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Regulation of CD4 and CD8 T Cell Responses during Vaccination and Viral Infection

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Regulation of CD4 and CD8 T Cell Responses during Vaccination and Viral Infection

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Abstract

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By Junghwa Lee

To design more effective therapeutic strategies and vaccines with the aim of restoring or enhancing functional T cell responses against chronic viral infection and cancer, it is important to better understand T cell responses to vaccine vectors and how T cells can be modulated to recover their functions under persistent antigenic stimulation.

In the first part of this work, we characterize CD4 T cells elicited by Adenovirus serotype 5 (Ad5) vectors. We examined CD4 T cell responses following immunization of mice with Ad5 vectors encoding full-length lymphocytic choriomeningitis virus (LCMV)-glycoprotein (GP) compared to those induced by an acute LCMV infection. In contrast to LCMV infection where balanced CD4 T helper 1 (Th1) and T follicular helper (Tfh) responses were generated, Ad5 immunization resulted in significantly reduced Th1 differentiation. Analysis at early time points after immunization with Ad5 vectors revealed a defect in the expression of CD25 (interleukin-2[IL-2] receptor alpha chain) on Ad5-elicited CD4 T cells and administration of exogenous IL-2 following Ad5 immunization partially restored Th1 responses. These results suggest that impairment of Th1 commitment after Ad5 immunization could be due to reduced IL-2-mediated signaling.

In the second part of this work, we address how blockade of cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and programmed cell death-1 (PD-1) affects differentiation of virus-specific CD4 and CD8 T cells in a adoptive transfer system during chronic LCMV infection. Both CTLA-4 and PD-1 are inhibitory receptors induced by T cell activation with high expression levels on exhausted T cells. Blocking these negative regulatory pathways can rescue exhausted T cells during chronic infection and cancer. Both CTLA-4 and PD-1 pathway blockade generated more virus-specific Th1 cells. However, in contrast to PD-1 pathway blockade, which had a pronounced effect in rescuing exhausted CD8 T cells in terms of both quantity and quality, CTLA-4 blockade combined with CD4 T cell transfer did not further promote LCMV-specific CD8 T cell responses or viral control compared to CD4 T cell transfer alone. Thus, our results show that individual inhibitory receptors can differentially regulate CD4 and CD8 T cell responses during chronic viral infection, with important implications for combination immunotherapy.

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Chapter 1: Introduction

I. CD4 T cell responses during viral infection

Upon recognition of virus-derived peptides on activated antigen presenting cells (APCs) along with costimulatory and cytokine-mediated signals, naïve CD4 T cells become activated, undergo massive clonal expansion and differentiate into distinct subsets of effector cells. Various subsets of effector CD4 T cells have been defined including T helper 1 (Th1), Th2, T follicular helper (Tfh), Th17 and regulatory T cells (Tregs)(1). Priming environment can vary depending on the nature of viral infection. During priming, multiple factors such as pattern recognition receptor (PRR)-mediated signaling, activation status of dendritic cells (DCs) and T cell receptor (TCR) signal strength influence the fate determination of CD4 T cells. Especially, cytokine milieu is a major determinant that drives differentiation of naïve CD4 T cells toward particular effector subsets. Cytokine-mediated signals and subsequent signal transducer and activator of transcription (STAT) activation turn on the lineage-specific transcription factors that direct differentiation program and commitment to corresponding effector cells. Each effector subset produces hallmark cytokines and has specialized functions.

1. Th1 and Tfh differentiation

Following virus infection, CD4 T cells predominantly develop into Th1 and Tfh cells. Th1 differentiation is promoted by proinflammatory cytokines induced during early viral infection, such as type I interferons (IFNs), interleukin-12 (IL-12) or IFNγ. Type I IFNs or IFNγ induces expression of T-bet, a master transcription factor for Th1 cells,

through STAT1 activation(2). T-bet drives expression of IL-12 receptor β (IL-12Rβ), rendering CD4 T cells responsive to IL-12. Then IL-12-mediated signaling via STAT4 activates IFNγ production and induces the second wave of T-bet expression which also transactivates IFNγ(3, 4). This autoregulatory feedback loop reinforces Th1 differentiation. Th1 cells produce large amounts of IFNγ. IFNγ activates macrophages to enhance their killing of phagocytosed microbes, to secrete proinflammatory cytokines, and to elevate expression of major histocompatibility complex (MHC) and costimulatory molecules. IFNγ also induces chemokines that recruit effector CD8 T cells or innate effector cells to the infection site. Th1 cells express CXC chemokine receptor 3 (CXCR3), allowing them to traffic to the peripheral tissue of inflammation where CXC chemokine ligand 9 (CXCL9), CXCL10, and CXCL11 are produced(5). Thus, Th1 cells play an important role in immune defense against intracellular pathogens such as virus and also cancer by activating cell-mediated immunity.

Tfh cells are a specialized CD4 T cell subset that provides help to B cells. Tfh cells regulate almost every stage of B cell differentiation and play an essential role in generating humoral immunity. Tfh cells are characterized by expression of B cell lymphoma 6 (Bcl6), a master regulator of the Tfh lineage(6), B cell follicle homing receptor CXCR5, signaling lymphocytic activation molecule (SLAM)
-associated protein (SAP), high-level of programmed cell death-1 (PD-1) and inducible T cell co-stimulator (ICOS) and secretion of IL-21(7). In addition to Bcl6, several other transcription factors are involved in Tfh differentiation, including interferon regulatory factor 4 (IRF4), Maf, basic leucine zipper transcription factor ATF-like (BATF) and T-cell factor 1 (TCF1)(8). Compared to Th1, the process of Tfh differentiation is more

complex, involving multisteps and multisignals. Several factors such as IL-6, ICOS, and IL-2 have been shown to regulate early Tfh differentiation(8). IL-6 induces Bcl6 in a STAT3-dependent manner and Bcl6 subsequently induces CXCR5 expression. ICOS expression by CD4 T cells during priming is essential for Bcl6 expression and initiation of Tfh differentiation. IL-2 also acts early but suppresses Tfh differentiation. The role of IL-2 in regulating Th1 and Tfh differentiation will be discussed in more detail later in this section. Early process of Tfh differentiation during priming can be initiated by DCs independently of B cells, but in order to maintain and complete the Tfh differentiation program, the interaction with cognate B cells is required. Primed CD4 T cells upregulating CXCR5 and downregulating CC chemokine receptor 7 (CCR7) and P-selectin glycoprotein ligand 1 (PSGL1) migrate to T-B border and B cell follicles. Subsequent interaction with cognate B cells stabilizes Bcl6 and CXCR5 expression. In the B cell follicles, B cells are the primary APCs presenting antigens to CD4 T cells and providing costimulation via ICOS ligand (ICOSL). ICOS-mediated signal is critical in the process of Tfh differentiation as shown by impaired Tfh development in ICOS-deficient(9) or ICOSL-deficient(10) mice. Tfh cells are further polarized to germinal center (GC) Tfh cells. GC Tfh cells reside in GCs and help GC B cells undergo affinity maturation and differentiation into long-lived high affinity plasma cells and memory B cells.

IL-2 plays an important role in CD4 T cell differentiation with a decisive influence on regulating Th1 versus Tfh differentiation(11, 12). IL-2 signals are mediated through STAT5 or phosphatidylinositol-3-kinase (PI3K) pathway. IL-2 signals via STAT5 upregulate IL-12Rβ and CD25 (IL-2 receptor alpha chain [IL-2Rα]) expression,

thereby increasing responsiveness to IL-12 and potentiating IL-2 signaling(13). IL-2 activation of STAT5 has also been shown to inhibit STAT3 binding to Bcl6 locus. IL-2 signaling inhibits Bcl6 exprssion by inducing B lymphocyte-induced maturation protein-1 (Blimp-1), which is an antagonist of Bcl6. Blimp-1 promotes CD4 T cell differentiation toward non-Tfh cells while suppressing Tfh generation. A recent report has demonstrated that IL-2-mediated induction of Blimp-1 is dependent on PI3K-Akt-mammalian target of rapamycin (mTOR) pathway(14). Therefore, IL-2 signals regulate reciprocal differentiation of Th1 and Tfh cell lineages(15, 16). Another evidence for the role of IL-2 in fate decision of Th1 versus Tfh is that differential CD25 expression during CD4 T cell priming is associated with Th1 and Tfh lineage commitment. Following acute infection, Th1 versus Tfh bifurcation occurs by 48-72 hours after infection and two populations are distinguishable by differential CD25 expression. Expression of CD25 during priming positively correlates with Blimp-1 expression and inversely correlates with Bcl6 and CXCR5 expression.

2. Role of CD4 T cells during viral infection

During viral infection, CD4 T cells mainly function to help CD8 T cells and B cells induce optimal responses and also can exert direct effector functions. Furthermore, CD4 T cells enhance innate immunity. For example, CD4 T cells activate phagocytes such as macrophages and natural killer (NK) cells, and promote their cytotoxic activities. CD4 T cell-mediated APC activation induces proinflammatory cytokines and chemokines that recruit innate effector cells to the inflamed tissue. Here, I will focus on the roles of CD4 T cells in orchestrating humoral and cellular responses during viral infection.

2.1. CD4 T cell-mediated CD8 T cell help

CD4 T cells help CD8 T cells promote optimal antiviral responses. Multiple mechanisms underlying CD4 T cell-mediated help have been implicated. One mechanism is through licensing of DCs. Following recognition of specific antigens. CD4 T cells stimulate DCs, via CD40L/CD40 interaction(17-19), to upregulate costimulatory molecules and enhance cytokine secretion such as, type I IFNs, IL-1, IL-6 and IL-12. These DCs then become more competent to prime CD8 T cells and induce differentiation into effector and memory CD8 T cells. CD4 T cell-mediated DC licensing may not be essential for CD8 T cell priming following viral infections that generate potent proinflammatory mediators, thereby directly activating DCs through PRRs. However, in a setting where inflammatory signals are suboptimal or downregulated, CD4 T cell-mediated DC activation is probably critical for efficient priming of CD8 T cells. Therefore, requirement for this form of CD4 T cell help in the initiation of virus-specific CD8 T cell responses can be dependent on the context of viral infection.

Although it still remains controversial whether CD4 T cells are required for programming or maintenance of antiviral CD8 T cell responses(20), the role of CD4 T cells seems to be critical for the generation of functional memory CD8 T cell responses following viral infection (21, 22). CD8 T cells generated in the absence of CD4 T cell help have exhibited defective recall responses to secondary challenge due to significantly reduced proliferative capacity of memory CD8 T cells. Several reports have shown that CD8 T cells differentiated without CD4 T cell help overexpress TNF-related apoptosis-inducing ligand (TRAIL) and enhanced TRAIL-mediated apoptosis correlates with impaired recall capacities of memory CD8 T cells in the absence of CD4 T cell help (23).

However, other studies show defective memory responses without CD4 T cell help are TRAIL-independent(24, 25). Helpless memory CD8 T cells have also been shown to upregulate PD-1(26) and T-bet(27). PD-1 blockade or provision of IL-2 signals rescues impaired secondary responses after rechallenge. Overexpression of T-bet in helpless CD8 T cells results in aberrant memory differentiation with a defect in central memory generation. Therefore, these results suggest that CD4 T cells support CD8 T cells by regulating the expression of several molecules important for survival and memory differentiation. CD4 T cells have also been shown to promote the optimal formation of memory CD8 T cells, through direct CD40L ligation to CD40 on CD8 T cells(28).

CD4 T cells also regulate CD8 T cell trafficking. CD4 T cells can recruit CD8 T cells to the APCs and promote their cognate interactions(29). Upon the cognate interaction between CD4 T cells and DCs, CC chemokine ligand 3 (CCL3) and CCL4 are produced to attract CCR5-expressing CD8 T cells to DCs (30), promoting CD8 T cell priming. CD4 T cells also recruit effector CD8 T cells to the peripheral site of infection through secretion of IFNγ. In Herpes simplex virus-2 (HSV-2) infection in mice, CD4 T cell secretion of IFNγ induces CXCL9 and CXCL10 from surrounding tissues that attract CXCR3-expressing effector CD8 T cells to the inflamed tissues(31). Therefore, CD4 T cells modulate CD8 T cell trafficking during the effector phase as well as the priming phase.

CD4 T cells secrete cytokines such as IL-2 or IL-21 that have an impact on CD8 T cell responses. The principal function of IL-2 is to promote proliferation and survival of T cells. In acute viral infection, to receive optimal IL-2 signaling during priming is essential for CD8 T cells to induce robust secondary responses(32). The effects of IL-21

mainly relate to memory and recall responses and survival of CD8 T cells. In acute lymphocytic choriomeningitis virus (LCMV) infection, IL-21 is dispensable for the generation of effector and memory CD8 T cell responses. However, IL-21 promotes IL-2 production by CD8 T cells during acute LCMV infection. Furthermore, a mixed bone marrow chimera experiment has shown that IL-21R-deficient CD8 T cells result in reduced secondary responses compared to wild-type counterparts (33). Absence of IL-21R-mediated signaling compromises primary and secondary CD8 T cell responses in adenovirus vector immunization (34). IL-21R deficient CD8 T cells display impaired proliferative capacity and elevated expression of TRAIL. Therefore CD4 T cells support antiviral CD8 T cell responses via multiple mechanisms during viral infection.

2.2. CD4 T cell-mediated B cell help

Antibody response is critical for preventing many viral infections and also contributes to the resolution of infection. Tfh cells are involved in almost every process of B cell differentiation and functions of Tfh cells are essential for the establishment of long-term humoral immunity.

Tfh cells are required for the formation and maintenance of GCs. Tfh cells initially induce Bcl6 expression in activated B cells and stimulate GC B cell differentiation. SAP expression by Tfh cells is critical for CD4 T cell-B cell adhesion, stabilizing their interactions and subsequently for GC Tfh development and GC formation(35, 36). Once GCs are formed, Tfh cells provide proliferation and survival signals to GC B cells via multiple mechanisms including CD40 ligand (CD40L), IL-21 and IL-4. These signals from Tfh calls are important for the maintenance of GC B cells

because GC B cells highly express FAS and are prone to FAS-mediated apoptosis without prosurvival stimuli (37). Tfh cells play an essential role in B cell affinity maturation to select high-affinity B cells. Somatic hypermutation depends on the expression of activation-induced cytidine deaminase (AID) by GC B cells. How AID expression is regulated in GC B cells and how Tfh cells regulate this process have not been fully defined. Differentiation of GC B cells into plasma cells is also regulated by Tfh cells. Tfh secretion of IL-21 induces Blimp-1, a master regulator of plasma cell differentiation, in GC B cells(38, 39). Tfh cells also stimulate class switch recombination by producing IL-4, IL-21 and other additional cytokines dependent on the context of infection. The help signals provided by Tfh cells to B cells are still not completely understood, but mainly involve CD40L-CD40 interaction, IL-21 and IL-4.

2.3. CD4 T cell-mediated direct effector functions

Besides helper functions, CD4 T cells also have direct roles in protection against viral pathogens, through production of antiviral cytokines or direct cytotoxic activities. Antiviral cytokines such as IFNγ and tumor necrosis factor (TNF) produced by CD4 T cells can inhibit viral replication in the infected cells. CD4 T cell-mediated direct cytotoxic activity and its contribution to viral control have been demonstrated in many viral infections both in mice and humans. Several mechanisms underlying direct effector functions of CD4 T cells, including perforin, granzyme, FAS ligand (FASL), or TRAIL-mediated mechanisms have been reported(40). Effector CD4 T cells protect mice against lethal influenza virus infection in a IFNγ-independent and perforin-dependent manner(41). MHC class II expression is induced on lung epithelial cells, providing

targets for cytotoxic CD4 T cells. IFNγ-independent cytolytic activity of CD4 T cells has also been demonstrated in persistent γ-herpesvirus 68 (rHV68) infection(42). CD4 T cells provide protection against lethal infection with HSV-2 by killing target cells via FAS-induced apoptosis(43). CD4 T cell-mediated direct killing has also been found in LCMV infection, which is at least in part mediated by FASL/FAS interaction(44). The cytotoxic activity of CD4 T cells is limited to the target cells expressing MHC class II. However, depending on viral pathogens, epithelial or endothelial cells can induce expression of MHC class II molecules(45). IFNγ also plays a role in upregulation of MHC class II molecules(46).

2.4. Role of CD4 T cells during chronic viral infection

Virus-specific CD4 T cells play an essential role in maintaining effective antiviral immunity in chronic viral infection. The critical role of CD4 T cells has been demonstrated in chronic infection where antigen-specific CD4 T cell help lacks. Infection of mice with LCMV clone-13 infection leads to persistent infection that lasts 2-3 months and is eventually cleared from most peripheral tissues via CD4 T cell, CD8 T cell, and B cell-dependent mechanisms. However, when CD4 T cells are depleted at the onset of infection, mice do not develop antigen-specific CD4 T cells, fail to induce antibody responses, and generate more deeply exhausted CD8 T cells. These mice do not resolve the infection and become life-long carriers(47, 48). Furthermore, adoptive transfer of virus-specific CD4 T cells into these chronically infected mice restores effector function of exhausted virus-specific CD8 T cells, induces antibody production, and substantially

reduces viral load(49), showing the important role of CD4 T cells in regulating antiviral CD8 T cell and antibody responses during chronic viral infection.

In many persistent infections, a correlation is found between effective CD4 T cell responses and control of viral persistence. Robust proliferative hepatitis C virus (HCV)-specific CD4 T responses during early phase of HCV infection have been associated with the resolution of infection and early functional defects in HCV-specific CD4 T cells correlate with the development of chronic HCV(50). In human immunodeficiency virus (HIV) patients, HIV-specific CD4 T cells from elite controllers display higher proliferative capacity and polyfunctionality compared to those from patients with progressive diseases(51).

During chronic infection, Th1 differentiation is suppressed and persistent antigenic stimulation directs CD4 T cell differentiation toward Tfh cells(52, 53). It has been initially thought that virus-specific CD4 T cells under sustained antigenic stimulation lose their effector functions based on their reduced ability to produce Th1 cytokines such as IFNγ, TNF and IL-2(54). In contrast to impaired Th1 responses, accumulation of Tfh cells is found in chronic infections such as LCMV(52, 53), SIV(55), HIV(56), and HCV(57) infection, and now it is understood that instead of simply losing functions, CD4 T cell responses during chronic infection are skewed toward Tfh cells. This CD4 T cell subset can sustain antiviral immunity under persistent antigen stimulation and maintain its ability to help B cells and CD8 T cells, ultimately controlling persistent infection.

A recent study has shown that restoration of Th1 cells in chronically infected mice with no antigen-specific CD4 T cell help, enhances virus-specific CD8 T cell

responses and promotes viral control(58). This result indicates that defective Th1 responses during chronic infection contribute to CD8 T cell dysfunction that prevents viral clearance. Mechanistically, elevated expression of PD-L1 and IL-10 by suppressive DCs, which is induced by type I IFN signalings upon continuous antigenic stimulation, results in Th1 suppression. Concomitant blockade of PD-L1 and IL-10 restores Th1 responses.

During persistent infection, when proinflammatory signals are downregulated and immunosuppressive environment is formed, CD4 T cell-mediated DC activation possibly plays a critical role in activating CD8 T cell responses. Cytokines produced by CD4 T cells such as IL-2 and IL-21 impact virus-specific CD8 T cell responses during chronic infection. IL-2 administration to LCMV-chronically infected mice increases virus-specific CD8 T cells and leads to decreased viral load(59). In addition, IL-2 administration reduces the expression of inhibitory receptors and upregulates CD127(60), a memory T cell marker. In HIV-1 infection, IL-2 produced by virus-specific CD4 T cells plays an important role in maintaining the functionality of antiviral CD8 T cells(56). Several reports have shown that IL-21 produced by CD4 T cells is critical for sustaining virus-specific CD8 T cells and eventually controlling chronic LCMV infection(61-63). IL-21-producing CD4 T cells play a role in enhancing virus-specific CD8 T cell responses in HIV infection(64) and correlate with the control of chronic hepatitis B infection(65). Despite profound effects of CD4 T cells on antiviral CD8 T cell responses during chronic infection, the mechanisms whereby CD4 T cells support virus-specific CD8 T cells are not yet fully understood and remain to be further determined.

II. T cell exhaustion

When acute infections are cleared by the immune system, memory T cells are generated. These cells rapidly reactivate effector functions upon antigen re-encounter and persist long term via homeostatic proliferation, independently of antigen(66, 67)(229). These key properties of memory T cells allow them to provide long-term protective immunity. In contrast, when the immune system fails to clear the virus and virus persists over long periods, prolonged antigen stimulation and inflammation lead to loss of effector functions of virus-specific CD8 T cells in a progressive and hierarchical manner, even resulting in clonal deletion(48, 68). This process, originally found in chronic LCMV infection in mice, was termed T cell exhaustion and has since been demonstrated to occur in various chronic viral infections and cancer in human(67, 69). Exhausted CD8 T cells are characterized by defects in effector functions such as cytokine production and cytotoxic activity, reduced proliferative capacity, and elevated and sustained expression of inhibitory receptors(70, 71). Exhausted CD8 T cells are distinctly different not only from functional effector or memory cells, but also from anergic cells, representing a unique transcriptional program(72). They also display distinct epigenetic profiles and metabolism. This indicates that these dysfunctional T cells are not simply inert cells, but actually undergo altered pattern of differentiation. In contrast to memory T cells, exhausted CD8 T cells fail to undergo homeostatic turnover in the absence of their specific antigens and depend on antigen for their survival(69, 73).

Complex immunosuppressive mechanisms, including both intrinsic factors such as inhibitory receptor-mediated negative signaling and extrinsic factors such as immunosuppressive cytokines (IL-10 and transforming growth factor (TGF)-β) and

immunoregulatory cells (Tregs and myeloid-derived suppressor cells (MDSCs)(74)), can contribute to the establishment and maintenance of persistent infection and T-cell dysfunction. CD4 T cell help is also critical for limiting CD8 T cell dysfunction.

T cells have intrinsic regulatory mechanisms that limit their effector functions. Activated T cells transiently upregulate multiple inhibitory receptors that, upon ligation with respective ligands, deliver inhibitory signals to T cells in order to degrade excessive or unwanted T cell activation and protect the host from immune-mediated tissue damage. Under normal physiological conditions, these negative regulatory pathways are important for maintaining self-tolerance and preventing autoimmunity. However, pathogens or tumor takes advantage of these pathways to dampen host immune defense for their long-term persistence(229). Persistent antigen stimulation, such as in chronic infection or cancer, maintains elevated expression of inhibitory receptors on T cells and leads to T cell exhaustion. Therefore, these inhibitory pathways are an important determinant of the outcome of the T-cell response, regulating the balance between effective host defense, immunopathology and immune suppression, implicating the potential for manipulating the inhibitory receptor pathway against various human diseases. Indeed, the finding that blocking the interaction between inhibitory receptors and their respective ligands can reverse T cell dysfunction and restore their effector functions made a breakthrough in developing immunotherapeutic approaches against chronic infection and cancer.

More focused on CD8 T cells, exhaustion in CD4 T cells is relatively less defined but antigen-specific CD4 T cells under chronic antigen stimulation also have a reduced ability to produce effector cytokines and upregulate inhibitory receptors(54, 75). A recent study has shown that exhausted CD4 T cells are molecularly distinct from effector or

memory CD4 T cells and also from exhausted CD8 T cells(76). Although there are some features conserved between exhausted CD4 and CD8 T cells, exhausted CD4 T cells have unique characteristics in several key aspects including altered expression of costimulatory and coinhibitory molecules and transcription factors. Exhausted CD4 T cells are also distinct from other CD4 T cell effector subsets and seem heterogenous in terms of their transcriptional profiles. Like CD8 T cells, exhaustion in CD4 T cells reflects a unique state of differentiation. CD4 T cells during chronic infection show the altered functional profile such as enhanced IL-10 and IL-21 production and the loss of Th1 signature with a trend in skewing toward Tfh-lke cells(71, 76).

Thus, immunoregulatory roles of inhibitory receptors have been implicated in many aspects of immune responses but overexpression of inhibitory receptors is a hallmark of exhausted T cells and plays a critical role in regulating T cell responses in chronic infection and cancer. In the next section, I will discuss the role of inhibitory receptors in regulating T cell exhaustion, specially focusing on PD-1 and cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4). These two receptors are most well-known immune checkpoints and blocking antibodies against PD-1 or CTLA-4 (checkpoint inhibitors) are currently being used in the clinics to treat cancer patients.

III. T cell-based immunotherapy

T cell exhaustion is a common feature of chronic infections and cancer and has now become a key target of immunotherapy. Restoration of functional T cells can be achieved either by adoptive transfer or by reinvigoration of endogenous T cells by blocking inhibitory signals in T cells. Here I will discuss two key strategies of T cell-targeted immunotherapies, blockade of inhibitory receptors and adoptive T cell transfer.

1. Role of Inhibitory receptors in T cell exhaustion and therapeutic potential of manipulating inhibitory receptor pathways

1.1. PD-1

Section 1.1. PD-1, Reprinted from Forum on Immunopathological Diseases and Therapeutics, 2015, (1-2): 7-18, Junghwa Lee, Eunseon Ahn, Haydn T. Kissick, Rafi Ahmed, Reinvigorating Exhausted T Cells by Blockade of the PD-1 Pathway, Copyright (2017), with permission from Begell House, Inc.

The role of the PD-1 pathway in T cell exhaustion

PD-1 is expressed in various hematopoietic cells including T cells, B cells, NK cells, NK T (NKT) cells, monocytes, macrophages, and dendritic cells (DCs) following their activation(77). PD-1 binds to its two ligands: programmed cell death 1 ligand-1 (PD-L1; B7-H1; CD274)(78, 79) and PD-L2 (B7-DC; CD273)(80, 81), both of which are B7 family members. PD-L1 is constitutively expressed in a wide range of cells including hematopoietic and nonhematopoietic cells. In contrast, PD-L2 expression is restricted to

professional APCs (monocytes, macrophages, and DCs) and a certain subset of B cells. Inflammatory cytokines such as IFNs (α, β, and γ) are potent regulators of both PD-L1 and PD-L2 expression. The function of PD-1 is best characterized in T cells. Its expression is induced by activation of T-cell receptor (TCR) signaling and further up-regulated by cytokines(82). Upon engagement of PD-1 with its ligands, the SH2-domain containing tyrosine phosphatase 1 (SHP-1) and SHP-2 are recruited to the phosphorylated immunoreceptor tyrosine-based switch motif (ITSM) in the cytoplasmic domain of PD-1. Recruitment of SHP-1 and/or SHP-2 to ITSM motif of PD-1 induces dephosphorylattion of proximal signaling molecules such as ZAP70 and phosphatidylinositol-3-kinase (PI3K) to inactivate them and as a consequence, TCR- and CD28-mediated signaling is attenuated (83-85) (Figure 1). It has been shown that PD-1 pathway is involved in the regulation of T cell motility(86, 87), metabolism(88), and cell cycle(89). PD-1 has also been shown to impair T cell functions by upregulating BATF that inhibits expression of effector genes(90). However, many of these findings were obtained from in vitro studies, and therefore the precise mechanism by which PD-1 regulates T cell exhaustion in vivo following persistent antigen exposure remains to be further determined.

Our group previously found that PD-L1 has a differential role in hematopoietic cells and nonhematopoietic cells in regulating T-cell responses(91). In chronic LCMV infection, PD-L1 deficiency in hematopoietic cells enhanced the T-cell response in terms of both magnitude and function. In comparison, PD-L1 deficiency in nonhematopoietic cells had no effect on the T-cell response but resulted in better virus control. This indicates that the PD-1 pathway restrains T cells from killing virus-infected cells as well as attenuating T cell activation. The PD-1/PD-L1 interaction between T cells and infected

cells (or cancer cells) inhibits target cell elimination by T cells. Abrogating this interaction releases the brake on T cells and promotes their effector functions, killing of target cells (Figure 2). Therefore, the PD-1 pathway negatively regulates T cells during priming and also the effector phase when T cells act on the target cells. This presumably results in more profound "rescue" effects by the blockade of PD-1 than do other inhibitory receptor blockades.

During chronic infection and cancer, expression of both PD-1 and PD-1 ligands is abundant; continuous antigen stimulation maintains high levels of PD-1 expression on antigen-specific T cells and the expression of PD-1 ligands is also up-regulated by inflammatory stimulation. PD-1–mediated T-cell dysfunction strongly dampens antiviral or antitumor immune responses. The effect of interfering with the PD-1 pathway on the rescue of exhausted T cells has been shown in many animal models and human diseases.

The therapeutic potential of PD-1 pathway blockade in chronic viral infection

The dominant role of PD-1 in regulating T-cell exhaustion was first described by our group in a mouse model of chronic LCMV infection. In this model, we found that exhausted CD8 T cells had increased PD-1 expression. Furthermore, blockade of the PD-1 pathway restored effector functions of LCMV-specific CD8 T cells and significantly reduced viral load(92). This finding has been further extended to other types of chronic infections in mice, nonhuman primates, and humans.

In HIV infection, PD-1 expression on HIV-specific CD8 T cells was correlated with impairment of CD8 T-cell function, high viral load, disease progression, and reduced CD4 count. In vitro blockade of PD-1 enhanced proliferation, cytokine

production, and survival of HIV-specific CD8 and CD4 T cells(93, 94). The effect of blocking PD-1/PD-L interactions on HIV disease progression has been shown in vivo using the humanized mouse model of chronic HIV infection. In vivo administration of anti-PD-L1 antibody increased the number of both CD4 and CD8 T cells and reduced HIV plasma viral load in HIV-1 chronically infected mice(95). In addition to HIV, blocking the PD-1 pathway was also found to promote an antiviral immune response in simian immunodeficiency virus (SIV) infection of rhesus macaques. Proliferation and polyfunctionality of SIV-specific CD8 T cells were augmented upon PD-1 blockade, and improved antiviral immunity was followed by viral load reduction and prolonged survival of chronically infected rhesus macaques(96). The role of the PD-1 pathway has also been investigated in chronic HCV infection. In the initial stage of HCV infection, most HCV-specific T cells expressed PD-1. HCV-specific CD8 T cells from the patients who resolved the disease showed reduced PD-1 expression, whereas HCV-specific CD8 T cells from the patient with chronic disease maintained high level of PD-1 expression and exhibited dysfunctional phenotype. In vitro blockade of the PD-1 and PD-L interaction enhanced the proliferation and function of HCV-specific CD8 T cells(97, 98). One recent report demonstrated the impact of interrupting PD-1 signals in chronically HCV-infected chimpanzees. Following PD-1 blockade, one of the three animals had significantly reduced HCV viremia that was associated with restored intrahepatic CD4 and CD8 T-cell response. It has been suggested that preexisting virus-specific T cells are likely to be essential for the success of PD-1 blockade therapy in this model(99). Together, these preclinical studies show that PD-1 expression on virus-specific T cells is correlated with their functional defects, and interrupting PD-1 signaling can reverse this decline. The fact

that exhaustion is reversible and not an untreatable state indicates powerful therapeutic potential for manipulating the PD-1 axis to reinvigorate dysfunctional T cells in chronic viral infections.

Currently, one clinical trial has been reported on PD-1 blockade in chronic viral infection. Anti-PD-1 antibody (BMS-936558, a fully human monoclonal antibody targeting PD-1) was used to treat patients chronically infected with HCV. Following a single infusion, suppression of HCV replication was observed in 11.1% of patients (5/45)(100). Also in this trial, one patient who previously did not respond to IFN-α therapy had undetectable viral load for at least 1 year following administration of the anti- PD-1 antibody. This promising result warrants further exploration of PD-1 blocking agents for therapeutic use in human chronic viral infection.

The PD-1 pathway in antitumor immunity and PD-1-directed cancer immunotherapy

PD-1 and PD-L1 interaction in the tumor environment is a mechanism used by the tumor to resist destruction by the immune system. PD-L1 is expressed by many types of cancer cells and up-regulated by various inflammatory stimuli in the tumor environment(101, 102). Myeloid cells in tumors were shown to express PD-L1 and mediate inhibition of T cells(103). Tumor-infiltrating T cells express high levels of PD-1 due to prolonged exposure to the tumor antigen and immunosuppressive environment and exhibit similar functional and phenotypic properties as the exhausted T cells in chronic infection. This includes defects in effector cytokine production and upregulated expression of inhibitory receptors(104-106). Currently, the prevailing mechanism underlying the PD-1/PD-L1 axis in tumor sites is that the interaction of PD-L1 on tumor

cells with PD-1 on tumor-infiltrating lymphocytes (TILs) delivers negative signals and inhibits antitumor T-cell response, facilitating tumorigenesis.

The role of PD-1 in tumor immune evasion was first shown when P815 tumor cells were transfected with PD-L1 and they became less susceptible to cytotoxic T-cell-mediated killing. This report also showed that the growth of PD-L1+ myeloma cells was completely suppressed in syngeneic PD-1–deficient mice, whereas rapid tumor growth was observed in wild-type littermates(107). Multiple in vivo mouse studies have shown that the PD-1/PD-L1 interaction inhibits antitumor immunity, and abrogating this interaction enhances the T-cell-mediated antitumor response, leading to tumor regression(101, 107, 108). Encouraging results from preclinical studies and the therapeutic potential of blocking the PD-1 pathway have led to clinical development of several blocking antibodies against PD-1 or PD-L1. Currently, the results of clinical trials targeting the PD-1 pathway are very promising. Blockade of the PD-1 pathway using either anti- PD-1 or anti-PD-L1 antibodies has revealed high clinical response rates and was effective in patients with advanced cancer including metastatic melanoma, non-small cell lung cancer (NSCLC), renal cell cancer (RCC), bladder cancer, Hodgkin's lymphoma, head and neck cancer, and breast cancer(109-131). Clinical responses by PD-1 pathway blockade tended to be durable and were accompanied by less adverse effects than those seen with ipilimumab, a CTLA-4 blocking antibody used for treating metastatic melanoma. In the last couple of years, human monoclonal antibodies targeting PD-1 or PD-L1(pembrolizumab;Merck, nivolumab;Bristol-Myers Squibb, Atezolizumab;Genentech/Roche) have been approved by the Food and Drug Administration (FDA), for the treatment of unresectable or metastatic melanoma,

NSCLC, metastatic RCC, Hodgkin's lymphoma and recently bladder cancer (urothelial carcinoma).

Consistent with the concept that the tumor evades host immune response through engagement of PD-L1 with PD-1 on T cells, early studies suggested a correlation between PD-L1 expressed by the tumor and poor prognosis. However, several studies indicated a lack of correlation or even a positive association of PD-L1 expression on tumor cells with lymphocyte infiltration and better prognosis(102). A recent study reported a negative feedback loop, whereby activated T cells infiltrating the tumor environment produce proinflammatory cytokines, such as IFNγ, that induce the up-regulation of PD-L1 on tumor cells(132). Therefore, PD-L1 expression in tumor cells possibly indicates preexisting immune responses.

Based on the mechanism of PD-1/PD-L1 expression, PD-L1 expression by tumor cells has been suggested as a biomarker for predicting the clinical response to PD-1 blockade therapy. Several clinical studies evaluated a correlation between tumor-associated PD-L1 expression and the clinical response to PD-1 blocking agents, and there seemed to be a trend of positive association. However, tumor expression of PD-L1 is apparently not an absolute biomarker because not all patients with PD-L1+ tumors respond to PD-1 blockade, and some patients with PD-L1- (PD-L1 negative) tumors are still responsive to PD-1 therapy(133, 134). Considering the inducible nature of PD-L1 and the fact that many other PD-1/PD-L interactions are possibly affected by PD-1 pathway blockade along with tumor cells and TIL interactions, tumor PD-L1 expression as a single marker is not an optimal biomarker of the response to PD-1–targeted

immunotherapy. Therefore, it is imperative to identify reliable biomarkers to select patients who can benefit from this therapy.

It has been shown that PD-1 blockade rescues the less exhausted CD8 T cells expressing intermediate levels of PD-1, whereas exhausted cells with high levels of PD-1 respond poorly and are unlikely to be reversed by the treatment(135). Several studies have shown that a certain level of preexisting antigen-specific T cells is essential to better respond to blockade of the PD-1 pathway. Therefore, combining PD-1 pathway blockade with other therapies that possibly stimulate T-cell responses or interrupt other negative signaling pathways could generate a synergistic effect. It is important to assess different combination therapies for those who do not respond to PD-1 blockade therapy. Still, the molecular mechanisms associated with the PD-1 pathway regulating T-cell exhaustion and the way in which PD-1 signaling is altered upon blocking the PD-1/PD-L interaction to restore effector T cell function remain to be determined.

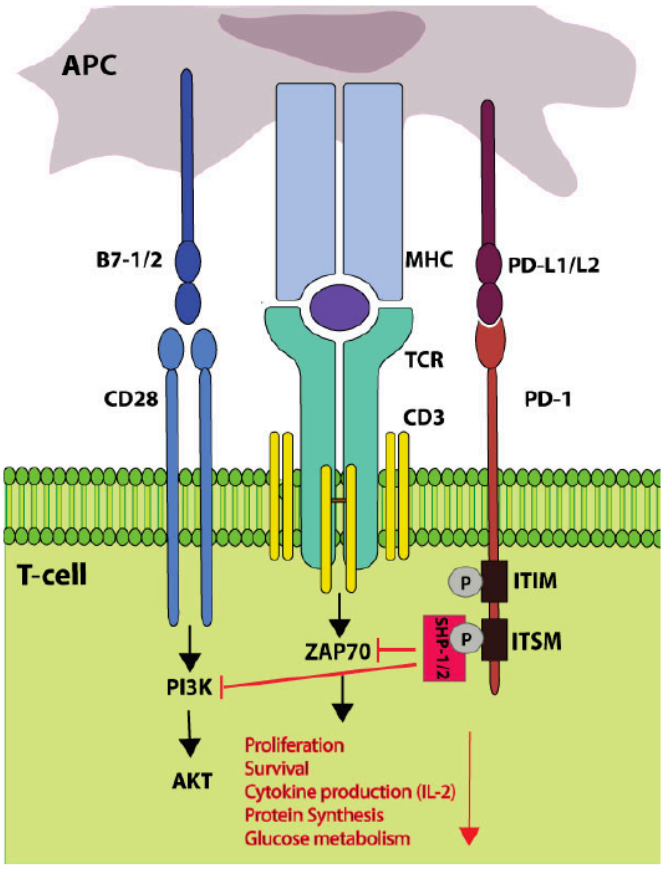


Figure 1. PD-1 signaling. PD-1 contains two tyrosine-based signaling motifs in the cytoplasmic domain: an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an ITSM. Upon engagement by PD-L1 or PD-L2, PD-1 is phosphorylated at both tyrosine residues. Phosphorylated ITSM recruits SHP-1 and SHP-2 that dephosphorylate effector molecules such as ZAP70 and PI3K activated by TCR and CD28 signaling. As a result, PD-1 signaling inhibits T-cell proliferation, survival, cytokine production, protein synthesis, and glucose metabolism. Reprinted from Forum on Immunopathological Diseases and Therapeutics, 2015, (1-2): 7-18, Junghwa Lee, Eunseon Ahn, Haydn T. Kissick, Rafi Ahmed, Reinvigorating Exhausted T Cells by Blockade of the PD-1 Pathway, Copyright (2017), with permission from Begell House, Inc.

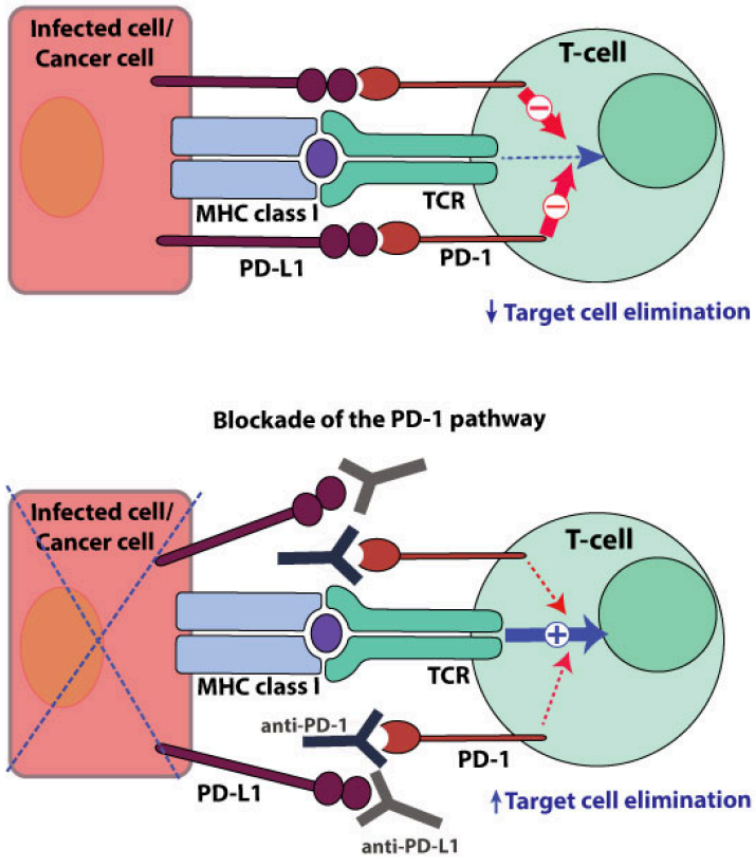


Figure 2. Blockade of PD-1/PD-L1 interactions between CD8 T cells and target cells. Antibody-mediated blockade of the PD-1 pathway promotes T cell-mediated elimination of target cells. Reprinted from Forum on Immunopathological Diseases and Therapeutics, 2015, (1-2): 7-18, Junghwa Lee, Eunseon Ahn, Haydn T. Kissick, Rafi Ahmed, Reinvigorating Exhausted T Cells by Blockade of the PD-1 Pathway, Copyright (2017), with permission from Begell House, Inc.

1.2. CTLA-4

CTLA-4 is another inhibitory receptor expressed on T cells and binds to B7 molecules (B7-1:CD80, B7-2L:CD86) on APCs. CTLA-4 shares its ligands with CD28, but binds with higher avidity and affinity and delivers inhibitory signals to T cells. Upon antigen recognition by TCRs, CD28:B7 costimulatory signals that lead to T cell proliferation and differentiation by production of IL-2 and upregulation of prosurvival genes, are required to fully activate T cells. However, CTLA-4 ligation to B7 molecules antagonizes CD28-mediated costimulation, attenuating many of these early outcomes of T cell activation(136). CTLA-4 deficient mice develop a fatal lymphoproliferative disorder(137, 138), indicating a critical immunoregulatory role of CTLA-4. CTLA-4 expression is primarily restricted to T cells. In conventional T cells, CTLA-4 is not found on naïve, resting T cells but its expression is induced on the surface upon TCR activation. On the other hand, Tregs constitutively express CTLA-4.

Numerous potential mechanisms of action of CTLA-4 have been suggested but the precise mechanism responsible for it's function remains unclear with controversial results. One model is that CTLA-4 counteracts CD28-mediated costimulation by ligand competition. Deletion or blockade of B7 ligands (by administration of CTLA-4-Ig) prevents CTLA-4 deficient mice from excessive lymphocyte activation and fatal diseases(139, 140). CTLA-4 has also been shown to increase T cell motility, limiting the formation of stable contacts between naïve T cells and DCs during priming(141). It has been originally thought that CTLA-4 inhibits TCR/CD28-mediated signals by recruiting protein phosphatase 2A (PP2A)(142, 143). However, other studies do not support this traditional model where CTLA-4 intrinsically mediates negative signaling(144) and

rather suggest that the function CTLA-4 is mainly to regulate the CD28 access to its ligands. In addition to T cell-intrinsic roles in regulating T cell responses, CTLA-4 also acts by T cell-extrinsic mechanisms. Upon ligation, CTLA-4 can deliver the reverse signals into B7-expressing APCs and induce indoleamine 2,3-dioxygenase (IDO) expression(145, 146). IDO functions to suppress T cell responses by reducing tryptophan levels or promoting the conversion of naïve T cells into Tregs (induced regulatory T cells, iTregs). CTLA-4 expression in Tregs has been shown to be critical for their suppressive functions(147) although this model is also challenged by contradictory reports in which the suppressive function of Tregs is not dependent on CTLA-4(148). Tregs have been shown to downregulate B7 molecules on APCs(149, 150) in a CTLA-4-dependent manner. Downregulation of B7 was abrogated by administration of anti-CTLA-4 antibodies or by deficiency of CTLA-4 in Tregs(151). Another study further explored the underlying mechanisms and found that CTLA-4 can remove B7 molecules on the surface of APCs by transendocytosis(152).

Blockade of CTLA-4 has demonstrated a significant anti-tumor activity in several types of murine tumor models and these promising preclinical studies led to the development of anti-human CTLA-4 antibodies(153, 154). The human anti-CTLA-4 monoclonal antibody, Ipilimumab, showed significant survival benefit in patients with advanced melanoma in Phase III clinical trials(155) and has been approved for the treatment of unresectable and metastatic melanoma. Ipilimumab was the first checkpoint inhibitor approved for use in cancer patients. Despite extensive studies and clinical application, the mechanism underlying CTLA-4 blockade remains ill-defined. The commonly observed outcome of CTLA-4 blockade is the increased ratio of effector T

cells to Tregs within the tumor. Antitumor effects of CTLA-4 blockade can be achieved by enhancing effector T cell responses and/or suppressing Treg functions. Recent papers have shown that CTLA-4 blockade also functions to deplete intratumoral Tregs through a Fc γ receptor (FcγR)-dependent mechanism(156, 157). ICOS/ICOSL pathway has been shown to be critical for the antitumor effect of CTLA-4 blockade, because tumor rejection was abrogated following the blockade of CTLA-4 in ICOS- or ICOSL-deficient mice(158). Recently CTLA-4 blockade combined with ICOS engagement using the tumor vaccine engineered to express ICOSL has been shown to result in a synergistic effect in enhancing antitumor responses and ultimately eradicating tumor in a mouse model(159).

During chronic infection, CTLA-4 expression is upregulated in virus-specific T cells and blocking CTLA-4 has been shown to enhance antiviral CD4 and CD8 T cell responses. Elevated CTLA-4 expression was found in HIV-specific CD4 T cells but not in CD8 T cells and in vitro blockade of CTLA-4 enhanced the functionality of HIV-specific CD4 T cells(160). During HCV infection, PD-1/CTLA-4 coexpression was correlated with the dysfunction of hepatic HCV-specific T cells that can be reversed by combined PD-1 and CTLA-4 blockade(161). However, during chronic LCMV infection, although CTLA-4 was one of the genes upregulated in exhausted CD8 T cells, in vivo blockade of CTLA-4, interestingly, had no significant effects on rescuing virus-specific CD8 T cells or reducing viral load(92). The effect of anti-CTLA-4 antibodies in chronic viral infection was first evaluated in a clinical trial where Tremelimumab, another anti-human CTLA-4 antibody, was administered to the patients with hepatocellular carcinoma

(HCC) and chronic HCV infection. The result of this pilot trial showed that this inhibitor can induce both antitumoral and antiviral effects and warrants further investigation.

Cancer clinical trials targeting the PD-1 or CTLA-4 pathway have achieved a very high rate of antitumor response. Currently, monotherapies using blocking antibodies against PD-1/PD-L1 or CTLA-4 and combination therapies with various immunotherapeutic strategies are being evaluated in different types of cancer. The clinical evaluation of these checkpoint blocking agents is currently focused on cancer treatment, but the therapies targeting the PD-1 or CTLA-4 pathway also have potential for treating chronic infections(229). It is also essential to identify the predictive biomarkers to personalize the therapy.

1.3. Combination of inhibitory receptor blockade

Section 1.3. Combination of inhibitory receptor blockade, Reprinted and Modified from Forum on Immunopathological Diseases and Therapeutics, 2015, (1-2): 7-18, Junghwa Lee, Eunseon Ahn, Haydn T. Kissick, Rafi Ahmed, Reinvigorating Exhausted T Cells by Blockade of the PD-1 Pathway, Copyright (2017), with permission from Begell House, Inc.

The severity of T-cell exhaustion has been shown to be correlated with coexpression of multiple inhibitory receptors including PD-1, CTLA-4, lymphocyte-activation gene 3 (LAG-3), T-cell immunoglobulin- and mucin-domain-containing molecule-3 (TIM-3), CD160, and 2B4(162). During chronic LCMV infection, Tim-3 or LAG-3 blockade alone had a minimal effect on rescuing virus-specific CD8 T cells, but

combining with PD-1 pathway blockade synergistically improved LCMV-specific CD8 T-cell response and virus control(162, 163). In addition, in murine cancer models, PD-1 pathway blockade in combination with blocking Tim-3, LAG-3, or CTLA-4 was more effective in restoring antitumor immunity and promoting tumor regression than targeting either pathway alone(164-166). An additive or synergistic effect on rescuing T cells by the combined blockade of different inhibitory receptors indicates their nonredundant roles and complex regulatory mechanisms underlying T-cell dysfunction. Consistent with preclinical data, clinical evaluation of PD-1 and CTLA-4 combination blockade reported a higher rate of clinical response than single therapy in patients with advanced melanoma(116, 167, 168). Combination therapy with PD-1 and CTLA-4 blocking antibodies (Nivolumab and Ipilimumab) is now approved for the treatment of advanced melanoma. Combined PD-1 and CTLA-4 blockade is also being tested in metastatic RCC (NCT01472081) and NSCLC (NCT01454102). Dual blockade of PD-1 and LAG-3 is being tested in solid tumors (NCT01968109).

2. Adoptive T cell immunotherapy

Adoptive transfer of autologous T cells involves isolation, ex vivo manipulation such as expansion or activation and reinfusion to the patients to treat human diseases(169, 170). This strategy allows T cells with desired specificity to expand in vitro to a large number and acquire effector properties. In cancer therapies, autologous T cells can be derived from naturally occurring tumor-reactive T cells (tumor infiltrating lymphocytes, TILs) that are isolated from the resected tumor. Adoptive transfer using expanded TILs has shown remarkable results primarily in melanoma(169). Adoptive transfer of heterogeneous T cells derived from TILs is advantageous in terms of targeting multiple antigens to prevent tumor escape. However, this approach has limitations in feasibility for clinical use. As a therapy for chronic infection and cancer, it may depend on the small number of antigen-specific T cells that might be exhausted and be not able to maintain effector functions. Alternatively, there have been efforts to use genetically modified T cells. T cells are derived from peripheral blood T cells and engineered to express a specific TCR or a chimeric antigen receptor (CAR). In the CAR technology, the antibody-derived single chain variable fragment (scFv) is combined to T cell signaling domains, which redirects the specificity and effector function of T cells. Since CAR-modified T cells can recognize T cells in a non-MHC restricted manner, this method can be used for all patients in a human leukocyte antigen (HLA)-unrestricted manner. The goal of these therapies with genetically modified T cells is to enhance specific binding of T cells to target cells and promote their effective killing.

Adoptive therapy has been used successfully against cancer and viral infection during allogeneic hematopoietic stem cell transplantation, specially protecting from the

reactivation of persistent viruses. Transfer of donor-derived EBV-specific CD8 T cells to bone marrow transplant recipients provided safe and durable protection against EBV-associated lymphoproliferation(171, 172). This therapy was effective as both treatment and prophylaxis. Infusion of EBV-specific CD8 T cells also showed clinical benefit in patients with EBV-associated cancers such as nasopharyngeal carcinoma(173) and Hodgkin's lymphoma(174). Similarly, transfer of donor-derived CMV-specific CD8 T cells reconstituted cellular immunity against the virus in immunocompromised patients with no evidence of toxicity(175). Adoptive transfer therapy has also been investigated in chronic infection such as HIV. In a clinical trial with HIV infected patients, transfer of CAR-transduced T cells resulted in a compartmental antiviral activity(176). CAR T cells trafficked to rectal tissues and decreased viral load. This study also demonstrated that adoptive therapy of retroviral transduced CAR T cells are safe to the HIV patients and persist in vivo. Adoptive therapy with CAR-modified T cells targeting CD19 has shown striking effects on tumor remission in patients with lymphoblastic leukemia or diffuse large B cells lymphoma(177, 178).

Along with T cell adoptive transfer therapy, host conditioning to improve the efficacy of adoptive T cell transfer has been studied. Lymphodepletion of immunosuppressive cells such as Tregs or MDSC or endogenous lymphocytes in order to create the niche for the adoptively transferred cells and to reduce the competition for the same survival factors or growth factors has been tested. A strong correlation between the intensity of lymphodepletion and efficacy of ACT-based cancer immunotherapy has been shown(179).

Cytokines can be exploited in concert with T cell transfer, to support the persistence or function of adoptively transferred cells(170). IL-2 is a potent T cell growth factor and recombinant human IL-2 is approved by FDA for the treatment of metastatic melanoma as a single agent. In cancer studies, T cell adoptive transfer therapy is often evaluated with concomitant administration of IL-2. However, because of its pleotropic roles, the clinical use of IL-2 should be carefully designed and tested. The effect of IL-7 and IL-15, cytokines important for T cell homeostasis, are currently being explored in preclinical and clinical studies.

Providing additional costimulatory signals or blocking inhibitory signals has been shown to improve the effects of adoptive transfer therapy. Combining adoptive transfer of TILs and anti-CD137 (4-1BB) antibody administration augmented the antitumor activity of TILs and significantly reduced tumor burden(180). Administration of an agonistic antibody to CD40 enhanced the expansion of adoptively transferred T cells and induced more potent antitumor responses(181). Combining T cell adoptive transfer with checkpoint blockade such as CTLA-4 or PD-1 pathway blockade is also a promising approach that possibly leads to additive or synergistic effects by enhancing both endogenous and transferred T cell responses. This combination therapy will be discussed in more detail in the next section.

Adoptive T cell therapy has been more focused on CD8 T cells because of their capability of directly killing target cells. However, adoptive transfer using CD4 T cells has also hown promising results in chronic infection and cancer(182). Transfer of autologous tumor-reactive CD4 T cells had significant effects on tumor regression or eradication. In a setting of post-bone marrow transplant, cotransferred CD4 T cells were

essential for the persistence of adoptively transferred CD8 T cells(183). Our group also showed that transfer of virus-specific CD4 T cells restored LCMV-specific antibody responses as well as endogenous CD8 T cell functions and led to marked reduction in viral load in chronically infected mice(49). Since the roles of CD4 T cells are critical in many aspects of immune responses against pathogens and cancer, orchestrating both humoral and cellular immunity, adoptive therapy using CD4 T cells has a strong potential to promote antiviral or antitumoral immune responses.

3. **Combination therapy of adoptive T cell transfer and inhibitory receptor blockade**

With the aim of developing more effective immunotherapeutic strategies that allow more patients to respond and apply to more types of cancers and various chronic infections, combination therapies with different immunological interventions have been explored. Here I will specially focus on the combination of adoptive T cell transfer and inhibitory receptor blockade.

Adoptive transfer of antigen-specific T cells is an effective immunotherapeutic approach to replenish the antiviral or antitumor immune responses. In the meanwhile, under the influence of continuous antigen exposure, adoptively transferred T cells also upregulate inhibitory receptors such as PD-1 or CTLA-4 and become dysfunctional. Therefore, blocking the interaction between the inhibitory receptors and their ligands can further augment the therapeutic efficacy of adoptively transferred cells. Moreover, it is thought that the effects of blocking inhibitory receptors can exert a potent activity in the

presence of some level of preexisting immunity. Thus, combining these two strategies allows to achieve superior outcomes than monotherapies (229).

In a murine tumor model using Her-2 transgenic mice, combined PD-1 blockade to CAR T cell transfer enhanced proliferation and function of transferred Her-2 tumor-specific T cells and led to more significant tumor regression compared to T cell transfer alone(184). Another study using a tumor mouse model has demonstrated that combining PD-1 pathway blockade to adoptive transfer of tumors-specific T cells facilitated immune cell infiltration by increasing the expression of IFNγ and the IFNγ-inducible chemokine at the tumor site, further promoting tumor regression than single treatment(185). In mice bearing melanoma, CTLA-4 blockade improved the outcome of adoptive therapy with tumor-reactive CD4 T cells, resulting in eradication of established tumors(186). In chronic LCMV infection, combination of adoptive transfer with virus-specific CD4 T cells and blockade of PD-1 pathway further augmented the functionality of transferred CD4 T cells and endogenous virus-specific CD8 T cells and resulted in more remarkable reduction in viral load compared to either therapy alone(49). Recently, combination of tumor-reactive CD8 T cells and CTLA-4 blockade led to durable complete remission in a patient with metastatic melanoma that was refractory to T cell transfer or CTLA-4 blockade monotherapy(187). Currently, clinical trials evaluating combination therapies for melanoma using adoptive transfer of autologous TILs and administration of blocking antibodies against PD-1 or CTLA-4 are underway.

Chapter 2: Adenovirus serotype 5 vaccination results in suboptimal CD4 T helper 1 responses in mice

Chapter 2, Adenovirus serotype 5 vaccination results in suboptimal CD4 T helper 1 responses in mice, Published in Journal of Virology, 2017, 91(5).

I. Summary

Adenovirus serotype 5 (Ad5) is one of the most widely used viral vectors and is known to generate potent T cell responses. While many previous studies have characterized Ad5-induced CD8 T cell responses, there is a relative lack of detailed studies that have analyzed CD4 T cells elicited by Ad5 vaccination. Here, we immunized mice with Ad5 vectors encoding lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP) and examined GP-specific CD4 T cell responses elicited by Ad5 vectors compared to those induced by an acute LCMV infection. In contrast to LCMV infection where balanced CD4 T helper 1 (Th1) and T follicular helper (Tfh) responses were induced, Ad5 immunization resulted in a significantly reduced frequency of Th1 cells. CD4 T cells elicited by Ad5 vectors expressed decreased levels of Th1 markers such as Tim3, SLAM, T-bet, and Ly6C, had lower amounts of cytotoxic molecules like granzyme B, and produced less interferon-gamma than CD4 T cells induced by LCMV infection. This defective CD4 Th1 response appeared to be intrinsic for Ad5 vectors and not a reflection of comparing a nonreplicating vector to a live viral infection since immunization with a DNA vector expressing LCMV-GP generated efficient CD4 Th1 responses. Analysis at early time points (day 3 or 4) after immunization with Ad5 vectors revealed a defect in

the expression of CD25 (interleukin-2 [IL-2] receptor alpha chain) on Ad5-elicited CD4 T cells and administration of exogenous IL-2 following Ad5 immunization partially restored CD4 Th1 responses. These results suggest that impairment of Th1 commitment after Ad5 immunization could be due to reduced IL-2-mediated signaling.

Significance

During viral infection, generating balanced responses of Th1 and Tfh cells is important to induce effective cell-mediated responses and provide optimal help for antibody responses. In this study, to investigate vaccine-induced CD4 T cell responses, we characterized CD4 T cells after immunization with Ad5 vectors expressing LCMV-GP in mice. Ad5 vectors led to altered effector differentiation of LCMV-GP-specific CD4 T cells compared to that during LCMV infection. CD4 T cells following Ad5 immunization exhibited impaired Th1 lineage commitment, generating significantly decreased Th1 responses than those induced by LCMV infection. Our results suggest that suboptimal IL-2 signaling possibly plays a role in reduced Th1 development following Ad5 immunization.

II. Introduction

Recombinant adenovirus (Ad) vectors are one of the most favorable vaccine platforms as they have certain potential advantages such as strong immunogenicity, large transgene capacity and good safety profile. Ad-based vaccine vectors can induce strong T cell-mediated and humoral immune responses to the encoded transgenes. Currently Ad vectors from multiple species and serotypes are being explored, either alone or as a prime-boost strategy, for a number of infectious diseases including human immunodeficiency virus (HIV), tuberculosis, malaria, Ebola, hepatitis C and influenza as well as cancer. Preclinical and clinical studies in mice, non-human primates and humans have shown promising results (188, 189). Human adenovirus serotype 5 (Ad5) is the best studied and the most commonly used adenovirus vector in vaccine development because of its superior immunogenicity compared to other Ad serotype vectors. Although preexisting antivector immunity in the human population and the lack of protection against HIV infection have raised concerns over the clinical application of Ad5 vectors (190, 191), Ad5 is still one of the most frequently used viral vectors in clinical studies, as significant efficacy has been shown against a broad range of pathogens and cancers (188, 189).

Ad5 vectors have been shown to induce high-frequency CD8 T cell responses in preclinical and clinical studies. More detailed studies on the characteristics of CD8 T cells have demonstrated that Ad5 immunization induces sustained CD8 T cell responses with more effector-like phenotypes (192), probably due to prolonged transgene expression (193). Assessment of Ad5-induced T cell responses has been more focused on CD8 T cells while relatively less is known about CD4 T cell responses elicited by Ad5

immunization. Previous studies have shown that CD4 T cells are critical for transgene-specific CD8 T cell and antibody responses elicited by Ad vector immunization (194-196). However, the phenotypic and functional properties of CD4 T cells induced by Ad vectors remain less well investigated.

CD4 T cells orchestrate immune responses against various types of pathogens by differentiating to diverse effector subsets with unique effector functions (1, 197). During viral infection, for example lymphocytic choriomeningitis virus (LCMV) infection in mice, both T helper 1 (Th1) and T follicular helper (Tfh) cells are generated (198). Th1 cells produce their signature cytokine, interferon-gamma (IFN-γ) and play a critical role in cell-mediated immunity and the host defense against intracellular pathogens (1, 197). Tfh cells are specialized in providing help to cognate B cells and essential for the initiation and maintenance of germinal center reactions and the generation of high-affinity antibodies, long-lived plasma cells and memory B cells (8). Given the critical roles of CD4 T cells in immune protection, it is crucial to better understand vaccine-induced CD4 T cell responses.

In this study, we examined CD4 T cell responses following immunization of mice with Ad5 vectors encoding full-length LCMV-glycoprotein (GP). We characterized LCMV-GP-specific CD4 T cells elicited by Ad5 vectors in comparison to those induced by infection with the original virus, LCMV. Ad5-elicited CD4 T cells undergo a distinct differentiation program which leads to suboptimal Th1 responses, at least partly, due to reduced interleukin-2 (IL-2) signaling.

III. Results

Ad5 immunization results in suboptimal CD4 Th1 responses. To examine Ad5-elicited CD4 T cell responses, we utilized SMARTA T cell receptor (TCR) transgenic cells specific for the major histocompatibility complex (MHC) class II-restricted epitope of LCMV GP_{66-77} and Ad5 vectors encoding full-length LCMV GP. SMARTA CD4 T cells (CD45.1+) were transferred into naïve C57BL/6 mice (CD45.2+) that were subsequently immunized with Ad5-LCMV-GP vectors or infected with LCMV Armstrong that causes an acute infection. Following Ad5 immunization or LCMV infection, congenically marked donor cells were analyzed in the spleen. Both Ad5 immunization and LCMV infection led to similar expansion and contraction of SMARTA CD4 T cells with the peak response at day 8. However, the magnitude of CD4 T cell responses elicited by Ad5 was significantly low compared to that induced by LCMV and the difference was maintained throughout the course of immunization/infection (Fig. 1A). To access differentiation of CD4 T cells following Ad5 immunization, the phenotypes of SMARTA CD4 T cells were analyzed at the peak of the response (day 8). Consistent with the previous report, balanced responses of two CD4 effector T cell subsets, Th1 and Tfh, were generated during LCMV infection (198). Approximately 45 to 50% of SMARTA CD4 T cells in the spleen were differentiated into Tfh cells that expressed CXCR5 and downregulated Th1-associated molecules in LCMV infection (Fig. 1B and 1C). The other half of SMARTA CD4 T cells ($CXCR5^{-}$) upregulated granzyme B and Ly6C and the majority of them expressed high-level of Tim3, SLAM and T-bet, thus representing Th1 cells. In comparison, dramatically reduced Th1 cells were found following Ad5 immunization; granzyme B and Tim3 expression levels were minimal and approximately

10 to 15% of SMARTA CD4 T cells expressed SLAM, T-bet and Ly6C. Mean fluorescence intensity (MFI) of Th1-associated molecules was also significantly lower in Ad5-elicited SMARTA CD4 T cells than those induced by LCMV infection (Fig. 1D). On the other hand, a high proportion of SMARTA CD4 T cells was differentiated into $CXCR5^+$ Tfh cells following Ad5 immunization (Fig. 1B and 1C). To further characterize CD4 T cells elicited by Ad5 vectors, cytokine production was measured after ex vivo stimulation with cognate peptide. SMARTA CD4 T cells produced substantially less IFN-γ after Ad5 immunization than after LCMV infection (Fig. 1E). These results demonstrate that immunization with Ad5 vectors resulted in significantly reduced Th1 differentiation.

We asked whether the reduced Th1 responses following Ad5 immunization compared to those after LCMV infection are due to the differences between nonreplicating vaccine vectors and live virus infection. To address this, we compared CD4 T cell responses induced by Ad5 vectors and DNA vectors expressing the same antigen, LCMV-GP, at day 8 post-immunization. In contrast to Ad5 vectors, DNA immunization generated more balanced Th1 and Tfh responses. DNA vectors induced SMARTA CD4 T cells that produced significantly more IFN-γ than Ad5 vectors and this was comparable to the levels after LCMV infection (Fig. 1F). Expression of granzyme B, SLAM and T-bet was also significantly elevated in SMARTA CD4 T cells following DNA immunization, compared to levels after Ad5 immunization (data not shown). Therefore, impaired Th1 development seems to be an intrinsic property of Ad5 vectors, rather than an outcome of using nonreplicating vectors.

Next, to determine whether similar CD4 T cell responses were observed in other tissues, we examined SMARTA CD4 T cells in inguinal lymph nodes (LNs), liver and blood after immunization with Ad5 vectors. Following Ad5 immunization, expansion of SMARTA CD4 T cells was remarkably reduced in all tissues examined compared to that after LCMV infection (Fig. 2A). After acute viral infection, Tfh cells are predominantly found in secondary lymphoid tissues and blood whereas a very small population of CXCR5+ cells is found in the nonlymphoid tissues where, instead, Th1 responses are dominant (198). Consistent with these localization patterns, both $SLAM^+$ Th1 and $CXCR5^+$ Tfh effector cells were found in LNs and blood while the majority of CD4 T cells were Th1 in the liver at day 8 post-LCMV infection (Fig. 2B and 2C). In Ad5-immunized mice, development of Th1 cells was greatly reduced in the LNs where the majority of CD4 T cells were Tfh cells. There was also a significantly lower frequency of Th1 cells in liver and blood compared to the frequencies in LCMV-infected animals. Taken together, these data indicate that Ad5 immunization led to substantially reduced Th1 responses in all tissues examined including lymphoid and nonlymphoid organs.

Immunization with Ad5 vectors does not generate CD4 Th2 or Th17 cells. In LCMV infection, virus-specific CD4 T cells predominantly develop into Th1 and Tfh cells with minimal generation of other CD4 T cell subsets such as Th2 or Th17. Given that Ad5 immunization possibly induces different environmental conditions such as the cytokine milieu during CD4 T cell activation, we wanted to determine whether other CD4 T cell subsets besides Th1 and Tfh were generated following Ad5 immunization. We assessed Th2- and Th17-associated transcription factors and cytokines at day 8 post-immunization. SMARTA CD4 T cells elicited by Ad5 vectors did not express GATA3 or

RORγt and did not produce IL-4 or IL-17 upon cognate peptide stimulation (Fig. 3A and 3B), indicating that no Th2 and Th17 differentiation occurred.

Similar to SMARTA CD4 T cells, Ad5-elicited endogenous GP_{66-77}-specific CD4 T cells display significantly decreased Th1 responses. To confirm whether endogenous LCMV-GP-specific CD4 T cells behave similarly to the transgenic CD4 T cells, we analyzed endogenous polyclonal LCMV GP_{66-77}-specific CD4 T cells by tetramer staining after immunization with Ad5-LCMV-GP vectors. Endogenous GP_{66-77}-specific CD4 T cell responses were analogous to the responses of SMARTA transgenic CD4 T cells and displayed similar kinetics (Fig. 4A). Similar to the responses of SMARTA CD4 T cells, Ad5 induced significantly lower endogenous GP_{66-77}-specific CD4 T cell responses than did LCMV, generating approximately 4.5-fold fewer GP_{66-77}-specific CD4 T cells at the peak of the expansion (day 8). Consistently, endogenous GP_{66-77}-specific CD4 T cells following Ad5 immunization exhibited a significantly lower frequency of Th1 cells than those generated by LCMV infection (Fig. 4B and 4C). Ad5 vector-induced CD4 T cells also resulted in decreased production of cytokines such as IFN-γ, tumor necrosis factor (TNF), and IL-2 (Fig. 4D). Therefore, endogenous polyclonal LCMV GP-specific CD4 T cell responses were similar to those of transgenic monoclonal CD4 T cells and resulted in suboptimal Th1 development after Ad5 immunization.

Next, to examine the kinetics of Th1 and Tfh cells following Ad5 immunization, we tracked GP_{66-77}-specific CD4 T cells by day 30 post-immunization. With Ad5 immunization, after the peak response at day 8, Th1 cells, identified by their high expression of SLAM, T-bet or Ly6C, were decreased over time whereas CXCR5+ Tfh

cells were relatively stably maintained (Fig. 4E). Consequently, at day 30 post-Ad5 immunization, transgene-specific CD4 T cells exhibited a further decline in Th1 responses, with responses skewing toward Tfh cells. The kinetics of Th1 and Tfh responses following Ad5 immunization was similar to that observed in LCMV infection. This analysis shows suboptimal Th1 responses at the memory phase following Ad5 immunization.

Ad5 vectors generate GP-specific antibody responses. Immunization with Ad5 vectors generated a high frequency of Tfh cells. Since the major function of Tfh cells is to provide help to B cells in generating optimal antibody responses, we sought to determine Ad5-elicited GP-specific antibody responses. Following immunization with Ad5 vectors, the GP-specific serum antibody titer was very low at day 8 post-immunization compared to that after LCMV infection (Fig. 5). However, a greater increase in the antibody titer was detected between day 8 and 15 post-Ad5 immunization and the difference in the titers between Ad5 and LCMV became smaller by day 30. The differences in antibody titers and kinetics between Ad5 immunization and LCMV infection could be attributed to the significantly low magnitude of Ad5-elcitied CD4 T cell responses and/or different conditions of antigen load and persistence during Ad5 immunization versus LCMV infection.

Ad5 vectors administered via the intravenous (i.v.) route result in a greater decrease in Th1 responses than intramuscular (i.m.) immunization. The route of immunization and the subsequent delivery of antigens to different sites can impact on the phenotypes of vaccine-induced T cell responses. In the experiments above, we administered Ad5 vectors through i.m. injections, a standard route of vaccination.

Alternatively, in this experiment, we immunized mice with Ad5 vectors intravenously and examined the impacts of the route of administration on vaccine-induced CD4 T cell responses. SMARTA CD4 T cells were transferred into C57BL/6 mice that were subsequently given Ad5 vectors via the i.m or i.v. route. 8 days later, Ad5-elicited CD4 T cells were analyzed in the spleen. There was a trend for slightly higher numbers of SMARTA CD4 T cells found following i.v. administration of Ad5 vectors than after i.m. immunization (data not shown). Compared to i.m. immunization, i.v. administration of Ad5 vectors generated even lower Th1 responses (almost absent) in the spleen and the majority of SMARTA CD4 T cells were differentiated into Tfh cells (Fig. 6A and B). We also compared endogenous GP_{66-77}-specific CD4 T cell responses following i.m. and i.v. immunization of Ad5 vectors. Besides further decreasing endogenous Th1 cells, i.v. immunization of Ad5 vectors also increased Tfh cells compared to i.m. immunization (Fig. 6C and D). Therefore, Ad5 immunization via either the i.m. or i.v. route led to significantly reduced Th1 responses compared to those induced by LCMV infection. Interestingly, Th1 development was further impaired following i.v. immunization compared to i.m. immunization of Ad5 vectors.

Regardless of the vector dose administered, Ad5 immunization generates suboptimal Th1 responses compared to those induced in LCMV infection. The dose of vector administered can also influence vaccine-induced immune responses. All experiments so far were performed with administration of 10^{10} virus particles (vp) of Ad5 vectors . To examine the effects of the vector dose on the quantity and quality of Ad5-elicited CD4 T cells, mice were given 10^8, 10^9, or 10^{10} vp of Ad5 vectors and CD4 T cell responses were assessed at day 8 post-immunization. Interestingly, there were no

significant dose-dependent effects on the magnitude of transgene-specific CD4 T cell responses (Fig. 7A). However, varying doses of Ad5 vectors generated CD4 T cells with somewhat different phenotypes. Ad5 vectors at the lower doses tended to generate more Th1 cells and fewer Tfh cells (Fig. 7B and C). Still, the lower doses of Ad5 did not raise effective Th1 responses above those induced in LCMV infection. Although the increased frequency of T-bet^{hi} CXCR5- or IFN-γ producing SMARTA CD4 cells at 10^8vp of Ad5 vectors, was similar to that in LCMV infection, SLAM and Ly6C expression of Ad5-elicited CD4 T cells was lower than that in LCMV infection. In particular, granzyme B and Tim3 expression levels on Ad5-elicited SMARTA CD4 T cells were significantly lower than those in LCMV infection, irrespective of the dose administered. Similar results were obtained when endogenous GP_{66-77}-specific CD4 T cells were analyzed following administration of the lower doses (10^8 or 10^9 vp) of Ad5 vectors. More GP_{66-77}-specific Th1 cells and fewer Tfh cells were observed with 10^9 vp as well as 10^8 vp of Ad5 vectors compared to those at 10^{10} vp (data not shown). Therefore, the dose of Ad5 vectors impacted Th1/Tfh differentiation to some degree; however, regardless of the dose administered, immunization with Ad5 vectors resulted in suboptimal Th1 responses compared to those in LCMV infection.

Impaired CD4 Th1 commitment following Ad5 immunization. We observed that Ad5-elicited CD4 T cell counts were substantially lower in magnitude than LCMV-induced CD4 T cell counts. Consequently, we sought to determine whether the decreased expansion of Ad5-elicited CD4 T cells was due to slow proliferation. Cell trace violet-labeled SMARTA CD4 T cells were transferred into naïve recipients that were subsequently immunized with Ad5-LCMV-GP vectors, infected with LCMV, or

remained uninfected (Fig. 8A). 3 and 4 days later, early proliferation of SMARTA CD4 T cells was examined. Ad5-induced SMARTA CD4 T cells were less proliferative than those in LCMV infection and this difference was more significant at day 4 post-immunization, as indicated by slow decay of cell-trace violet intensity (Fig. 8B, left). As a result, significantly fewer SMARTA CD4 T cells were detected in the spleen at day 4 post-Ad5 immunization (Fig. 8B, right).

Fate decisions of naïve CD4 T cells occur within the first few rounds of cell division (15, 16). In LCMV infection, it has been shown that CD4 T cells rapidly bifurcate into a Th1 versus Tfh differentiation program by day 3 post-infection. To confirm whether early commitment of CD4 T cells marks their effector phenotypes, we analyzed differentiation of SMARTA CD4 T cells 3 and 4 days after Ad5 immunization. At these early time points, SMARTA CD4 T cells elicited by Ad5 immunization or LCMV infection displayed remarkably different phenotypes which reflected their effector differentiation (Fig. 9A). In LCMV infection, both Th1-like and Tfh-like SMARTA CD4 T cells were found in the spleen at day 3 and 4 post-infection. Strikingly, however, following Ad5 immunization, SMARTA CD4 T cells did not upregulate Th1-associated molecules such as Tim3, SLAM, and Ly6C and expressed significantly lower levels of T-bet compared to SMARTA CD4 T cells after LCMV infection, both at day 3 and 4 post-immunization. On the other hand, both Ad5 immunization and LCMV infection induced a similar frequency of SMARTA CD4 T cells expressing CXCR5. Following Ad5 immunization, the majority of SMARTA CD4 T cells expressed a high level of folate receptor 4 (FR4), which is also a Tfh marker (199). Expression of the cytotoxic molecule, granzyme B, was minimal in SMARTA CD4 T cells following Ad5

immunization (Fig. 9B). Ad5-induced SMARTA CD4 T cells also produced less amount of IFN-γ and TNF. IL-2 production was not significantly different between Ad5-elicited and LCMV-elicited SMARTA CD4 T cells. Together, while antigen-specific CD4 T cells exhibited distinguishable Th1 and Tfh populations early after LCMV infection, Ad5-elicited CD4 T cells showed impaired Th1 commitment.

Notably, expression of CD25, a high-affinity IL-2 receptor alpha chain (IL-2Rα), was minimal on SMARTA CD4 T cells after Ad5 immunization whereas SMARTA CD4 T cells markedly upregulated CD25 following LCMV infection (Fig. 9C). Given that IL-2 receptor (IL-2R)-mediated signaling is known to be required for inducing Th1 differentiation while negatively regulating Tfh development (11, 12), a reduction in IL-2 signals could contribute to suboptimal Th1 responses and skew responses toward Tfh differentiation after Ad5 immunization.

IL-2 administration following Ad5 immunization restores CD4 Th1 differentiation. To determine whether IL-2/IL-2R signaling actually plays a role in regulating Ad5-elicited Th1 and Tfh differentiation, IL-2 was administered following immunization with Ad5 vectors. Mice were given 15,000 IU of recombinant IL-2 or PBS twice daily from the day of immunization and until day 7 post-immunization before sacrificed at day 8 (Fig. 10A). IL-2 administration did not significantly affect expansion of SMARTA CD4 T cells; the numbers of SMARTA CD4 T cells in IL-2-treated and PBS-treated groups were similar at day 8 post-immunization (data not shown). However, administration of IL-2 following Ad5 immunization significantly promoted Th1 differentiation, as shown by the increased frequency of SMARTA CD4 T cells expressing granzyme B, SLAM, and T-bet, whereas the frequency of Tfh cells was

decreased by IL-2 treatment (Fig. 10B, C, and D). Increased generation of Th1 cells by exogenous IL-2 indicates that attenuated IL-2 signaling in Ad5 immunization possibly plays a role in reduced Th1 responses.

IV. Discussion

Despite the crucial role of CD4 T cells in protective immunity, differentiation of transgene-specific CD4 T cells following Ad5 immunization has not been well described. In this study, we characterized Ad5-elicited CD4 T cell responses after immunizing mice with Ad5 vectors encoding LCMV-GP. Immunization with Ad5 vectors generated significantly lower Th1 responses than did LCMV infection. These distinct differentiation phenotypes were also observed at early time points, indicating that commitment to Th1 cells was impaired after Ad5 immunization. Our results suggest that this impaired Th1 development is, at least partly, mediated by the attenuation of IL-2 signaling in Ad5 immunization.

CD25 (IL-2Rα) is rapidly and transiently upregulated on antigen-specific T cells following TCR activation and required for the responsiveness to IL-2 by forming high-affinity IL-2Rs along with CD122 (IL-2Rβ) and γ_c (the common cytokine receptor γ-chain, CD132) (11, 12). Expression of CD25 is not only regulated by TCR stimulation but also highly dependent on IL-2. IL-2 signaling through STAT5 can directly upregulate CD25 whose expression is, thus, enhanced via a positive feedback loop. IL-2 signaling has a decisive influence on regulating Th1 versus Tfh differentiation. IL-2-induced activation of signal transducer and activator of transcription 5 (STAT5) upregulates IL-12Rβ, increasing responsiveness to the Th1-driving cytokine IL-12 and T-bet, the Th1 master regulator (13). On the other hand, IL-2 signaling via STAT5 and the phosphatidylinositol-3-kinase (PI3K) pathway inhibits expression of B cell lymphoma 6 (Bcl6), the transcription factor directing Tfh generation (6), through several mechanisms including induction of B lymphocyte-induced maturation protein-1 (Blimp-1) (14, 200),

an antagonist of Bcl6 (201). Therefore, IL-2 signaling promotes development of Th1 cells while suppressing Tfh differentiation. In acute infection, it has been shown that expression of CD25 during CD4 T cell priming strongly correlates with Blimp-1 expression but inversely correlates with Bcl6 expression (15, 16). Following Ad5 immunization, we found that CD25 expression was markedly low on the majority of transgene-specific CD4 T cells, consistent with reduced effector Th1 differentiation. Increased Th1 responses after exogenous IL-2 administration following Ad5 immunization further confirmed that suboptimal Th1 responses were attributable, at least in part, to the decrease in IL-2 signaling.

An important question for this study was what initially causes the attenuation of IL-2 signaling and reduced CD25 expression in Ad5-elcitied CD4 T cells. Of note, significantly more FoxP3+ regulatory T cells (Tregs) were found at early time points (day 2 to day 4) and day 8 following Ad5 immunization than after LCMV infection. One of the suppressive functions of Tregs is to consume IL-2 secreted by other cells, which can limit IL-2 availability to effector T cells. Tregs constitutively express high levels of CD25 which renders them highly accessible to IL-2. A relatively low proportion of effector CD4 T cells and a high proportion of Tregs with Ad5 immunization could reduce local IL-2 concentrations and lead to attenuated IL-2 signaling during CD4 T cell priming. A recent report showed that transforming growth factor beta (TGF-β) acts to suppress CD25 expression on virus-specific CD4 T cells, thereby restricting IL-2 signaling and resulting in CD4 T cell differentiation toward Tfh from Th1 cells (202). Tregs also produce TGF-β and therefore possibly have a negative impact on IL-2 signaling. In a pilot study, to determine the role of Tregs in Ad5 immunziation, we

depleted Tregs in FoxP3^{DTR} knock-in mice in which FoxP3+Treg cells were specifically depleted by diphtheria toxin (DT) administration. Elimination of Tregs following Ad5 immunization partly restored Th1 responses, suggesting that Tregs contribute to suboptimal Th1 responses elicited by Ad5 vectors. We also observed that CD25 expression was upregulated on Ad5-elcitied CD4 T cells in DT-treated mice, indicating that those cells were receiving more IL-2 signals upon removal of Tregs (data not shown).

The innate immune environment including inflammatory cytokines or antigen presentation by dendritic cells (DCs) can influence CD4 T cell differentiation. Type I IFNs have been shown to promote Th1 differentiation by enhancing CD25 expression and STAT5 activation while inhibiting Tfh development (203). CD4 T cell fate decisions occur during DC priming (15). Antigen display by DCs and the duration of T-DC interactions can impact on the strength of TCR signaling and CD4 T cell lineage determination (204-206). Consistent with this concept, the lower doses of Ad5 vectors tended to generate more Th1 cells and fewer Tfh cells. Further studies will be needed to determine other mechanisms, such as whether type I IFNs or DC priming could contribute to differentiation of CD4 T cells following Ad5 immunization.

Similar CD4 T cell responses were observed when SMARTA transgenic CD4 T cells and endogenous GP_{66-77}-specific CD4 T cells were analyzed, but certain differences were also found between these two types of cells. In terms of the magnitude of CD4 T cell responses, at the peak of the response, 45-fold fewer SMARTA CD4 T cells were detected after Ad5 immunization than after LCMV infection, whereas the number of endogenous GP_{66-77}-specific CD4 T cells was 4.5-fold lower following Ad5 immunization

compared to that after LCMV infection. In term of phenotypes, SMARTA CD4 T cells displayed relatively lower percentages of Th1 cells and higher percentages of Tfh cells compared to endogenous cells. This could be explained by different properties of TCRs on SMARTA CD4 T cells and endogenous GP_{66-77}-specific CD4 T cells (such as different TCR affinities/avidities), since SMARTA CD4 T cells bear monoclonal TCRs whereas endogenous GP_{66-77}-specific CD4 T cells are polyclonal. For example, SMARTA CD4 T cells exhibit a mean affinity which is ~10-fold higher than that of endogenous GP_{66-77}-specific cells(207). The clonal differences in TCRs can lead to various degree of proliferative capacity or lineage commitment following immunization or infection.

In this study, we investigated CD4 T cell responses induced by adenovirus vectors. Ad5 immunization resulted in suboptimal Th1 differentiation due to impaired commitment to Th1 cells. Our results demonstrate that Ad5 vectors can mediate altered effector differentiation of transgene-specific CD4 T cells compared to the original pathogen. We suggest reduced IL-2 signaling as one of the potential mechanisms that result in suboptimal Th1 responses following Ad5 immunization. IL-2 signaling has been shown to play a critical role in regulating Th1 versus Tfh differentiation in acute viral infections. Our study also demonstrates the importance of IL-2 signals in vaccine-induced CD4 T cell responses, implicating the potential of manipulating IL-2 signaling to drive favorable vaccine-induced CD4 T cell responses. Additionally, it will be interesting to examine CD4 T cell responses after immunization with alternative serotype Ad vectors in comparison to Ad5, as they have different biological properties and have been shown to elicit distinct immune responses (208, 209).

V. Materials and Methods

Mice and immunization/infection Six- to eight-week-old female C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). SMARTA mice bearing the transgenic T cell receptor (TCR) specific to the GP_{66-77} epitope of LCMV (210) were bred in house on a C57BL/6 background. For Ad5 immunization, C57BL/6 mice were immunized intramuscularly (i.m.) with 10^{10} virus particles (vp) of replication-incompetent (E1/E3 deleted) Ad5 vectors expressing full-length LCMV-GP (Ad5-LCMV-GP). For the experiments on alternative routes of immunization, mice were given 10^{10} vp of Ad5 vectors intravenously (i.v.). For the dose experiments, the lower doses (10^8 or 10^9 vp) of Ad5 vectors were administered to mice. Ad5 vectors were produced in the Fred Hutchinson Cancer Research Center and verified by restriction analysis, sequencing, and immunostaining. In parallel, mice were infected with 2 x 10^5 plaque-forming unit (pfu) of LCMV Armstrong intraperitoneally (i.p.). For analysis at early time points (day 3 and 4), mice were immunized with 10^{10} vp of Ad5 vectors i.v. or infected with 2 x 10^6 pfu of LCMV Armstrong i.v. to facilitate synchronization of the activation of CD4 T cells. For DNA immunization, 200μg of DNA vectors expressing full-length LCMV-GP was administered i.m. All experiments were conducted in accordance with the Emory University Institutional Animal Care and Use committee guidelines.

Cell transfer To generate SMARTA chimeric mice, SMARTA CD4 T cells were isolated from the spleens of naïve SMARTA mice by using a CD4+ T cell isolation kit (Miltenyi Biotech, San Diego, CA). For the analysis at day 8 and later time points, 1 x 10^5 purified SMARTA CD4 T cells were transferred i.v. into C57BL/6 mice 1 day before Ad5 immunization or LCMV infection. For early proliferation experiments, 1 x 10^6

purified SMARTA CD4 T cells were transferred to C57BL/6 mice after labeling with cell trace violet (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

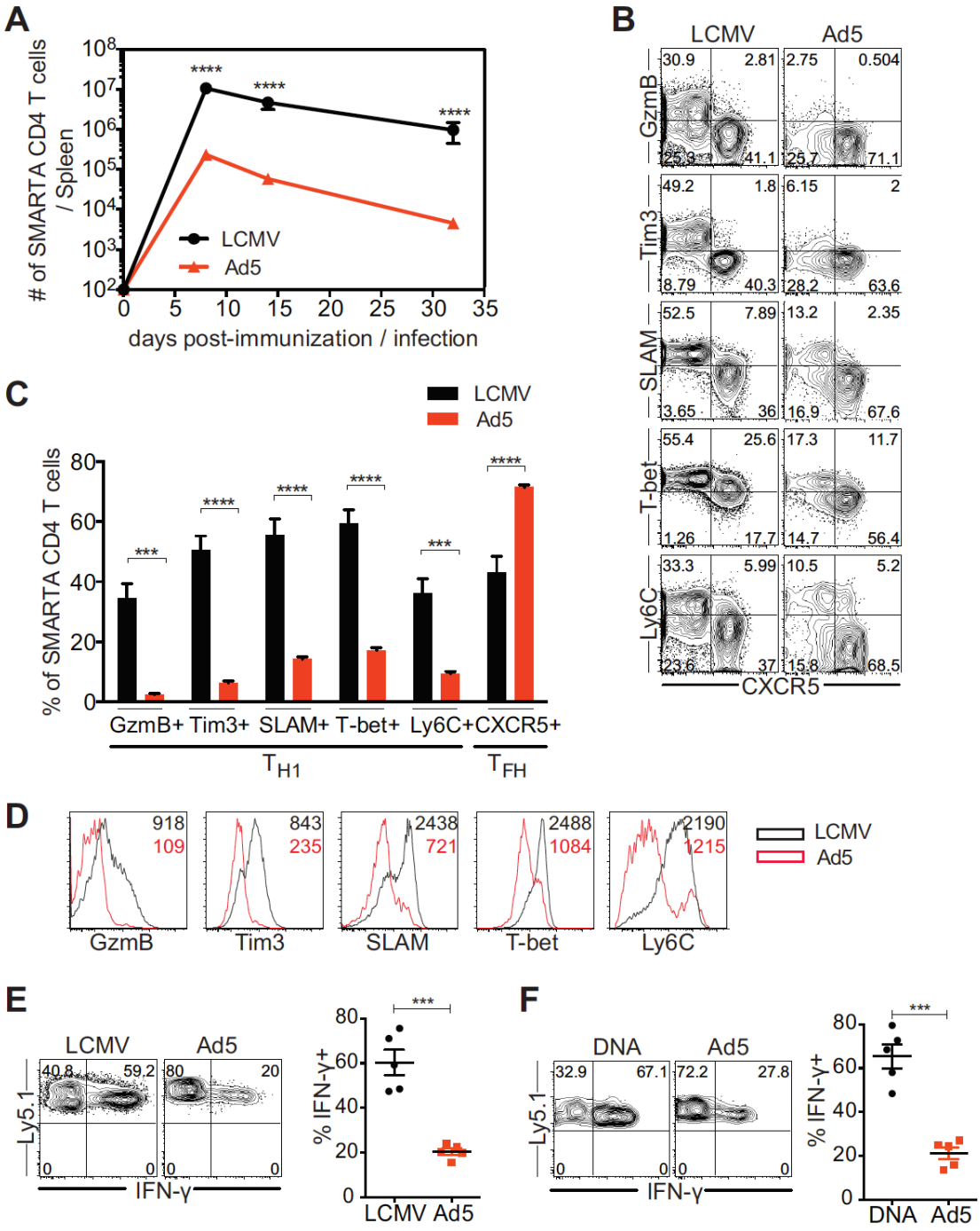
Antibodies and flow cytometry All antibodies were purchased from BD Biosciences (San Jose, CA) except for CD45.1 (Biolegend, San Diego, CA), granzyme B (Invitrogen), T-bet (eBiosciences, San Diego, CA), and Tim3 (R&D Systems, Minneapolis, MN). CXCR5 staining was performed using a three-step staining protocol as described previously (201). Transcription factors were stained using the FoxP3/transcription factor staining buffer set (eBiosciences). Intracellular cytokine staining was performed after 5h of stimulation with GP_{61-80} peptide as described previously (211). Endogenous LCMV GP_{66-77} –specific CD4 T cell responses were measured by staining with I-A^b GP_{66-77} tetramers (DIYKGVYQFKSV, National Institutes of Health [NIH] Tetramer Core Facility, Emory University, Atlanta, GA) at 37°C for 2h. Dead cells were excluded by using Live/Dead fixable dead cell stain kits (Invitrogen). Samples were acquired using a FACSCanto II or LSR II flow cytometer (BD Biosciences). Flow cytometry data were analyzed using FlowJo software (Tree Star, Ashland, OR).

IL-2 administration A dose of 15,000 IU of recombinant human IL-2 (Amgen, Thousand Oaks, CA) diluted in PBS containing 0.1% normal mouse serum was administered i.p. to the mice, twice daily (every 12 h) from the day of Ad5 immunization (day 0), for 8 consecutive days, until day 7 post-immunization.

Enzyme-link immunosorbent assay (ELISA) LCMV glycoprotein-specific antibodies were measured by ELSIA. The plates were coated with LCMV glycoprotein and incubated at 4°C overnight. Serially diluted serum was added to the plates and

incubated for 1.5 h. Bound serum antibodies were detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) (SouthernBiotech, Birmingham, AL). The antibody titers were determined by endpoint titration.

Statistical analysis Data were analyzed using Prism 6 software (GraphPad, La Jolla, CA). Statistical significance was determined by using two-tailed unpaired Student's t-tests. P values of less than 0.05 were considered statistically significant.


A
# of SMARTA CD4 T cells / Spleen
days post-immunization / infection
LCMV
Ad5
****
B
LCMV
Ad5
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SLAM
T-bet
Ly6C
CXCR5
C
% of SMARTA CD4 T cells
GzmB+ Tim3+ SLAM+ T-bet+ Ly6C+ CXCR5+
T_H1
T_FH
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IFN-γ
% IFN-γ+
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F
DNA
Ad5

Figure 1. Ad5 immunization leads to suboptimal Th1 differentiation. CD45.1$^+$ SMARTA transgenic CD4 T cells specific for the LCMV GP_{66-77} epitope were transferred into C57BL/6 mice (CD45.2$^+$) that were subsequently immunized with Ad5 vectors expressing full–length LCMV-GP or infected with LCMV Armstrong strain. Congenically marked (CD45.1$^+$) donor cells were analyzed in the spleen. (A) Kinetics of SMARTA CD4 T cells. (B-F) Analysis was performed at day 8 post-immunization or infection. (B) Representative fluorescence-activated cell sorting (FACS) plots showing the phenotype of SMARTA CD4 T cells. (C) The graph shows the frequency of SMARTA CD4 T cells expressing Th1 markers (granzyme B [GzmB], Tim3, SLAM, T-bet, Ly6C) or a Tfh marker (CXCR5). (D) Representative histograms of the indicated molecules expressed by SMARTA CD4 T cells. The numbers indicate the MFI of the indicated molecules. (E) Cytokine production of SMARTA CD4 T cells after ex vivo stimulation with GP_{61-80} peptide. Representative FACS plots show IFN-γ production of SMARTA CD4 T cells (left). The frequency of IFN-γ^+ cells in SMARTA CD4 T cells (right). (F) SMARTA chimeric mice were generated and immunized intramuscularly with Ad5 or DNA vectors expressing full–length LCMV-GP. Analysis was performed at day 8 post-immunization. Cytokine production was assessed after ex vivo stimulation with GP_{61-80} peptide. Representative FACS plots show IFN-γ production of SMARTA CD4 T cells (left). The frequency of IFN-γ^+ cells in SMARTA CD4 T cells (right). Data are representative of 2 independent experiments with 4-5 mice per group per experiment. Error bars indicate standard errors of means (SEM). ***, $P < 0.001$; ****, $P < 0.0001$.

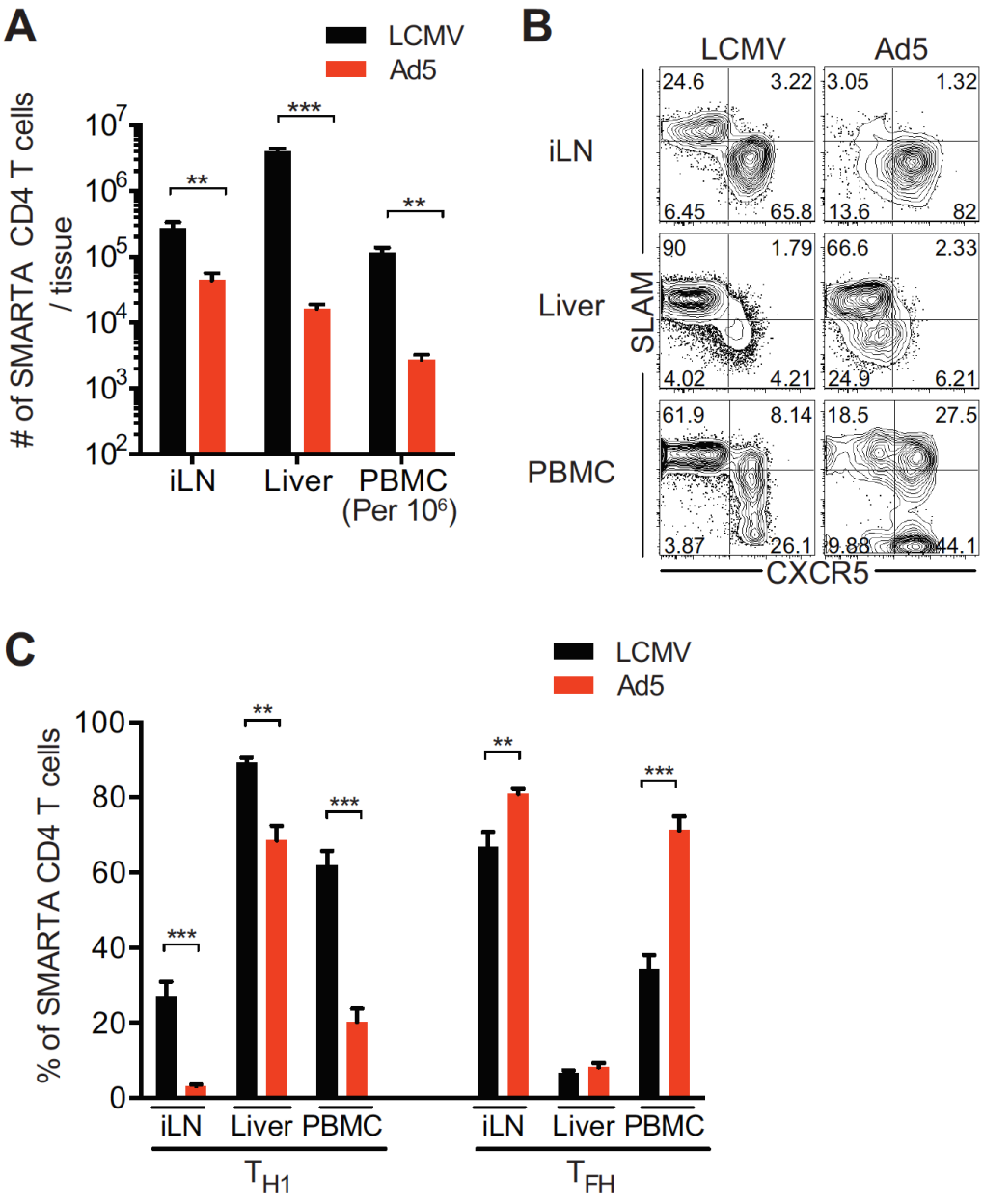

Figure 2. Suboptimal Th1 responses are found in tissues including lymphoid and nonlymphoid organs after Ad5 immunization. Same experimental set up as Figure 1. SMARTA CD4 T cells were analyzed in inguinal lymph nodes (iLN), liver and peripheral blood mononuclear cells (PBMC) 8 days after Ad5 immunization or LCMV infection. (A) Numbers of SMARTA CD4 T cells in the indicated tissues. Numbers of SMARTA CD4 T cells in blood were calculated per one million PBMCs. (B) Representative FACS plots showing the phenotype of SMARTA CD4 T cells. (C) Percentages of $SLAM^{+}CXCR5^{-}$ Th1 or $CXCR5^{+}$Tfh SMARTA CD4 T cells in the indicated tissues. Data are representative of 2 independent experiments with 4-5 mice per group per experiment. Error bars indicate SEM. **, $P < 0.01$; ***, $P < 0.001$.

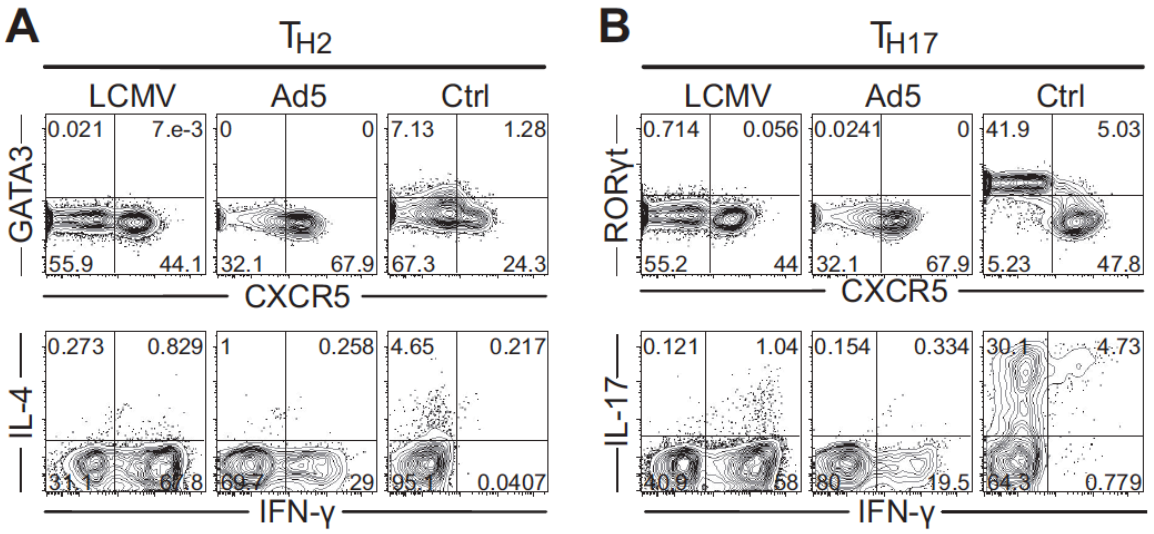

Figure 3. Immunization with Ad5 vectors does not generate Th2 or Th17 cells. SMARTA chimeric mice were generated and immunized with Ad5-LCMV-GP vectors or infected with LCMV. At day 8 post-immunization or infection, SMARTA CD4 T cells were analyzed for Th2-associated (A) or Th17-associated (B) transcription factors and cytokines. (A) Representative FACS plots show GATA3 and IL-4 expression. (B) Representative FACS plots show RORγt and IL-17 expression. Th2 positive control cells expressing GATA3 and IL-4 and Th17 positive control cells expressing RORγt and IL-17 were analyzed together. Cytokine production was measured after ex vivo stimulation with GP_{61-80} peptide.

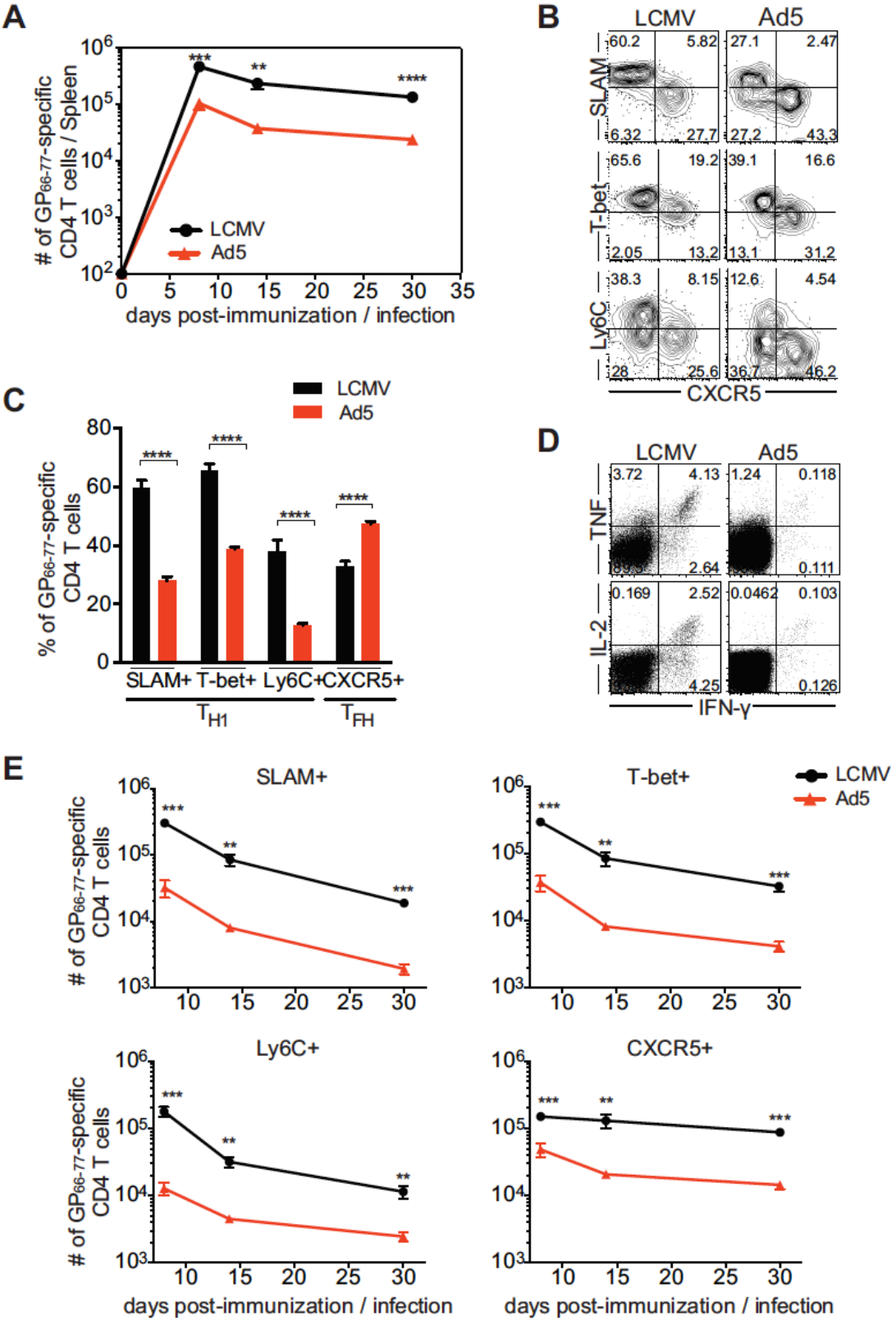
A
# of GP66-77-specific CD4 T cells / Spleen
days post-immunization / infection
LCMV
Ad5
B
LCMV
Ad5
SLAM
T-bet
Ly6C
CXCR5
C
% of GP66-77-specific CD4 T cells
SLAM+ T-bet+ Ly6C+ CXCR5+
TH1
TFH
D
TNF
IL-2
IFN-γ
E
SLAM+
T-bet+
Ly6C+
CXCR5+
# of GP66-77-specific CD4 T cells
days post-immunization / infection

Figure 4. Ad5-elicited endogenous GP_{66-77}-specific CD4 T cells exhibit significantly reduced Th1 responses. Endogenous polyclonal GP_{66-77}-specific CD4 T cells were analyzed in the spleen after immunizing C57BL/6 mice with Ad5-LCMV-GP vectors or infecting mice with LCMV. (A) Kinetics of GP_{66-77}-specific (MHC class II tetramer+) CD4 T cells. (B-D) Analysis was performed 8 days after Ad5 immunization or LCMV infection. (B) Representative FACS plots showing the phenotype of GP_{66-77}-specific CD4 T cells. (C) The graph shows the frequency of GP_{66-77}-specific Th1 cells (CXCR5- cells expressing T-bet, SLAM, or Ly6C) or Tfh cells (CXCR5+ cells). (D) Cytokine production of CD4 T cells after ex vivo stimulation with GP_{61-80} peptide. Representative FACS plots show IFN-γ, TNF, and IL-2 production with gating on total CD4 T cells. (E) The graphs show the number of Th1 cells (CXCR5- cells expressing T-bet, SLAM, or Ly6C) and Tfh cells (CXCR5+ cells) in the spleen by day 30 post- immunization or infection. Data are representative of 2 independent experiments with 4-5 mice per group per experiment. Error bars indicate SEM. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

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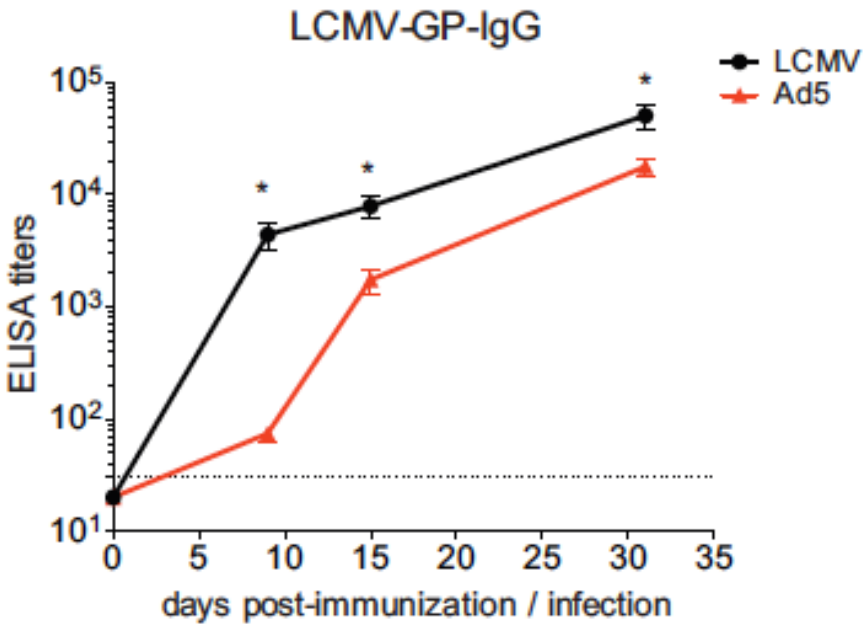


Figure 5. GP-specific antibody responses elicited by Ad5 vectors. Serum GP-specific antibody titers were measured by ELSIA at day 8, 15, and 30 following immunization with Ad5 vectors or LCMV infection. The dotted line indicates the lower limit of detection. Data are representative of 2 independent experiments with 4-5 mice per group per experiment. Error bars indicate SEM. *, $P < 0.05$.

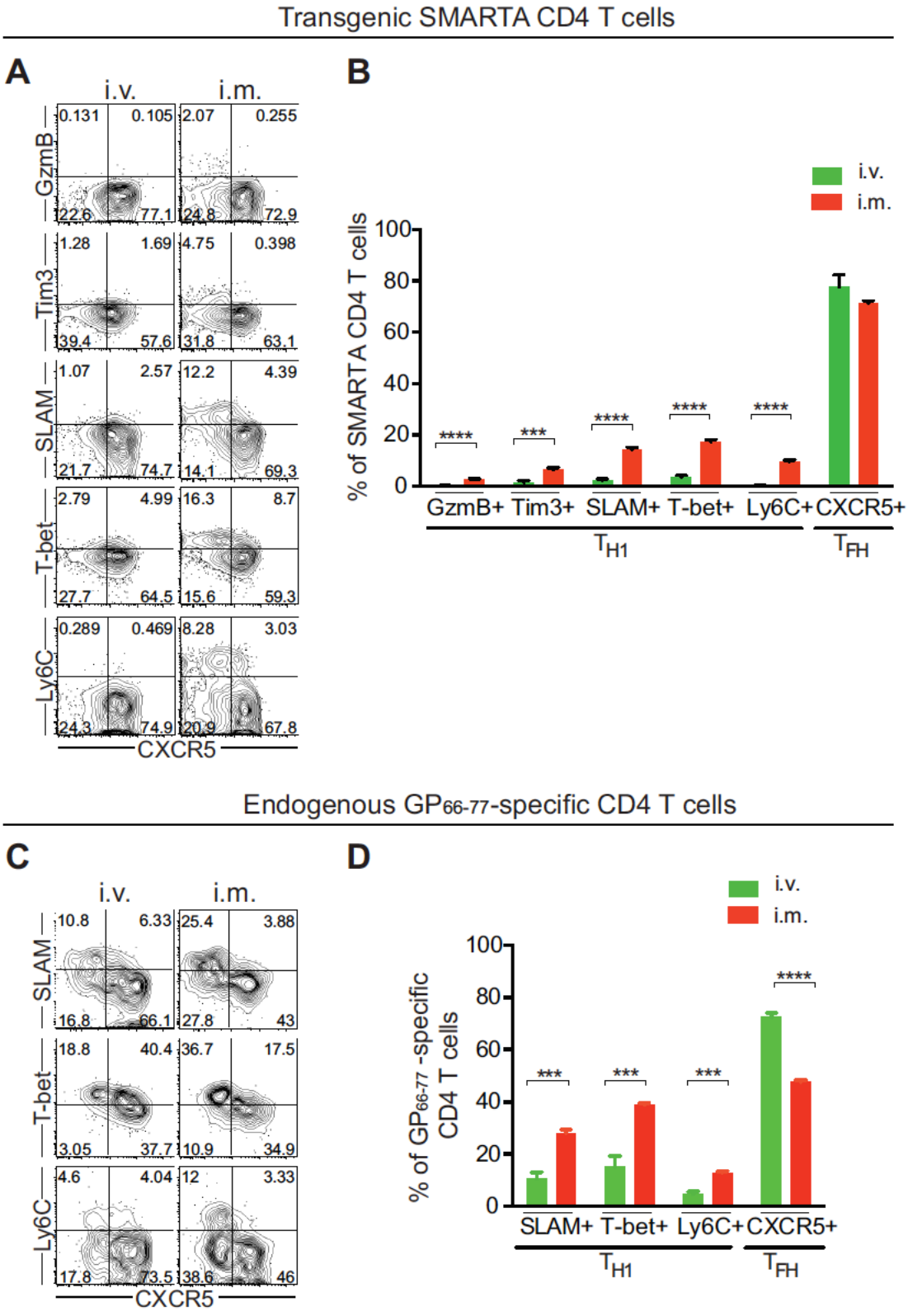

Transgenic SMARTA CD4 T cells
A
i.v.
i.m.
GzmB
Tim3
SLAM
T-bet
Ly6C
CXCR5
0.131
0.105
22.6
77.1
2.07
0.255
24.8
72.9
1.28
1.69
39.4
57.6
4.75
0.398
31.8
63.1
1.07
2.57
21.7
74.7
12.2
4.39
14.1
69.3
2.79
4.99
27.7
64.5
16.3
8.7
15.6
59.3
0.289
0.469
24.3
74.9
8.28
3.03
20.9
67.8
B
i.v.
i.m.
% of SMARTA CD4 T cells
100
80
60
40
20
0
****
***
****
****
****
GzmB+
Tim3+
SLAM+
T-bet+
Ly6C+
CXCR5+
TH1
TFH
Endogenous GP66-77-specific CD4 T cells
C
i.v.
i.m.
SLAM
T-bet
Ly6C
CXCR5
10.8
6.33
16.8
66.1
25.4
3.88
27.8
43
18.8
40.4
13.05
37.7
36.7
17.5
10.9
34.9
4.6
4.04
17.8
73.5
12
3.33
38.6
46
D
i.v.
i.m.
% of GP66-77 -specific CD4 T cells
100
80
60
40
20
0
***
***
***
****
SLAM+
T-bet+
Ly6C+
CXCR5+
TH1
TFH

Figure 6. Ad5 vectors administered via the i.v. route impair Th1 development further than via i.m. immunization. (A-B) SMARTA chimeric mice were generated and immunized with Ad5 vectors via the i.m. or i.v. route. (A) Representative FACS plots showing the phenotype of SMARTA CD4 T cells in the spleen at day 8 post-immunization. (B) The graph shows the frequency of Th1 or Tfh SMARTA CD4 T cells. (C-D) Following i.m. or i.v. administration of Ad5 vectors, endogenous GP_{66-77}-specific (MHC class II tetramer+) CD4 T cells were analyzed in the spleen at day 8 post-immunization. (C) Representative FACS plots showing the phenotype of GP_{66-77}-specific CD4 T cells. (D) The graph shows the frequency of GP_{66-77}-specific Th1 or Tfh cells. Data are representative of 2 independent experiments with 3-5 mice per group per experiment. Error bars indicate SEM. ***, $P < 0.001$; ****, $P < 0.0001$.

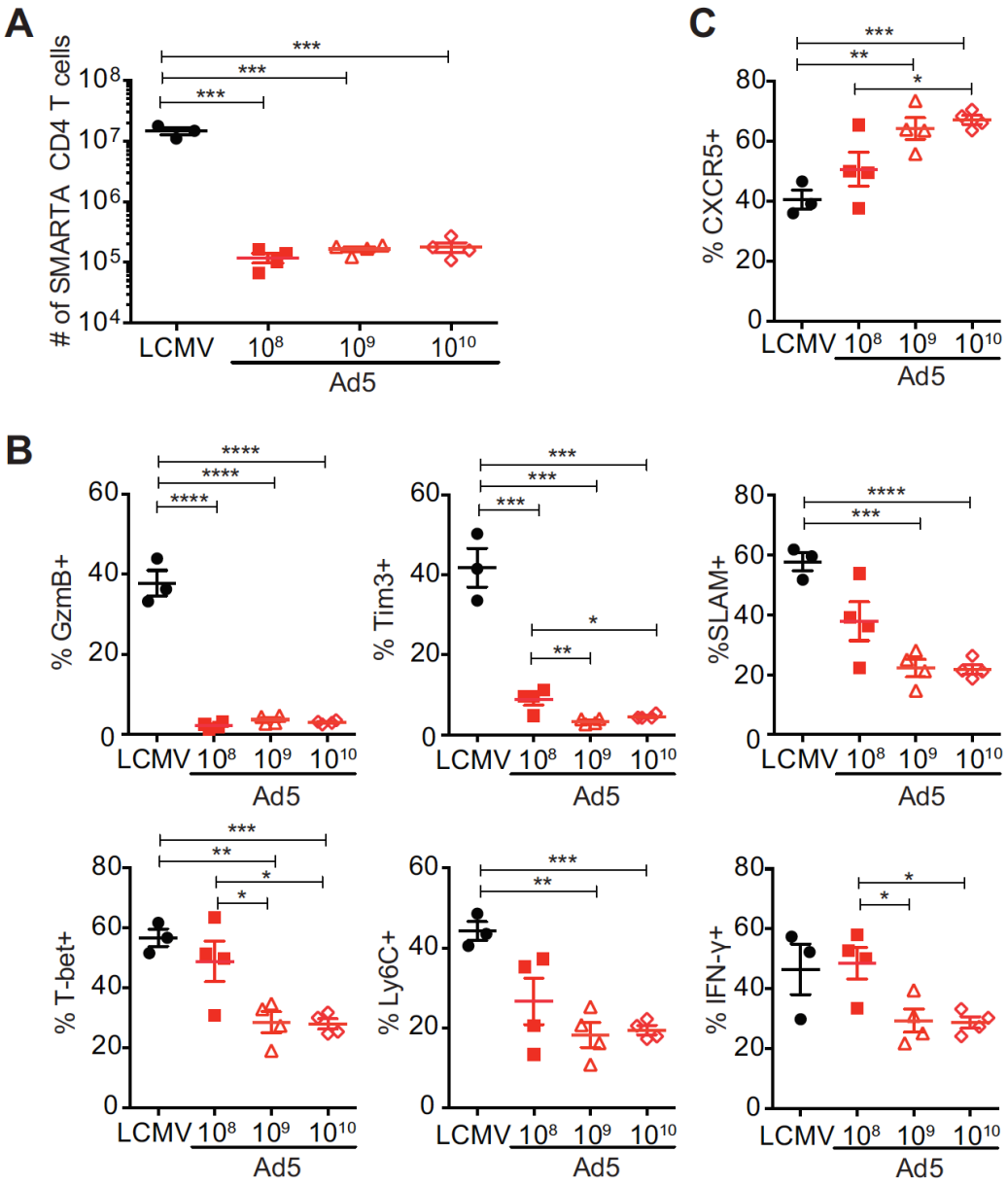


Figure 7. Regardless of the dose delivered, Ad5 vectors do not raise effective Th1 responses compared to those induced with LCMV infection. SMARTA chimeric mice were generated and immunized with 10^8, 10^9, or 10^{10} vp of Ad5 vector or infected with LCMV Armstrong. SMARTA CD4 T cells were analyzed in the spleen at day 8 post-immunization or infection. (A) The graph shows total number of SMARTA CD4 T cells. (B) The graphs demonstrate the frequency of Th1 cells (CXCR5- SMARTA CD4 T cells expressing granzyme B [GzmB], Tim3, SLAM, T-bet and Ly6C or SMARTA CD4 T cells producing IFN-γ after ex vivo stimulation with GP_{61-80} peptide). (C) The graph shows the frequency of CXCR5+ Tfh cells. Data are representative of 2 independent experiments with 4 mice per group per experiment. Error bars indicate SEM. *, $P < 0.05$. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

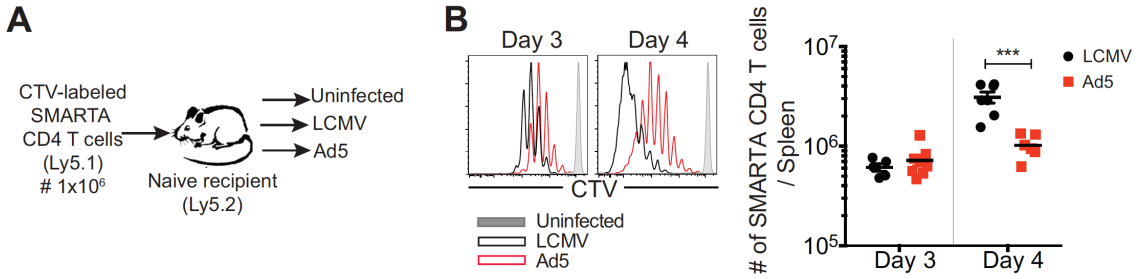


Figure 8. Ad5-elicited CD4 T cells are less proliferative than those induced by LCMV infection. Cell trace violet (CTV)-labeled SMARTA CD4 T cells ($CD45.1^+$) were transferred into C57BL/6 mice ($CD45.2^+$) that were subsequently immunized with Ad5-LCMV-GP vectors, infected with LCMV, or remained uninfected. 3 and 4 days later, proliferation of SMARTA CD4 T cells from the spleen was analyzed. (A) Experimental set up. (B, left) Proliferation of SMARTA CD4 T cells. Representative histograms gated on SMARTA CD4 T cells show the cell trace violet intensity distribution. (B, right) The graph shows absolute number of SMARTA CD4 T cells. Data are representative of 2 independent experiments with 2-4 mice per group per experiment. Error bars indicate SEM. ***, $P < 0.001$.

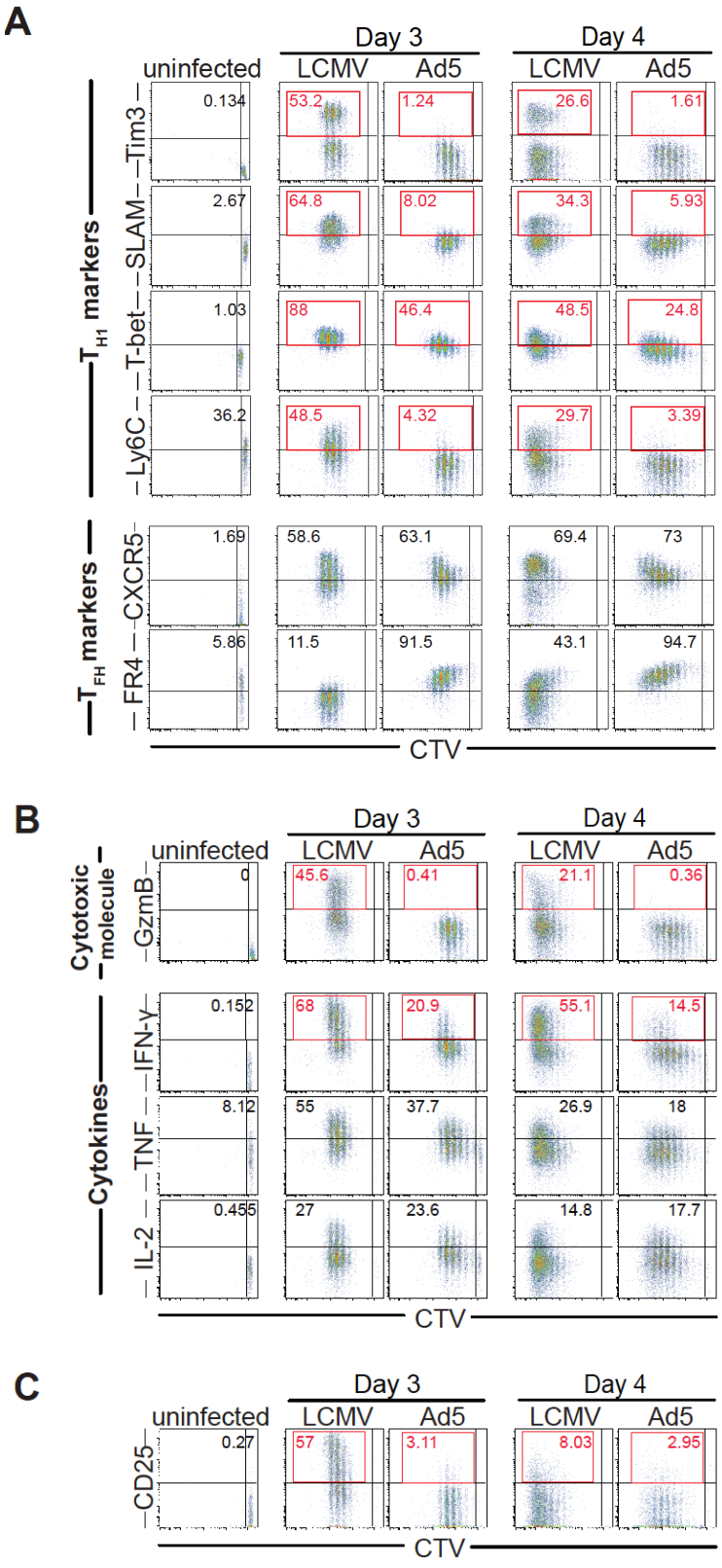

A
Day 3
Day 4
uninfected
LCMV
Ad5
LCMV
Ad5
Tim3
SLAM
T-bet
Ly6C
T_H1 markers
0.134
53.2
1.24
26.6
1.61
2.67
64.8
8.02
34.3
5.93
1.03
88
46.4
48.5
24.8
36.2
48.5
4.32
29.7
3.39
CXCR5
FR4
T_FH markers
1.69
58.6
63.1
69.4
73
5.86
11.5
91.5
43.1
94.7
CTV
B
Day 3
Day 4
uninfected
LCMV
Ad5
LCMV
Ad5
GzmB
Cytotoxic molecule
0
45.6
0.41
21.1
0.36
IFN-γ
TNF
IL-2
Cytokines
0.152
68
20.9
55.1
14.5
8.12
55
37.7
26.9
18
0.455
27
23.6
14.8
17.7
CTV
C
Day 3
Day 4
uninfected
LCMV
Ad5
LCMV
Ad5
CD25
0.27
57
3.11
8.03
2.95
CTV

Figure 9. Impaired Th1 commitment following Ad5 immunization. Same experimental set up as Figure 8. 3 or 4 days after Ad5 immunization or LCMV infection, differentiation of SMARTA CD4 T cells was analyzed in the spleen. Representative FACS plots gated on SMARTA CD4 T cells show the expression of Th1 and Tfh markers (A), cytotoxic molecule and cytokines (B), and CD25 (C), together with CTV dilution. The numbers in the flow plots indicate the percentages of cells corresponding to the upper right quadrant (uninfected) or the upper left quadrant (day 3 and 4). Data are representative of 2 independent experiments with 2-4 mice per group per experiment.

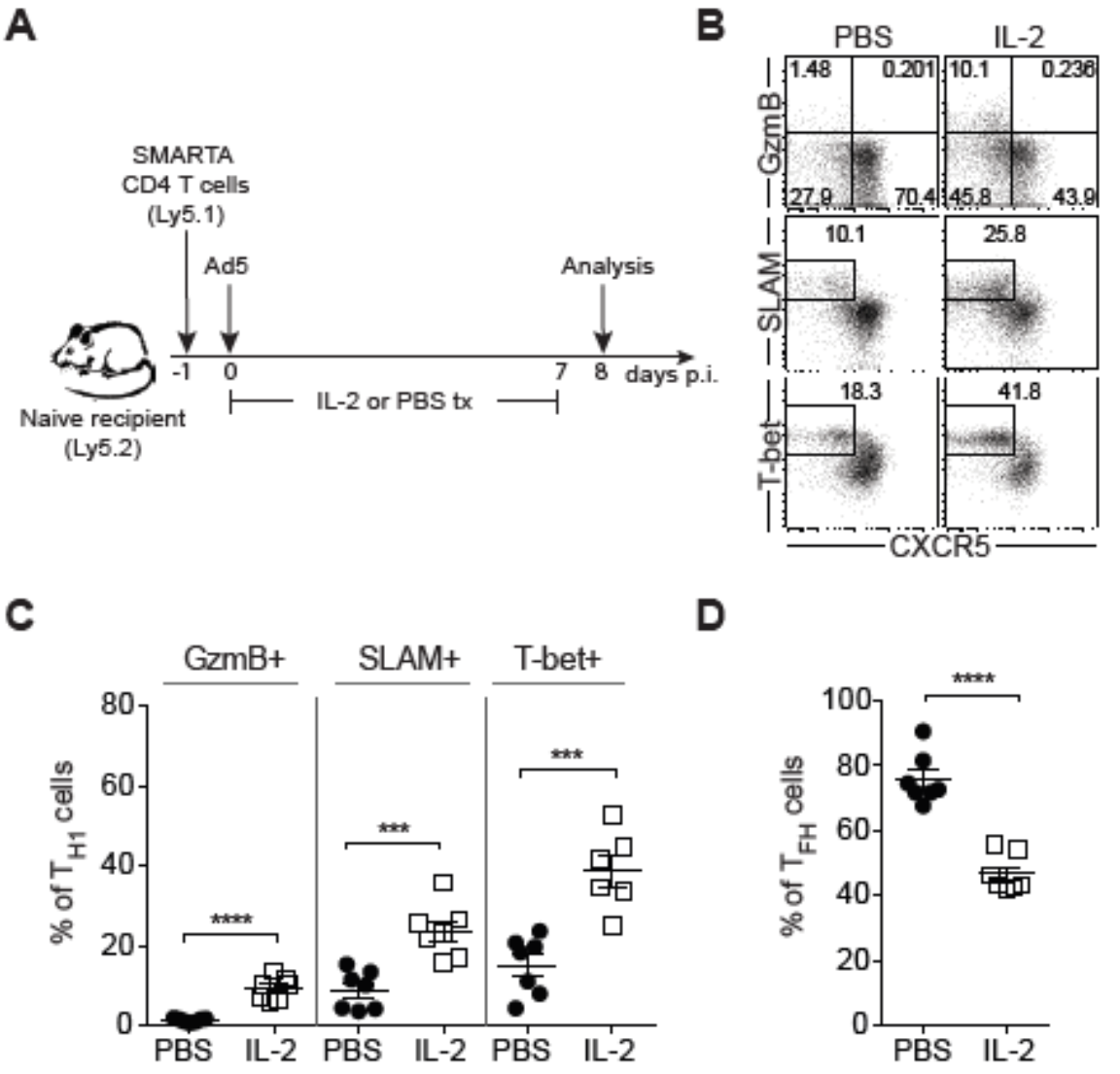


Figure 10. IL-2 administration following Ad5 immunization restores Th1 differentiation. (A) Experimental set up. SMARTA CD4 T cells were transferred into C57BL/6 mice that were subsequently immunized with Ad5 vectors. A dose of 15,000 IU of recombinant IL-2 or PBS was injected intraperitoneally (i.p.) into the mice every 12 h from the day of immunization and until day 7 post-immunization before sacrificed at day 8. (B) Representative FACS plots showing the phenotype of SMARTA CD4 T cells in the spleen. (C-D) The graph shows the frequency of Th1 (C) or Tfh (D) SMARTA CD4 T cells. Data are representative of 2 independent experiments with 3-4 mice per group per experiment. Error bars indicate SEM. ***$P < 0.001$; ****$P < 0.0001$.

Chapter 3: Differential effects of blocking PD-1 and CTLA-4 pathways in regulating virus-specific CD4 and CD8 T cells during chronic LCMV infection

I. Summary

Harnessing the immune system to control viral infections and cancer has resulted in successful outcomes. Two immunotherapeutic approaches that have been extensively studied to restore functional T cells are: (1) adoptive cell transfer and (2) blockade of inhibitory pathways. Adoptive transfer of T cells promotes endogenous immune responses but both are limited by expression of inhibitory receptors. Elevated expression of inhibitory receptors such as cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and programmed cell death-1 (PD-1) is a key feature of exhausted T cells in cancer as well as chronic viral infection. In this study, we address the differential effects of CTLA-4 or PD-1 pathway blockade on virus-specific CD4 and CD8 T cell responses in a adoptive transfer system during chronic lymphocytic choriomeningitis virus (LCMV) infection. Blocking either CTLA-4 or PD-L1 enhanced the functionality of transferred LCMV-specific CD4 T cells and promoted differentiation into CD4 T helper 1 (Th1) cells. However, in contrast to PD-L1 blockade, which had a pronounced effect in rescuing exhausted CD8 T cells in terms of both quantity and quality, CTLA-4 blockade combined with CD4 T cell transfer did not further promote LCMV-specific CD8 T cell responses or viral control compared to CD4 T cell transfer alone. Furthermore, PD-L1 blockade improved the efficacy of CD8 T cell adoptive transfer by enhancing the expansion and functionality of transferred CD8 T cells whereas CTLA-4 blockade had a

minimal effect in combination with CD8 T cell transfer. Theses results indicate that individual inhibitory receptors can differentially regulate virus-specific CD4 and CD8 T cell responses during persistent antigenic stimulation.

II. Introduction

T cell exhaustion or dysfunction is a common feature of chronic infections and cancer and has now become a key target of immunotherapy(70). Restoration of functional T cell responses can be achieved either by adoptive transfer of virus- or tumor-specific T cells or by reinvigoration of endogenous T cells by blockade of inhibitory signals in T cells(212, 213). Adoptive transfer involves the isolation, ex vivo manipulation such as expansion or activation, and reinfusion of autologous lymphocytes. Adoptive transfer therapy has been successfully used against viral infection during allogeneic hematopoietic stem cell transplantation and in some cancers(214). Adoptive immunotherapy has been more focused on CD8 T cells, predominantly because of their ability to directly kill virus-infected or cancer cells. However, CD4 T cell adoptive transfer therapy has also demonstrated significant efficacy(182); transfer of autologous CD4 T cells specific to tumor antigen resulted in regression or eradication of metastatic tumor (215, 216) and transfer of virus-specific CD4 T cells restored endogenous antiviral responses and substantially improved virus control during chronic lymphocytic choriomeningitis virus (LCMV) infection(49). CD4 T cells have also been shown to be required for the persistence of transferred virus-specific CD8 T cells in allogeneic bone marrow transplant recipients(183). During chronic infection, CD4 T cells are specially essential for maintaining CD8 T cell function and eventually leading to viral clearance. CD4 T cells coordinate various arms of immune systems, regulating innate immune responses as well as cellular and humoral immunity.

Another strategy is the blockade of immune checkpoints. T cells have intrinsic regulatory mechanisms that limit their effector functions. Activated T cells transiently

upregulate multiple inhibitory receptors that, upon ligation with respective ligands, deliver inhibitory signals to T cells to limit T cell activation. However, in the presence of persistent antigen stimulation, such as in chronic infection and cancer, elevated expression of the inhibitory receptors is maintained, resulting in an attenuation of T cell activation and effector function. To remove these inhibitory signals and restore T cell responses, antibodies that abrogate the interactions between inhibitory receptors and their ligands have been used (checkpoint inhibitors). Programmed cell death-1 (PD-1) is the best-known inhibitory receptor that plays a major role in regulating T cell exhaustion. PD-1 pathway blockade was first shown to restore the function of exhausted CD8 T cells in vivo during chronic LCMV infection(92). PD-1 pathway blockade also demonstrated significant efficacy for reducing tumor burden and controlling chronic viral infections in humans (95, 99, 134). Currently, PD-1 checkpoint inhibitors have been approved for use in cancer patients and various anti-PD-1 or PD-L1 antibodies are being evaluated in ongoing clinical trials for the treatment of many different types of cancers. Cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) blockade has also demonstrated significant antitumor activities and anti-human CTLA-4 antibody, Ipilimumab, was the first checkpoint blocking antibody to be approved for clinical use in metastatic melanoma (217, 218). Still, the mechanism underlying the action of CTLA-4 blockade remaines ill-defined, although the increased ratio of effector T cells to regulatory T cells (Tregs) in tumor-infiltrating lymphocytes is the commonly observed outcome following blockade of CTLA-4. In chronic infections, such as HIV and HCV, in vitro CTLA-4 blockade has been shown to enhance virus-specific CD4 and CD8 T cell responses (160, 161).

However, interestingly, in chronic LCMV infection, in vivo blockade of CTLA-4 had no significant effects on rescuing virus-specific CD8 T cells or reducing viral load(92).

Our previous study has shown that transfer of virus-specific CD4 T cells into mice with established chronic LCMV infection restored endogenous CD8 T cell and B cell responses, leading to a marked decrease in virus titer(49). Furthermore, PD-L1 blockade combined with CD4 T cell therapy enhanced the function of transferred CD4 T cells and this combination therapy had a synergistic effect in further augmenting the LCMV-specific CD8 T cell responses and viral clearance. Thus, checkpoint blockades are able to not only restore endogenous T cell responses but also enhance the function of the adoptively transferred T cells. Hence, there is a strong rationale for combining adoptive transfer therapy and checkpoint blockade to control persistence infection or eradicate tumor cells.

A study in a mouse melanoma model has demonstrated that adoptive transfer of tumor-reactive CD4 T cells exerted a superior antitumor activity in combination with CTLA-4 blockade, leading to complete rejection of established melanoma(186). In this study, CTLA-4 blockade increased the expansion of transferred CD4 T cells and reduced the accumulation of Tregs. Therefore, we wanted to examine whether CTLA-4 blockade can improve the efficacy of CD4 T cell transfer in chronic LCMV infection, by enhancing the expansion or functionality of transferred CD4 T cells and subsequently further promoting endogenous antiviral CD8 T cell and antibody responses. Previously, we did not observe any effect of CTLA-4 blockade on virus-specific CD8 T cells in chronic LCMV infection where specially cognate CD4 T cell help was lacking. Hence, we sought to study the effects of CTLA-4 blockade on LCMV-specific CD4 T cells in an

adoptive transfer model. We also wanted to determine whether CTLA-4 and PD-1 inhibitory pathways have differential roles in regulating virus-specific CD4 versus CD8 T cells. We found that while both CTLA-4 and PD-L1 blockade enhanced LCMV-specific CD4 T cell responses, the effects of these checkpoint inhibitors differed with regards to restoration of CD8 T cell responses. We show that in contrast to PD-L1 blockade, CTLA-4 blockade had only minimal effects in restoring virus-specific CD8 T cell responses during chronic LCMV infection.

III. Results

Either CTLA-4 or PD-L1 blockade partially rescues CD4 T helper 1 (Th1) development in established chronic infection. For CD4 T cell adoptive transfer model, we utilized SMARTA TCR transgenic cells specific for the major histocompatibility complex (MHC) class II-restricted epitope of LCMV GP_{66-77}. Recipient mice were chronically infected mice (at least >45 days post-infection) with no antigen-specific CD4 T cell help due to transient depletion of CD4 T cells prior to LCMV CL-13 infection(47). In this chronic infection model, CD4 T cells recover to normal numbers after a few weeks, but mice lack antigen-specific CD4 T cells, fail to mount a humoral response and have severely exhausted CD8 T cells, and thus become life-long carriers with uncontrolled viremia. We transferred naïve SMARTA CD4 T cells into chronically infected mice, followed by treatment with anti-CTLA-4, anti-PD-L1 blocking antibodies or PBS, starting on the day of transfer, every 3rd day for 2 weeks (Fig. 1A). To examine the kinetics of SMARTA CD4 T cells, we tracked the congenically marked SMARTA CD4 T cells in blood. When transferred into chronically infected hosts, SMARTA CD4 T cells expanded with a peak response between 1-2 weeks post-transfer and then contracted and persisted (Fig. 1B)(49). Blocking CTLA-4 or PD-L1 enhanced expansion of SMARTA CD4 T cells, resulting in approximately 2-fold higher number of SMARTA CD4 T cells at day 8 post-transfer compared to PBS treated recipients (Fig. 1B and C). Following either CTLA-4 or PD-L1 blockade, SMARTA CD4 T cells declined to a similar level to PBS treated group by day 15 post-transfer, and were maintained thereafter (Fig. 1B). In contrast to our findings in the blood, the number of SMARTA CD4 T cells in the spleen 8 days post-transfer was not significantly affected by CTLA-4 or PD-L1

blockade, albeit a slightly higher number of SMARTA CD4 T cells was found after blocking either CTLA-4 or PD-L1 (Fig. 1D). Similar to the spleen, in the lung and liver, there was a trend towards elevated numbers of SMARTA CD4 T cells following CTLA-4 and/or PD-L1 blockade (data not shown).

Next, we further investigated how transferred CD4 T cells differentiated in the host with established chronic infection by characterizing their phenotype and functionality. It has been described that chronic viral infection favors T follicular helper (Tfh) differentiation over Th1(52). Specially when CD4 T cells are primed in an established chronic infection, Th1 development is suppressed(53, 58). Consistently with these findings, we also observed that SMARTA CD4 T cells transferred into chronically infected mice exhibited defective Th1 differentiation. 60h post-transfer (Supplemental Fig. 1A), we found low expression of CD25 and Th1 markers such as granzyme B, Tim3, Ly6C and T-bet in SMARTA CD4 T cells transferred to chronically infected mice (Supplemental Fig. 1B). In contrast, SMARTA CD4 T cells substantially upregulated Th1-associated molecules 60h after acute LCMV infection. On the other hand, CXCR5 expression was similar in SMARTA CD4 T cells during chronic and acute LCMV infection. This impaired Th1 commitment in established chronic infection led to significantly reduced Th1 responses at day 8 post-priming while CD4 T cells following acute infection generated both effective Th1 and Tfh responses (Supplemental Fig. 1C)

At day 8 post-transfer, SMARTA CD4 T cells transferred into chronically infected mice (PBS treated recipients) showed a very small Th1 population expressing Tim3, SLAM, Ly6C or T-bet, whereas cells with Tfh phenotype developed in high frequency (Fig. 2A, B and C). However, blocking CTLA-4 or PD-L1 increased the

frequencies and total numbers of Th1 cells, even though Tfh cells were not significantly affected by either CTLA-4 or PD-L1 blockade. In addition, blocking CTLA-4 or PD-L1 substantially raised the frequency and number of SMARTA CD4 T cells expressing the cytotoxic molecule, granzyme B and producing IFNγ, also indicating enhanced Th1 differentiation and functionality (Fig. 2D and E). More effective Th1 responses upon CTLA-4 or PD-L1 blockade were also found in nonlymphoid organs. Expression of granzyme B (Fig. 3A and B) and SLAM (data not shown) was elevated in SMARTA CD4 T cells following CTLA-4 blockade in the lung and following CTLA-4 and PD-L1 blockade blockades in the liver. Therefore, these results show that combining SMARTA CD4 T cell transfer with either CTLA-4 or PD-L1 blockade augmented differentiation of Th1 cells in established chronic infection.

FoxP3+ Tregs remain unchanged by SMARTA CD4 T cell transfer and CTLA-4 or PD-L1 blockade during chronic infection. FoxP3+ Tregs play a critical role in T cell exhaustion and chronic infection(219). In addition, CTLA-4 and PD-1-mediated signals have been shown to play an important role in regulating Tregs(211, 220, 221). Hence we wanted to determine whether SMARTA CD4 T cells could differentiate into Tregs and whether it would be modulated by CTLA-4 or PD-L1 blockade. SMARTA CD4 T cells showed minimal expression of FoxP3 after adoptive transfer into mice with established chronic infection, and there was no effect of CTLA-4 or PD-L1 blockade (Fig. 4A). Likewise the number of endogenous Tregs was not significantly different in mice receiving SMARTA CD4 T cells with or without CTLA-4 or PD-L1 blocking antibodies, compared to untreated mice (Fig. 4A and B). Therefore, adoptive transfer of

CD4 T cells and the combination therapy with either CTLA-4 or PD-L1 blockade did not have an impact on Tregs in our model.

Unlike PD-L1 blockade, CTLA-4 blockade does not enhance the effect of SMARTA CD4 T cell transfer on endogenous LCMV-specific CD8 T cell responses. Next, we examined whether the combination therapy of SMARTA CD4 T cell transfer and blockade of CTLA-4 or PD-L1 has an additive or synergistic effect on rescuing exhausted virus-specific CD8 T cells during chronic infection. In chronically infected mice (untreated), the frequency of GP33-specific CD8 T cells was very low and hardly detected in the blood (Fig. 5A and B). Following SMARTA CD4 T cell transfer, an increase in the number of endogenous GP33-specific CD8 T cells was detected as early as day 8 post-transfer. After the peak response at day 14, GP33-specific CD8 T cells contracted but remained elevated when compared to untreated mice for the time course examined. Consistent with our previous findings, combination of SMARTA CD4 T cell transfer with PD-L1 blockade resulted in a substantial increase in GP33-specific CD8 T cells compared to SMARTA CD4 T cell transfer. In contrast, CTLA-4 blockade did not further increase virus-specific CD8 T cells compared to SMARTA CD4 T cell transfer alone, showing similar frequencies and kinetics of GP33-specific CD8 T cells.

Increased virus-specific CD8 T cells were also found in the spleen 8 days following SMARTA CD4 T cell transfer and PD-L1 blockade further increased GP33-specific CD8 T cells (Fig. 6A). However, combining CTLA-4 blockade to SMARTA CD4 T cells transfer did not show a significant effect on further promoting GP33-specific CD8 T cell expansion. Similar trends were also observed when GP276-specific CD8 T cells were analyzed in the spleen (Fig. 6B). Likewise, in the lung, GP33-specific CD8 T

cells were further elevated by SMARTA CD4 T cell transfer in combination with PD-L1 blockade but not with CTLA-4 blockade (Fig. 6C). To assess the functionality of LCMV-specific CD8 T cells following combination therapies, IFNγ production was measured after ex vivo restimulation. Transfer of SMARTA CD4 T cells increased the number of IFNγ-secreting CD8 T cells which was further augmented by combination with PD-L1 blockade (Fig. 6D). However, CTLA-4 blockade combination had no significant effects on further improving cytokine production by LCMV-specific CD8 T cells, compared to SMARTA CD4 T cell transfer alone. Therefore, in contrast to PD-L1 blockade, combination of CTLA-4 blockade to SMARTA CD4 T cell transfer did not have a significant effect on further promoting CD8 T cell responses compared to SMARTA CD4 T cell transfer alone.

SMARTA CD4 T cell transfer combined with PD-L1 blockade, but not with CTLA-4 blockade, has a synergistic effect on reducing viral load in chronic infection. We finally determined whether these combination therapies further improved viral control. Virus titer from serum and multiple tissues was measured at day 14 post-transfer. Mice receiving SMARTA CD4 T cells significantly reduced serum virus titer by day 14 post-transfer (Fig. 7). Combined SMARTA CD4 T cell transfer and CTLA-4 blockade didn't have a further effect on viral load compared to SMARTA CD4 T cell transfer alone whereas PD-L1 blockade led to a further decrease in virus titer. Similar results were observed in the spleen, lung (Fig. 7) and liver (data not shown). The combination therapy of SMARTA CD4 T cell transfer and PD-L1 blockade already showed a significant reduction in viral load at day 8 post-transfer compared to other groups while SMARTA CD4 T cell transfer with or without CTLA-4 blockade

maintained a similar level of virus titer to that of untreated mice (data not shown). Therefore, although CTLA-4 blockade augmented the functionality of SMARTA CD4 T cells and induced more effective Th1 responses, it did not lead to further enhancement of virus-specific CD8 T cell responses and virus control. In contrast, SMARTA CD4 T cell transfer combined with PD-L1 blockade resulted in a synergistic effect on the rescue of CD8 T cell responses, resulting in faster and more significant viral clearance.

CTLA-4 blockade does not improve the efficacy of CD8 T cell adoptive transfer whereas PD-L1 blockade enhances the responses of transferred CD8 T cells during chronic infection. Single blockade of CTLA-4 or CTLA-4 blockade combined with SMARTA CD4 T cell transfer did not have an impact on the rescue of exhausted LCMV-specific CD8 T cells in chronic infection. Here, we combined CD8 T cell transfer and CTLA-4 blockade and examined whether CTLA-4 blockade has any effect on naïve CD8 T cells that differentiate in an established chronic infection. For comparison, PD-L1 blockade was also combined with CD8 T cell adoptive transfer. We transferred P14 CD8 T cells specific to the GP_{33-41} epitope of LCMV into chronically infected mice that were subsequently treated with CTLA-4 or PD-L1 blocking antibodies (Fig. 8A). We first monitored P14 CD8 T cells in the blood. Blocking CTLA-4 did not have any effects on P14 CD8 T cell expansion, but PD-L1 blockade resulted in a huge expansion of P14 CD8 T cells with the peak response at day 8 (Fig. 8B and C). Following blockade of PD-L1, a much higher number of P14 CD8 T cells was also found in the tissues, such as spleen (Fig. 8D) and lung (data not shown) whereas CTLA-4 blockade led to a slight or minimal increase in P14 CD8 T cells for the time course we examined.

When transferred into chronically infected hosts, P14 CD8 T cells expressed high-level of PD-1, Tim3 and Eome (data not shown) but were able to produce IFNγ and expressed granzyme B (Fig. 9A to D). CTLA-4 blockade did not increase cytokine production or granzyme B expression of P14 CD8 T cells. In contrast, after PD-L1 blockade, a significantly higher proportion of P14 CD8 T cells produced IFNγ. Polyfunctionality of P14 CD8 T cells was greatly enhanced by blocking PD-L1, generating higher numbers of P14 CD8 T cells secreting both IFNγ and TNF or IL-2. Likewise, granzyme B expression was also upregulated in P14 CD8 T cells after PD-L1 blockade. In addition, PD-L1 blockade increased T-bet expression and decreased Eomes in P14 CD8 T cells (data not shown). Therefore, P14 CD8 T cells had enhanced functionality and less exhausted phenotype following PD-L1 blockade. Consistent with enhanced P14 CD8 T cell responses, there was a significant decrease in viral load following PD-L1 blockade by day 8 post-transfer compared to mice receiving P14 CD8 T cells with or without CTLA-4 blockade (Fig. 9E). In contrast, but consistent with our data, we found no significant difference in virus titer when CTLA-4 blockade was combined to P14 transfer . Therefore, CTLA-4 blockade did not enhance the efficacy of CD8 T cell adoptive transfer whereas PD-L1 blockade significantly improved transferred CD8 T cell responses in terms of both expansion and functionality, and also promoted viral control during chronic infection.

IV. Discussion

When SMARTA CD4 T cells differentiated in established chronic infection, generation of Th1 cells was inhibited. Either CTLA-4 or PD-L1 blockade partially rescued this inhibition, leading to generation of a higher number of Th1 cells compared to SMARTA CD4 T cell transfer alone. We had previously shown that blocking CTLA-4 has no direct effects on virus-specific CD8 T cells in chronic LCMV infection where antigen-specific CD4 T cell help was absent, but in this study, we show that CTLA-4 blockade works on CD4 T cells, resulting in more effective Th1 responses. However, combined SMARTA CD4 T cell transfer and CTLA-4 blockade did not have a significant effect on further recovering exhausted CD8 T cells or reducing virus titer compared to SMARTA CD4 T cell transfer alone, showing no correlation between enhanced Th1 responses and endogenous CD8 T cell responses or virus control. It is possibly because the increase in Th1 cells was significant but moderate, which did not result in providing optimal help to CD8 T cells or exerting cytotoxic activities. Th1 suppression during viral persistence is probably mediated by multiple mechanisms among which we found that both CTLA-4 and PD-1 pathways play a role in this inhibition. A recent paper has also demonstrated that elevated expression of PD-L1 and IL-10 in suppressive dendritic cells is responsible for impeded Th1 generation during persistent infection, by showing the restoration of Th1 cells following simultaneous blockade of PD-L1 and IL-10(58). Another possibility is that Tfh cells generated during viral persistence provide help to CD8 T cells, for example by secreting IL-21 which plays a role in sustaining virus-specific CD8 T cells during chronic infection (61-63). In contrast to CTLA-4 blockade, combined PD-L1 blockade further elevated LCMV-specific CD8 T cells as well as

promoting Th1 generation. This is probably because PD-1 pathway blockade itself directly acts on reversing exhausted CD8 T cells, considering the result of combined SMARTA CD4 T cell transfer and CTLA-4 blockade therapy. We observed that blocking CTLA-4 or PD-1 pathway increased Th1 responses when naïve CD4 T cells differentiated during established chronic infection. Whether during chronic LCMV infection, these treatments can rescue already exhausted CD4 T cells and result in the restoration of Th1 responses needs to be further investigated.

In this study, we further characterized SMARTA CD4 T cells following transfer into chronically infected recipients. The majority of CD4 T cells (60-80%) differentiated into Tfh cells and a very small population (less than 10%) represented Th1 cells. Transfer of SMARTA CD4 T cells promoted antibody responses (data not shown)(49), indicating that Tfh cells developed during virus persistence played a role in helping B cells. In addition, CD4 T cells substantially enhanced endogenous virus-specific CD8 T cell responses, but whether Th1 and/or Tfh subsets contribute to CD8 T cell rescue remains unclear and requires further studies.

CTLA-4 blockade had no significant effects on Tfh responses. On the other hand, both Tfh cells and germinal center (GC) B cells were decreased upon PD-L1 blockade at day 14 post-transfer (Supplemental Fig. 2). The lower number of GC B cells, but not Tfh cells, was also observed at day 8 post-transfer, following PD-L1 blockade (data not shown). Reduced GC B cells due to the blockade of PD-1 pathway might limit Tfh cells(222, 223). This is also possibly because of decreased virus titer following PD-L1 blockade.

Antitumor effects of CTLA-4 blockade have been shown to be also mediated by depleting intratumoral Tregs through a Fc γ receptor (FcγR)-dependent mechanism(156, 157). We also assessed Tregs following SMARTA CD4 T cell transfer with or without checkpoint blockade but found no significant change in the number of Tregs.

Our results have shown that both CTLA-4 and PD-L1 blockade effectively enhanced LCMV-specific CD4 Th1 responses. However, CTLA-4 blockade had a minimal effect on CD8 T cells whereas PD-L1 blockade was more pronounced on promoting virus-specific CD8 responses in chronic LCMV infection. Eventually SMARTA CD4 T cell transfer combined with PD-L1 blockade resulted in significant viral load reduction, correlated with enhanced virus-specific CD8 T cell responses. In this model, virus control correlated with virus-specific CD8 T cells, but not with Th1 responses or antibody responses. Additionally, we performed CD8 T cell adoptive transfer in combination with either CTLA-4 or PD-L1 blockade in chronic LCMV infection. CTLA-4 blockade had a slight or minimal effect on promoting P14 CD8 T cells and virus control whereas PD-L1 blockade had a marked effect on enhancing P14 CD8 T cells, leading to substantial reduction in viral load. This result further confirms that CTLA-4 blockade is not effective in augmenting naïve CD8 T cells differentiated in established persistent infection as well as already exhausted CD8 T cells. Distinct effects of CTLA-4 blockade on virus-specific CD4 versus CD8 T cells might be related to different levels of CTLA-4 expression in CD4 and CD8 T cells following adoptive transfer. At day 8 after SMARTA transfer, around 50-60% of SMARTA CD4 T cells expressed CTLA-4 whereas ~20% of GP33-specific CD8 T cells expressed CTLA-4. Transcriptional analysis has shown that CTLA-4 expression is higher in virus-specific

CD4 T cells than in virus-specific CD8 T cells, referred as a CD4 T cell-biased inhibitory molecule (76). On the other hand, almost all of SMARTA CD4 T cells and LCMV-specific CD8 T cells upregulated PD-1 at day 8 post-transfer. Our results indicate that individual receptors can play differential roles in regulating CD4 and CD8 T cell responses during persistent antigenic stimulation.

V. Materials and Method

Mice and infections Six- to eight-week-old female C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). SMARTA mice bearing the transgenic T cell receptor (TCR) specific to the GP_{66-77} epitope of LCMV (210) were bred in house on a C57BL/6 background. P14 mice bearing transgenic TCR specific to the GP_{33-41} epitope of LCMV were bred in house on a C57BL/6 background. Mice were infected with 2 x 10^6 plaque-forming unit (pfu) of LCMV clone 13 intravenously (i.v.). Transient CD4 T cell depletion was performed at the onset of infection, by injecting anti-CD4 depleting antibodies intraperitoneally (i.p.) at day -2 or day 0 (the day of infection). For acute LCMV infection, mice were infected with 2 x 10^5 pfu of LCMV Armstrong strain i.p. for day 8 analysis and with 2 x 10^6 pfu of Armstrong i.v. for 60h analysis. All experiments were conducted in accordance with the Emory University Institutional Animal Care and Use committee guidelines.

Cell transfer For CD4 T cell adoptive transfer, SMARTA CD4 T cells were isolated from the spleens of naïve SMARTA mice by using a CD4+ T cell isolation kit (Miltenyi Biotech, San Diego, CA). 2~4 x 10^6 purified SMARTA CD4 T cells were transferred i.v. into chronically infected mice (at least >day 45 post-infection). For early time point experiments, 1 x 10^6 purified SMARTA CD4 T cells were labeled with cell trace violet (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and then transferred to chronically infected mice, or uninfected C57BL/6 mice that were followed with infection with Armstrong or remained uninfected. For the comparison of CD4 T cell differentiation between chronic and acute infection at day 8 post-priming, 1 x 10^3 purified SMARTA CD4 T cells were transferred into chronically infected mice or

uninfected C57BL/6 mice that were followed with Armstrong infection. For CD8 T cell adoptive transfer, P14 cells were isolated from the spleens of naïve P14 mice by using a CD8+ T cell isolation kit (Miltenyi Biotech) and 1 x 10^4 purified P14 CD8 T cells were transferred to chronically infected mice (at least >day 45 post-infection).

In vivo antibody blockade 200 microgram of hamster anti-mouse CTLA-4 antibodies (9H10, BioXcell) or rat anti-mouse PD-L1 antibodies (10F.9G2) was administered i.p. from the day of transfer (day 0), every 3rd day, total 5 times. The mice that received SMARTA CD4 T cells alone were treated with PBS.

Antibodies and flow cytometry All antibodies were purchased from BD Biosciences (San Jose, CA) except for CD45.1 (Biolegend, San Diego, CA), granzyme B (Invitrogen), T-bet (eBiosciences, San Diego, CA), Tim3 (R&D Systems, Minneapolis, MN) and PNA (Vector Laboratories). CXCR5 staining was performed using a three-step staining protocol as described previously (201). Transcription factors were stained using the FoxP3/transcription factor staining buffer set (eBiosciences). MHC class I tetramers were prepared and used as previously described(211). Intracellular cytokine staining was performed after 5h stimulation with GP_{61-80} peptide or a LCMV peptide pool consisting of GP33-41, GP276-286, GP92-101, NP205-212, NP235-249, and NP396-404 as described previously (211). Dead cells were excluded by using Live/Dead fixable dead cell stain kits (Invitrogen). Samples were acquired using a FACSCanto II or LSR II flow cytometer (BD Biosciences). Flow cytometry data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Measurement of virus titer Viral titer was determined by plaque assay on Vero cells as previously described(224).

Statistical analysis Data were analyzed using Prism 6 software (GraphPad, La Jolla, CA). Statistical significance was determined by using two-tailed unpaired Student's t-tests. P values of less than 0.05 was considered statistically significant.

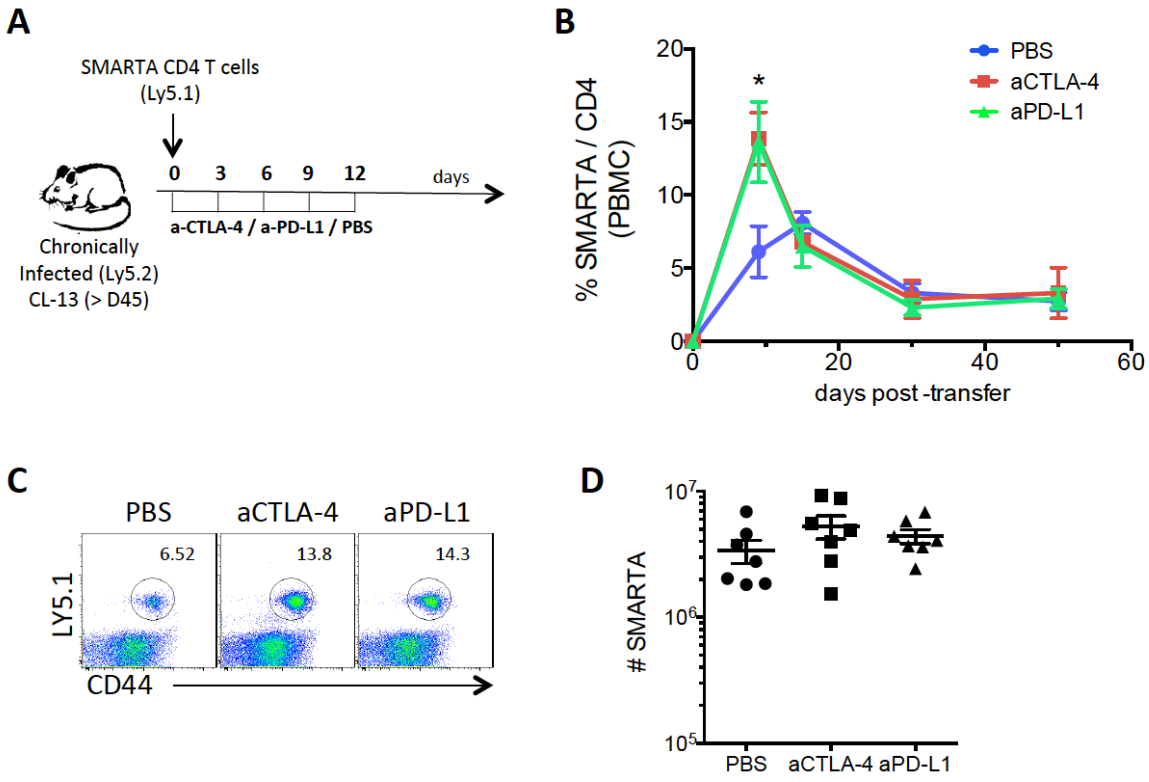


Figure 1. Expansion of SMARTA CD4 T cells in chronically infected mice following combination with CTLA-4 or PD-L1 blockade. (A) Experimental setup. (B) The graph shows the frequency of SMARTA CD4 T cells in blood. (C) Representative fluorescence activated cell sorting (FACS) plots show the frequency of SMARTA CD4 T cells in peripheral blood CD4 T cells at day 8 post-transfer (D) The graph shows the number of SMARTA CD4 T cells in the spleen at day 8 post-transfer. Data are representative of 2 independent experiments with 4-6 mice per group per experiment. Error bars indicate standard errors of means (SEM).

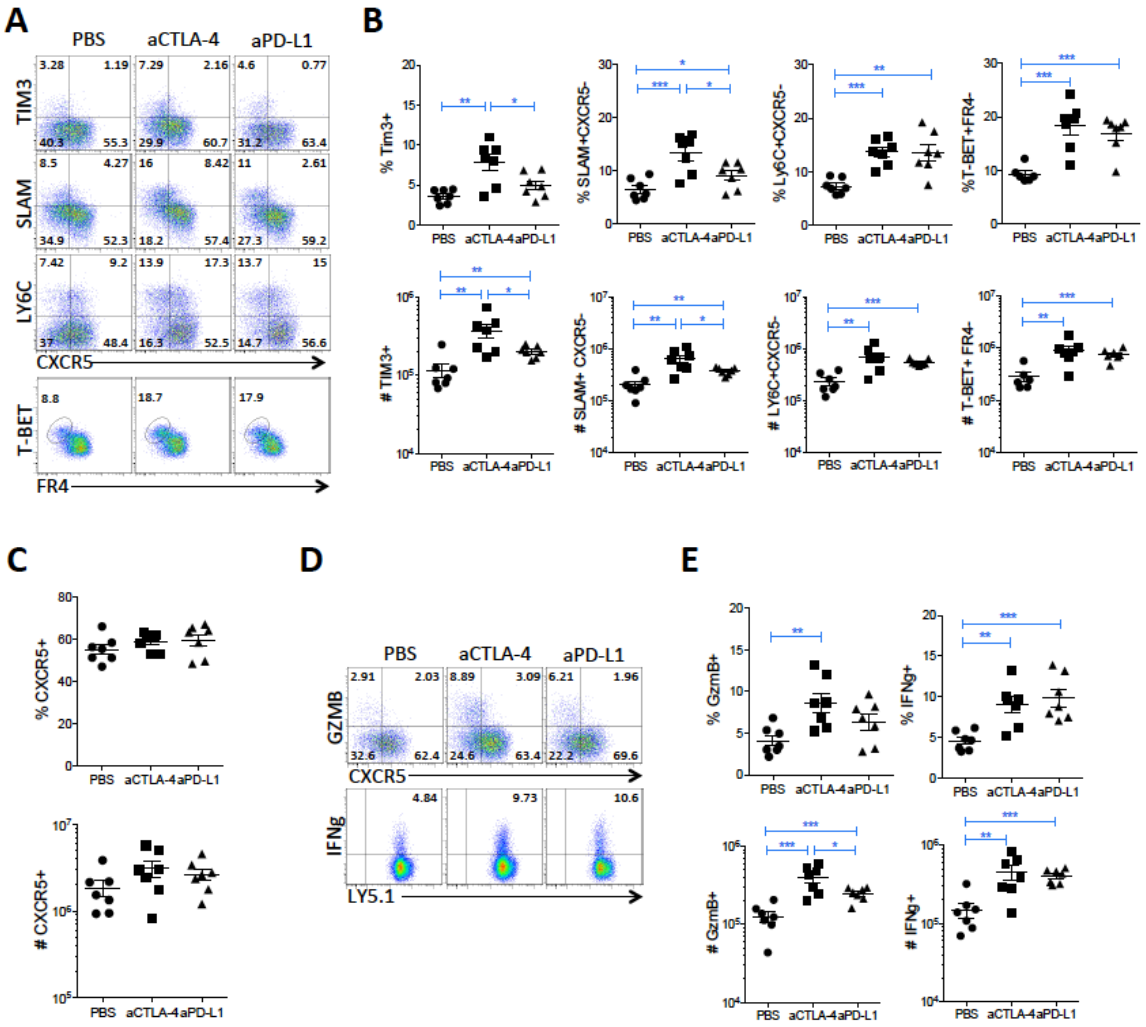


Figure 2. Either CTLA-4 or PD-L1 blockade partially rescues Th1 differentiation during established chronic infection. Same experimental set up as Figure 1. SMARTA CD4 T cells were analyzed in the spleen at day 8 post-transfer. (A) Representative FACS plots show the phenotype of SMARTA CD4 T cells. (B) Graphs show the frequency (upper row) and number (bottom row) of SMARTA CD4 T cells expressing Th1 markers (Tim3, SLAM, Ly6C, T-bet). (C) Graphs show the frequency (upper row) and number (bottom row) of SMARTA CD4 T cells expressing a Tfh marker, CXCR5. (D) Representative FACS plots show granzyme B (GzmB) expression and IFNγ production of SMARTA CD4 T cells after ex vivo stimulation with $GP_{61\text{-}80}$ peptide. (E) Graphs show the frequency (upper row) and number (bottom row) of SMARTA CD4 T cells expressing granzyme B or producing IFNγ. Data are representative of 2 independent experiments with 4-6 mice per group per experiment. Error bars indicate standard errors of means (SEM). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

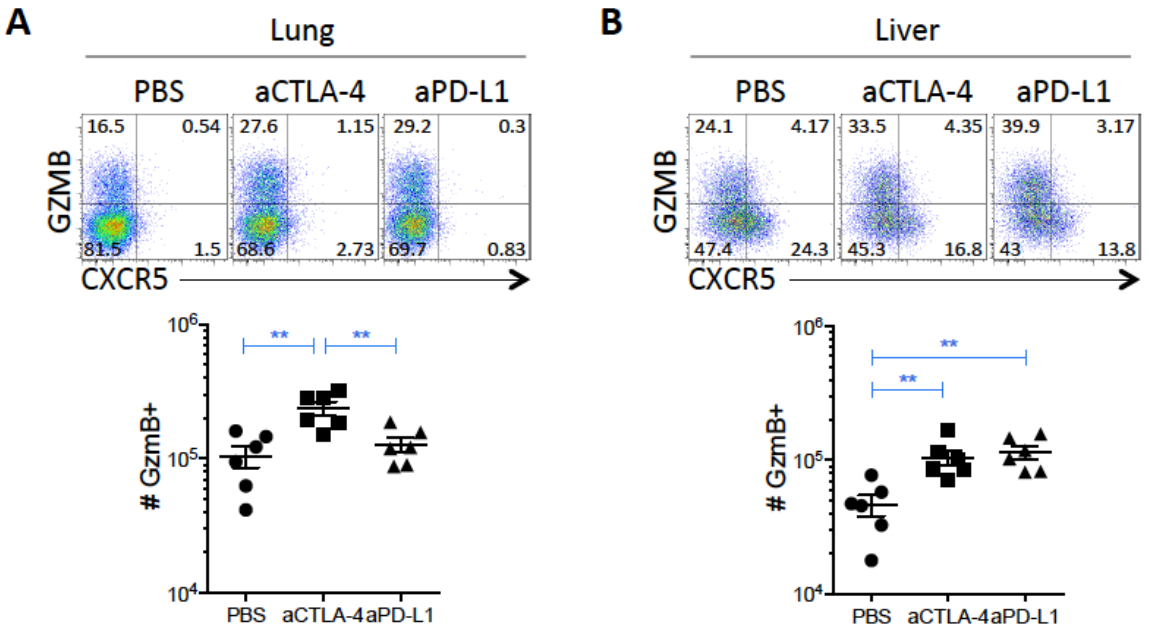


Figure 3. Elevated Th1 responses in nonlymphoid tissues following CTLA-4 or PD-L1 blockade. Same experimetal set up as Figure 1. Analysis was performed at day 8 post-transfer. Representative FACS plots show granzyme B and CXCR5 expression on SMARTA CD4 T cells in the lung (A) and liver (B). Data are representative of 2 independent experiments with 4-6 mice per group per experiment. Error bars indicate standard errors of means (SEM). $^{**}P < 0.01$.

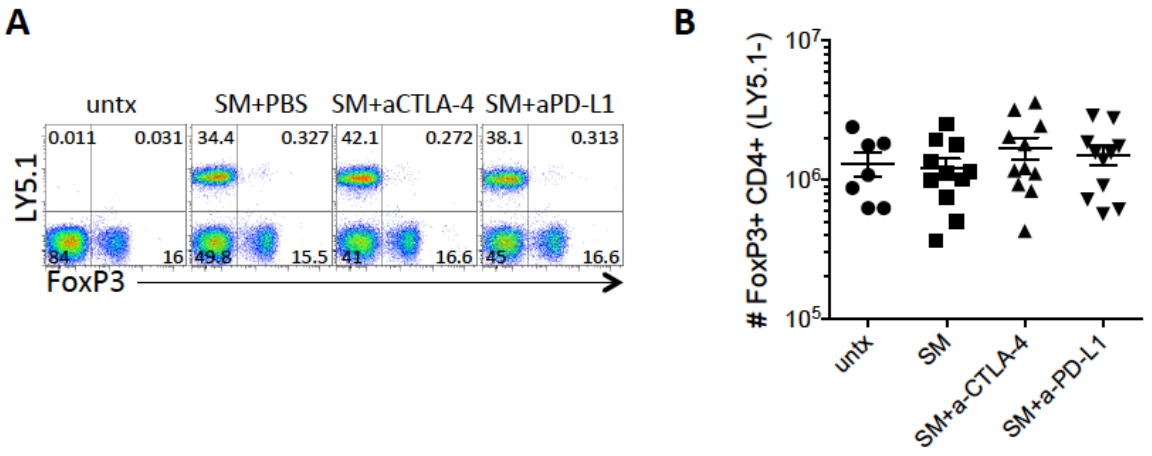


Figure 4. FoxP3+ Tregs remain unchanged during chronic infection after SMARTA CD4 T cell transfer or combined CTLA-4 or PD-L1 blockade. Same experimental set up as Figure 1. Analysis was performed in the spleen 8 days after transfer. (A) Representative FACS plots show FoxP3 and Ly5.1 expression in CD4 T cells. (B) The graph shows the number of endogenous (Ly5.1-) FoxP3+ Tregs. Data are representative of 2 independent experiments with 4-5 mice per group per experiment. Error bars indicate standard errors of means (SEM).

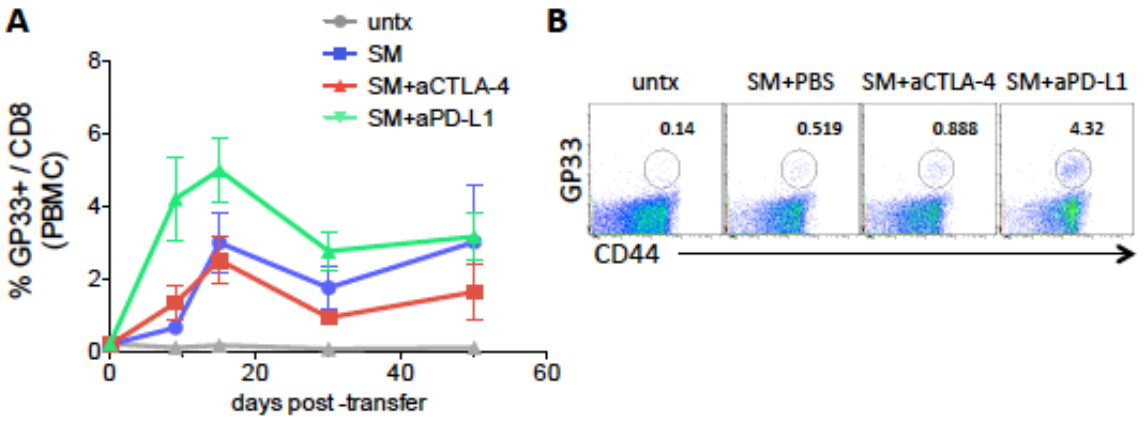


Figure 5. Expansion of endogenous LCMV-specific CD8 T cells following SMARTA CD4 T cell transfer with or without CTLA-4 or PD-L1 blockade. Same experimental set up as Figure 1. (A) Kinetics of GP33-specific CD8 T cells in blood. (B) Representative FACS plots show the frequency of GP33-specific CD8 T cells in peripheral blood at day 8 post-transfer. Data are representative of 2 independent experiments with 4-6 mice per group per experiment. Error bars indicate standard errors of means (SEM). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

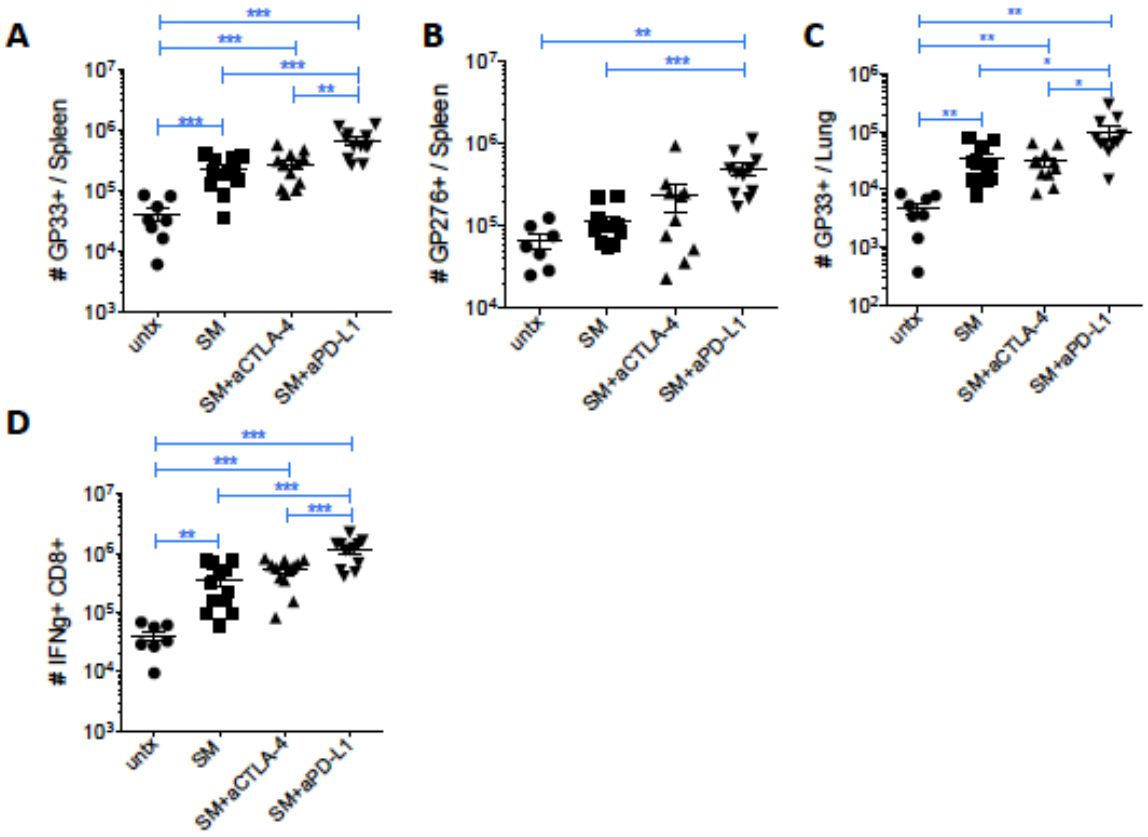


Figure 6. Endogenous LCMV-specific CD8 T cell responses are significantly enhanced with PD-L1 blockade but not with CTLA-4 blockade compared to SMARTA CD4 T cell transfer alone. Same experimental set up as Figure 1. (A-C) The graphs show the number of GP33- or GP276- specific CD8 T cells in the indicated tissues. (D) The graph shows the number of CD8 T cells producing IFNγ after ex vivo stimulation with a pool of LCMV peptides consisting of GP_{33-41}, $GP_{276-286}$, $NP_{205-212}$, $NP_{235-249}$, and $NP_{396-404}$. Data are representative of 2 independent experiments with 4-6 mice per group per experiment. Error bars indicate standard errors of means (SEM). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

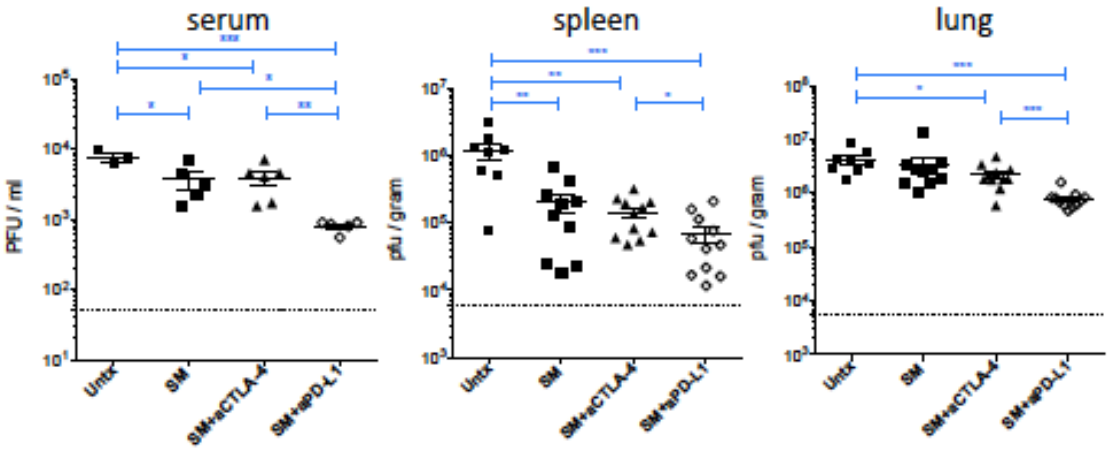


Figure 7. SMARTA CD4 T cell transfer combined with PD-L1 blockade, but not with CTLA-4 blockade has a synergistic effect on reducing viral load. Same experimental set up as Figure 1. Virus titer was measured from serum, spleen or lung at day 14 post-transfer. PFU= plaque forming units. Data are representative of 2 independent experiments with 4-5 mice per group per experiment. Error bars indicate standard errors of means (SEM). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

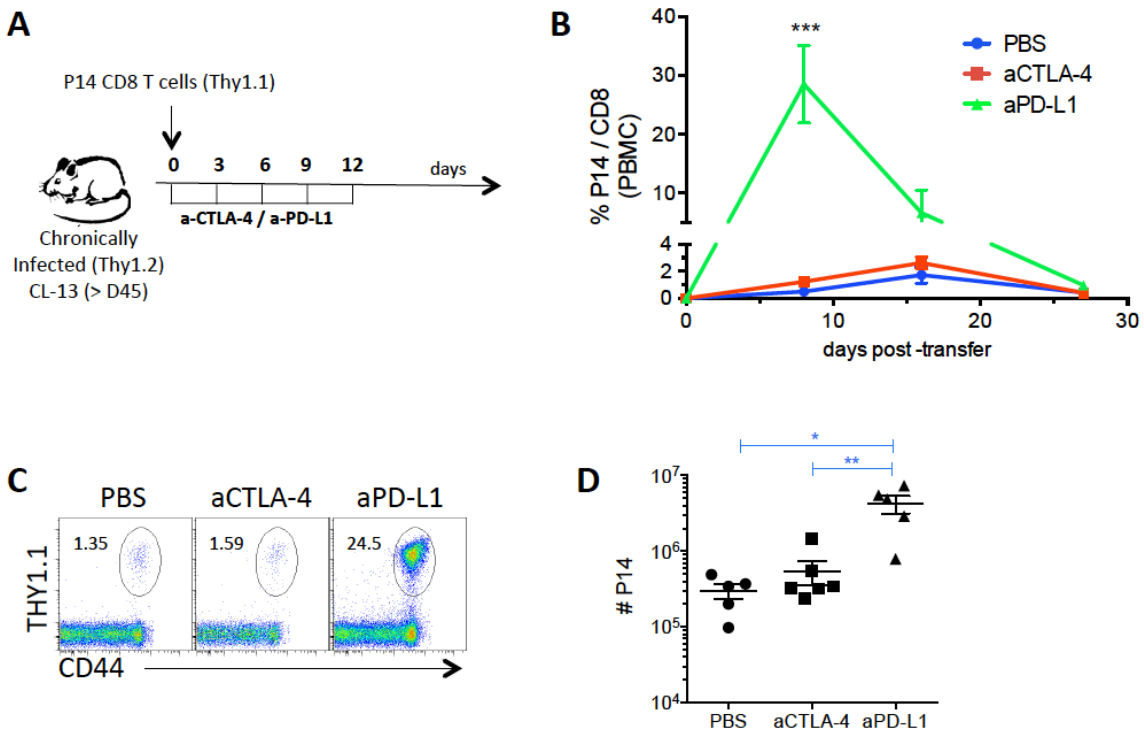


Figure 8. PD-L1 blockade in chronically infected mice, but not CTLA-4 blockade, promotes expansion of transferred naïve P14 CD8 T cells. (A) Experimental set up. (B) The graph shows the frequency of P14 CD8 T cells in peripheral blood. (C) Representative FACS plots show the frequency of P14 CD8 T cells in peripheral blood at day 8 post-transfer. (D) The graph shows total numbers of P14 CD8 T cells in the spleen at day 8 post-transfer. Data are representative of 2 independent experiments with 4-5 mice per group per experiment. Error bars indicate standard errors of means (SEM). $*P < 0.05$; $**P < 0.01$.

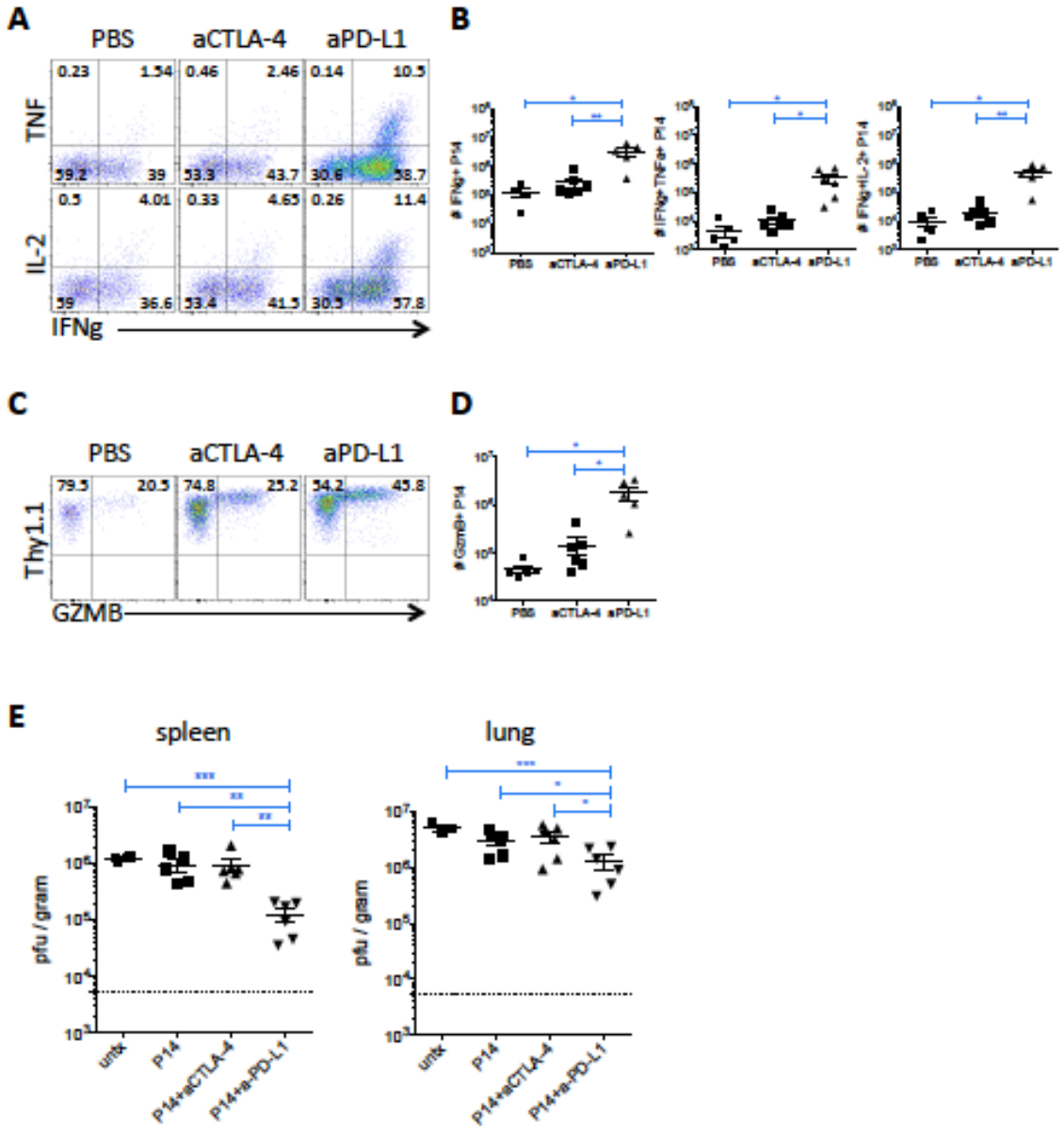
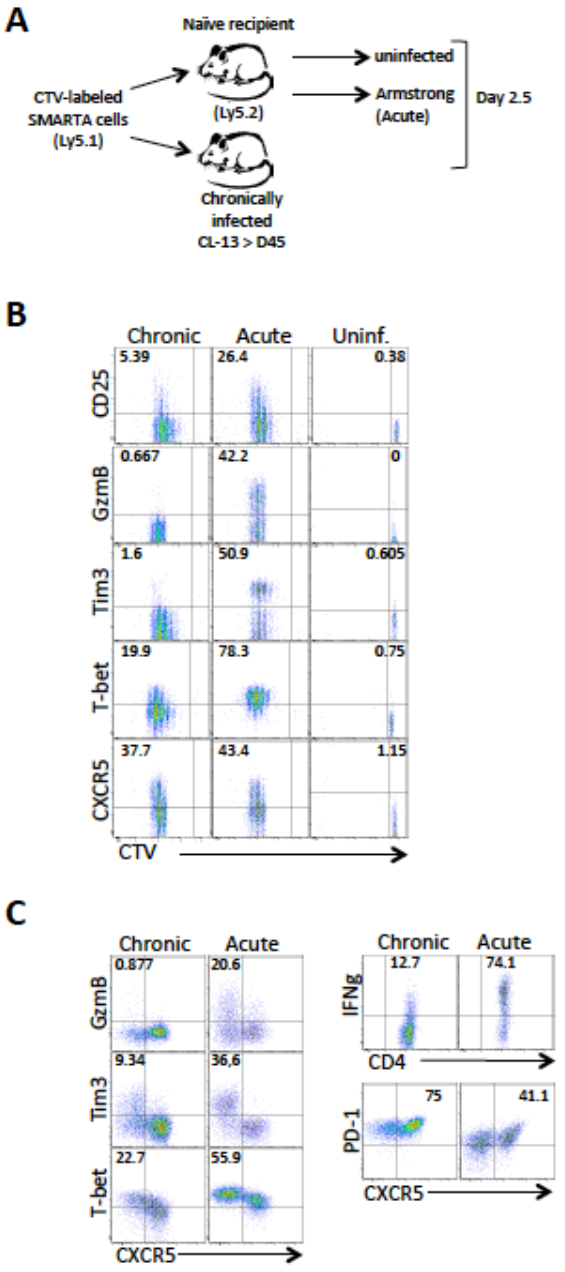
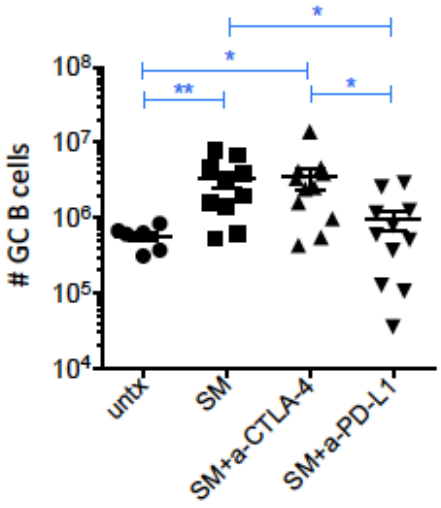


Figure 9. PD-L1 blockade promotes increased functionality of P14 CD8 T cells and leads to substantial reduction in viral load whereas CTLA-4 blockade has minimal effects. Same experimental set up as Figure 8. P14 CD8 T cells were analyzed in the spleen at day 8 post-transfer. (A) Representative FACS plots show cytokine secretion of P14 CD8 T cells after ex vivo stimulation with GP_{33-41} peptide. (B) Graphs show numbers of P14 CD8 T cells producing IFNγ or co-producing IFNγ and TNFa or IL-2. (C) Representative FACS plots show granzyme B expression in P14 CD8 T cells. (D) The graph shows numbers of granzyme B-expressing P14 CD8 T cells. (E) Graphs show virus titer in spleen and lung at day 8 post-transfer. Data are representative of 2 independent experiments with 4-5 mice per group per experiment. Error bars indicate standard errors of means (SEM). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.



Supplemental Figure 1. Th1 generation is inhibited when CD4 T cells are primed in established chronic infection. (A) Experimental set up. (B) Representative FACS plots gated on SMARTA CD4 T cells present in the spleen 60h post-transfer show cell divisions (dilution of cell trace violet dye) and expression of different markers associated with T cell activation and differentiation. (C) Representative FACS plots gated on SMARTA CD4 T cells present in the spleen, 8 days post-transfer, show expression of the markers associated with Th1 and Tfh differentiation in chronic and acute infection. Data are representative of 2 independent experiments with 4-5 mice per group per experiment.



Supplemental Figure 2. GC B cells following SMARTA CD4 T cell transfer with or without CTLA-4 or PD-L1 blockade. Same experimental set up as Figure 1.The graph shows the number of GC B cells (PNA+FAS+B220+CD19+) in the spleen. Data are representative of 2 independent experiments with 4-6 mice per group per experiment. Error bars indicate standard errors of means (SEM). *$P < 0.05$; **$P < 0.01$.

Chapter 4. Discussion

I. CD4 T cell responses following immunization with Ad5 vectors

Recombinant adenovirus vectors are potent vaccine platforms because of robust immunogenicity and good safety profile. Adenovirus vectors have been shown to induce both humoral and cellular immune responses against various infectious pathogens, especially generating strong CD8 T cell responses to vector-encoded transgenes. Therefore adenovirus vectors are considered a promising candidate for vaccine development against intracellular pathogens and cancer where cell-mediated immunity is critical for the protection. Ad5 vector is the most frequently used adenovirus serotype owing to its superior immunogenicity compared to other adenovirus serotype vectors. However, the major limitation of using Ad5 vectors for vaccination is high prevalence of preexisting anti-vector immunity in human populations. Nearly all individuals are exposed to adenoviruses during their lives and have preexisting immunity, particularly neutralizing antibodies against the most common adenovirus serotypes. The presence of these vector-specific neutralizing antibodies has been shown to decrease the vaccine efficacy. Furthermore, the HIV vaccine trial with Ad5-based vaccines encoding HIV-1 antigens (gag/pol/nef) showed no protective efficacy in seropositive individuals (190, 191). With the concerns over the clinical use of Ad5 vectors, alternative human serotypes and adenoviruses from nonhuman species have been increasingly used in preclinical and clinical studies. Still, Ad5-based vaccine vectors have shown efficacy against a broad range of infectious diseases such as tuberculosis, malaria, and influenza and cancer (188, 189).

Ad5 vectors are well known to induce high-magnitude CD8 T cell responses. However, several reports have demonstrated that Ad5-elicited CD8 T cells display more effector-like or partially exhausted phenotype. It seems that the low level of transgene expression persists for prolonged periods following Ad5 immunization(193, 209).

While more efforts have been made to understand Ad5-elicited CD8 T cell responses, less is known about CD4 T cell responses induced by Ad5 vectors. Recent papers have shown that CD4 T cells are critical for transgene-specific CD8 T cell and antibody responses following immunization with adenovirus vectors (195, 196), but the phenotypic and functional properties of Ad5-elicited CD4 T cells are not well defined.

In the chapter 2, I characterized CD4 T cell responses elicited by Ad5 vectors expressing LCMV-GP compared to those induced by LCMV infection. Ad5 vectors resulted in significantly reduced Th1 responses compared to LCMV infection. Early time point analysis demonstrated that Th1 commitment was impaired following Ad5 immunization. Ad5-elicited CD4 T cells expressed minimal or very low levels of Th1-associated molecules at day 3 and 4 post-immunization whereas LCMV-induced CD4 T cells significantly upregulated Th1 markers. I also found that Ad5-elicited CD4 T cells minimally expressed CD25 during early priming, indicating that attenuated IL-2 signals may play a role in impaired Th1 differentiation. To further investigate this mechanistic aspect, mice were injected with IL-2 following immunization with Ad5 vectors. Exogenous IL-2 administration was able to partially restore Th1 responses following Ad5 immunization. Theses results show that impaired Th1 commitment is, at least in part, mediated by reduced IL-2 signaling in Ad5 immunization.

Interestingly, we also found that more Tregs are present following Ad5 immunization than after LCMV infection (~3-fold [day 2 to day 4] or ~7-fold [day 8] higher number of Tregs after Ad5 immunization compared to in LCMV infection). Tregs can negatively impact on IL-2 signals by directly consuming IL-2 and/or by secreting TGFβ. Tregs do not secrete significant amounts of IL-2 but are highly dependent on IL-2 for their survival and maintenance(225, 226). IL-2 consumption by Tregs depletes local IL-2 concentration. TGFβ has been shown to negatively regulate CD25 expression on virus-specific CD4 T cells(202). Therefore, it is also possible that Tregs limit IL-2 signaling by secretion of TGFβ. Treg depletion experiment confirmed the role of Tregs in Ad5 immunization. Following immunization with Ad5 vectors, removal of Tregs promoted the generation of Th1 cells and upregulated CD25 expression on transgene-specific CD4 T cells. These results indicate that Tregs contribute to reduced IL-2 signaling and impaired Th1 differentiation following Ad5 immunization.

Therefore, I propose suboptimal IL-2 signaling as one of the potential mechanisms underlying impaired Th1 differentiation following Ad5 vaccination. IL-2 acts early during priming and promotes Th1 differentiation while suppressing Tfh development. Our results indicate that IL-2 signals play an important role in the fate determination of Th1 versus Tfh lineages in Ad5 vaccination, implicating the potential of manipulating IL-2 pathway to drive favorable vaccine-induced CD4 T cell responses.

Reduced Th1 responses following Ad5 immunization compared to those after LCMV infection are not simply due to the differences between non-replicating vaccine vectors and live virus infection. DNA vectors expressing the same antigen LCMV-GP induced a significantly higher frequency of Th1 cells than Ad5 vectors, leading to more

balanced Th1 and Tfh responses, like LCMV infection. In addition, non-replicating modified vaccinia virus ankara vectors have been shown to induce good Th1 responses(227). Therefore, impaired Th1 development is rather due to an intrinsic property of Ad5 vectors.

Vector doses and routes of administration impacted on the phenotype of Ad5-elicited CD4 T cells at some degree, but regardless of different conditions of antigen load, Ad5 still did not raise effective Th1 responses compared to those after LCMV infection. Furthermore, consistent with the concept that strong TCR signal strength favors Tfh differentiation, the higher doses of Ad5 vectors tended to generate more Tfh cells and fewer Th1 cells. These results also further support that suboptimal Th1 responses are intrinsic for Ad5 vectors.

Ad5 vectors expressing LCMV-GP induced a high frequency of GP-specific CD8 T cells, which was comparable to that induced by LCMV infection (unpublished work, Sarkar et al.). However, in terms of quality, Ad5 generated suboptimal effector and memory CD8 T cells. GP-specific effector CD8 T cells elicited by Ad5 vectors displayed reduced functionality such as cytokine production and cytotoxicity, compared to CD8 T cells after LCMV infection. Ad5-elicited memory CD8 T cells also showed reduced polyfunctionality and recall proliferative potential compared to memory CD8 T cells induced by LCMV infection, and failed to undergo homeostatic proliferation. Distinct phenotypes of Ad5-elicited CD8 T cells were also found at early time points after Ad5 immunization and prolonged antigen stimulation further compromised the formation of optimal memory CD8 T cells following Ad5 immunization. This study also found reduced CD25 expression on Ad5-elcitied CD8 T cells and therefore, differences in IL-2

signals after Ad5 immunization compared to LCMV infection may affect CD8 T cell responses as observed in CD4 T cells responses. As Th1 cells are classically considered to provide help to CD8 T cells, impaired Th1 responses with Ad5 immunization may further negatively impact on CD8 T cell effector differentiation.

In LCMV infection, it has been shown that Th1 and Tfh memory cells maintain the ability to preferentially recall their previously programmed lineage-specific features and functions upon reactivation(198). After the peak response at day 8 post-immunization, the number of Ad5-elicited Th1 cells was decreased over time while the number of Tfh cells was relatively stably maintained. We observed that by day 30 post-immunization, Ad5-elicited Th1 cells further declined and Tfh cells were dominant. Although this study mainly focused on characterizing Ad5-induced effector CD4 T cell responses, it will be intriguing to analyze memory Th1 and Tfh cells following Ad5 immunization. It needs to be considered that prolonged transgene expression induced by Ad5 vectors may influence the longitudinal CD4 T cell responses.

CD4 T cells are essential for generating functional memory CD8 T cells that efficiently induce recall responses upon secondary challenge. CD4 T cells also play a critical role in supporting optimal secondary responses of memory CD8 T cells specially against persistent and high levels of antigen stimulation(228). Hence, with the aim of developing vaccines that provide effective CD4 T cell help, to better understand vaccine-induced CD4 T cell responses is important.

II. Differential effects of blocking PD-1 and CTLA-4 pathways in regulating virus-specific CD4 and CD8 T cells during chronic LCMV infection

Our previous study has shown that adoptive transfer of LCMV-specific CD4 T cells (SMARTA) into chronically infected mice restored endogenous virus-specific CD8 T cell and antibody responses. Combining of SMARTA CD4 T cell transfer and PD-L1 blockade enhanced the functionality of transferred SMARTA CD4 T cells and further promoted CD8 T cell responses and virus control compared to SMARTA CD4 T cell transfer alone. In the chapter 3, I characterized differentiation of transferred SMARTA CD4 T cells in chronically infected mice. I also examined the effects of SMARTA CD4 T cell transfer combined with CTLA-4 blockade in regulating antiviral CD4 and CD8 T cell responses in comparison to SMARTA CD4 T cell transfer and PD-L1 blockade combination. We previously showed that CTLA-4 blockade has no significant effects on LCMV-specific CD8 T cells during chronic infection. However, this was observed from chronically LCMV infected mice that lack antigen-specific CD4 T cells and therefore the effect of CTLA-4 blockade on virus-specific CD4 T cells was not addressed. Hence, in this study, I sought to determine the effects of CTLA-4 blockade on LCMV-specific CD4 T cells in an adoptive transfer system during chronic infection.

Following transfer into the mice with established chronic infection, naïve virus-specific CD4 T cells predominantly differentiated into Tfh cells while differentiation to Th1 cells was significantly suppressed. Blocking of CTLA-4 or PD-1 pathway partially restored Th1 responses. Both CTLA-4 and PD-L1 blockade increased the number of Th1

cells and enhanced functionality of Th1 cells as shown by increased IFNγ and granzyme B production.

Next, I examined the effect of combined therapies on endogenous virus-specific CD8 T cells. With enhanced Th1 responses by the combination therapy of CD4 T cell transfer and CTLA-4 blockade, I hypothesized that endogenous CD8 T cell responses are further promoted compared to SMARTA CD4 T cell transfer alone. However, LCMV-specific CD8 T cell responses were not further enhanced in combination with CTLA-4 blockade and were similar to those after SMARTA CD4 T cell transfer alone. In contrast, consistent with previous results, combination of CD4 T cell transfer and PD-L1 blockade further enhanced LCMV-specific CD8 T cell responses in terms of both quantity and function. This result shows that promoted Th1 responses upon the blockade of CTLA-4 did not result in the enhancement of virus-specific CD8 T cell responses. Therefore, further augmented CD8 T cell responses in combination with PD-L1 blockade than CD4 T cell transfer alone were mainly due to the additional effect of PD-L1 blockade directly acting on CD8 T cells. Thus, CTLA-4 and PD-L1 blockade display similar effects on virus-specific CD4 T cells, promoting Th1 differentiation, but have differential effects in regulating virus-specific CD8 T cells during chronic viral infection.

A recent study has shown that transfer of in vitro polarized Th1 cells into chronically infected mice significantly promoted virus-specific CD8 T cell responses and virus control(58). However, in our system, increased Th1 cells by the blockade of inhibitory receptors did not recover CD8 T cells. In theses two cases, the quality or functionality of Th1 cells generated by in vitro polarization and Th1 cells generated during viral persistence may be different. In vitro polarized Th1 cells are likely to have a

more potent ability to promote virus-specific CD8 T cells. It is possible that the increase in Th1 cells by the blockade of inhibitory receptors was significant but this change may be modest and may not be sufficient to promote endogenous virus-specific CD8 T cell responses.

Multiple mechanisms are probably involved in Th1 suppression during viral persistence, and manipulating one pathway may not lead to substantial effects in restoring Th1 responses. Snell et al.(58) also showed that prolonged type I IFN signaling during persistent infection induced elevated expression of IL-10 and PD-L1 in suppressive DCs and inhibited Th1 priming. Simultaneous blockade of IL-10R and PD-L1 completely restored Th1 generation.

CTLA-4 blockade did not have any significant effects on LCMV-specific CD8 T cell responses during chronic infection (92), but I found that CTLA-4 blockade effectively increases the generation of LCMV-specific Th1 cells. Differential effects of CTLA-4 blockade on virus-specific CD4 and CD8 T cells may be associated with differential expression of CTLA-4 in CD4 versus CD8 T cells. 8 days after transfer into chronically infected recipients, SMARTA CD4 T cells express higher level of CTLA-4 than LCMV-specific CD8 T cells. The other report has also shown that CTLA-4 is more preferentially expressed in virus-specific CD4 T cells than in CD8 T cells during viral persistence(76).

The differential effects of combination therapies with CTLA-4 or PD-L1 blockade on virus-specific CD4 and CD8 T cells were observed at day 8 post-transfer. There was no change in virus titer in all groups by day 8. Significant reduction in viral load was

observed from day 14. Therefore, the effects of combination therapies we found at day 8 post-transfer were not due to reduced viral load.

Combined CTLA-4 blockade did not further impact Tfh differentiation or LCMV-specific antibody responses compared to CD4 T cell transfer alone. Combined PD-L1 blockade had no effect on Tfh cells at day 8 post-transfer but resulted in a decrease in Tfh cells, GC B cells and LCMV-specific antibody titers at day 14 compared to SMRATA CD4 T cell transfer alone. Slightly reduced GC B cells were observed from day 8. Therefore, reduced GC B cells by blocking PD-1 pathway may negatively regulate Tfh cells(222, 223). Decreased Tfh cells may result from reduced virus titer at day 14. Combination of CD4 T cell transfer and PD-L1 blockade most significantly lowered virus titer at day 14 compared to other groups.

Transferred virus-specific CD4 T cells provided a long-lasting effect in restoring antiviral CD8 T cell responses in chronically infected hosts. The mechanisms by which CD4 T cells reinvigorate exhausted CD8 T cells and help to enhance virus control are not fully understood and remain to be further determined. Provision of cognate CD4 T cells may help CD8 cells by DC licensing, which probably occurs before CD4 T cells are fully polarized. Classically Th1 cells are thought to be responsible for helping CD8 T cells. Another possibility is that when Th1 responses are suboptimal under persistent antigenic stimulation, dominant Tfh cells may play a role in supporting CD8 T cells, by producing cytokines such as IL-21.

The mechanisms whereby CD4 T cells enhance the expansion and function of CD8 T cells in the presence of high level of antigen and whether a particular CD4 T cell subset is favorable to rescue CD8 T cell dysfunction and control persistent infection are

important questions that remain to be solved to develop more effective therapeutic strategies and vaccines against chronic infection.

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