lysosome- and proteasome-dependent degradation[142, 143].

How IL-7Rα expression is regulated in Ag-specific $CD8^+$ T cells during immune responses is less well known. Naive CD8 T cells for the most part uniformly express IL-7Rα. TCR-mediated activation markedly down-regulates IL-7Rα in mature $CD8^+$ T cells, an effect that has been previously reported both *in vitro* by 6hr after stimulation[144] and *in vivo* by d4 after LCMV infection[44, 128]. In the latter, both the frequency of IL-7Rα-expressing cells and their levels of IL-7Rα steadily decreased until d6 or 7 postinfection, when IL-7Rα presented in only 15% responding $CD8^+$ T cells with very low levels[44, 128]. Intriguingly, by d8 when the virus is cleared and effector expansion peaks, a considerable recovery was observed in both the frequency and expression levels of IL-7Rα, which occurred in $KLRG1^{lo}$ but not $KLRG1^{hi}$ cells and correlated well with *Il7ra*

mRNA abundance[44, 128], together implicating an acute re-expression of IL-7Rα in a small subset of late effectors. In a prevailing model, wherein TCR engagement is sufficient to cause IL-7Rα down-regulation and that the strength/duration of Ag exposure affects the ability of corresponding T cells to re-express IL-7Rα, the expression of IL-7Rα during Ag responses is regulated solely by Ag-dependent signals[145] through the opposing function of transcription factors GABP and Gfi-1[128],. However, it has been reported *in vitro* that IL-2 plays an important role in maintaining the down-regulation initiated by TCR activation[146], and that hormone glucocorticoids can antagonize TCR activation-mediated downregulation of IL-7Rα [147, 148], arguing against TCR stimulation being the only extrinsic regulator of IL-7Rα during $CD8^+$ T cell responses. Notably, glucocorticoid has been indicated to recover diminished IL-7Rα expression in simian immunodeficiency virus–infected macaques[149], raising the prospect of modulating IL-7Rα expression by extrinsic stimuli during T cell responses.

We undertook the present study to elucidate, from several possibilities, the underlying mechanisms by which CD27 costimulation promotes IL-7Rα-expressing effector $CD8^+$ T cells. Several layers of questions have been addressed. First, is the increase in IL-7Rα-expressing effector $CD8^+$ T cells a direct effect of CD27 triggering on $CD8^+$ T cells or other cell populations? Second, does CD27 costimulation support IL-7Rα via antagonizing its acute down-regulation or via facilitating its recovery? And third, does CD27 costimulation selectively promote the proliferation/survival, or drive the development of, IL-7Rα-expressing effector cells? Finally, does CD27 costimulation promote cell responsiveness to IL-7 via enhancing IL-7Rα? To address these questions, we have used agonistic/antagonistic monoclonal antibodies and transgenic models,

utilized *in vitro* and *in vivo* systems, and performed imaging flow cytometry and quantitative PCR to monitor the IL-7Rα profile of activated-$CD8^+$ T cells.

4.3 Materials and Methods

4.3.1 Animals. C57Bl/6 mice were obtained from National Cancer Institute (Frederick, MD). OT-I mice, expressing TCRs specific for $OVA_{257-264}$ peptide in complex with $H\text{-}2K^b$, were purchased from Taconic (B6.129S7-*Rag1*^{tm1Mom} Tg(TcraTcrb)1100Mjb N9+N1, model # 4175), and crossed on $Thy1.1^+$ (B6.PL-*Thy1*^{a}/CyJ stock # 000406) mice obtained from the Jackson Laboratory. CD27KO mice were provided by Dr. Steven Schoenberger (La Jolla Institute of Immunology and Allergy), with the permission of Dr. Jannie Borst (Netherlands Cancer Institute). Mice were maintained in specific pathogen-free facilities and were treated in accordance with the guidelines established by the Animal Care and Use Committee at the University of Virginia.

4.3.2 Virus and peptides. Recombinant vaccinia expressing OVA (OVA-vac) was provided by J. Yewdell (National Institute of Allergy and Infectious Diseases), and was propagated on $HuTK^2$ cells. Recombinant vaccinia expressing OVA (OVA-adeno) was either purchased from Gene Transfer Vector Core (University of Iowa) or provided by Dr. Young Hahn (University of Virginia), and was propagated on 293A fibroblasts. Synthetic peptide $OVA_{257-264}$ (SIINFEKL) was purchased from GenScript (Piscataway, NJ). Endotoxin was removed by Detoxi-Gel endotoxin-removal kit (Pierce, Rockford, IL).

4.3.3 ***Antibodies.*** Agonistic AT124.1 anti-CD27 has been described[88] and endotoxin was removed as above. FR70-blocking anti-CD70 has been described before[18]. Control Rat IgG was purchased from Sigma (St Louis, MO). GK1.5-depleting anti-CD4 was obtained from ATCC.

4.3.4 In vitro T cell stimulation. Splenocytes from naïve Thy1.1^{+} OT-1 mice were either co-cultured with irradiated, OVA257-pulsed WT C57Bl/6 splenocytes (as Ag-presenting cells), or stimulated with αCD3/28 antibodies (eBioscience). αCD27 and control IgG were given in some assays as indicated. Cells were cultured in murine T cell culture medium for 48hr to down-regulate IL-7Rα, and then analyzed with flow cytometry. In some assays, after 48hr stimulation cells were labeled with CellTrace Violet proliferation dye (life technology, Grand Island, NY), and adoptively transferred into OVA-adeno pre-primed C57Bl/6 recipients.

4.3.5 in vivo adoptive transfer and Immunization. In some assays (in Figure 1), 1,000 naïve OT-1 cells were transferred into WT or CD27$^{-/-}$ C57Bl/6 mice 1d before 1*10^{7} pfu OVA-vac i.v. priming. CD27 stimulation was performed by injecting 50 μg AT124.1 i.p. on d0, 3 and 6. CD70 blockade was performed by injecting 500 μg FR70 i.p. on d0, 2, 4 and 6. Where indicated, mice were depleted of peripheral CD4^{+} T cells by i.p. injection of 200 μg GK1.5 7 and 3d before priming and confirmed by tail vain bleed. 7d after priming, spleen, lymph nodes and bleeds were harvested and analyzed by flow cytometry. In the other assays, WT C57Bl/6 mice were primed i.p. with 2*10^{8} pfu OVA-adeno, and 1*10^4 *in vitro*-stimulated, IL-7Rα-down-regulated OT1 cells were transferred at d2. Mice were treated with AT124.1 or control IgG i.p. several hours after the adoptive transfer and then 2d later. 2~4d after the transfer, splenocytes were harvested and analyzed with flow cytometry, or sorted for OT-1 cells for qPCR.

4.3.6 Flow cytometry. Lymphocytes were isolated from blood or homogenized spleens/lomph nodes and first stained with Aqua vital dye (life technologies; Carlsbad, CA) for 20 min at 4°C. In some experiments cells were then incubated with OVA$_{257-264}$-specific H2-K^{b} dextramer-APC (Immudex, Denmark) for 20 min at 4°C to identify

endogenous effector $CD8^+$ T cells. After Fc blockade, surface markers were stained for 30 min at 4°C, with antibodies used in various combinations including anti-CD8-ef450 (53-6.7), anti-CD8-PerCP(53-6.7), anti-CD4-PE(GK1.5), anti-Thy1.1-FITC (HIS51), anti-KLRG1-PE (2F1), anti-IL-7Rα-PerCP-Cy5.5 (A7R34), anti-IL-7Rα-PE (A7R34), anti-γc-PE(TUGm2), anti-CD122-PE (5H4), anti-IL-15Rα-PE(DNT15Ra) and anti-IL-21Rα-PE(eBio4A9) all from eBioscience (San Diego, CA). For Annexin V staining, cells were incubated with anti-Annexin V-Alexa Fluor 647 (life technologies) in room temperature in dark for 15min, and then run in a flow cytometer within 1hr. For pSTAT5 staining, splenocytes were pre-incubated with IL-7 *in vitro* for 20min, and permeabilized with Phosflow Perm Buffer III (BD) on ice for 1h, and incubated with anti-pSTAT5-Alexa Fluor 647(Cell Signaling) for 30min. Cell counts were assessed by adding counting beads (life technologies) at the end of staining. Staining was assessed by flow cytometry on a FACS Canto II (Becton Dickinson; Franklin Lakes, NJ) and analyzed using FlowJo 7.6.5 Software (Treestar, OR).

4.3.7 ***Imaging flow cytometry.*** The staining followed the same protocol as for regular flow cytometry, except cells were gently permabilized with 1% saponin (Sigma) for 20min before stained for IL-7Rα or the isotype control. DAPI was applied at the end of the staining to identify nuclear compartment. Staining was assessed by Amnis ImageStreamX mark II and analyzed using IDEAS software.

4.3.8 qPCR. Transferred stimulated-OT1 cells were sorted from recipient spleens 3~4d after the transfer (therefore 5~6d after priming the recipient with OVA-adeno). Total mRNA was extracted and subjected to reverse transcription, and the resulting cDNA was analyzed by CFX Connect real-time PCR detect system (BioRad) using Sso SYBR Green supermix. *Il7ra* primers were synthesized by life technologies, with the forward

primer sequence 5'-GGAACAACTATGTAAGAAGCCAAAAACG-3' and the reverse primer sequence 5'-AAGATCATTGGGCAGAAAACTTTCC-3'. Data were normalized to two validated reference genes RPS18 and Rpl13a from BioRad PrimePCR Reference Genes system.

4.3.9 Statistical analysis. Statistical analysis was performed with Prism 5 (GraphPad Software, Inc., La Jolla, CA) and data were presented as the Mean ± SD. Comparisons between groups were performed by either unpaired two-tailed Student's *t* test or one-way ANOVA. Statistical significance was determined for 95% confidence interval.

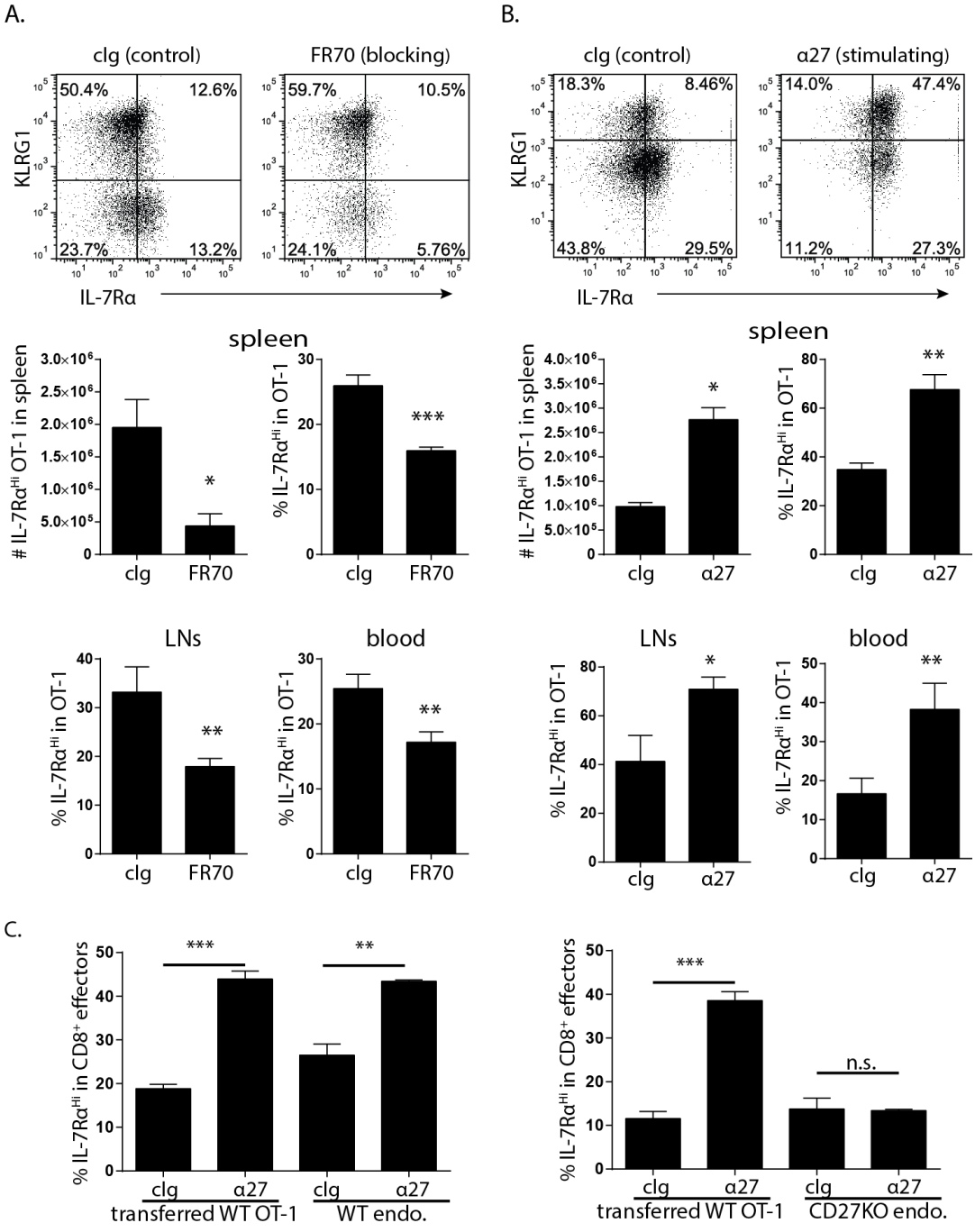
4.4 Results

4.4.1 Direct CD27 costimulation of CD8$^+$ T cells during primary responses is critical for the generation of IL-7Rα-expressing effector cells

In agreement with our previous finding in mouse spleens[80], abolishment of CD27 costimulation by blocking mAb FR70 during primary CD8$^+$ T cell responses to vaccinia virus led to a substantial reduction in the proportion of IL-7Rα-expressing effectors at D7 in secondary lymphoid tissues (spleen and lymph nodes) and peripheral blood (Figure 4.1A). Reciprocally, stimulation of CD27 by agonistic mAb AT-124 during primary responses in CD4-depleted animals resulted in a significant increase in the proportion of IL-7Rα-expressing effectors (Figure 4.1B). Importantly, CD27 costimulaiton promoted not only the proportion, but also the absolute number of IL-7Rα-expressing effectors in spleen (Figure 4.1A, B), indicating the formation of an enlarged memory precursor pool. Consistent with this were the outcomes of highly compromised CD8$^+$ T cell memory upon CD70 blockade and the rescue of 'helpless' memory by exogenous CD27 stimulaiton[80], supporting the mechanistic theme that CD27 costimulation promotes CD8$^+$ T cell memory at least partially via enhancing IL-7Rα-expressing memory precursor

Figure4.1 Direct CD27 costimulation of $CD8^+$ T cells during primary response is critical for the generation of IL-7Rα-expressing effector cells. (A, B) 1,000 OT-1 cells were adoptively transferred into WT recipients 1d before vaccinia-OVA priming. Mice were then treated with either (A) CD70 blocking antibodies (FR70) or (B) CD27 stimulating antibodies (α27), in comparison with control IgG (cIg). Spleens, or lymph nodes and blood, were harvested at d7, and OT-1 cells were analyzed by flow cytometry for surface expression of IL-7Rα. (C) The same experimental setting as (B), but adding $CD27^{-/-}$ recipients in compared to WT, and both OT-1 and endogenous OVA-specific $CD8^+$ T cells were analyzed by d7 for surface expression of IL-7Rα. Dot plots was pre-gated on OT1 cells (live $Thy1.1^+$ $CD8^+$) and showed representative mouse for each group. Bar charts showed combined results in each group from a representative experiment. Experiments were independently repeated 2~5 times with 3 mice per group. Student t-test for bar charts in (A) and (B), and one-way ANOVA for (C). *p<0. 05, **p<0. 01, and ***p<0. 001.

Figure 4.1


A.
cIg (control)
FR70 (blocking)
KLRG1
IL-7Rα
50.4%
12.6%
23.7%
13.2%
59.7%
10.5%
24.1%
5.76%
spleen
IL-7Rα^Hi OT-1 in spleen
% IL-7Rα^Hi in OT-1
cIg
FR70
LNs
blood
B.
cIg (control)
α27 (stimulating)
18.3%
8.46%
43.8%
29.5%
14.0%
47.4%
11.2%
27.3%
α27
C.
% IL-7Rα^Hi in CD8+ effectors
transferred WT OT-1
WT endo.
CD27KO endo.
n.s.

effector cells (MPECs). It's noteworthy that no difference was found in homeostatic level of IL-7Rα comparing CD27KO to WT mice, and levels of IL-7Rα on naïve $CD8^+$ T cells are not sensitive to additive CD27 stimulation *in vivo* (data not shown), suggesting the impact of CD27 signal on IL-7Rα is specific for effector but not quiescent $CD8^+$ T cells.

To explore the cellular mechanisms by which CD27 signal impacts MPEC differentiation, we first sought to identify the cellular target of CD27 stimulation that enhances IL-7Rα-expressing $CD8^+$ effectors during acute viral infection. CD27 is broadly expressed on T[150], B[26] and NK[151] cells and stimulation of CD27 contributes to their activation. We hypothesized that it is the direct stimulation of CD27 on $CD8^+$ T cells, given our previous finding that direct CD27 stimulation promoted $CD8^+$ T cell memory and that CD27-driven promotion of IL-7Rα-expressing effectors was independent of both $CD4^+$ T cells and NK cells (data not shown). To test this hypothesis, we adoptively transferred small amount (500) of WT OT1 $Thy1.1^+$ cells into WT and CD27KO recipients a day prior adeno-OVA priming and treated the mice with either αCD27 or control IgG during primary responses. Substantial responses were derived from both transferred OT1 and endogenous $CD8^+$ T cell components in d7 spleen (data not shown). The proportion of IL-7Rα-expressing WT OT1 was increased 2~4 fold upon CD27 stimulation in both WT and CD27KO recipients, indicating that the expression of CD27 on host population is dispensable for IL-7Rα expression on OT1 cells (Figure 4.1C, left). Conversely, unlike WT endogenous effectors, endogenous effectors in CD27KO mice failed to increase their IL-7Rα frequency in response to αCD27 treatment, indicating that the expression of CD27 on $CD8^+$ T cells is necessary (Figure 4.1C, right). Together these data indicate that CD27 stimulation works intrinsically on the responding $CD8^+$ T cells to enhance IL-7Rα-expressing effector population.

4.4.2 Early CD27 costimulation during first 48hr does not prevent the acute down-regulation of IL-7Rα upon TCR signaling

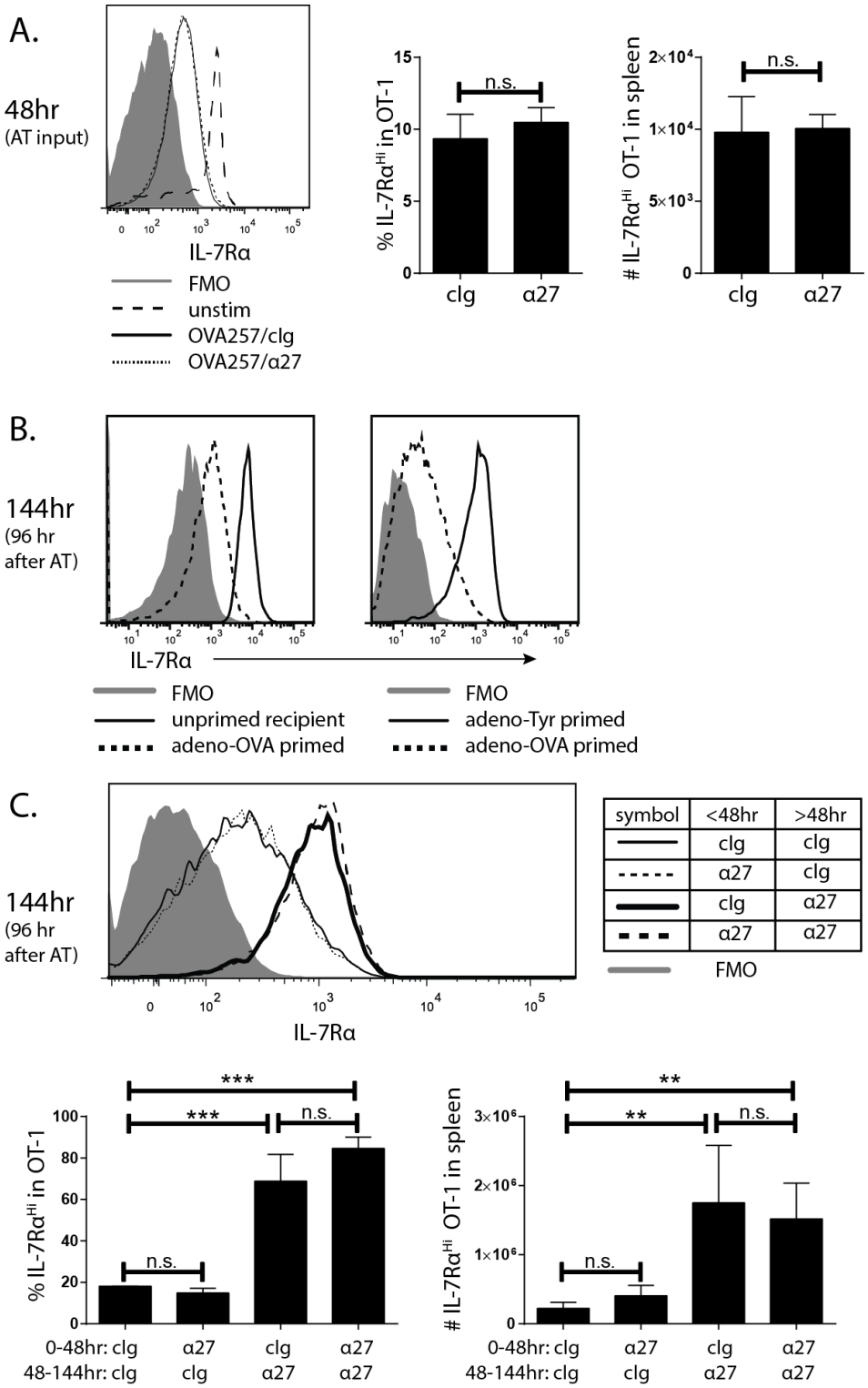
During acute viral infection, IL-7Rα is markedly down-regulated by TCR signaling at early stage of primary responses (e.g. as early as 6hr *in vitro*[144] and during the first 4-5 d of LCMV infection[56]), and the expression is regained in a small subset by the peak of primary responses upon Ag clearance (e.g. by d 8 after LCMV infection[56]). The CD27-driven promotion of IL-7Rα-expressing effectors at the peak of primary responses could be a consequence of either alleviated IL-7Rα down-regulation, and/or accelerated recovery during IL-7Rα re-expression stage. To define the mechanisms by which CD27 signal enhances IL-7Rα-expressing effectors, we began with examining whether CD27 stimulation prevented the acute down-regulation of IL-7Rα upon TCR stimulation as reported for glucocorticoid stimulation *in vitro*. Splenocytes from OT1 mice were co-cultured with $OVA_{257-264}$ congenic peptide in the presence of either αCD27 or control IgG, and IL-7Rα was assessed at 48hr by flow cytometry. In both groups IL-7Rα was dramatically down-regulated, and CD27 stimulation showed no impact in either the proportion or absolute number of IL-7Rα-expressing early effectors (Figure 4.2A). Thus, CD27 costimulation has no impact on IL-7Rα down-regulation in early effectors.

4.4.3 CD27 costimulation after 48hr facilitates IL-7Rα re-expression

Unlike *in* vivo during infection where the down-regulation of IL-7Rα is sustained in the majority of $CD8^+$ effector T cells at the peak of the primary response, mouse $CD8^+$ T cells stimulated *in vitro* only transiently (within 48hr) repress IL-7Rα and then regain it uniformly (% IL-7Rα >90% by 120hr), implying certain determinants that prevent the universal re-expression of IL-7Rα *in vivo* are deficient *in vitro*. Two potential determinants that may account for this discrepancy are Ag and inflammatory signals.

Figure 4.2 Early CD27 stimulation has no impact on IL-7Rα down-regulation, yet late CD27 stimulation facilitates IL-7Rα re-expression. (A) OT-1 cells were co-cultured with irradiated APC pulsed with OVA_{257} peptide, in the presence of either CD27 stimulating antibodies (α27) or control IgG (cIg). Frequency and absolute number of IL-7Rα-expressing OT-1 cells were assessed at 48hr. (B) 48hr *in vitro*-stimulated, IL-7Rα-down-regulated OT-1 cells described in (A) were adoptively transferred into mice pre-primed with adeno-OVA, or adeno-Tyr (adenovirus recombinant with tyrosinase) or plain PBS when indicated. Spleens were harvested 96 hr later after the adoptive transfer and the expression of IL-7Rα on OT-1 cells were identified by flow cytometry. (C) Similar setting as in (B), except recipient mice were infected with Adeno-OVA, and after the adoptive transfer they were then treated with either CD27 stimulating antibodies (α27) or control IgG (cIg). Spleens were harvested 96 hr later after the adoptive transfer and the expression of IL-7Rα on OT-1 cells were identified by flow cytometry. Each experiment was independently repeated 2 or 3 times with 3 mice per group. Student t test for bar charts in (A), and one-way ANOVA for bar charts in (C). **p<0. 01, and ***p<0. 001.

Figure 4.2


A.
48hr
(AT input)
IL-7Rα
FMO
unstim
OVA257/clg
OVA257/α27
% IL-7Rα^Hi in OT-1
n.s.
clg
α27
IL-7Rα^Hi OT-1 in spleen
n.s.
clg
α27
B.
144hr
(96 hr
after AT)
IL-7Rα
FMO
unprimed recipient
adeno-OVA primed
FMO
adeno-Tyr primed
adeno-OVA primed
C.
144hr
(96 hr
after AT)
IL-7Rα
symbol
<48hr
>48hr
clg
clg
α27
clg
clg
α27
α27
α27
FMO
% IL-7Rα^Hi in OT-1
***
***
n.s.
n.s.
0-48hr: clg
48-144hr: clg
α27
clg
clg
α27
α27
α27
IL-7Rα^Hi OT-1 in spleen
**
**
n.s.
n.s.
0-48hr: clg
48-144hr: clg
α27
clg
clg
α27
α27
α27

Supporting this, we found that adoptively transferring *in vitro* stimulated, IL-7Rα-down-regulated OT1 cells into adeno-OVA pre-primed but not naïve B6 recipients led to restricted recovery of IL-7Rα (Figure 4.2B, left). Furthermore, the restricted recovery could not be achieved by pre-priming recipients with adenovirus recombinant with tyrosinase (adeno-Tyr) (Figure 4.2B, right), indicating Ag signals are necessary while bystander inflammation is not sufficient for the sustained down-regulation of IL-7Rα *in vivo*.

We therefore switched to an *in vivo* system for further studies of IL-7Rα re-expression after 48hr. After co-culturing OT1 splenocytes with $OVA_{257-264}$ cognate peptide in the presence of either αCD27 (referred to as 'early' CD27 stimulation) or control IgG for 48hr, we adoptively transferred these *in vitro* stimulated, IL-7Rα-down-regulated OT1 cells into adeno-OVA pre-primed B6 recipients. We then treated the mice with αCD27 (referred to as 'late' CD27 stimulation) or control IgG, and assessed IL-7Rα at 144hr. Along with the finding that early CD27 costimulation had no influence in IL-7Rα down-regulation in early effectors (Figure 4.2A), the early CD27 costimulation did not alter either the proportion or absolute number of IL-7Rα-expressing effectors later around the peak of primary responses (Figure 4.2C). In contrast, late CD27 stimulation dramatically promoted both the proportion (~ 3.8 fold) and absolute number (~ 7.8 fold) of IL-7Rα-expressing effectors by 144hr (Figure 4.2C), indicating that additive late CD27 signal impacts IL-7Rα re-expression stage to enhance the IL-7Rα-expressing effector $CD8^+$ T cells.

4.4.4 CD27 costimulation has minimal impact on survival/proliferation of IL-7Rα-expressing effector $CD8^+$ T cells

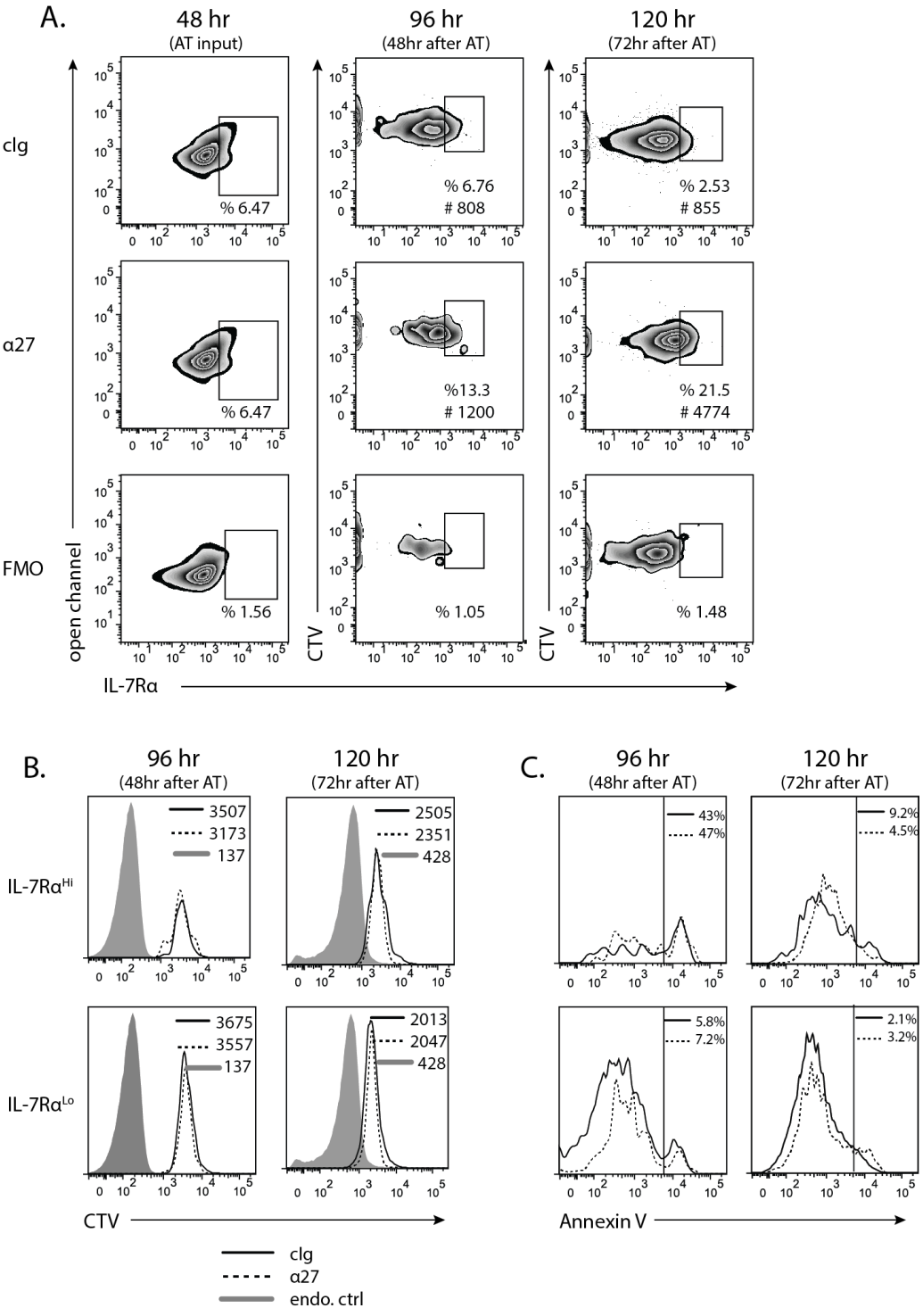
The change in frequency and absolute number of IL-7Rα-expressing cells induced by CD27 stimulation can be explained by two competing yet not mutually exclusive mechanisms. On one hand, CD27 costimulation has been regarded as an anti-apoptotic signal, which contributes to effector expansion and survival in multiple immunization and infection models. Thus CD27 signal may differentially promote survival and/or proliferation of IL-7Rα-re-expressing cells, leading to enrichment in this subset. On the other hand, CD27 signal may directly regulate IL-7Rα re-expression.

To formally identify whether CD27 costimulation makes IL-7Rα-re-expressing cells divide faster, we stimulated OT1 *in vitro* for 48hr to down-regulate their IL-7Rα, labeled them with proliferation dye and adoptively transferred into adeno-OVA pre-primed B6 recipients, and then assessed IL-7Rα expression and cell proliferation at 96 and 120hr. Consistent with the result at 144hr (Figure 4.2C), both the frequency and the absolute number of IL-7Rα-expressing cells were markedly enhanced in the presence of CD27 stimulation as early as 96hr (48hr post-adoptive transfer) compared to controls, and were further enhanced at 120hr (Figure 4.3A). This occurred without a detectable increase in cell proliferation of IL-7Rα-expressing (IL-7Rα^{Hi}) effector CD8^{+} T cells (Figure 4.3B, upper), indicating that the increased number of IL-7Rα-expressing cells induced by CD27 stimulation is not a consequence of accelerated cell divisions. Moreover, effector CD8^{+} T cell subset lacking of IL-7Rα (IL-7Rα^{Lo}) tended to proliferate more than their IL-7Rα^{Hi} counterparts at 120hr, and CD27 stimulation did not hinder their proliferation (Figure 4.3B, bottom), further indicating that the increased frequency of IL-7Rα is not a secondary consequence of selective expansion.

To determine whether CD27 costimulation makes IL-7Rα-re-expressing cells less prone to activation-induced cell death, we assessed cell apoptosis by Annexin V staining

Figure 4.3 The increased frequency and absolute number of IL-7Rα-re-expression cells driven by CD27 stimulation are not associated with accelerated cell division or enhanced cell survival. After 48hr *in vitro* stimulation to down-regulate their IL-7Rα, OT-1 cells were then labeled with CTV proliferation dye and adoptively transferred into adeno-OVA pre-primed recipients. Mice were then treated with either CD27 stimulating antibodies (α27) or control IgG (cIg). 48 and 72hr after the adoptive transfer, (A) IL-7Rα expression on OT-1 cells, and (B) proliferation and (C) cell apoptosis in OT-1 subsets were analyzed by flow cytometry. Flow cytometry plots showed representative data from duplicates, with subset percentage, cell number, or fluorescence geometric mean indicated. Similar experiments were done twice.

Figure 4.3


A.
48 hr
(AT input)
96 hr
(48hr after AT)
120 hr
(72hr after AT)
clg
α27
FMO
% 6.47
% 6.76
# 808
% 2.53
# 855
% 6.47
%13.3
1200
% 21.5
# 4774
% 1.56
% 1.05
% 1.48
open channel
CTV
CTV
IL-7Rα
B.
96 hr
(48hr after AT)
120 hr
(72hr after AT)
IL-7Rα^Hi
3507
3173
137
2505
2351
428
IL-7Rα^Lo
3675
3557
137
2013
2047
428
CTV
clg
α27
endo. ctrl
C.
96 hr
(48hr after AT)
120 hr
(72hr after AT)
43%
47%
9.2%
4.5%
5.8%
7.2%
2.1%
3.2%
Annexin V

at 96 and 120hr. Interestingly, though IL-7 signaling is well known for its anti-apoptotic effects, we did not observe a survival advantage in the IL-7Rα^{Hi} subset of effector CD8^{+} T cells compared to their IL-7Rα^{Lo} counterparts. In fact, at 96hr IL-7Rα^{Hi} CD8^{+} T cells were much more apoptotic than IL-7Rα^{Hi} CD8^{+} T cells, which could not be alleviated by adding exogenous CD27 stimulation (Figure 4.3C). Moreover, the Annexin V staining was nearly identical regardless the presence or absence of additive CD27 stimulation, indicating that the increased number and frequency of IL-7Rα-expressing cells induced by CD27 stimulation is not a consequence of enhanced cell survival.

Combined, these data demenstrate that the increased IL-7Rα is not a secondary consequence of selective subset expansion/maintenance.

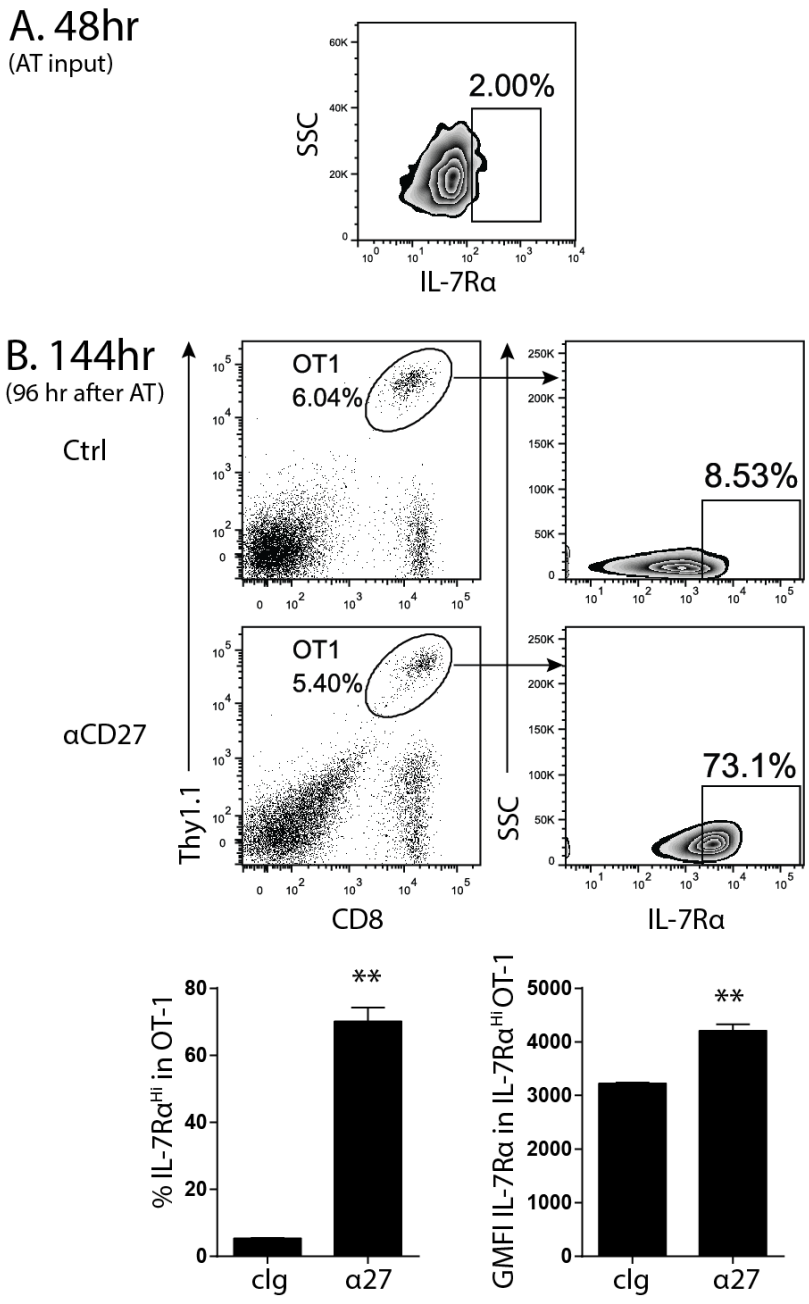
4.4.5 CD27 stimulation directly modulates IL-7Rα re-expression

To formally identify whether CD27 stimulation enhances IL-7Rα-expressing effectors by direct regulation of IL-7Rα re-expression, we stringently sorted for OT1 with minimal IL-7Rα expression after 48hr *in vitro* stimulation with OVA$_{257-264}$ peptide (Figure 4.4A), transferred them into adeno-OVA pre-primed B6 recipients and assessed IL-7Rα re-expression by 144hr. In contrast to control group that showed limited increase in IL-7Rα frequency (~5%) compared to 48hr input (~2%), the majority (~70%) of OT1 from αCD27-treated recipients re-expressed IL-7Rα (Figure 4.4B). Furthermore, within those late effectors that re-expressed IL-7Rα, CD27 costimulation induced 30% higher levels of IL-7Rα in a per cell base (Figure 4.4B). These data, together with our previous finding that CD27 stimulation profoundly upregulated the expression of IL-7Rα in differentiated KLRG1-expressing cells[80], indicates that additive CD27 stimulation can directly promote IL-7Rα re-expression.

4.4.6 CD27 costimulation has minimal impact on IL-7Rα trafficking

Figure 4.4 CD27 stimulation directly modulates IL-7Rα re-expression. (A) 48hr *in vitro*-stimulated OT-1 cells were stringently sorted for minimal IL-7Rα expression, and then adoptively transferred into mice pre-primed with adeno-OVA. Mice were then treated with either CD27 stimulating antibodies (α27) or control IgG (cIg). (B) Spleens were harvested 96hr later after the adoptive transfer and the percentage of IL-7Rα-re-expressing OT-1 cells, and per-cell levels of IL-7Rα within the IL-7Rα-re-expressing OT-1 cells were estimated by flow cytometry. Bar charts showed the combined result of three mice per group from a representative experiment. Experiments were independently repeated 3 times. Student t test, **p<0. 01.

Figure4.4


A. 48hr
(AT input)
2.00%
SSC
IL-7Rα
B. 144hr
(96 hr after AT)
Ctrl
αCD27
OT1
6.04%
8.53%
OT1
5.40%
73.1%
Thy1.1
CD8
SSC
IL-7Rα
% IL-7Rα^Hi in OT-1
**
clg
α27
GMFI IL-7Rα in IL-7Rα^Hi OT-1
**
clg
α27

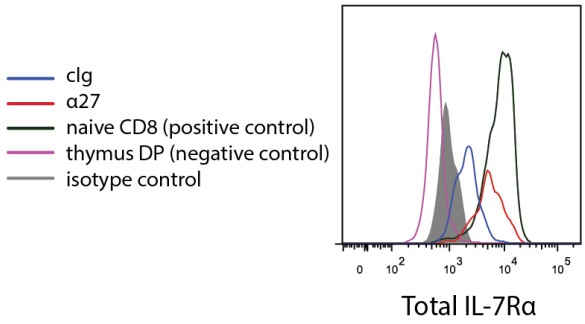
We then sought to identify the underlying mechanisms by which CD27 costimulation promotes IL-7Rα. To this point, we had measured the surface expression of IL-7Rα, while several recent studies have revealed a considerable intracellular expression of IL-7Rα in $CD8^+$ T cells. More intriguingly, it has been reported that the surface expression of IL-7Rα could be regulated post-translationally by targeting protein trafficking[142, 143] (internalization/recycling) or degradation[143, 152]. We therefore determined the impact of CD27 on IL-7Rα protein distribution. FACS staining of permeabilized cells showed that CD27 stimulation led to 2~3 fold increase of total (surface plus intracellular) IL-7Rα protein in later effectors, implying CD27 regulation of IL-7Rα is not restricted to surface expression of the protein but rather in a general manner (Figure 4.5A). To visually confirm the result and directly identify whether CD27 costimulation has differentially contributes to the surface versus intracellular expression of IL-7Rα, we performed imaging flow cytometry analysis. The IL-7Rα staining in most control Ig-treated cells was barely visible by imaging. In the few cells where IL-7Rα was visible, the staining was distributed both on the cell surface and in cytoplasm (Figure 4.5B, last row). Consistent with regular flow cytometry analysis, we found that CD27 costimulation substantially enhanced the expression of total (surface plus intracellular) IL-7Rα (Figure 4.5B). Further analysis (Figure 4.5C) revealed that the enhancement occurred concomitantly in both surface and intracellular compartments, and that CD27 costimualtion did not skew the distribution of IL-7Rα expressed in each compartment. Thus CD27 stimulation promotes the surface expression of IL-7Rα by enhancing its total protein expression rather than by regulation of IL-7Rα trafficking onto cell surface.

4.4.7 CD27 costimulation regulates IL-7Rα re-expression by promoting its mRNA

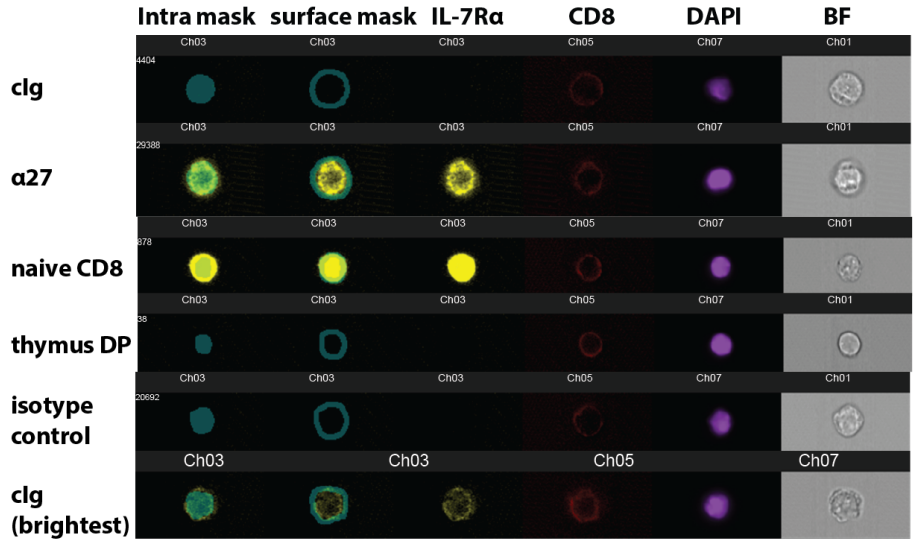
Figure 4.5 CD27 stimulation concomitantly promotes IL-7Rα expression in both surface and intracellular compartments without skewing the proportion of each compartment. To assess the total (surface + intracellular) expression of IL-7Rα, splenocytes were harvested from either CD27 stimulating antibodies (α27) or control IgG (cIg) treated mice, permeabilized and stained for IL-7Rα. (A) Levels of IL-7Rα on OT-1 cells identified by regular flow cytometry. (B) The imaging of IL-7Rα expression by imaging flow cytometry using the identical samples from (A). (C) Further analysis of (B) revealed the proportion of surface IL-7Rα in the total expression. Similar experiments were independently repeated twice, with duplicates per group.

Figure4.5

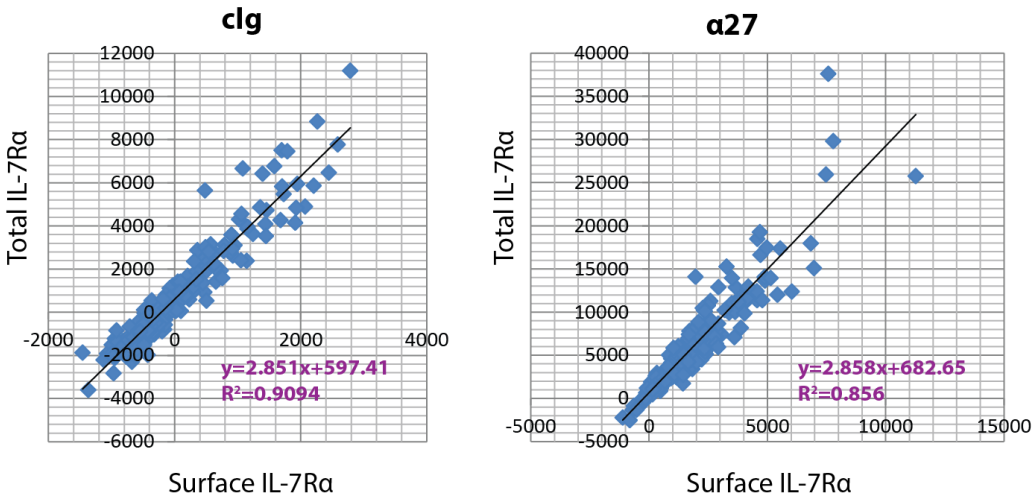
A.



B.



C.



The regulation of IL-7Rα has been reported primarily via transcriptional modulation in T cell development and during circulating in the periphery. To identify whether CD27 regulates *il7ra* mRNA, we sorted late effectors from control Ig or αCD27-treated mice and compared their *il7ra* mRNA level by qPCR. Consistent with the 3~4 fold higher induction of IL-7Rα re-expression (Figure 4.2C), CD27 stimulation led to a ~3.5 fold increase in *il7ra* mRNA. Thus CD27 signal promotes IL-7Rα at least partially by enhancing its mRNA abundance (Figure 4.6).

4.4.8 CD27 costimulation enhances both IL-7Rα and γc expression on effector CD8$^+$ T cells and promotes their responsiveness to IL-7

Finally, we sought to directly address whether CD27-driven IL-7Rα promotion has functional relevance. Interestingly, CD27 stimulation promoted not only IL-7Rα but also γc of the receptor (Figure 4.7A). To establish whether the change of IL-7R upon CD27 signals subsequently led to a change in cell responsiveness to IL-7, we measured the levels of pSTAT5 - the downstream component of IL-7R along the signaling pathway, after a short *in vitro* stimulation of IL-7 on later effectors harvested from mouse spleens. We found elevated levels of pSTAT5 in effectors from αCD27-treated mice (Figure 4.7A) and, conversely, decreased levels of pSTAT5 from FR70-treated mice (Figure 4.7B), compared with those from control Ig-treated animals. Thus the change in the downstream signaling mediated by pSTAT5 correlates with the change in their IL-7R expression.

Importantly, CD27 costimulation exhibited marginal effect on high-affinity (alpha chain) receptor subunits specific for other γc cytokines such as IL-21 and IL-15 (Figure 4.8A), and preliminary data suggested that no substantial change occurred in the subsequent pSTAT signaling in response to these cytokines (data not shown). Thus the

Figure 4.6 CD27 signals regulate IL-7Rα re-expression by promoting its mRNA. d5~6 effector $CD8^+$ T cells from CD27 stimulating antibodies (α27) or control IgG (cIg) treated mice were sorted and their *il7ra* mRNA levels were compared by quantitative PCR. Data showed combined results from 3 independent experiments, with duplicates or triplicates in each experiment. Student t test, ***p<0. 001.

Figure 4.6

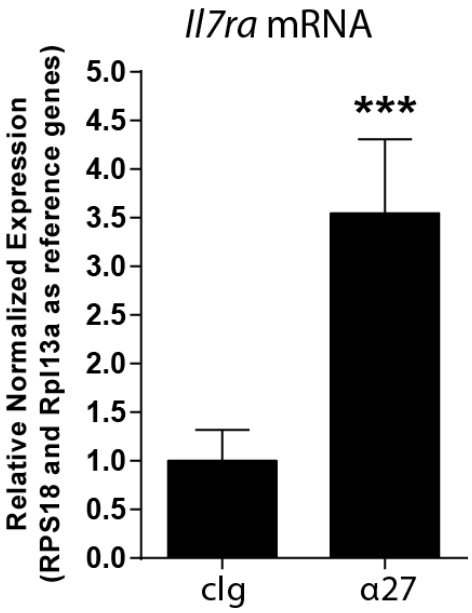
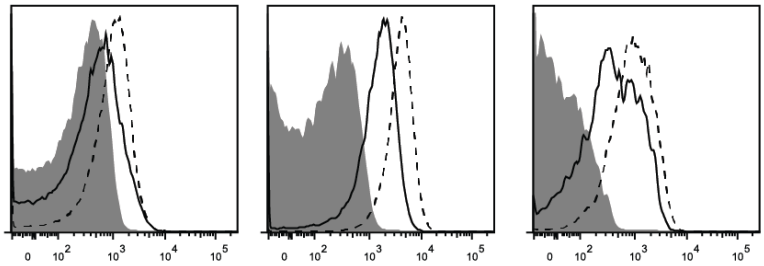

Il7ra mRNA
Relative Normalized Expression
(RPS18 and Rpl13a as reference genes)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
clg
α27

Figure 4.7 CD27 costimulation enhances both IL-7Rα and γc expression on effector CD8$^+$ T cells and promotes pSTAT5-mediated signaling. Adeno-OVA primed mice were treated with either (A) CD27 stimulating antibodies (α27) or (B) CD70 blocking antibodies (FR70) in comparison with control IgG (cIg). Spleens were harvested at d7, and OVA-specific CD8 $^+$ T cells were analyzed by flow cytometry for surface expression of IL-7Rα and γc, and levels of pSTAT5 upon in vitro stimulation of IL-7. Overlapping histograms showed representative data from triplicates per group. Experiments were independently repeated > 3 times.

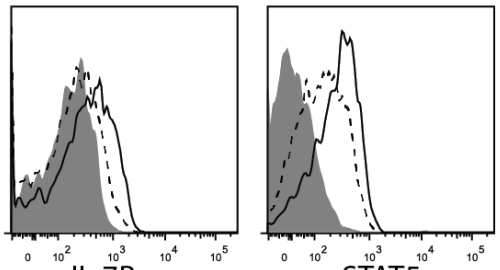
Figure 4.7

A.



	IL-7Rα	γc	pSTAT5
FMO	106	-12.7	1.70
cIg	284 (± 22)	1170 (± 87)	374 (± 36)
a27	714 (± 28)	3084 (± 328)	747 (±56)

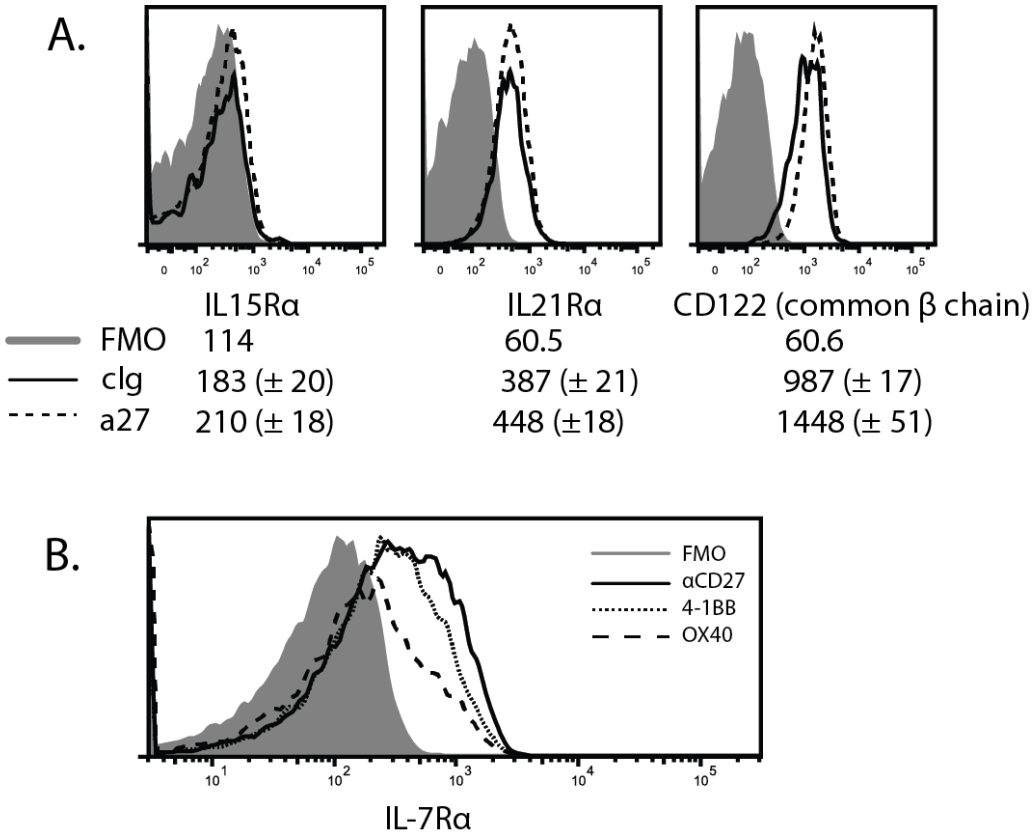
B.



	IL-7Rα	pSTAT5
FMO	125	10.6
cIg	270 (± 23)	219 (± 11)
FR70	168 (± 14)	140 (±13)

Figure 4.8 The ability of CD27 stimulation in modulating other γc cytokine receptors, and the ability of other TNF receptor super-family members in inducing IL-7Rα. (A) Adeno-OVA primed mice were treated with either CD27 stimulating antibodies (α27) or control IgG (cIg), and d7 spleens were harvested and effector $CD8^+$ T cells were analyzed by flow cytometry for surface expression of cytokine receptors. Experiments were independently repeated 2~3 times. (B) OVA protein immunized mice were treated with stimulating antibodies of CD27, 4-1BB and OX40, respectively, and d7 expression of IL-7Rα was assessed by flow cytometry. Representative data were showed from a singular experiment with duplicates per group.

Figure 4.8


A.
IL15Rα
IL21Rα
CD122 (common β chain)
FMO 114 60.5 60.6
clg 183 (± 20) 387 (± 21) 987 (± 17)
a27 210 (± 18) 448 (±18) 1448 (± 51)
B.
FMO
αCD27
4-1BB
OX40
IL-7Rα

enhancement in γc expression driven by CD27 costimulation could be critical yet not sufficient to augment cell responsiveness to IL-7, while the enhancement in IL-7Rα is essential. Of note, preliminary work revealed that CD27 regulation of IL-7Rα is superior compared to another TNFR family member 4-1BB, while the ability of OX-40 to promote IL-7Rα is very limited (Figure 4.8B).

4.5 Discussion

Our study demonstrates that CD27 signals regulate IL-7Rα re-expression to promote IL-7Rα-expressing effector $CD8^+$ T cells. Using vaccinia virus, we previously reported a positive correlation between CD27 signals and IL-7Rα expression during primary $CD8^+$ T cell responses[80], which has also been noted by others in more recent studies using other infectious models[76, 153, 154]. However, as CD27 costimulation has been regarded as an anti-apoptotic signal, which contributes to effector survival and expansion in multiple immunization and infection models, it should be anticipated that CD27 costimulaition might promote IL-7Rα-expressing MPECs by facilitating their survival and/or proliferation. In the current study we have formally tested and ruled out this possibility. Furthermore, the adoptive transfer assays using stringently sorted, IL-7Rα-down-regulated *in vitro*-generated CD8 effectors indicate not only a >5 fold increase in the frequency of IL-7Rα-expressing later effectors induced by CD27 stimulation, but also a striking enhancement in IL-7Rα levels among those expressing IL-7Rα. These taken together strongly argue for a Signal 3 role of CD27 costimulation that regulates MPEC differentiation, independent of its previously well-established Signal 2 role that promotes survival and/or proliferation of effector populations. Importantly, CD27 regulation of IL-7Rα is superior compared to another TNFR family member 4-1BB, while the ability of OX-40 to promote IL-7Rα is very limited. Taking this dissimilarity into

consideration during adjuvant choosing may lead to the development of more potent vaccine.

We should note that, discrete from *in vitro*, the re-expression of IL-7Rα and MPEC differentiation during primary $CD8^+$ T cell responses *in vivo* is not a default pathway. The re-expression of IL-7Rα does not occur in majority of later effectors, and our data attribute this to the impact of Ag signals but not inflammation *in vivo*. It is currently unknown how Ag signals *in vivo* would differ from Ag stimulation *in vitro*, as the latter fails to prevent the universal re-expression of IL-7Rα even when applied repeatedly *in vitro* (data not shown). It has been reported that KLRG1 marks effector cells committed not to re-express IL-7Rα and thus becoming SLECs[44]. Given induction of KLRG1 is only observed *in vivo* and so far is not achievable *in vitro* (Susan Kaech, Yale University, email communication), we speculated CD27 signals promote IL-7Rα re-expression *in vivo* by counteracting KLRG1-mediated effect. This could provide a potential explanation for our previous finding that CD27 stimulation profoundly up-regulated the expression of IL-7Rα in differentiated KLRG1-expressing cells[80]. However, we found that KLRG1 remained un-inducible on OT1 cells that had been stimulated *in vitro* and then adoptively transferred into pre-primed animals (data not shown), yet IL-7Rα re-expression is limited and CD27 signals are capable of promoting it. Therefore, the sustained repression of IL-7Rα *in vivo* is dependent on Ag signal and does not require induction of KLRG1, while CD27 costimulation provides an instructive signal to induce IL-7Rα re-expression. Importantly, we believe the enhanced re-expression is not simply due to a quicker clearance of Ag by CD27 stimulation, as stimulation of its close family member 4-1BB induced a larger CD8 T cell response (data not shown) – and therefore unlikely to have more Ag load, led to weaker IL-7Rα re-expression.

Furthermore, the current study indicates that CD27 costimulation regulates IL-7Rα re-expression by enhancing *Il7ra* mRNA abundance rather than facilitating the protein trafficking on to the cell surface. Much work has been done to study the regulation of IL-7Rα expression at transcriptional levels, and recent studies start to elucidate mechanisms involving posttranslational regulation. Based on the imaging flow cytometry analysis that IL-7Rα protein distribution (surface versus intracellular) has not been substantially changed by CD27 stimulation, we speculate CD27 signals are unlikely involved in the mobilization or degradation of IL-7Rα protein. In contrast, the increase of surface expression of IL-7Rα correlates well with an increase in *IL7ra* mRNA, a consequence of either accelerated gene transcription or enhanced mRNA stability. Control of IL-7Rα expression in T cells has been suggested by most evidence to be mediated primarily through changes in *Il7rα* gene transcription. *IL7ra* transcription is controlled by the opposing actions of the transcription factor pairs GABPα1/Gfi-1[128] and Foxo1[129, 155] /Foxp1[130], suggesting a role for CD27 signaling in the activity of these factors. Induction of IL-7Rα by GABPα plays a key role in naïve T cell homeostasis[156] and has been shown to be required for IL-7Rα expression in MPECs during acute viral infection[128]. GABP has been indicated as a downstream target of JNK (c-Jun N-teriminal kinase) – which is a main mediator of downstream signaling pathways induced by CD27 costimulation besides NF-κB [26, 27, 36], and JNK phosphorylates GABPα and GABPβ in *vitro*[156]. We are currently investigating whether CD27 costimulation activates GABPα by phosphorylating it via JNK, which then leads to up-regulation of IL-7Rα transcription and re-expression. Alternatively, CD27 signals may restrict Gfi-1, a transcription factor induced by IL-7 or TCR engagement and required for stable IL-7Rα repression in effector $CD8^+$ T cells by antagonizing GABPα binding[128]. Taken together, future studies

are expected to elucidate whether there is a transcriptional cassette that is elicited by CD27 stimulation that directly accounts for IL-7Rα re-expression.

The finding of augmented expression of γc upon CD27 stimulation is novel and implicates CD27 costimulation may facilitate IL-7 signaling by regulating multiple components along the pathway. Preliminary data indicate that consistent with the *in vivo* phenotype, stimulation of CD27 *in vitro* substantially promoted the expression of γc on $CD8^+$ T cells while there was no increase in mRNA levels, suggesting that CD27 costimulation modulates γc by distinctive and independent mechanism(s) from its regulation of IL-7Rα. IL-7 initiates cell signaling by inducing the physical approximation of IL-7Rα and γc proteins on the cell surface, which causes transactivation of Jak1 and Jak3 in the cytosol. Intriguingly, besides its well-established role in transcriptional down-regulation of IL-7Rα, TCR stimulation has been reported to impair IL-7 signaling by cleaving the cytosolic tail of γc to dissociate Jak3 from surface IL-7R[157], and by inducing expression of the microRNA miR-17 to target Jak1 that is associated with IL-7Rα[158]. Whether CD27 costimulation regulates microRNA or Jak1/3 to modulate IL-7 signaling remains an open question. Activated Jak1 and Jak3 phosphorylate monomeric STAT5 to induce its dimerization and translocation to nucleus to induce targeting genes. The levels of pSTAT5 in response to *in vitro* IL-7 stimulation are elevated in effectors from anti-CD27-treated mice compared to control mice and we implicate it as a consequence of enhanced IL-7R expression on the cell surface. However, it could also be a consequence of increased Jak1/3 activation or alternatively higher abundance of STAT5 in the cytosol.

Finally, our findings of CD27-induced enhanced IL-7R (both α and γc subunits) and augmented pSTAT5-mediated signaling highlight the potential of targeting CD27-CD70

axis to enhance IL-7 signaling for antiviral/antitumor immunotherapy. IL-7 is required for $CD8^+$ T cell development in thymus[159-161] and for homeostatic maintenance of naïve $CD8^+$ T cells in periphery[162-164]. Importantly, IL-7 plays an essential role in the long-term survival of Ag-specific $CD8^+$ T cells during contraction[56]. The pre-clinical and clinical application of rIL-7 has been reported to result in enhanced T cell immunity, raising the prospect that IL-7 signaling could mediate therapeutic benefits in the treatment of acute and chronic infections and cancer[132, 133, 165]. Given the published clinical experience with the use of rhIL-7 therapy so far is modest[166], enhancing IL-7Rα by targeting CD27 may synergize with rhIL-7 therapy to achieve optimized IL-7 signaling especially in the elderly[167], leading to more favorable outcomes. Moreover, in comparison to rhIL-7 therapy that primarily targets steady-state T cell populations and improves immune reconstitution through increasing thymic output and though Ag-independent homeostatic driven proliferation in the periphery[165], CD27 stimulation promotes IL-7Rα in effector $CD8^+$ T cell population in an Ag-dependent manner and contributes to their long-term maintenance. Therefore, CD27 regulation of IL-7Rα present a suitable candidate therapeutic strategy to enhance the effectiveness of adoptive immunotherapy for cancer; and probably more importantly, this strategy could be applied to clinical settings where deleterious off-target effects obscure the efficacy of rIL-7 therapy – such as in HIV infection where rhIL-7 has been reported in clinical trials to promote viral persistence during antiretroviral therapy by inducing survival and expansion of HIV-1-latently infected memory CD4(+) T lymphocytes[168, 169]. In addition, our finding that CD27 stimulation is capable to induce IL-7Rα on KLRG1-expressing cells raises the prospect that targeting CD27 could boost the endogenous responses in patients with chronic infection and

cancer by altering the exhausted characteristics of Ag-specific $CD8^+$ T cells to promote their maintenance and function.

Chapter 5. Discussion

5.1 Concluding remarks

CD27-CD70 interactions are a critical functional component of $CD4^+$ helper T cell-mediated DC activation, and our previous work and others' have shown significant contributions of CD27-CD70 interactions to primary CTL responses and $CD8^+$ T cell memory. However, the understanding of their function in $CD8^+$ T cell activation and fate decisions is incomplete and currently obscured by the variability in their requirement during different stages of $CD8^+$ T cell responses depending on the nature of the immunogen. In this work, by enforcing or abrogating CD27 function by genetic or protein intervention in murine models, we have assessed the function of CD27 costimulation in the primary and secondary CTL responses to both subunit vaccine and replicating virus, and identified a complex interplay between CD27 costimulatory and inflammatory cytokine pathways that influences the activation and fate decisions of $CD8^+$ T cells.

In the context of a subunit vaccine model, we have demonstrated that CD27 costimulation on $CD8^+$ T cells is an obligatory component of the activity of CD40 stimulation and that CD27 stimulation can substitute CD40 stimulation in the synergy with TLR agonists to drive functional primary and secondary CTL responses. The infusion of CD27 stimulation supports sustained expansion of primary $CD8^+$ T cells, and enhances per-cell IFN-γ production. Mechanistically, we have identified that the responses are dependent on the cooperation of CD27 stimulation and IFN-1 (but not IL-12) at the level of $CD8^+$ T cells, and that the interaction between CD27 and IFN-1 is imprinted by optimized regulation of fate decision transcription factors, including CD27-mediated induction of Eomesodermin and IFN-1-mediated induction of T-bet.

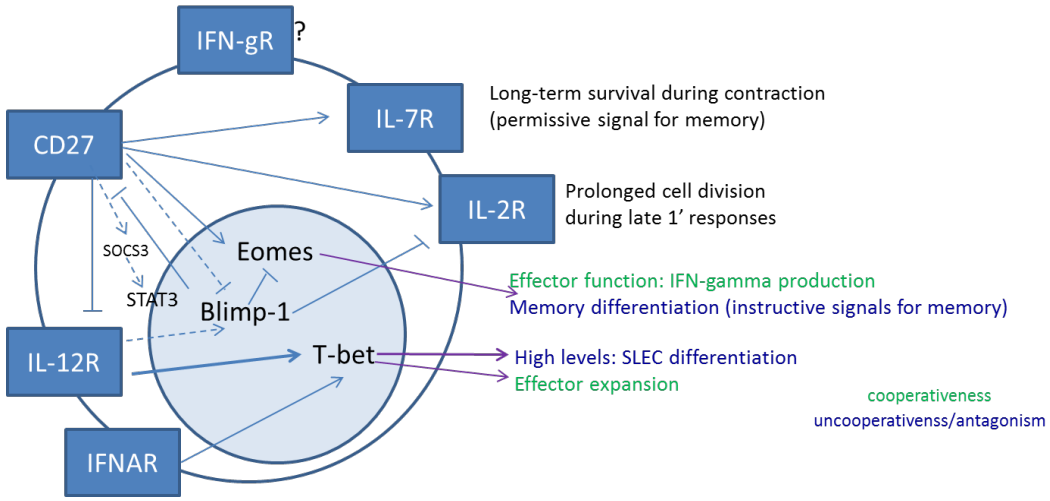
In the vaccinia infection model where $CD4^+$ T cell help is critical for $CD8^+$ T cell memory, we have demonstrated that a main defect in $CD8^+$ T cell memory occurring in the absence of $CD4^+$ helper T cells can be caused by inadequate CD27 costimulation, and that CD27 costimulation during primary $CD8^+$ T cell responses programs the capability to mount secondary $CD8^+$ T cell responses. Mechanistically, we have indicated that CD27 costimulation regulates the re-expression of IL-7Rα, which has been shown to be necessary for memory $CD8^+$ T cell survival; and that CD27 stimulation induces Eomesodermin, a key transcription factor directing memory development. Moreover, we have identified IL-12 as the cause of loss-of-memory in the absence of $CD4^+$ T cell help, and demonstrated that CD27 stimulation rescues helpless memory via restricting the impact of IL-12.

Enhancing IL-7 signaling by rIL-7 therapy has been reported to result in enhanced T cell immunity and favorable pre-clinical and clinical outcome in the treatment of acute and chronic infections and cancer. We have shown that CD27 stimulation promotes not only IL-7Rα, but also the common γ chain of the receptor and the downstream signaling mediated by pSTAT5. Constitutive STAT5 activation proufoundly enhances effector and memory $CD8^+$ T cell survival and augments homeostatic proliferation, Akt activation and Bcl2 expression[170]. Therefore it is of particular interest to elucidate the mechanism(s) by which CD27 costimulation supports IL-7Rα expression, and by extension, IL-7 signaling. We have formally tested two competing yet not mutually exclusive hypotheses – CD27 stimulation (1) selectively promotes the proliferation/survival, and/or (2) drives the development of IL-7Rα-expressing effector cells. Our data demonstrate that exogenous CD27 stimulation directly regulates IL-7Rα re-expression during late stages of priming by

enhancing its mRNA abundance, while its impact is marginal on the proliferation and survival of $CD8^+$ T cells attaining IL-7Rα.

In the remaining Chapter 5, I will discuss the following four topics: (5.2) convergent effects of CD27 signals, CD40 signals and $CD4^+$ T cell help; (5.3) CD27 costimulation-driven determinants for the regulation of $CD8^+$ T cell activation and fate decisions; (5.4) interactivity between CD27 costimulatory and inflammatory cytokine signals; (5.5) divergent effects of CD27 signals, CD40 signals and $CD4^+$ T cell help. Because some points in my following discussion are based on new data, they are included as supplemental figures under discussion. In summary, our proposed model for the role of CD27 costimulaiton in the activation and fate decisions of $CD8^+$ T cells are attached below as Figure 5.1.

Figure 5.1 the role of CD27 costimulaiton in the activation and fate decisions of $CD8^+$ T cells.



5.2 Convergent effects of CD27 signals, CD40 signals and $CD4^+$ T cell help

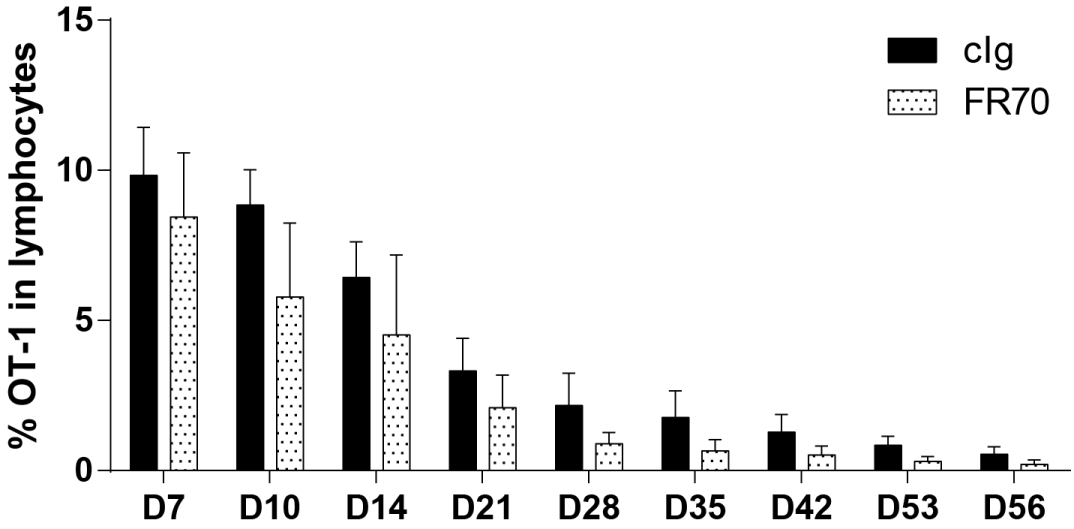
The understanding of the mechanisms by which $CD4^+$ helper T cells promote $CD8^+$ T cell responses is incomplete. Several lines of evidence in our data have indicated that CD27 stimulation, CD40 stimulation and $CD4^+$ T cell help have considerable overlapping effects on $CD8^+$ T cell activation and fate decisions. First, abrogating CD27-CD70 interactions by antagonistic CD70 antibodies dramatically reduced CD40-driven CTL responses (by ~75%) in the subunit vaccine setting, and within the remaining CTLs there was a substantial loss of KLRG1-expressing SLECs, a phenotype that also occurs in the absence of $CD4^+$ T cells in vaccinia infections. Furthermore, abrogating CD27-CD70 interactions during primary responses to vaccinia infection resulted in a moderate reduction in the primary $CD8^+$ T cell responses while the secondary responses were almost completely abolished, which again recapitulated the phenotypes occurring in the absence of $CD4^+$ T cells in the identical infection model. Importantly, IL-12 was found to be accountable for both the 'helpless' memory to vaccinia infection and the loss-of-memory upon CD70 blockade. These together lead us to the conclusion that CD27-CD70 interactions are a main functional downstream consequence of $CD4^+$ T cell help that is transmitted via DC activation, which is further confirmed by the ability of CD27 stimulation to substitute CD40 stimulation in driving $CD8^+$ T cell responses in the subunit vaccine setting and its ability to rescue 'helpless' $CD8^+$ T cell memory in the viral infection setting. Importantly, induction of CD70 on DC occurs 48~72hr after CD40 stimulation, implying $CD4^+$ T cell-DC contact via CD40L-CD40 interactions and DC-$CD8^+$ T cell contact via CD27-CD70 interactions occur in a sequential temporal order rather than concomitantly. This fits nicely with the **sequential two-cell model** for $CD4^+$ T cell help (Figure 1.1), and is consistent with a recent study from the Schoenberger

laboratory[76], which proposed that the $CD4^+$ T-cell help signal is transmitted from DC to $CD8^+$ T cells via CD27-CD70 interactions.

5.3 CD27 costimulation-driven determinants for the regulation of $CD8^+$ T cell activation and fate decisions

What determinants does CD27 costimulation provide to regulate CTL activation and fate decision? To start pursuing this central question, we have demonstrated that CD27 costimulation plays a critical role in the generation of IL-7Rα-expressing effector $CD8^+$ T cells by modulating *Il7ra* mRNA. IL-7Rα is functionally required for the long-term survival of $CD8^+$ T cells through the contraction stage after clearance of pathogens, and long-lasting memory cells are enriched in the small subset of $CD8^+$ T cells that attain IL-7Rα expression by the peak of primary responses – namely memory precursor effector cells (MPECs). This notion is in accord with the fact that blockade of CD70 during vaccinia infection leads to a more profound contraction (Supplemental Figure1) and subsequently inadequate quantity of memory $CD8^+$ T cells compared to the control group, which is associated with a substantial reduction in the frequency of IL-7Rα and the levels of pSTAT5-mediated corresponding signaling in effector $CD8^+$ T cells. Therefore, the current data strongly support that CD27 costimulation promotes $CD8^+$ T cell memory in part by restricting contraction via modulation of IL-7Rα expression. To definitively identify whether the reduction in IL-7Rα contributes to the loss-of-memory in antagonistic CD70 antibodies-treated animals, we could enforce IL-7Rα expression in CTLs by transducing OT-1 cells *in vitro* with retroviral vector recombinant with IL-7Rα, then adoptively transfer transduced OT-1 cells into B6 recipient and assess whether the defects in $CD8^+$ T cell memory upon CD70 blockade can be alleviated by correcting the impeded IL-7Rα arm.

Supplemental Figure 1. Blockade of CD70 during primary CD8$^+$ T cell responses to vaccinia infection leads to more profound contraction. OT-1 chimeric mice (n=3 per group) were challenged with OVA-vac and were treated with control IgG (clg) or antagonistic anti-CD70 (FR70) during the expansion of the primary response. Data showed the percentage of OT-1 in lymphocytes in the peripheral blood during indicated time course. The experiment was performed once.



This experiment would determine the relative contribution of IL-7Rα regulation to function of CD27 costimulation in $CD8^+$ T cell memory.

Nevertheless, it is particularly significant to note that the expression of IL-7Rα is necessary but not sufficient to support the long-term maintenance of memory $CD8^+$ T cells, as no advanced $CD8^+$ T cell memory can be achieved just by constitutive overexpression of IL-7Rα in CTLs[123]. Notably, the increased IL-7Rα in IL-7Rαtg CTLs did not alter levels of the common γ chain[123], the other subunit of the receptor. However, IL-7 initiates cell signaling by inducing the physical approximation of IL-7Rα and γc, and γc is required for the signaling transduction to the downstream pathways - primarily the JAK/STAT and PI3K/AKT pathways[171]. Along this line, enforcing IL-7Rα in KLRG1-expressing SLECs was not able to degrade $p27^{kip}$ – the cell cycle inhibitor downstream of the PI3K/AKT pathway, implying a defect in IL-7 signaling transduction[123]. These together raise the question whether IL-7Rα overexpression fails to promote $CD8^+$ T cell memory further because it fails to further induce IL-7 signaling due to the limited availability of γc. To date the functional significance of γc quantity in $CD8^+$ T cell memory has not been explicitly addressed. As a start, concurrent overexpression of IL-7Rα and γc could be generated, and its capability in enhancing IL-7-mediated $p27^{kip}$ degradation and long-term maintenance of KLRG1-expressing SLECs would be assessed and compared to overexpression of either IL-7Rα or γc, respectively.

Of note, in our hands CD27 stimulation not only promotes the surface expression of IL-7Rα on CTLs, but also strongly enhances levels of γc expression. If in the preceding proposed experiment advanced $CD8^+$ T cell memory can be achieved by constitutive overexpression of γc together with IL-7Rα, then it is reasonable to speculate that both the increased IL-7Rα and γc induced by CD27 costimulation contribute to $CD8^+$ T cell

memory by co-operatively enhancing cell responsiveness to IL-7 during contraction. This is an attractive hypothesis as it could reconcile some discrepancies in the current work. First, though abolishing CD27 signals by blockade of CD70 results in 3~5-fold decrease in IL-7Rα-expressing cells at the peak of primary responses, its impact on memory cell population is much more profound (~30-fold difference) – potentially due to inadequate levels of γc compared to controls. In addition, though formation of IL-7Rα–expressing MPEC appears unimpeded in CD4-depleted animals, the 'helpless' memory is highly comprised – again possibly due to inadequate levels of γc of CTLs upon CD4-depletion (so far we haven't assessed the impact of CD4-deficiency on γc levels of CTL but are planning to). Importantly, the current data demonstrate that exogenous CD27 stimulation rescues 'helpless' memory to vaccinia infection, which correlates with elevated levels of γc on CTLs at the peak of primary responses. Given CD27 stimulation favors IL-7Rα^{Hi}KLRG1^{Hi} subset differentiation, our current working hypothesis is that CD27 stimulation potentiates KLRG1-expressing CTLs to become long-term memory cells not by merely inducing IL-7Rα expression (which has been demonstrated to be insufficient[123]), but by concomitantly enhancing their γc levels. To start to test this possibility, initial work would be required to confirm the enhanced γc in the IL-7Rα^{Hi}KLRG1^{Hi} CTL subset upon CD27 stimulation, and subsequently the high potential of this population to become memory cells. If promising, it would highlight the mechanistic prospect of targeting CD27-CD70 axis to potentiate terminally differentiated CTLs (i.e determine whether expression of a functional IL-7R complex is sufficient to overcome the senescent aspects of KLRG1-expressing cells).

The γc subunit is shared by the receptor complexes for IL-7, IL-2, IL-4, IL-15 and IL-21 family of cytokines, which have been shown to play critical roles in CD8^{+} T cell

responses by mechanisms including regulation of cell survival, proliferation and memory differentiation. Therefore it's evolutionarily meaningful that γc is universally expressed on $CD8^+$ T cells, and in turn supports the notion that expression levels of γc could be critical for cell responsiveness to these 'fate-decision' cytokines, including but not restricted to IL-7. In fact, it has been shown that the survival of KLRG1-expressing SLECs normally depends on IL-15[44]. We have proposed above that the failure of IL-7Rα overexpression to rescue KLRG1-expressing SLECs could be due to insufficient γc to couple with IL-7Rα and transduce functional signaling upon IL-7 stimulation. Alternatively, the enforced IL-7Rα achieved in other studies may compete with IL-15Rα/β for γc binding, leading to increased responsiveness to IL-7 (as suggested by increased induction of pSTAT5, but not $p27^{kip123}$) at the expense of responsiveness to IL-15, the combined outcome of which could be neutral in cell survival. In contrast to genetically enforcing IL-7Rα expression, our current work indicates that CD27 costimulation not only promotes IL-7Rα, but also enhances the levels of γc and likely the common β chain of IL-15R complex as well. It suggests a potential function of CD27 signals in promoting CTL responsiveness to IL-15, which has been demonstrated to induce memory differentiation and support homeostatic proliferation of memory $CD8^+$ T cells.

Combined, CD27 costimulation supports $CD8^+$ T cell memory in part by modulating the expression of cytokine receptors that influence the differentiation and long-term survival of memory $CD8^+$ T cells. It is also noteworthy that CD27 signals have been reported to enhance primary $CD8^+$ T cell survival via preventing Fas-mediated cell death[32]. Naïve $CD8^+$ T cells up-regulate Fas upon TCR-mediated activation, and Fas is maintained at high levels on memory $CD8^+$ T cells. Notably, IL-7 is known to increase Fas expression on resting T and B cells, making cells more susceptible to Fas-mediated

apoptosis[172-174]. IL-7Rα-expressing MPECs outcompete during the contraction stage by acquiring IL-7-mediated pro-survival signaling, which could in turn result in Fas up-regulation on the cell surface and increased cell sensitivity to Fas-L. These together lead to another potential mechanism as to how CD27 signals restrain $CD8^+$ T cell contraction[175]: CD27 costimulation may not only enhance the quantity of MPECs by regulation of IL-7R, but also upgrade their memory potential by preventing apoptotic pathways in those cells. Pertaining to this, while abolishing CD27 signals led to a significant decrease in MPECs, its impact on memory cell population is much more profound. To test whether CD27 signals make IL-7R-expressing effector $CD8^+$ T cells less susceptible to Fas-mediated cell death, we could harvest $CD8^+$ T cells during the contraction stage from mice pre-treated with either antagonistic CD70 or control antibodies, and assess Ag-specific $CD8^+$ T cells for their Fas expression levels and caspase induction upon Fas stimulation in *vitro*. Alternatively, CD27 signals could restrict FasL expression on cell populations like $CD4^+$ T cells to prevent Fas-mediated cell death[32] of MPECs.

IL-2 signaling has been demonstrated to play a critical yet paradoxical role in $CD8^+$ T cell expansion and differentiation *in vivo*. Several lines of evidence indicate that IL-2 signals during priming are crucial in driving secondary expansion of memory $CD8^+$ T cells[11], and that IL-2-producing effector $CD8^+$ T cells have higher secondary responsiveness upon rechallenge [Anke Redeker, Leiden University Medical Center; Shannon Kahan, University of Alabama Birmingham; personal communication during Keystone Symposia poster session]. Consistent with data from the Borst group[30], we have shown that exogenous CD27 stimulation induced autocrine IL-2 production of CTLs. However, we have also indicated that blockade of CD70 led to inadequate

quantity of memory $CD8^+$ T cells without impeding their secondary expansion capacity. Thus it is unlikely CD27 costimulation supports $CD8^+$ T cell memory by enhancing production of autocrine IL-2 in effector $CD8^+$ T cells upon vaccinia infection. Of note, IL-2 production of CTLs in CD4-depleted animals is not impeded in our hands, which is inconsistent with Feau *et al*'s data[69] but further supports the conclusion that the role of CD27-driven autocrine IL-2 in the rescue of 'helpless' memory during vaccinia infection is not significant.

Potentially reconcile these contradictory data, recent studies comparing LCMV and Listeria infection [Laurent Chorro, Albert Einstein College of Medicine, personal communication during Keystone Symposia poster session] indicate that there is variability in the requirement of IL-2 signaling for T cell proliferation *in vivo* during primary and secondary expansion. Pertaining to this, IL-2-signaling has been indicated to be critical for the prolonged proliferation of primary $CD8^+$ T cells in DC immunization model via PI3K-dependent induction of FoxM1[104]. In the agreement with this, when used as an adjuvant in the protein vaccine setting, agonistic CD27 antibodies lead to sustained induction of IL-2Rα (CD25**)** – the high-affinity IL-2 receptor by d5, and a subsequent prolonged expansion of effector $CD8^+$ T cells during d5~7, suggesting that CD27-mediated regulation of IL-2/IL-2R axis could functionally contribute to the regulation of $CD8^+$ T cell responses. On the other hand, sustained CD25 expression on effector $CD8^+$ T cells in infectious model has been implicated in the generation of terminally differentiated SLECs[12, 14], and our work shows that CD27-driven rescue of 'helpless' memory is associated with reduced levels of IL-2Rα in IL-7Rα-expressing MPECs during vaccinia infection, implying that CD27 signals could support $CD8^+$ T cell memory by restricting terminal differentiation pathways.

Taken together, further investigation is expected with regard to the paradoxical function of IL-2 signaling in $CD8^+$ T cell responses and the complexity of CD27-mediated modulation of IL-2 signaling. Markedly, it has been shown that IL-2 production and IL-2 consumption occur in reciprocal populations of effector $CD8^+$ T cells, implying that IL-2 acts in paracrine instead of autocrine manner [Shannon Kahan, University of Alabama Birmingham, personal communication during Keystone Symposia poster session]. In the agreement with this, by using systemic approaches and computational models, recent studies from Altan-Bonnet group have unraveled that while TCR signaling induces a positive feedback loop of IL-2 production, it governs a negative feedback to IL-2-induced pSTAT5-mediated signaling [Gregoire Altan-Bonnet, Memorial Sloan-Kettering Cancer Center, Keystone Symposia speaker]. Computational biochemical models combined with quantitative experimental validation would be a powerful tool for study of the nonlinear signaling transduction and dynamic feedback in immune regulation and thus facilitate future study of the functional outcomes, including CD27 regulation of IL-2 production and consumption, and the competition of cytokine-private receptors for γc as addressed above.

Aside from modulating the surface expression of receptors for cytokines that influence the differentiation and expansion/survival of $CD8^+$ T cells (such as IL-2, IL-7 and IL-15 as discussed above), we have demonstrated in both vaccine and infection settings that CD27 signals induce Eomes, a key T-box transcription factor dictating effector $CD8^+$ T cell function and fate. The mechanism of CD27-mediated Eomes induction has not been established yet. It has been indicated that STAT5 augments Eomes expression[13]. Our preliminary work indicates that abolishing CD27 signals in mice by CD70 blockade during vaccinia infection reduced steady state levels of pSTAT5

in CTLs (data not shown), which may reflect their decreased responsiveness to IL-2 (data not shown), suggesting the enhanced Eomes achieved with CD27 costimulation may be a consequence of its ability to regulate IL-2/IL-2R node. Alternatively, as CD27 signals promote IL-15Rβ and γc, the increased Eomes could be a downstream consequence of augmented IL-15 signaling. We have shown that when used as an adjuvant, agonistic CD27 antibodies augment IFN-γ production in CTLs on a per cell basis and enhance killing, which may be a function of the increased Eomes expression[103]. We have also indicated that the success in rescue of 'helpless' memory achieved by CD27 stimulation is associated with its ability to correct impeded Eomes expression, strongly suggesting that CD27 costimulation supports $CD8^+$ T cell memory via enhancing Eomes. To definitively identify the relative contribution of Eomes in the function of CD27 signals during $CD8^+$ T cell responses, we could utilize $Eomes^{flox/flox}$ $GranzymeB^{Cre}$ mice that deplete Eomes in CTLs, and assess to what extent CD27-driven responses is obstructed compare to WT counterparts.

5.4 Interactivity between CD27 costimulatory and inflammatory cytokine signals

The contribution of CD27 stimulation to $CD8^+$ T cell activation and fate decisions appears to be highly associated with the extent of inflammation depending on the type of immunization. We have demonstrated that CD27 costimulation synergizes with IFN-1 at the level of $CD8^+$ T cells to achieve robust primary $CD8^+$ T cell responses in the low-inflammatory, subunit vaccine setting while it antagonizes the impact of IL-12, consequentially promoting $CD8^+$ T cell memory in the high-inflammatory, replicating viral infection setting. We have identified that the complex interplay between CD27 costimulatory and inflammatory cytokine pathways leads to delicate regulation of T-box transcription factor pair Eomes and T-bet, an critical axis for the activation and fate

decisions of $CD8^+$ T cells[94]

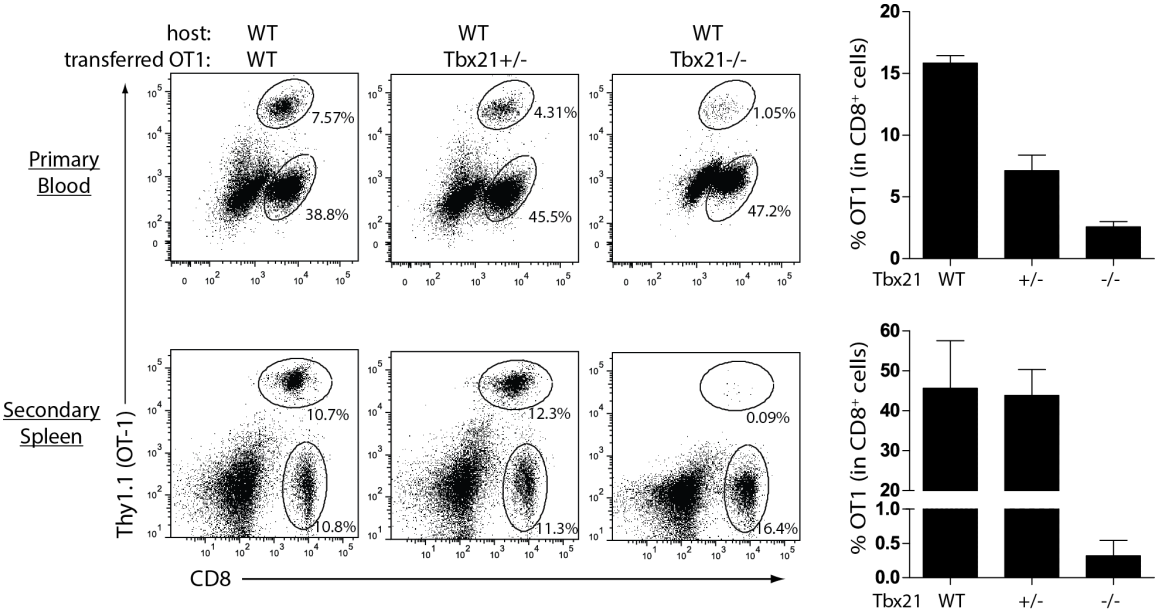
T-bet and Eomes inversely regulate effector and memory $CD8^+$ T cell fate. T-bet is typically induced by inflammatory signals like IL-12 and has been demonstrated to direct $CD8^+$ T cells to take the characteristics of SLECs[44], while its counterpart Eomes enables $CD8^+$ T cells to compete for the memory niche[60]. IL-12 has been demonstrated to induce T-bet and repress Eomes[59] in CTLs. Pertaining to this, we have identified that IL-12-mediated loss-of-memory in the absence of $CD4^+$ T cell help during the primary $CD8^+$ T cell response to vaccinia infection is associated with hindered Eomes induction, while CD27-mediated rescue of the 'helpless' memory is associated with increased Eomes levels. Therefore, either CD27 signaling reduces cell sensitivity to IL-12 via the reduction in IL-12R expression and thus abrogates IL-12-mediated suppression of Eomes, or Eomes is a novel downstream target of CD27 signaling. The latter is favored by our data in the subunit vaccine setting where agonistic CD27 antibodies were used as sole adjuvant and resulted in induction of Eomes. It has been indicated that Eomes to T-bet ratio is more critical than their respective absolute values in dictating memory/effector differentiation[176]. Consistent with this notion, the contribution of CD27 costimulation to $CD8^+$ T cell memory is more profound during vaccinia infection – which is high-inflammatory and thus putatively accompanied with high T-bet, than in DC or protein immunization settings – which are less-inflammatory and thus putatively accompanied with low T-bet. Pending the direct comparison of T-bet levels in those models, our work strongly supports that the requirement for CD27 and potentially other TNF-superfamily members such as 4-1BB (CD137, also a potent inducer of Eomes) [102, 127] for $CD8^+$ T cell memory development may be exacerbated by the presence of high levels of T-bet associated with inflammatory context during the initial expansion of the primary $CD8^+$ T

cell response.

T-bet and Eomes also play critical role in primary $CD8^+$ T cell expansion and effector function[103, 176]. We have demonstrated that the ability of pIC (and more specifically, IFN-1) to synergize with CD27 stimulation in a subunit vaccine is highly associated with its ability to induce T-bet, and that the magnitude of primary $CD8^+$ T cell responses closely correlates with the levels of T-bet in CTLs. In contrast to T-bet, Eomes has been indicated to promote effector function without significantly influence the primary expansion of $CD8^+$ T cells in viral infection models[103, 176], which is in agreement with our finding that the contribution of CD27 costimulation to the magnitude of primary $CD8^+$ T cell responses is much less significant than its role in $CD8^+$ T cell memory. However, we have also demonstrated that the primary expansion of $CD8^+$ T cells in response to less-inflammatory immunogens such as DC and protein immunizations is highly dependent on CD27 signals, suggesting a requirement for Eomes function. To reconcile these data, it has been proposed that some redundancy exists between T-bet and Eomes in the primary expansion of effector $CD8^+$ T cells[94, 103].

We currently hypothesize that T-bet is a principal regulator of the primary expansion of CTLs; Eomes is redundant when adequate T-bet is achieved (e.g. by inflammatory context during replicating viral infections), whereas it becomes important when the levels of T-bet is insufficient (e.g. due to low-inflammatory immunogens such as DC or protein immunization), which reduces the potential redundancy between T-bet and Eomes. Besides, it's noteworthy that though Eomes is a principal regulator of memory differentiation, it has been shown by our (Supplemental Figure 2) and others' data that

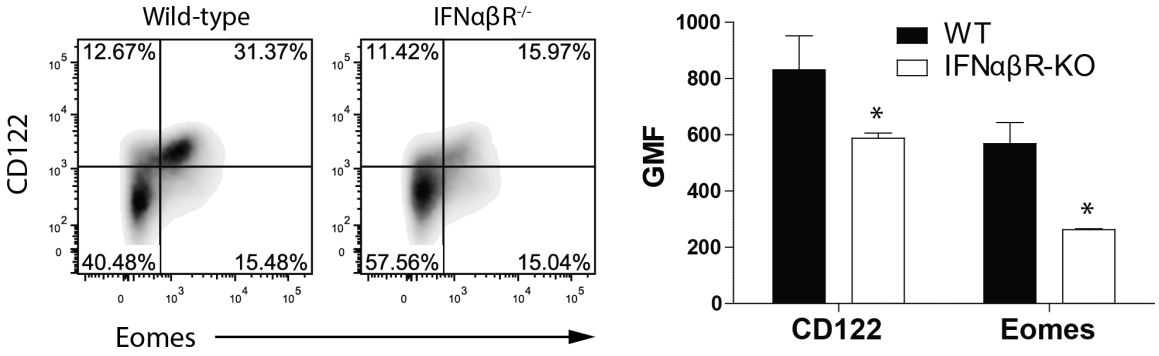
Supplemental Figure 2. Function of T-bet is required for the generation of CD8$^+$ T cell memory to vaccinia infection. OT-1 chimeric mice were generated by adoptively transferring 5k WT, Tbx21+/- (T-bet heterozygous) or Tbx21-/- (T-bet knockout) OT-1 cells into B6 recipients, respectively. Each cohort (n=3/cohort) was primed with OVA-vac. The primary responses were measured in periphery blood 7d later post-infection. The secondary responses were measured in spleen 5d after challenge with OVA-adeno, 35d after the initial priming. The experiment was performed once.



T-bet is also required for the generation of fully functional memory. This could be a potential explanation as to why in subunit vaccine setting CD27 signals alone induce potent Eomes expression yet still need to co-operate with pIC (and by extension IFN-1) to achieve competent secondary responses upon rechallenge. Taken together, the ability of CD27 costimulatory signals to promote primary and secondary $CD8^+$ T cell responses closely correlates with the induction of Eomes expression, whereas the ability of inflammatory cytokine signals to regulate primary and secondary $CD8^+$ T cell responses closely correlates with the induction of T-bet expression. We are currently generating the appropriate knockouts to test this directly.

However, the interactivity between CD27 costimulatory and inflammatory cytokine signals in $CD8^+$ T cell activation and fate decisions is certainly more complicated than the integration of CD27-mediated Eomes induction and inflammation-mediated T-bet induction. Several alternative mechanisms can explain the co-operativity of IFN-1 to CD27 signals. In particular, it has been shown that, in contrast to infectious models, subunit vaccine efficacy is highly dependent on IL-27[177, 178] and IL-15 (Ross Kedl, University of Colorado, personal communication), cytokines that are elicited by TLR agonists/IFN-1-stimulated DC and act directly on CTLs. IL-27R-mediated signaling in $CD8^+$ T cells has been indicated to induce Eomes and IL-15Rβ (CD122) [177], which is consistent with the $Eomes^{Lo}$ IL-$15R\beta^{Lo}$ phenotype found in IFNRαβKO $CD8^+$ T cells (Supplemental Figure 3). IL-15R-mediated signaling in $CD8^+$ T cells has been shown to promote cell activation and proliferation[122, 179]. Thus IFN-1 could play an indirect role via IL-27 and IL-15 to enhance Eomes and IL-15 signaling to regulate $CD8^+$ T cell differentiation, activation and proliferation. This is inconsistent with our adoptive transfer chimera data, which identifies that IFN-1 and CD27 signals synergize at the levels of

Supplemental Figure 3. Deficient Eomes and CD122 in IFNRαβKO. Expression of CD122 and Eomes on OVA257-specific CD8+ T cells responding to OVA+ αCD27/pIC immunization in wild-type or IFNαβR-/- mice. Data in plots show representative stains; data in histogram show combined data from 3 mice per group. *p<0.05 compared to wildtype (WT).



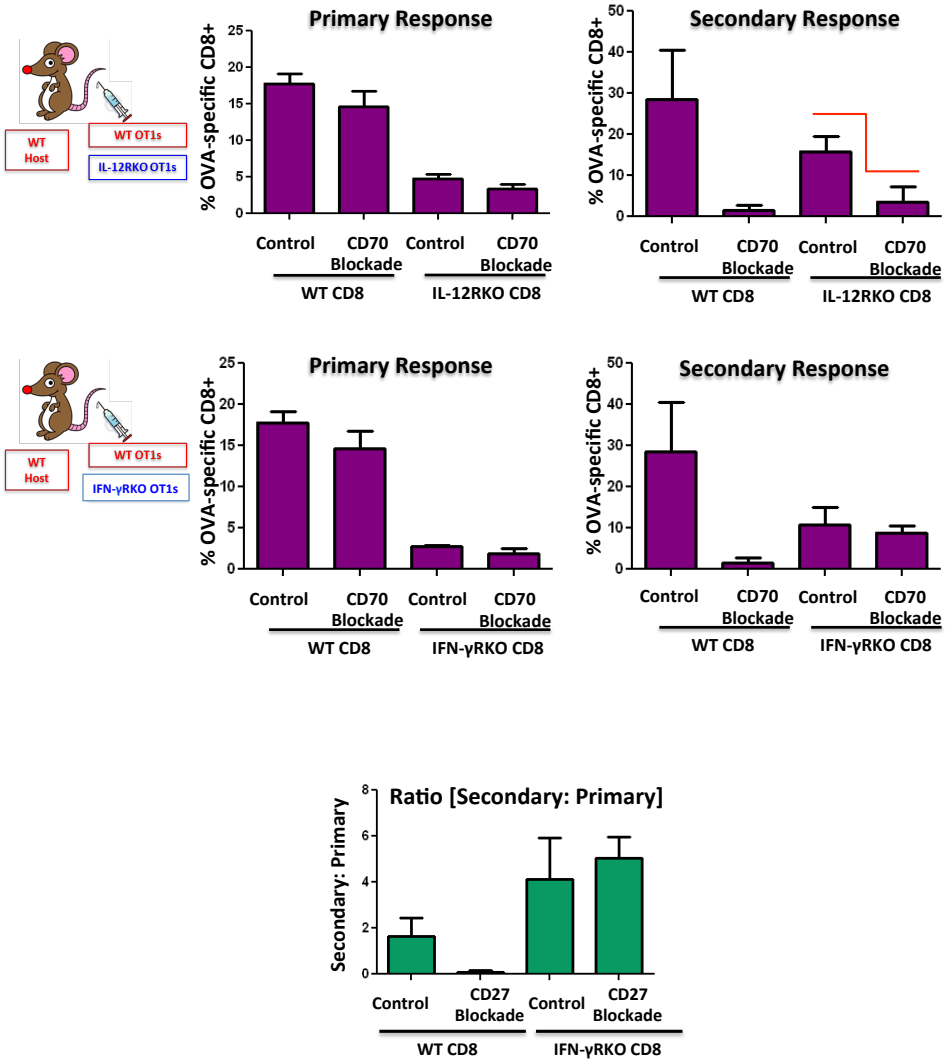
$CD8^+$ T cells. To try to reconcile this, we could directly compare Thy mismatched WT and IFNR$\alpha\beta$KO CTLs by generating 1:1 bone marrow chimera in CD45 mismatched WT and IFNR$\alpha\beta$KO recipient mice, respectively. Alternatively, IFN-1 has been shown to act directly on $CD8^+$ T cells and promote their survival via preventing NK cell-mediated cytotoxicity[100, 101]. Of note, CD27 costimulation has been shown to play critical role in NK cells activation and cytotoxic function[151, 180], suggesting IFN-1 may cooperate in a way that restricts the 'off-target' effect of CD27 costimulation. To test that, we could assess whether NK-depletion is able to promote the limited responses achieved by αCD27 alone. However, as our data strongly support that IFN-1 and CD27 signals impacts divergent stages of the primary expansion – IFN-1 promote the initial stages of the responses while CD27 signals facilitate the sustained expansion, we conclude these two would likely act via distinct rather than overlapping mechanisms.

What is the role of CD27 signals in the synergism with IFN-1? We have indicated that CD27 signals lead to prolonged expansion during d5-7 post protein immunization, which closely correlates with the sustained CD25 expression of CTLs on d5. Thus we conclude the main mechanism of CD27-driven $CD8^+$ T cell responses is by prolonged proliferation, likely via enhanced late IL-2 signaling[104]. Besides, CD27 has been demonstrated in our previous work to prevent PD-1-mediated cell death in tumor setting[88], and it would be of our future interest to identify whether CD27 involvement results in lower PD-1 expression and thus supports CTL survival during the late expansion.

We have reported that CD27 signals and IL-12 are unable to cooperate in subunit vaccine and that CD27 signals support $CD8^+$ T cell memory by restricting the impact of IL-12 in infectious models. IL-12 induces transcription factor Blimp-1 in $CD4^+$ T cells[181],

whereas Blimp-1 in CTLs has been shown to down-regulate CD27 expression and prevent Eomes induction[153]. Pertaining to this, we could assess in the subunit vaccine setting whether infusion of IL-12 leads to decreased CD27 and thus limits the binding to the agonistic CD27 antibodies. We have shown that during vaccinia infection CD27 costimulation restricts IL-12R expression on CTLs, implying the antagonism between CD27 costimulation and IL-12 signaling likely occurs at the level of $CD8^+$ T cells. SOCS3 has been identified as a negative regulator of IL-12 signaling in CTLs[17], and thus a reasonable hypothesis would be that the CD27 costimulation supports $CD8^+$ T cell memory via preventing the hypersensitivity to IL-12 by up-regulation of SOC3. However, we have contradictory data indicating that regardless of the alteration in IL-12R, CD27 signals had no substantial impact on IL-12-induced pSTAT4 or T-bet (data not shown), suggesting the detrimental effect of IL-12 on $CD8^+$ T cell memory could be indirect. IL-12 *in vivo* induces IFN-γ via a positive feedback loop[182], so we begun to directly address the question whether it is IL-12R- or IFNγR- mediated signaling in CTLs that impairs $CD8^+$ T cell memory in the absence of CD27 costimulation. To achieve this, we generated IL-12RKOxOT1 and IFNγRKOxOT1, and compared their functional memory formation with WT OT1 after respectively adoptively transferred into B6 recipients in vaccinia infection model with CD70 blockade. Our preliminary data (Supplemental Figure 4) supports that, in the absence of CD27 signals, IL-12-induced IFNγ acts on $CD8^+$ T cells and this compromises memory. The function of IFNγR-mediated signaling in CTLs to date has not been well established and is inconsistent[183-185]. Further investigations are required to elucidate how CD27 signals restricts the impact of IFNγ signaling in $CD8^+$ T cells (pending the reconfirmation of the preceding IFNγRKOxOT1 assay), with possible mechanisms including modulation of the negative

Supplemental Figure 4. IL-12 causes highly compromised CD8$^+$ T cell memory to vaccinia in the absence of CD27 costimulation likely via direct effect of IFN-γ on CD8$^+$ T cells. OT-1 chimeric mice were generated by adoptively transferring 5k WT, IL12RKO or IFNγRKO OT-1 cells into B6 recipients, respectively. Mice was primed with OVA-vac and then treated with either control IgG or antagonistic anti-CD70. The primary responses were measured in periphery blood 7d later post-infection. The secondary responses were measured in spleen 5d after challenge with OVA-adeno, 35d after the initial priming. Experiments were independently repeated twice.



regulator SOCS1[186]. Of note, recent work from Kaech group indicated that Treg-induced IL-10 helps to restrict inflammatory cytokine production *in vivo* and thus enhances memory differentiation of $CD8^+$ T cells via STAT3-dependent mechanisms [Brian Laidlaw, Yale University, personal communication during Keystone Symposia poster session]. Intriguingly, CD27 costimulation has been shown to have key contributions to Treg differentiation[187], activation and function[188], so it would be relevant to dissect whether CD27 signals restricts the impact of inflammation by reducing the *in vivo* secretion of inflammatory cytokines via Treg-dependent mechanisms. Nevertheless, as our data show that CD27 expression on $CD8^+$ T cells is sufficient for agonistic CD27 antibodies to rescue the 'helpless' memory, we speculate the Treg-mediated pathways may be relevant but not dominant.

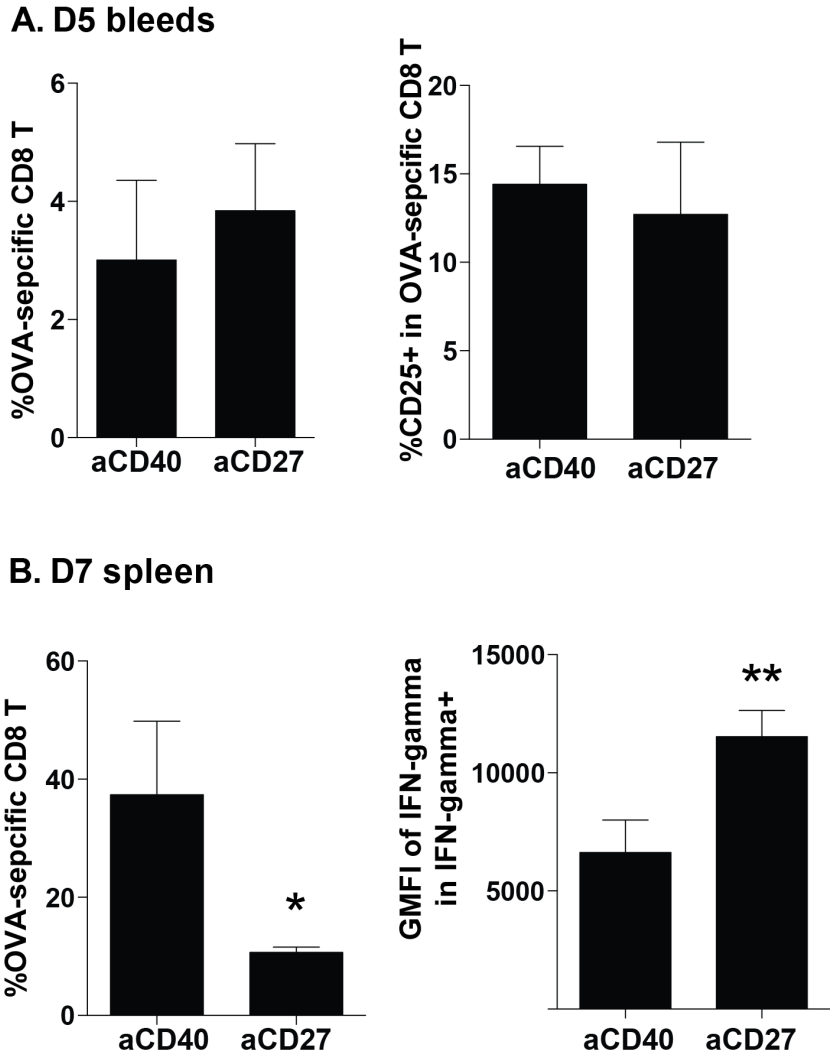
5.5 Divergent effects of CD27 signals, CD40 signals and $CD4^+$ T cell help

Extensive studies including this work have demonstrated that CD27 stimulation, CD40 stimulation and $CD4^+$ T cell help have considerable overlapping effects on $CD8^+$ T cell activation and fate decisions. However, sufficient evidence exists to indicate that they are unsurprisingly not completely synonymous. For instance, agonistic CD27 antibodies together with TLR agonists can drive a robust primary $CD8^+$ T cell response to protein immunization; however, the response is 2-fold less in frequency and 4-fold less in cellularity compared to that driven by agonistic CD40 antibodies. Despite the reduction in response magnitude, αCD27-driven effectors have higher capacity to produce IFN-γ on a per-cell basis (Supplemental Figure 5B, right), suggesting they have greater effector activity compared to the αCD40-driven counterparts. Moreover, in the vaccine setting CD27 stimulation favors SLEC (and $CD127^{Hi}KLRG1^{Hi}$ subset) differentiation while CD40 stimulation drives a more MPEC-like effector population. As

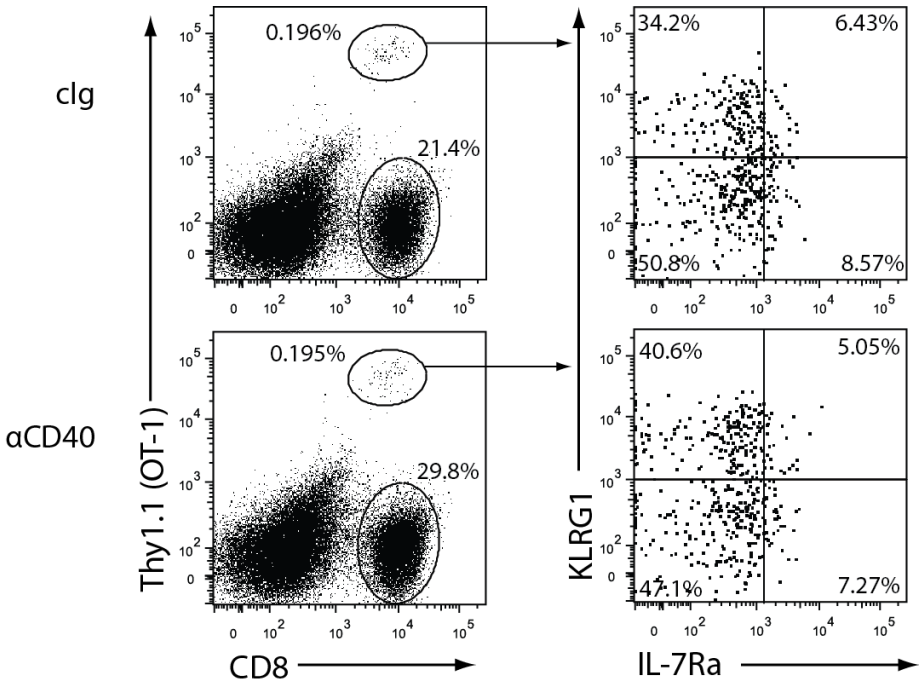
the discrepancies occur not only quantitatively (referring to effector number) but also qualitatively (referring to effector function and differentiation), and greater quantity does not go with better quality, we believe these differences cannot be simply attributed to distinct efficacy of the agonistic antibodies for CD27 and CD40. Instead, these are probably clues for biological differences between CD27 and CD40 stimulation pathways. In fact, αCD27- and αCD40-driven responses are comparable in magnitude by d5, and greater expansion occurs between d5~7 in the latter, which accounts for the increased magnitude by d7 (Supplemental Figure 5A left, 5B left). The discrete response kinetics further speaks to mechanistic differences.

How does CD40 promote effector expansion at late stages of primary responses beyond the induction of CD70? As illustrated by the **direct two-cell model** (Figure 1.1 Model I), direct stimulation of $CD8^+$ T cells by $CD4^+$ T cells (prominently via CD40L-CD40 interactions) have been indicated to enhance $CD8^+$ T cell activation and survival[7, 8]. Therefore one potential explanation could be the contributions of CD40 triggering on $CD8^+$ T cells. To test this hypothesis, we adoptively transferred WT OT-1 cells into CD40KO recipients, primed the mice with either αCD40 or cIg on top of αCD27 vaccine (OVA+αCD27/pIC), and assessed the impact of CD40 stimulation (restricted to OT-1 cells in this setting) to CD27-driven primary CTL responses. Preliminary data from a singular experiment indicates that the infusion of CD40 stimulation led to no changes in the magnitude of OT-1 responses compared to those driven by αCD27 vaccine alone (Supplemental Figure 6). Furthermore, no changes in MPEC/SLEC differentiation were observed, with the CD27-associated SLEC phenotype predominating (Supplemental Figure 6). Taken together, we conclude that the role of CD40 stimulation directly on $CD8^+$ T cells is insignificant.

Supplemental Figure 5. Comparison of CD27 and CD40 stimulation-driven CD8$^+$ T cell responses, CD25 expression and per-cell IFN-γ production. CD4-depleted B6 mice (n=3/group) were primed with OVA+pIC, in combination with either agonistic anti-CD40 (aCD40) or anti-CD27 (aCD27). D5 and D7 responses were measured by OVA257-specific MHC Dextramer, and the expression of CD25 and levels of per-cell IFN-γ were analyzed within Dextramer+ gating. The experiment was repeated 1-2 times.

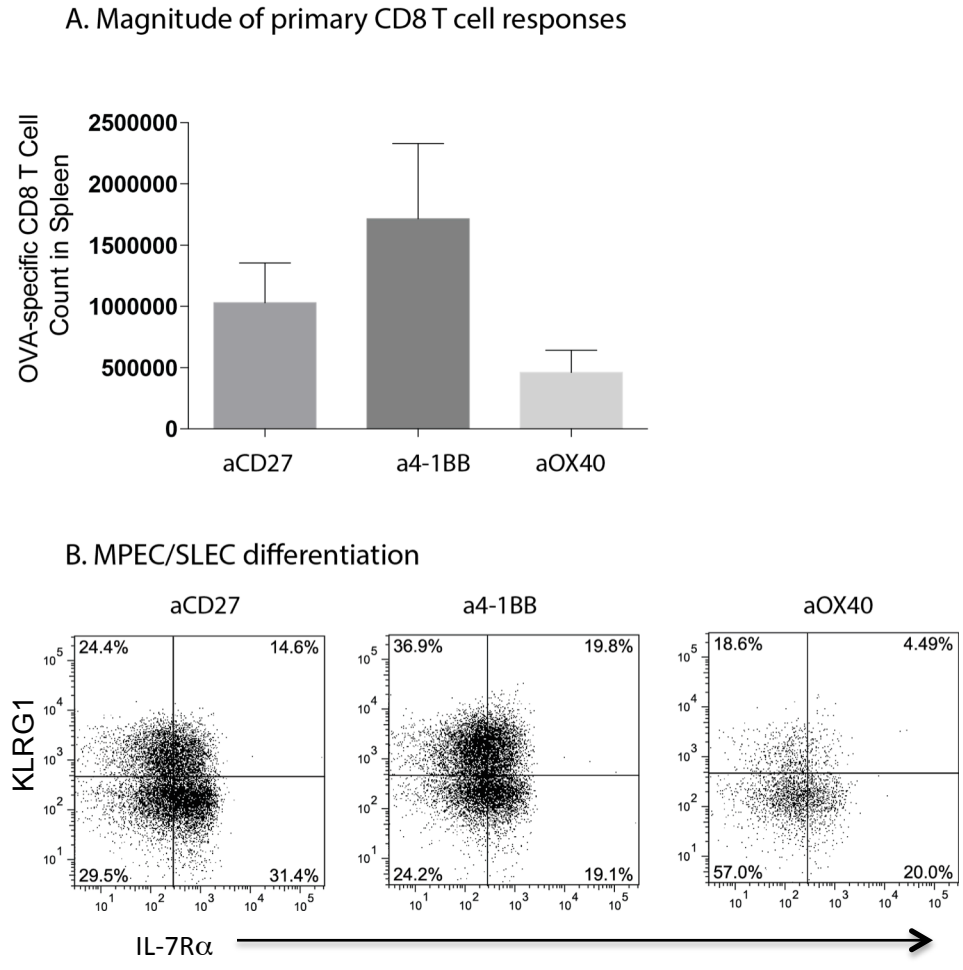


Supplemental Figure 6. Insignificant role of direct CD40 stimulation of CD8$^+$ T cells in the primary expansion and MPEC/SLEC differentiation of CD8$^+$ T cells during subunit vaccine. 500 wild-type OT-1 cells were adoptively transferred into CD40KO recipients. Mice were primed with OVA+pIC+αCD27, in combination of either cIg or αCD40. D7 spleen OT-1 responses were measured as well as IL-7Rα/KLRG1 phenotypes in OT-1. The experiment was performed once.



Given the lack of direct impact of CD40 on the emergent $CD8^+$ T cell responses (pending the reproducibility of the aforementioned experiment), an alternative explanation could be that CD40-'licensed' DC raise certain cofactors besides induction of CD70 to support $CD8^+$ T cell expansion and regulate their memory differentiation. One reasonable candidate would be the induction of other TNFSF members on CD40-stimulated DC, such as 4-1BBL and OX40L[189]. Like CD27 costimulation, intensive studies have highlighted significant contributions of 4-1BB and OX40 in driving CTL activation and differentiation[189-191], and it is thought that the type of pathogens attribute to the timing and coupling of distinct costimulatory pathways elicited by a variety of TNFSF members. To support this, a direct comparison of CD27, 4-1BB and OX40 stimulation in the subunit vaccine setting by our preliminary work revealed differential impact of them on both the magnitude of CTL responses and the differentiation profiles of $CD8^+$ T cells (Supplemental Figure 7). Particularly, consistent with the phenotypes of CD40-driven CTLs, 4-1BB stimulation led to in increased magnitude of $CD8^+$ T cell responses compared to those driven by CD27 stimulation, and OX40 stimulation resulted in less differentiated phenotype (lower KLRG1, and lower IL-7Rα) compared to αCD27-driven effectors. Therefore it is feasible that CD40 stimulation functions by eliciting a combined signal of TNFSF stimulations. To test this hypothesis, blocking and stimulating multiple arms of TNFSF could be performed, and the readouts of $CD8^+$ T cell activation/differentiation/function would be compared to abrogating or enforcing CD40 stimulation in both vaccine and/or infectious settings. These experiments will further our understanding of the relative significance of distinct TNFSF costimulatory pathways not only in CD40-driven vaccine, but also in $CD4^+$ T cell help-dependent CTL responses to natural pathogens such as virus.

Supplemental Figure 7. Differential impact of TNF receptor super-family members CD27, 4-1BB and OX40 on CD8$^+$ T cell responses and differentiation during subunit vaccine. Mice were primed with OVA+pIC, in combination with agonistic antibodies for different TNFR superfamily members including CD27, 4-1BB and OX40, respectively. D7 spleen responses were measured as well as IL-7Rα/KLRG1 phenotypes in OVA257-specific effector CD8$^+$ T cells. The experiment was performed once.



Another likely candidate to explain the discrepancy between CD27 and CD40 stimulation could be IL-15/IL-15R axis. Like IL-27, IL-15 is a critical factor for the proliferation and activation of $CD8^+$ T cells in response to subunit vaccines (Ross Kedl, University of Colorado, personal communication), and plays a critical role in inhibition of activation-induced cell death and the persistence of memory $CD8^+$ T cells. Activation of CD40 on DC has been indicated to strongly promote their expression of IL-15Rα[192-194], an action that is critical for trans-presentation of IL-15 to $CD8^+$ T cells. This is an attractive explanation as to which cofactors CD40 stimulation elicits to temper CD70-driven differentiation in the meanwhile of enhancing $CD8^+$ T cell expansion. To test whether the up-regulation of IL-15R on DC is the other main functional downstream consequence of CD40-'licensed' DC, we can first examine whether CD40 stimulation drives significantly higher IL-15Rα on DC than CD27 stimulation. If promising, we can then block IL-15 to identify whether the CD40-driven responses are IL-15-dependent. Particularly, as CD70 blockade severely diminishes yet not completely abolishes the CD40-driven responses, we speculate the remaining responses could be IL-15-dependent.

Combined, our data to data are consistent with our initial hypothesis: that CD27-CD70 interactions represent a main 'help' signal from $CD4^+$ T cell-activated DC to regulate CTL activation and fate decisions. However, it is not surprising that other mechanisms by which the 'help' is provided from $CD4^+$ T cells support the activity of CD27. Furthermore, reasonable evidence indicates that CD27 costimulation can be a critical regulator of the CTL responses that are primarily independent of CD40[25] stimulation and/or $CD4^+$ helper T cells[40]. Thus, though considerably overlapping with CD40 stimulation and $CD4^+$ T cell help, CD27 stimulation may govern some divergent

regulatory mechanisms for CTL activation and fate decisions. For instance, although MPEC formation appeared unimpeded in CD4-depleted animals, extensive evidence from this work strongly suggests that part of the mechanism of CD27 regulation of $CD8^+$ T cell responses is by modulating the expression of IL-7Rα on CTLs.

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