Mechanisms of Ad5-Induced Immune Dysfunction

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Abstract

The failure of an Adenovirus 5 (Ad5)-based human immunodeficiency virus type 1 (HIV-1) vaccine in the STEP trial warranted a detailed evaluation of the immunological properties of Ad5. Previous studies have revealed that immunization with Ad5 induces a partially exhausted T cell response but the mechanism of Ad5-induced immune dysfunction is unknown. Using classical animal models, it has been shown that altering antigen dose, and modulating the PD-1/PD-L1 signaling pathway, or modulating regulatory T cells (Tregs) can influence the quality of memory CD8 T cell. Therefore, we interrogated whether these factors play similar roles in Ad5-induced dysfunction. Here, we show that reducing Ad5 vaccine dose induces highly functional memory CD8 T cell responses, characterized by lower PD-1 expression, higher cytokine co-expression, and an improved recall expansion following a heterologous boost. Interestingly, we show that the dysfunctional recall of Ad5-primed T cells following high-dose immunization may partly be mediated by a PD-1-dependent CD8 T cell intrinsic phenomenon, as blockade of PD-L1 leads to a substantial improvement in anamnestic T cell expansion. Furthermore, we provide preliminary data suggesting that Treg may not play a crucial role in the development of Ad5-induced dysfunction. Overall, our data contribute to the understanding of the mechanism of Ad5-induced immune dysfunction, and may be relevant for improving vaccination modalities for HIV and other chronic viral infection.

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1. Chapter 1: Background

1.1 Introduction

Vaccines are one of the most important public health tools in the fight against infectious diseases. The goal of vaccines is to generate long-lived immunological protection, with memory immune response that can prevent natural infection or significantly lessen the disease burden[1]. Most of the currently licensed vaccines have primarily conferred this immunological protection via the induction of high-affinity neutralizing antibodies. However, developing highly effective vaccines against intracellular pathogens such as human immunodeficiency virus-1 (HIV-1), *Mycobacterium tuberculosis*, *Plasmodium falciparum*, hepatitis C virus (HCV) will also require the generation of potent memory CD8 T cells[2, 3, 4, 5, 6]. In order to elicit a potent cellular immune response, the vaccine antigen must be processed endogenously within antigen presenting cells and presented in the context of class I major histocompatibility complex (MHC-I) molecules to CD8 T cells. Until recently, achieving this goal has been hampered by a lack of vaccine platforms capable of such, which has led to the development of novel platforms including DNA vaccines and viral vectors.

DNA vaccines are genetically engineered plasmids containing the DNA sequence of the antigen of interest that can be delivered directly into the appropriate tissue[7]. DNA vaccines are safe and capable of eliciting protective cellular and humoral immune responses in small animal models[8, 9]. However, their application is limited by their low immunogenicity in human and other primates[9]. The suboptimal immunogenicity of DNA vaccines in primates may partly be

due to binding of the DNA to serum amyloid P component (SAP), a negative regulator of the innate immune response[10]. The limited efficacy DNA vaccines led to the development of more immunogenic viral vector vaccines.

In contrast to DNA vaccines, viral vectors have been shown to be highly immunogenic, both in preclinical and clinical studies[11]. They are typically attenuated viruses that are genetically modified to carry an antigen to which an immune response is being sought into the body. They also elicit very potent innate immune responses through the binding of specific Toll-like receptors (TLRs) leading to the generation of a cytokine milieu which modulate the adaptive immune response[12]. The adaptive immune response induced by these vectors differ quantitatively and qualitatively based on the distinct biological properties of the vector. Over the last couple of years, a wide range of virus families have been developed and tested as vaccine vectors for either human or veterinary use[11, 13, 14, 15]. Among these wide array of viruses which include canarypox virus[16], flavivirus[17], lentivirus[18], modified vaccinia virus Ankara[19] and sendai virus[20], adenovirus[21] has been the most widely studied replication-defective vaccine vectors. Of all the adenovirus serotypes isolated so far, adenovirus 5 (Ad5) is the best characterized[22].

However, following the failure of an Ad5-based HIV vaccine in clinical trials, a detailed evaluation of the immunological properties revealed that it elicited a dysfunctional immune response[23]. It has been suggested that this dysfunctional response, coupled with the prevalent pre-existing Ad5-vector immunity may have contributed to the failure of the Ad5-based vaccine. However, the possible biological or immunological parameters responsible for the

immune dysfunction is not fully understood. Although there are ongoing pre-clinical and clinical studies evaluating the immunogenicity and protective efficacy of other serotypes of adenoviruses[12], understanding the biological and immunological properties of Ad5 that may have contributed to the dysfunctional T cell response will help in the selection of novel vaccine vectors, some of which are phylogenetically related to Ad5.

1.2 Adenovirus Biology

Adenoviruses are a family of airborne viruses that infect virtually all major classes of vertebrates causing mild to severe diseases of the airways and gastrointestinal tracts[24]. Currently, more than 60 distinct adenovirus serotypes of human origin have been identified[25]. These viruses are subdivided into seven different serotypes (subgroups A through G) based on the neutralizing antibodies profiles and this corresponds to the divergence of nucleotide sequence of the capsid protein genes[25].

Adenoviruses are double stranded DNA viruses with a non-enveloped icosahedral shell, 70-100 nm in diameter, enclosing about ~26-43 kilobases long linear genomic material[26]. The genome is made up of early and late genes, classified based on the time of expression in relation to DNA replication. The early genes (E1a, E1b, E2a, E2b, E3, and E4) express proteins before DNA replication and they are involved in activating and modulating transcription, the cell-cycle, cell signaling, DNA repair and immune evasion[27, 28, 29]. The late genes (L1–L5) which are expressed following DNA replication, code mostly for structural protein[30]. E1, E2, and E4 gene products regulate transcription of late genes while E3 gene products subvert the host

immune response by affecting the intracellular trafficking machinery, preventing antigen presentation and modulating cytokine pathways[31]. Genetic manipulation of these early genes is often employed in order to adapt adenovirus for use as vectors

Structurally, the adenovirus capsid is icosahedral, with each icosahedron containing 12 copies of hexon trimers and a pentameric pentons located at the 12 vertices[32]. Projecting from the penton bases are trimeric fibers which are made up of a distal knob domain, a shaft, and an N-terminal tail[32]. The distal knob domain of the fibers selectively binds with high affinity to cellular receptors, leading to cellular transduction. Entry receptors identified so far include coxsackievirus B and adenovirus receptor (CAR), CD46, sialic acids, desmoglein 2, and CD80/86[33, 34, 35, 36, 37, 38]. The most characterized include the coxsackievirus B and adenovirus receptor (CAR), which is expressed mainly on epithelial and endothelial cells in human, non-human primates and mice [34]; and CD46, a regulatory protein in the complement system that is expressed on most cells in humans, yet restricted to the testis and sperm in mice[39]. Adenoviruses belonging to serotypes A, C, D, E, and F uses CAR for cellular attachment while CD46 is used by the adenoviruses in serotypes B and D[40]. In addition to the difference in receptor usage, adenoviruses also differ in their intracellular trafficking characteristics and association with the nuclear envelope, with some exiting the endosomal space rapidly following endocytosis and translocating immediately to the nucleus (subgroup C) while others traffic to the late endosome and therefore exhibit a slower association with the nucleus (subgroups B and D)[41, 42, 43, 44]. The differences in cellular receptor and intracellular trafficking influence cellular tropism, particularly the immune cell subsets these viruses interact with, the pattern

recognition receptors (PRRs) they activate, the specific tissue sites where the transgene are expressed, and therefore the characteristics of innate and adaptive immune response activated by these viruses. Appreciation of how these properties influence the immune response is particularly important when adenoviruses are used as vaccine vectors.

1.3 Adenovirus as a Vaccine Vector

Following their discovery over six decades ago[45], adenoviruses have evolved to be an important biological tool utilized as model system providing significant insights into complex cellular processes such as DNA replication and transcription, DNA repair responses, oncogenesis and alternate RNA splicing[46, 47]. Additionally, their ability to readily infect human and other mammalian cells have made them a popular choice for gene delivery[48]. Their application in gene therapy was however hindered by the robust immune response they induce leading to a shortened duration of transgene expression[49]. Interestingly, this strong immunogenicity has been exploited by vaccinologists with the development of many adenoviral vaccine vectors. Adenoviruses have some unique features that make them particularly suitable for use as vaccine vectors[12, 22]. First, they have a very broad cellular tropism with the ability to infect and express its transgene in a wide variety of rapidly dividing and non-dividing cells. Secondly, they are stable and can be readily grown and purified in large quantities. Thirdly, they cause relatively mild disease in human and compared to lentiviral vectors, they do not integrate into the host cell genome, reducing the risk of malignant transformation. Fourthly, their genome is well characterized and can be easily manipulated to render them replication-defective. This is typically achieved by deleting the early gene E1, which is required for adenovirus replication. In

addition, E3, another early gene important for host-vector immune interaction, is often deleted. Although not essential in E1-deleted adenovirus, deletion of E3 or E3/E4 regions offers the benefit of increasing the transgene insertion capacity to up to 7.5kbp to carry medium-sized protein transgenes[50, 51]. Furthermore, vectorized adenoviruses are thermostable and have been demonstrated in both preclinical and clinical studies to be safe for both systemic and mucosal surface application[22]. Finally, and most importantly, replication incompetent adenoviruses are highly immunogenic, inducing robust antibody and cell-mediated immunity to the inserted transgenes with the ability to elicit both systemic and mucosal immune response following parenteral delivery[52, 53, 54].

1.4 Human Adenovirus 5 Vaccine Vector

Ad5 is the most studied of the adenovirus vectors. It is a serotype C virus and transduces cells either through the use of the coxsackie-adenovirus receptor (CAR)[34] or in a CAR-independent pathway[55]. As a stand alone vaccine vector, it is arguably the most immunogenic human adenovirus serotype. Ballay A. *et al* was the first to report that recombinant replication–competent Ad5 carrying hepatitis B surface elicits antibody response against the transgene in rabbits[51]. Not long afterwards, the ability of E1-deleted replication-incompetent Ad5 vectors expressing rabies virus glycopeptide (GP) to induce protective titers of neutralizing antibodies and rabies-virus specific CD8 and CD4 T cells was demonstrated[56]. Following this important finding, replication-incompetent Ad5 has been tested in several other preclinical and clinical studies and shown to elicit both neutralizing antibodies and cellular immune responses

against pathogens such as dengue virus, ebola virus, hepatitis C virus, Epstein-Barr virus, *Streptococcus pneumonia*, *Mycobacteria tuberculosis, Plasmodium falciparum*, HIV among others[57, 58, 59, 60, 61, 62, 63].

In the HIV field, Ad5 expressing simian immunodeficiency virus (SIV) Gag peptide conferred a protective immune response following challenge of rhesus monkeys with pathogenic SIV/HIV chimera SHIV 89.6P10, but failed to protect against a more stringent, neutralization-resistant strain SIVmac239[64, 65]. Subsequently, two large Phase IIb clinical trials (STEP in North America and Phambili trials in South Africa) were commenced[66, 67]. In these studies, the Merck Ad5-Gag-Pol-Nef vaccine was administered three times to volunteers that had either none or moderate-to-high titers of neutralizing antibodies against Ad5 with the aim of eliciting HIV-specific T-cell responses capable of providing complete or partial protection from HIV-1 infection or a decrease in viral load set points post-infection. Both studies were stopped and unblinded before completion of enrollment following an interim analysis of the STEP trial that showed a lack of efficacy[67]. Even more alarming was a subsequent analysis that revealed a trend toward higher HIV-1 acquisition in vaccine recipients compared to placebo controls, particularly in a subgroup of uncircumcised men with high baseline Ad5 seropositivity[67]. Although not completely proven, a probable explanation for this unexpected finding is that the vaccine led to an increase in the number of HIV-1 target cells, resulting from the activation of Ad5-specific CD4 T cells at mucosal surfaces[68, 69]. Furthermore, a second Ad5-based Phase IIb HIV vaccine clinical trial (HVTN 505) did not show protective efficacy against HIV-1 infection in human[70]. It is

important to note however, that the Ad5 used in this study was different in design from the Merck Ad5 vector, and was used as a boosting vector following a DNA vaccine prime.

The failure of the Ad5 vector in these clinical trials led to a renewed interest in two areas of Ad vector research. First is the development and evaluation of rarer adenovirus serotypes of human origin[50, 71], as well as serotypes derived from other species such as chimpanzees[72, 73, 74] and rhesus monkeys[75], and structurally modified Ad5 vector expressing heterologous adenovirus hexons[76] in order to circumvent the problem of pre-existing Ad5 vector immunity. The other area of intense research is the analysis of the biologic properties of Ad vector-induced immune responses[23, 77, 78, 79, 80, 81, 82]. These detailed analyses have shed more light on the inherent properties of Ad5-induced T cell responses, highlighting how these responses substantially differ from those elicited by alternative serotype Ad vector such as adenovirus 26 (Ad26) or adenovirus 35 (Ad35).

1.5 Ad5-induced Immune Dysfunction

The most surprising finding from the comprehensive evaluation of the phenotype and quality of Ad vector-induced immune response was that Ad5, compared to other serotype adenoviruses, induces a partially impaired immune response. This observation was first reported by Yang *et al* when they analyzed the phenotypic profile of the immune responses elicited by an Ad5 vaccine in mice[83]. They observed that Ad5-elicited CD8 T cells exhibit a protracted effector phenotype with a delayed contraction phase, a phenomenon they argued may be due to the prolonged antigen presentation seen following Ad5 immunization[83]. In line

with this observation, others have shown that, compared to alternative serotype Ad vectors, Ad5 induces a partially dysfunctional T cell response in mice. Ad5-induced memory T cells were found to express low levels of IL-7 alpha chain receptor CD127, lymphoid homing receptor CD62L, antiapoptotic molecule Bcl-2, as well as high levels of inhibitory receptors PD-1 and Tim 3[23, 81, 82, 84]. Although Ad5 immunization induces a high magnitude primary T cell response, these T cells are less polyfunctional with reduced capability to secrete IFN-γ, TNF-α, and IL-2, compared to alternative serotype adenovirus[23, 82]. Furthermore, circulating and tissue resident antigen-specific memory CD8 T cells induced by Ad5 proliferate less robustly following a secondary exposure (boosting) to antigens[81]. Similar findings have also been observed in non-human primates[79]. It is important to note that although recombinant E1-deleted Ad5 vector does not replicate, it induces a memory CD8 T cell population that to some extent – except for its functionality – is reminiscent of an exhausted T cell response seen in models of chronic infection or cancer characterized by persistent antigenic stimulation[85], and as such suggests that Ad5-induced CD8 T cells may be "partially exhausted" (Table A).

1.6 Factors Influencing Ad5-induced Immune Dysfunction

In order to understand why Ad5-induced memory CD8 T cells exhibit such dysfunctional or "partially exhausted" phenotypes, it is I tt mportant to consider the virological and immunological cues that influence the development and maintenance of exhausted CD8 T cells in the classical immune exhaustion model of chronic infection, and assess whether these factors play similar roles in mediating Ad5-induced immune dysfunction, and more importantly,

if modulation of these parameters will lead to an improvement in the phenotype and quality of Ad5-induced memory CD8 T cells. Extrapolating from this classical model and relying on our current understanding of the biology of Ad5, possible factors that may favor the development of Ad5-induced immune dysfunction include 1) vector dose, 2) immuno-inhibitory receptors, 3) CD4 T cells - conventional and regulatory T cells and 4) antigen presenting cells and expression of co-stimulatory and/or co-inhibitory molecules. (Figure. A).

1.6.1 Vector Dose

The amount and duration of antigen exposure impact antigen-specific CD8 T cells both in terms of quantity and quality. In an acute infection model, antigen dose affects the number of naïve CD8 T cells recruited for activation and their differentiation into effector and memory CD8 T cells[86]. While in chronic infections, such as the chronic model of lymphocytic choriomeningitis virus (LCMV) infection in mice, differentiation of primed CD8 T cells into either an exhausted or memory phenotype is dependent on the amount and duration of antigen exposure[87, 88]. Likewise, in Ad vector vaccine research, the dose of Ad vector impacts the quality of transgene-specific CD8 T cell differentiation and functionality[78]. High dose Ad5 immunization has been shown to induce transgene specific CD8 T cells bearing features of a dysfunctional T cell population: reduced functional CD8 T cells as evidenced by lower per cell expression of IFN-γ, lower fraction of cells coproducing TNF-α or IL-2 and IFN-γ; impaired memory conversion with lower expression of CD127 and high expression of KLRG-1; and an absence of a contraction phase[78, 89]. Conversely, reducing the dose of Ad5 significantly improves some of these

impairments[78]. However, the effect of lowering the priming dose of Ad5 on recall expansion following antigen re-exposure has not been investigated to date. In addition, it is also not known whether lowering the dose will lead to a reduced expression of immunoinhibitory receptors, suggesting a reduced immune exhaustion.

1.6.2 Immunoinhibitory Receptors

Immunoinhibitory receptors such as PD-1 (Programmed Cell Death 1), Tim-3 (T-cell immunoglobulin and mucin-domain containing-3), and 2B4 (CD244) are induced on T cells immediately following activation, and at this stage, they serve as safeguards to curtail excessive immune response[23, 85]. However, the sustained up-regulation of immunoinhibitory receptors has been recognized as a prominent feature of exhausted CD8 T cells, where they play a significant role in negatively regulating T cell function[90]. The most widely studied of these receptors is PD-1, a member of the CD28 superfamily, which is inducibly expressed on T cells, B cells, natural killer T (NKT) cells, and some myeloid cells[91]. Like other members of the family, PD-1 transduces signals only in the context of T cell receptor (TCR) crosslinking following a TCR-antigen-MHC engagement. PD-1 has two ligands: PD-L1 and PD-L2. PD-L1, also known as B7-H1, has a broad tissue distribution and is expressed on B cells, DCs, macrophages, BM-derived mast cells, and T cells and peripheral epithelial and endothelial cells upon stimulation by proinflammatory cytokines, such as interferons and tumor necrosis factor[92, 93, 94]. PD-L2 or B7-DC is more restrictively expressed on dendritic cells and macrophages [93]. Upon interaction with its ligands, PD-1 sends signals that limit proliferation, interfere with cytotoxic activity and IFN-γ

production through the up-regulation of the transcription factor BATF in CD8 T cells[95, 96]. The suppressive function of PD-1 is also achieved via the reduction in the duration of DC:T cell contact, and the direct dephosphorylation of proximal TCR signaling through the recruitment of SHP1 and SHP2 phosphatases by the immunoreceptor tyrosine-based switch (ITSM) motif on the intracellular cytoplasmic tail of the PD-1 receptor[97, 98, 99]. Other immunoinhibitory receptors that are up-regulated on exhausted T cells include CTLA4, Tim-3, LAG-2, CD160 and 2B4 and these receptors have been shown to act either independently or in synergy with one another and/or with PD-1 to influence the degree of T cell exhaustion[85]. Partial restoration of T cell function can be achieved by blocking the signaling pathways of these receptors (known as immune checkpoint blockade)[100].

As mentioned earlier, Ad5 vector elicited greater percentages of transgene-specific CD8 T cells co-expressing both PD-1 and Tim-3 or singly-positive for either PD-1 or Tim-3 [23] (Penaloza-MacMaster, P. et al. unpublished data). Indeed, PD-1 and Tim-3 expression on Ad5-induced T cells is associated with less functionality (Penaloza-MacMaster, P. et al, unpublished data) similar to findings in the chronic model of LCMV infection in mice[85] or HIV infection in humans[101]. Additionally, the higher co-expression of PD-1 and Tim-3 correlates with reduced levels of memory markers on CD8 T cells, possibly explaining the poor anamnestic potential seen with Ad5 immunization. Similarly, increased PD-1 expression was also observed on "helpless" memory CD8 T cells compared to memory CD8 T cells primed in the presence of CD4 T cells[102]. Interestingly, PD-1 blockade during antigen re-exposure enhanced expansion of these 'helpless' memory CD8 T cells[102], indicating that PD-1 may limit memory CD8 T cell expansion.

Furthermore, PD-1 blockade has been shown to restore pulmonary CD8 T effector functions (degranulation and cytokine production) and enhanced viral clearance in a model of respiratory virus reinfection[103]. It is not known whether a similar improved recall expansion and restoration of polyfunctionality will be seen following checkpoint blockade in the context of Ad5 immunization.

1.6.3 Conventional and Regulatory CD4 T cells

It is well established that conventional CD4 T cell help is required for memory CD8 T cell differentiation following natural infection[104, 105] or vaccination[106]. It has been reported that Ad5 induces a dysfunctional CD4 T cell response characterized by high PD-1 expression and IL-10 expression[80], while an alternative serotype adenovirus, Ad26, which induces a more favorable CD8 T cell response with better phenotypic and qualitative profile efficiently elicits functional CD4 T response[79]. The impaired CD4 T cell help associated with Ad5 may therefore be responsible for Ad5-induced CD8 T cell dysfunction. On the other hand, regulatory CD4 T cells (Tregs), whose primary role is to control and dampen immune response, have been shown to help in maintaining CD8 T cell exhaustion, and the ablation of Tregs in mice chronically infected with LCMV led to a significant rescue and expansion of exhausted LCMV-specific CD8 T cells[107]. Since Ad5-induced immune dysfunction exhibits some exhausted T cell phenotype, it is not impossible that regulatory T cells may play a role in driving this exhaustion. This is however yet to be determined.

1.6.4 Antigen Presenting Cells, Co-stimulatory and Co-inhibitory Molecules

Adenoviral vectors differentially interact with antigen presenting cells (APCs) such as dendritic cells (DCs)[55] due to their distinct *in vivo* biological properties[108, 109]. It has been shown that Ad vectors form immune complexes with specific neutralizing antibodies which affect transduction efficiency and maturation of dendritic cells in a FcR and Toll-like receptor 9 (TLR9) interaction[68]. Comparative evaluation of the potency of immune complexes formed by Ad5 and alternative serotype adenovirus vectors such as Ad26 and Ad36 showed that immune complexes of Ad5 were stronger inducers of DC maturation (as measured by the up-regulation of co-stimulatory molecules and production of proinflammatory cytokines) compared to Ad26 and Ad35 immune complexes[68]. It is however not known if the stronger DC induction combined with the more protracted persistence of Ad5 influences the impaired immune response seen in Ad5. The role of co-stimulatory (e.g. B7-1) and co-inhibitory molecules (e.g. PD-L1) expressed on antigen presenting cells following Ad vector transduction and how they may contribute to the impaired immune response is not fully understood.

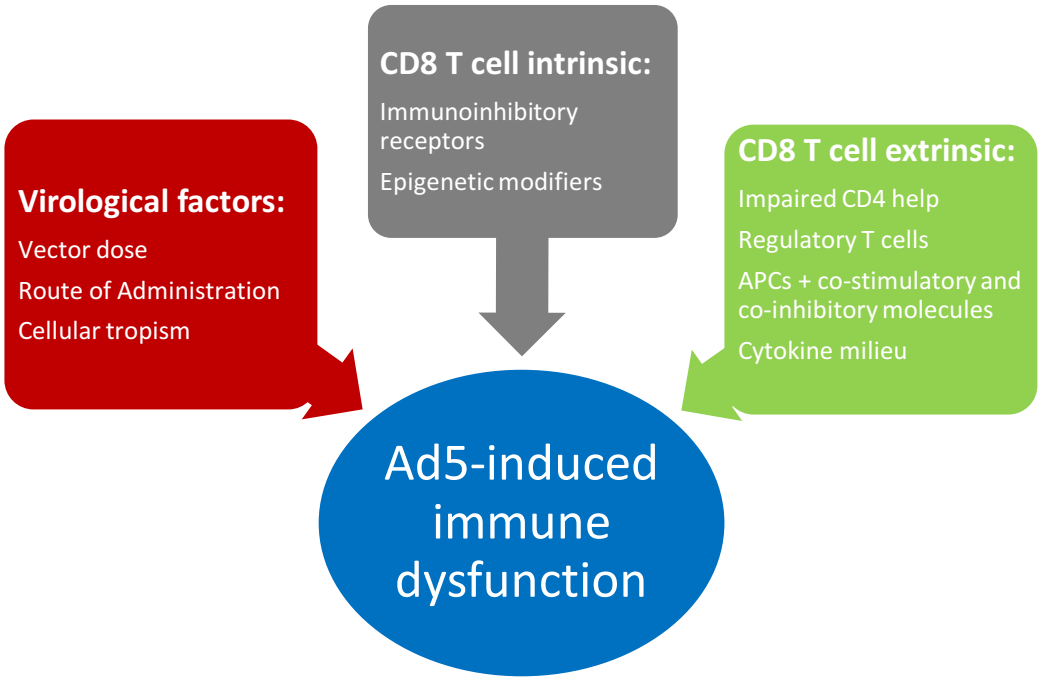
1.7 Summary

Vaccine-mediated protection against intracellular infections such as HIV-1, malaria and tuberculosis will most likely require the generation of potent humoral and cellular immune responses. Until its failure in the HIV-1 vaccine trial (STEP trial), Ad5 was one of the most promising vectors used for eliciting such responses. Evidence is accumulating that the immune response elicited by Ad5-based vaccine vectors exhibits a dysfunctional phenotype, and that

this dysfunction may have contributed to its failure. Assessing the immunological and biological cues that affect a similarly impaired T cell response in the chronic viral infection (LCMV) model may help us elucidate the mechanism of Ad5-induced immune dysfunction.

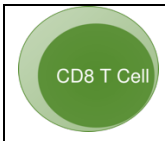
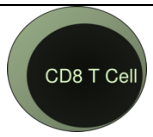
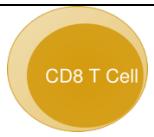
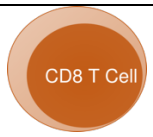
1.8 Figures

1.8.1 Figure A: Potential factors influencing Ad5-induced immune dysfunction



A schematic representation of the possible factors influencing the development and maintenance of Ad5-induced immune dysfunction

Table A: Spectrum of exhausted CD8 T states

	 *Fully Functional Memory CD8 T cells (e.g. Acute LCMV infection, Ad26-Induced memory CD8 T cells)*	 *Ad5-induced memory CD8 T cells*	 *Partially exhausted CD8 T cells (e.g. Early chronic LCMV infection)*	 *Severely Exhausted CD8 T cell (e.g. Late Chronic LCMV infection)*
Antigen load	--	?-/+	++	++++
CD4 help	+++	?++	++	---
IL-2	++	-	-	-
TNF-α	+++	++/-	+/-	-
IFN-γ	+++	++	++	-
Cytotoxicity	+++	++	+	-
Inhibitory Receptors	+	++	++	+++
Proliferative Potential	+++	+	+	-
Apoptosis	-	-	+/-	++++

Classical immune exhaustion in chronic infections and cancers is characterized by a step-wise and progressive loss of effector capabilities, the sustained upregulation of inhibitory receptors, and the loss of self-renewal capabilities. Ad5-elicited CD8 T cells lie in a spectrum between fully functional memory CD8 T cells generated following the clearance of an acute infection (e.g. Acute LCMV infection or alternative serotype adenoviral (e.g. Ad26) immunization) and fully exhausted CD8 T cells from chronic LCMV infection. The role of vector load/persistence and CD4 T cell help in modulating Ad5-induced immune exhaustion has not yet been fully determined. (Adapted and modified from Kahan SM *et al.* Virology. 2013[85])

2. Chapter 2: Data and Methods

2.1 Introduction

Replication-incompetent human adenovirus serotype 5 (Ad5) vector elicits potent and protective CD8+ T cell responses in preclinical studies. However, a detailed evaluation of the immunological properties of Ad5 in mouse and non-human primates has revealed that Ad5 elicits a partially exhausted T cell response, characterized by low frequency of polyfunctional T cells, high expression of immunoinhibitory receptors, dysfunctional cytokine secretion, and an impaired memory recall. Recently, it has been shown that factors such as vector priming dose or antigen load, expression of immunoinhibitory receptors, and regulatory T cells may modulate the phenotype, quality and anamnestic potential of memory T cells. In this study, we investigated the possible role of vector dose, PD-1 signaling pathway, and regulatory T cells in modulating Ad5-induced cellular immune response.

2.1.1 Hypothesis

Vector dose, PD-1 signaling and regulatory T cells modulate Ad5-induced cellular immune response.

2.1.2 Specific research questions:

1. Is it possible to overcome the impaired anamnestic immune response associated with Ad5 by altering the priming dose?
2. What is the effect of immunoinhibitory receptor blockade on the recall of Ad5-induced memory CD8 T cells?
3. Is there a role for regulatory T cells in modulating the Ad5-elicited immune dysfunction?

2.2 Materials and Methods

2.2.1 Mice and immunizations

Six to 8-week-old female C57BL/6 mice (from Jackson Laboratories) were used for all immunization experiments. Replication-incompetent, E1/E3-deleted Ad5, Ad26 and Ad35 vectors expressing SIVmac239 Gag were prepared as previously described[71, 110], while modified vaccinia Ankara (MVA) expressing SIV Gag-Pol-Env was prepared by the National Institutes of Health (NIH). Mice were immunized intramuscularly in both hind leg muscles (quadriceps) with escalating doses (10^7, 10^8, 10^9 or 10^{10} viral particles (vp)) of Ad5 or Ad26 vectors per mouse. Mice were boosted 60 days post prime intramuscularly with MVA at a dose of $\leq 10^7$ plaque-forming units (PFU) or Ad35-SIVmac239 Gag (10^9 vp). Intramuscular (i.m) injections were administered in 100 µl phosphate-buffered saline (PBS) solution (50 µl per quadricep). Animals were housed at the Beth Israel Deaconess Medical Centre (BIDMC) animal facility and all experiments were performed according to approved IACUC protocol.

2.2.2 Isolation of lymphocytes

Single-cell suspensions of blood and tissues were generated as previously described[71, 111]. Briefly, mouse blood was collected in RPMI 1640 containing 40U/ml heparin. PBMCs were isolated from whole blood using Ficoll-Hypaque density centrifugation at 1900 rpm for 20 min. Liver, lymph node and spleen were homogenized to single cell suspension by forcing tissue chunk through a 100µm sterile cell strainer using the barrel of a 5-cc syringe. Red blood cells were lysed using ammonium chloride (ACK) Lysing Buffer (ThermoFischer). Resulting cell

suspension was pelleted, re-suspended in RPMI containing 10% fetal calf serum and penicillin/streptomycin. Cells were counted thereafter. Homogenized liver tissue was applied to a 44/67% Percoll gradient and centrifuged at 2,000 rpm for 10 min at 20^{o}C. The interface containing the intrahepatic lymphocyte population was harvested and washed, and the resulting lymphocytes suspension was re-suspended in RPMI containing 10% fetal calf serum and penicillin/streptomycin, and counted.

2.2.3 Tetramer binding assay and flow cytometry

MHC class I tetramer staining was performed on single cell suspension using an H-2D^{b} tetramer folded around the immunodominant SIV Gag AL11 epitope (AAVKNWMTQTL) as described previously[112]. Biotinylated class I monomers were obtained from the National Institutes of Health Tetramer Core Facility (Emory University, GA) and tetramerised in-house. Background staining of CD8 T cells from naïve animals was ≤ 0.1%. Surface staining was performed with anti-CD8a (53- 6.7), -CD44 (IM7), -CD127 (A7R34), -CD62L (MEL-14), -KLRG1 (2F1) and -PD-1 (RMP1-30). Transcription factor staining was performed by first permeabilizing the cells with the FoxP3 Fixation/Permeabilization Kit (eBioscience) and subsequently staining with anti-T-bet (4B10), –Eomes (Dan11mag) and –FoxP3 (150D/E4). All Antibodies were purchased from BD Biosciences, eBioscience, or BioLegend. Live/Dead Fixable Near-IR staining kit was obtained from Life Technologies. After staining, cells were washed in PBS containing 2% fetal bovine serum (FBS) and fixed using cytofix/cytoperm buffer (BD Bioscience). Samples were acquired on an LSR II flow cytometer (BD Biosciences), and data were analyzed using FlowJo

version 9.7.7 (Tree Star).

2.2.4 Intracellular cytokine staining (ICS) assays

Cytokine expression of Gag-specific cellular immune responses in immunized mice was assessed by multiparameter intracellular cytokine staining (ICS) assays as previously described[110], with some modifications. Briefly, lymphocytes isolated from the blood (~$5x10^5$), spleen (10^6) or the liver (10^6) were incubated for 5 hours at 37 °C with 0.2 µg/ml of SIV Gag peptide pool (together with brefeldin A (GolgiPlug) and monensin (GolgiStop). After incubation, cells were stained with anti-CD8 (53-6.7), -CD44 Pacific Blue (IM7), -CD4 (RM4-5) and Live/Dead Fixable Near-IR staining kit. Afterwards, cells were fixed and permeabilized with Cytofix/Cytoperm prior to intracellular staining with anti-IFN-γ (XMG1.2), anti-TNF (MP6-XT22), and anti-IL-2 (JES6-5H4). All ICS reagents were purchased from BD Biosciences.

2.2.5 Monoclonal antibodies treatments

PD-L1 blockade was achieved via injection of 200 microgram 10F.9G2 (BioXCell) at the time of priming or boosting immunization. The regimen consisted of five doses every 3 days as previously described[100], with the first dose given a day prior to immmunization. All antibodies (ab) were diluted in DPBS and administered intraperitoneally (i.p). Rat IgG2b (BioXCell) was used as control. Memory CD8 T cell recall responses were assessed in blood on day 7, and in blood and tissues on day 15 post boost.

2.2.6 *In vitro* Treg suppression assay

In vitro Treg suppression assay was performed as previously described[113], with some modifications. Briefly, CD4 T cells were enriched by negative magnetic selection from the spleen of adenoviral vector-immunized Foxp3^{gfp} mice (pooled from 2 spleens per group) using an EasySep™ Mouse CD4 T Cell Isolation Kit (Stemcell Technologies). After enrichment of CD4 T cells, FoxP3^{+}GFP^{+} CD4 T cells were sorted using a FACSAria II cell sorter (BD Bioscience). Naive CD8 T cells were isolated from the spleen of C57BL/6 mice, labeled with Cell Trace Violet (CTV) dye (5 µM; Life Technologies) for 10 min at room temperature in PBS and washed with complete media before culture. For the suppression assay, 2.5 x 10^4 CTV-labelled CD8 T cells were co-cultured with Dynabeads® Mouse T-Activator CD3/CD28 beads (ThermoFischer Scientific) at a ratio of 4 to 5 (beads:CD8 T cells), and varying numbers (0, 1.5x 10^3, 3x10^3, 6x10^3, 1.25x10^4, 2.5x10^4) of sorted FoxP3^{+}GFP^{+} CD4 T cells per well in a 96-well flat bottom plate. After 72 h of incubation at 37^{o}C, cells were harvested, stained with anti-CD8a (53-6.7), -CD44 (IM7), and -CD4 (RM4-5), -CD25 (3C7) and Live/Dead Fixable Near-IR staining kit (RMP1-30). CD8 T cells were analyzed for CTV dilution by flow cytometry.

2.2.7 Statistical analysis

Statistical analysis was performed on GraphPad Prism version 6.0h using two-tailed nonparametric Mann-Whitney U test.

2.3 Results

2.3.1 The kinetics, phenotype and functionality of vaccine-induced CD8 T cells following vaccination with different doses of Ad5

We initiated studies by assessing the magnitude, phenotype and quality of transgene-specific CD8 T cells elicited by different doses of Ad5 vector. We immunized 6-8 week old C57BL/6 mice intramuscularly (i.m) with escalating doses of Ad5 (10^8, 10^9 and 10^{10} vp) expressing the Gag antigen from SIV strain mac239. Gag-specific CD8 T cell responses in peripheral blood were assessed longitudinally using an MHC class I tetramer loaded with the immunodominant epitope AL11[112]. Mice vaccinated with 10^{10} vp of Ad26 expressing the same antigen also were included as control (Fig. 1A). Consistent with previous reports[78, 81], all doses of Ad5 elicited similar peak CD8 T cell responses at day 15 post-prime, but the low dose Ad5 prime (10^8 vp) was associated with more pronounced CD8 T cell contraction compared to high dose Ad5 prime (10^{10}) ($p<0.001$) (Fig. 1B-1C).

Next, we examined the phenotype of D^b-AL11+ CD8 T cells following vaccination with different doses of Ad5. It has previously been shown that Ad5, compared to alternate serotype Ad vectors such as Ad26 and Ad35, leads to an up-regulation of the immunoinhibitory receptor, PD-1 on vaccine-elicited CD8 T cells[23, 81]. We therefore sought to assess whether PD-1 up-regulation on Ad5-elicited CD8 T cells is dose-dependent. We noticed that antigen-specific CD8 T cells in mice primed with low dose Ad5 expressed significantly lower PD-1 relative to high dose Ad5-primed mice at the peak of the primary response (day 15 post immunization (p.i))

(p<0.0001; Fig. 2A) and at memory time-point (day 60 p.i) (p=0.0059; Fig. 2B), suggesting that PD-1 expression on Ad5-elicited CD8 T cells is dose-dependent.

We further assessed the cell surface expression of killer cell lectin-like receptor G1 (KLRG1) and IL-7 receptor alpha-chain (CD127), two markers often used to delineate terminal ($KLRG1^{+}$ $CD127^{-}$) and memory precursor ($KLRG1^{-}CD127^{+}$) effector CD8 T cells in mice[114, 115]. We found that, on day 60 post immunization, the proportion of $KLRG1^{+}CD127^{-}$ memory precursor effector cells (MPECs) in blood was reduced in the low dose Ad5-immunized group compared with the high dose group (Ad5 10^{8} vp, 18% MPECs;Ad5 10^{10} vp, 10% MPECs; p<0.05), while there was a corresponding higher proportion of $KLRG1^{+}CD127^{-}$ short lived effector cells (SLECs) in mice immunized with high dose Ad5 relative to low dose Ad5-immunized mice (Ad5 10^{8} vp, 45% MPECs ;Ad5 10^{10} vp, 50% MPECs; p<0.05) (Fig. 2C). This suggests that there is a preferential differentiation of vaccine-induced effector CD8 T cells towards the memory precursor lineage following low dose Ad5 immunization compared with high dose Ad5 immunization.

Polyfunctional T cells are T cells which secrete multiple cytokines concurrently. They have been shown to play an essential role in the control of HIV infection, and correlate with protection in some vaccine models[2, 116, 117, 118]. Therefore, we assessed the ability of vaccine-elicited memory CD8 T cells to secrete multiple cytokines following *ex-vivo* antigen re-stimulation with an overlapping SIV Gag peptide pool. We observed that low dose Ad5 induces a higher frequency of Gag-specific CD8 T cells co-expressing two (IFN-γ and TNF-α) or three (IFN-γ, TNF-α and IL-2) cytokines, while high dose Ad5 leads to the induction of more CD8 T

cells expressing only IFN-γ relative to low dose Ad5 (P=0.0029 (for Ad5 10^8 vp versus Ad5 10^{10} vp; IFN-γ, TNF-α and IL-2 co-producers); P<0.0001 (for Ad5 10^8 vp versus Ad5 10^{10} vp; IFN-γ and TNF-α co-producers); P<0.0001 (for Ad5 10^8 vp versus Ad5 10^{10} vp; IFN-γ only) (Fig. 2D). For all parameters examined, the phenotype and quality of CD8 T cells induced by low dose Ad5 closely resembles those of Ad26-induced CD8 T cells (Fig. 1B-1C; Fig. 2A-2D). Altogether, these findings build on previous reports by showing that low dose Ad5 vector is as similarly immunogenic as high dose Ad5 and yet elicits a more functional transgene-specific CD8 T cell population characterized by lower PD-1 expression, enhanced memory conversion and improved functionality.

2.3.2 Improved anamnestic CD8 T cell responses after vaccination with low dose Ad5 vector

It has previously been shown that, compared with alternative adenovirus serotype, Ad5-induced CD8 T cells exhibit reduced anamnestic expansion following a heterologous vector boost[23, 81] or a secondary antigen challenge[119]. We therefore sought to assess the recall expansion of the phenotypically favorable CD8 T cell induced by low dose Ad5 compared with CD8 T cells induced by high dose Ad5. Mice vaccinated with escalating doses of Ad5 or a fixed dose of Ad26 vectors expressing SIV Gag were boosted with Ad35 vector expressing the same SIV Gag transgene (Fig. 2A). Intriguingly, we observed a robust recall expansion of low-dose Ad5 primed CD8 T cells compared with high dose Ad5 prime by day 7 post-boost (difference between pre and post-boost for low dose Ad5 was 5.37-fold; for high dose Ad5, 1.92-fold; a 2.8-fold difference, p=0.0012) (Fig. 3B-3D). Similarly, on day 14 and 30 post boost, there was a

trend towards a higher number of Gag-specific CD8 T cells in the group primed with low dose Ad5 compared with high dose Ad5-primed mice, although this was not significant (data not shown)

On day 7 post boost, we analyzed the phenotype of antigen-specific CD8 T cells in blood. We observed a higher expression of Ki-67, a marker of proliferative potential, on Gag-specific CD8 T cells from low dose Ad5-primed mice compared with high dose Ad5-primed (Fig. 2E) ($p= 0.029$), correlating with the improved recall expansion seen in the low dose group. Additionally, granzyme B expression on Gag-specific CD8 T cells in low dose Ad5-vaccinated mice was greater than those of high dose Ad5-vaccinated mice following boost, suggesting improved cytotoxic potential (Fig. 2F) ($p= 0.029$). Furthermore, antigen-specific CD8 T cells in mice primed with low dose Ad5 expressed significantly lower PD-1 relative to high dose Ad5-primed mice following boost (Fig. 3G) ($p< 0.0005$). No significant differences were observed among dose groups in the expression of other phenotypic markers such as CD127, KLRG-1 or the lymphoid homing receptor CD62L (data not shown).

To determine the functionality of vaccine-elicited CD8 T cells following heterologous boost, we isolated splenocytes on day 30 post boost and analyzed their ability to express IFN-γ, TNF-α and IL-2 concurrently after re-stimulation with Gag peptide. We showed that the number of individual vaccine elicited CD8 T cells co-expressing three cytokines was significantly greater in low dose Ad5-primed mice compared with high dose Ad5-primed mice (Fig. 4A-4C). In line with this finding, we also observed that the frequency of Gag-specific CD8 T cells co-producing IFN-γ and TNF-α in mice primed with low dose Ad5 was 2.4% compared with 0.8% in low dose

Ad5-primed mice, a 3 fold difference (*P* = 0.031), while the frequency of Gag-specific CD8 T cells co-producing IFN-γ and IL-2 was 4 fold higher in low dose Ad5-primed mice than in high dose Ad5 primed mice (% of CD8 T cells co-producing IFN-γ and IL-2: mean (low dose Ad5) = 0.3%, mean (low dose Ad5) = 0.07%; *P* = 0.0079). Representative flow cytometry plots of cytokine co-expression on Gag-specific CD8 T cells are shown (Fig. 4D). Furthermore, we found that the proportion of cells co-expressing all three cytokines (IFN-γ, TNF-α and IL-2) was significantly higher in the low dose group compared with the high dose (P=0.008) (Fig. 4E). However, there was no difference in the per cell expression of these cytokines (data not shown). Similar findings were seen following an MVA boost (data not shown). Taken together, these data suggest that lowering the dose of Ad5 at prime will improve anamnestic recall of vaccine-induced memory CD8 T cells, their cytotoxic potential, and functionality, without compromising the magnitude of its primary response.

2.3.3 Excessive up-regulation of PD-1 on Ad5-induced memory CD8 T cells impairs their recall response

Higher PD-1 expression on memory CD8 T cells has previously been shown to correlate with impaired memory recall in some models of immune exhaustion[102]. We therefore reasoned that the higher PD-1 expression on high dose Ad5-induced memory CD8 T cells may be responsible for the blunting of recall response. Data from pre-clinical and clinical studies have shown that PD-1 blockade can be achieved by *in vivo* administration of either anti–PD-1 or anti–PD-1 ligand (aPD-L1)[100, 120, 121, 122, 123, 124]. To assess the functional significance of PD-1 expression

on Ad5 induced memory CD8 T cells, we inhibited the PD-L1:PD-1 pathway by administering anti-PD-L1 blocking antibody (aPD-L1) at the time of boosting with a heterologous vector, Ad35 (Fig. 5A). Mice vaccinated with Ad26 were used as control since CD8 T cells elicited by this vector are not associated with high PD-1 expression[23]. Treatment with anti-PD-L1 significantly enhanced the recall response of Ad5-induced memory CD8 T cells following boost, as compared with controls (3.8 fold expansion in PD-L1 blockade group, compared with 1.6 fold expansion in isotype control group, *P*=0.0317) (Fig. 5B-5C). Similar treatment of Ad26-induced memory CD8 T cells does not alter memory recall. This suggests that the improvement in recall response observed following anti-PD-L1 treatment can only occur in the context of high PD-1 expression. We also did not observe any differential up-regulation of PD-L1 expression on T cells or DCs following Ad5 vaccination compared to Ad26 immunization (data not shown), suggesting that the effect is a CD8 T cell intrinsic phenomenon. There were no differences in expression profiles of phenotypic markers such as CD62L, CD127, or KLRG-1 on the expanded Gag-specific memory CD8 T between both treatment groups (data not shown) . On day 14 post boost, we isolated splenocytes and assessed the functionality of Gag specific CD8 T cells as described earlier. In the Ad5-immunized group, we found a higher frequency of IFN-γ-producing Gag-specific CD8 T cells (*P*=0.04) and TNF-α producing Gag-specific CD8 T cells (P =0.03) following anti-PD-L1-treatment compared to untreated (Fig. 5D and 5E). There was no significant difference in frequency of IL-2-producing Gag-specific CD8 T cells between both groups (Fig. 5F). However, there was a trend towards a more polyfunctional antigen-specific CD8 T cells in the treatment group compared with those untreated (Fig. 5G).

To determine the effect of blocking PD-1 signaling pathway on the primary immune responses of Ad5 vector and assess if this early blockade will lead to a similar improvement in memory recall as seen when the PD-1 pathway was blocked at a later time-point, we administered anti-PD-L1 treatment at the time of Ad5 prime (Fig. 6A). Mice were followed longitudinally to assess tetramer kinetics in blood, and boosted with Ad35 on day 60 post immunization to assess memory recall. We observed that PD-1 blockade does not alter the kinetics of primary response in both Ad5- or Ad26-primed mice. In addition, there was no difference in expression level of surface markers like CD62L, CD127 and KLRG-1 (data not shown), and cytokine co-expression on gag-specific CD8 T cells was similar in both treated and control group (Fig. 6C). In addition, PD-L1 blockade at prime does not lead to a significant enhancement of memory recall following a heterologous Ad35 boost (Fig. 6E and 6F). Taken together, these data suggest that the establishment of an "immune dysfunction" state characterized by up-regulation of PD-1 is required for PD-1 blockade to have a significant effect on recall response

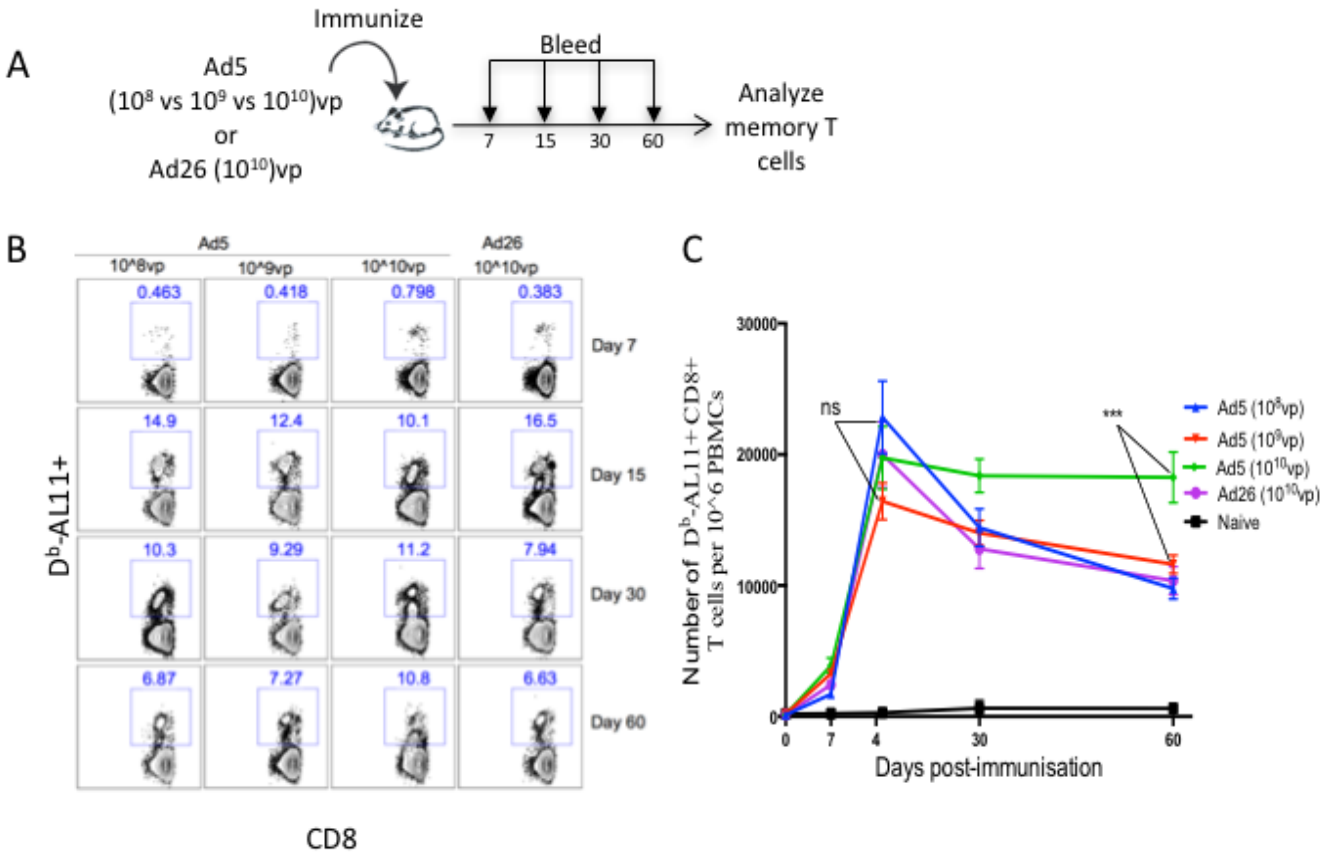
2.3.4 Similar Tregs frequency and suppressive function in Ad5- and Ad26-immunized mice

Regulatory T cells have been shown to modulate CD8 T cell activation, proliferation, differentiation and memory recall[125]. Increased frequency and suppressive function of FoxP3+ CD4 T cells have been reported in LCMV clone 13 infection, a mouse model of chronic infection, in which PD-1 is up-regulated[107, 126]. As a prelude to determining whether Tregs may play a role in the impaired memory recall associated with Ad5-elicited CD8 T cells, we sought to determine

whether Tregs following Ad5 immunization are differentially activated compared to alternative serotype Ad vector immunized mice. We vaccinated FoxP3^{gfp} mice with Ad5 expressing SIV Gag or Ad26 expressing SIV Gag and assessed the frequency of FoxP3+ CD4 T cells at the peak of infection (day 15 p.i) (Fig. 7A). Tregs isolated from naïve mice or LCMV clone 13 infected mice served as controls. As expected, the frequency of Tregs was significantly higher in LCMV clone 13 infected mice compared to naïve or Ad-immunized mice. However, the number and frequency of FoxP3+ CD4 T cells in the spleen and blood of Ad5 immunized, Ad26 immunized and naïve mice was similar. Representative flow cytometry plots are shown in Fig. 7A. To assess whether there is a difference in the suppressive capacity of Tregs induced by these vectors, we isolated splenocytes from Ad5 or Ad26 vaccinated mice, enriched for CD4 T cells followed by FACS sorting for GFP+ Tregs. We co-culture isolated Tregs with Cell Trace Violet (CTV)-labeled naive CD8 T cells (target cells) isolated from naïve mice, in the presence of antiCD3/antiCD28 beads, and assessed CD8 T cell proliferation 72 hours following incubation. Interestingly, we observed that at peak primary response (day 15 post immunization), Tregs from Ad5 immunized and Ad26 immunized mice were similarly suppressive of CD8 proliferation, with both being less suppressive compared with naïve, and Tregs from Clone 13 infected mice being the most suppressive amongst all groups (Fig. 7B-7C). Although more rigorous investigations are required, these results suggest that regulatory T cells may not play an important role in the impaired immune recall seen with Ad5, further supporting the hypothesis that the impaired immune response seen following Ad5 immunization is probably a CD8 T cell intrinsic phenomenon.

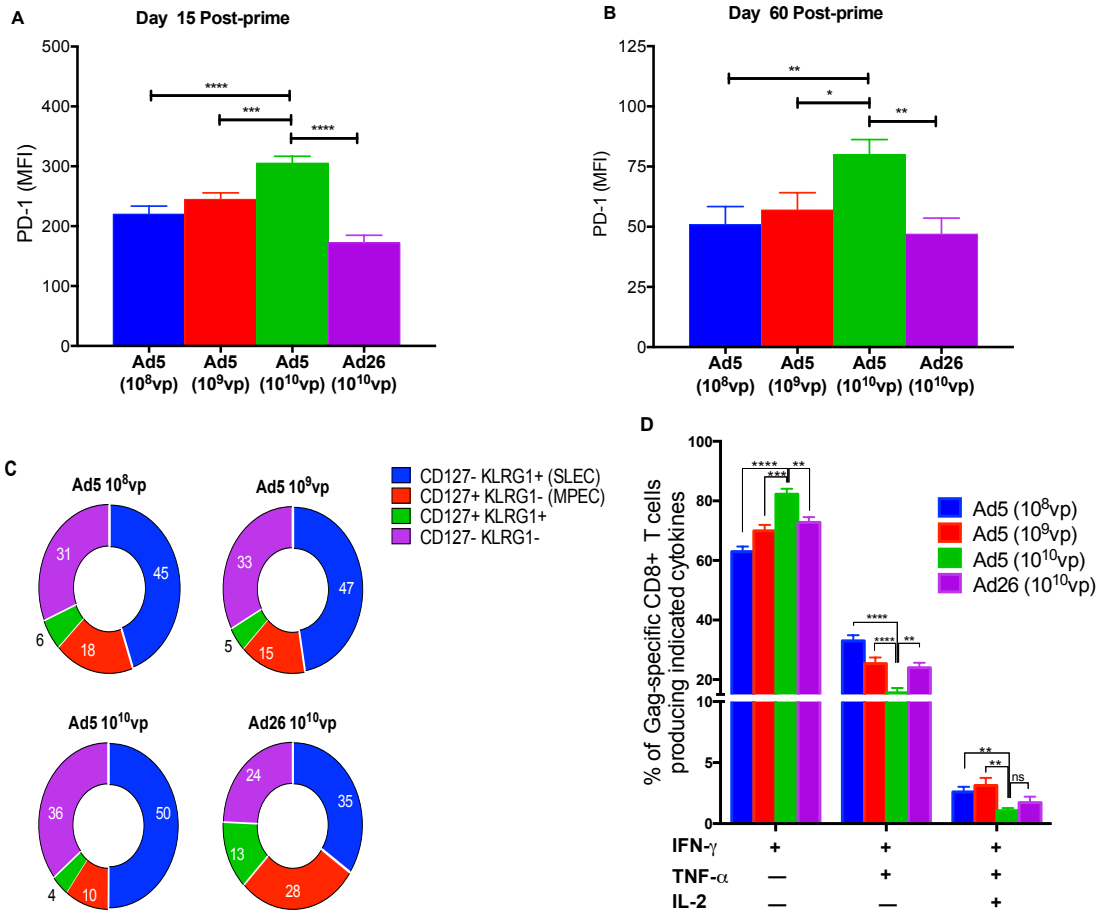
2.4 Figures

2.4.1 Figure 1: Kinetics of vaccine-elicited CD8 T cell following vaccination with different doses of Ad5



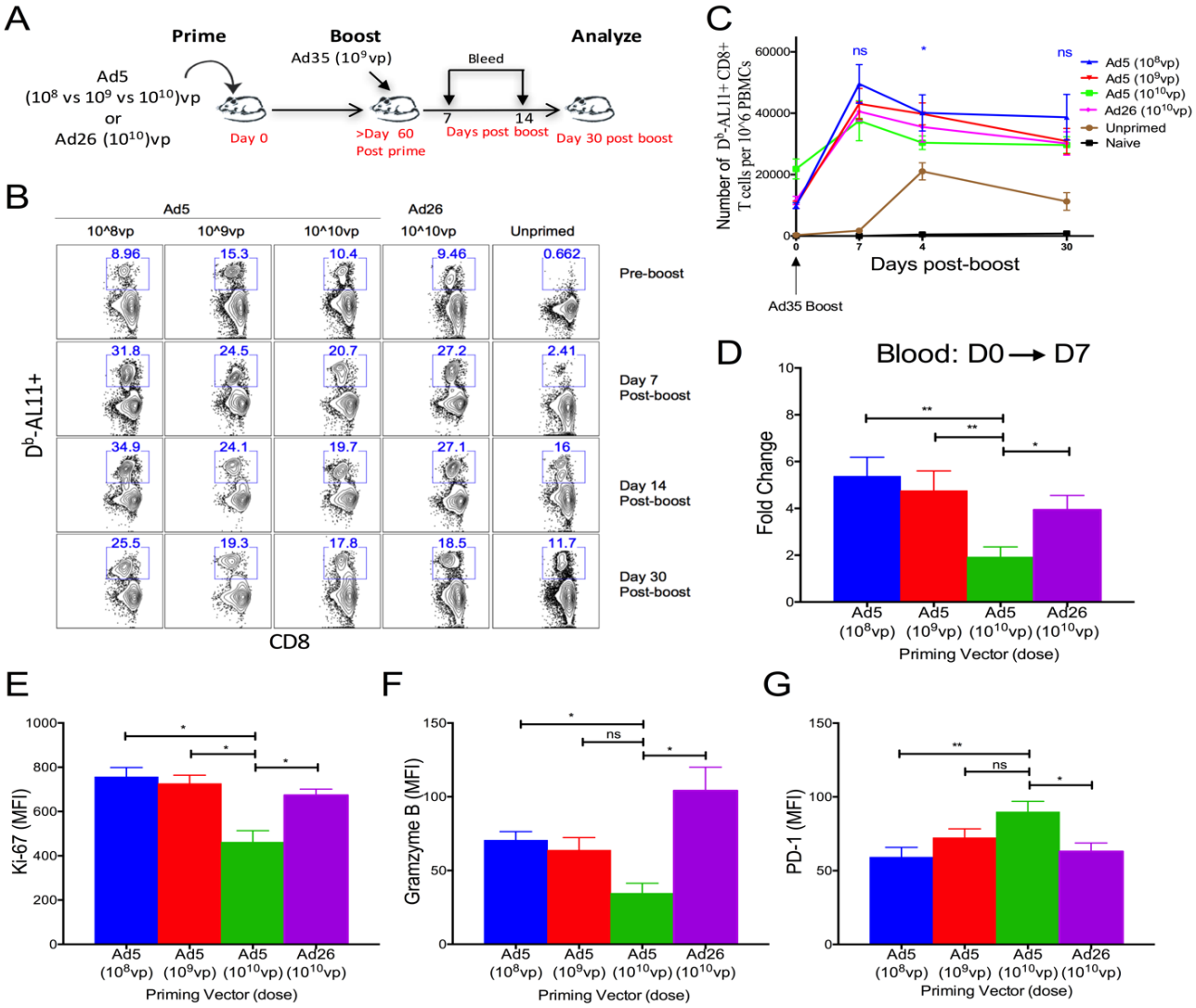
(A) Experimental outline. **(B)** Representative flow cytometry plots showing the percentages of Gag-specific CD8 T cells in blood following immunization with Ad5-SIVGag (10^8, 10^9, and 10^{10}) vp or Ad26-SIVGag (10^{10} vp) **(C)** Number of Gag-specific CD8 T cells in blood. Data are representative of at least three independent experiments, with n=4-5 mice per group per experiment. Error bars represent standard errors of the means (SEM).; ***, $P < 0.001$; ns; not significant.

2.4.2 Figure 2: Phenotype and polyfunctionality of vaccine-induced CD8 T cell following vaccination with different doses of Ad5.



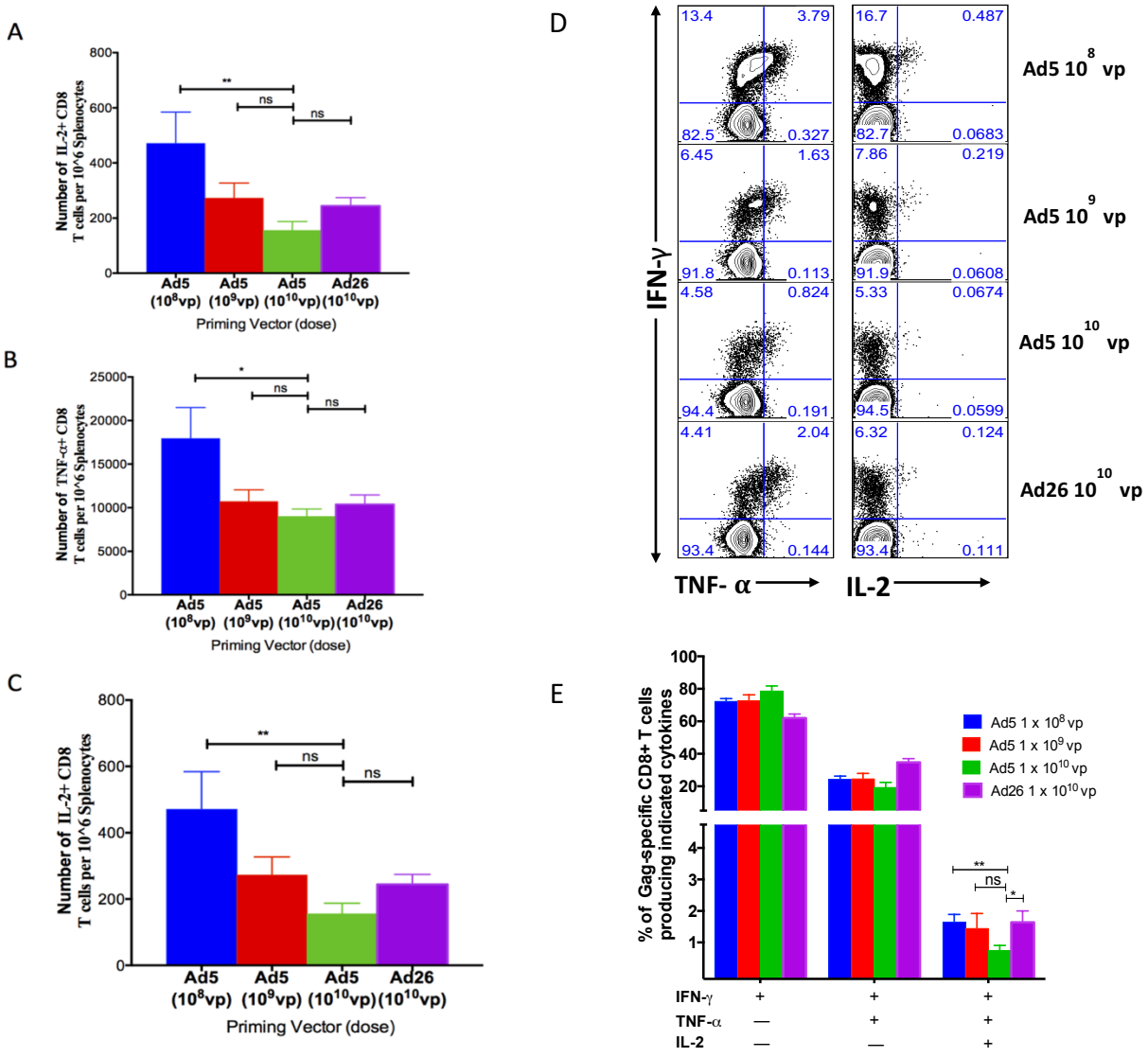
(A and B) MFI of PD-1 on Gag-specific CD8 T cells in blood at (A) peak time point (Day 15 p.i) and at (B) memory time point (Day 60p.i) following immunization with indicated doses of Ad5 and Ad26. **(C)** Proportion (%) of Gag-specific CD8 T cells that express various combinations of CD127/KLRG1 at day 60 following Ad5 or Ad26 vaccination**. (D)** Proportion (%) of total Gag-specific CD8 T cells in blood co-expressing 3 (IFN-γ, TNF-α and IL-2) cytokines, 2 (IFN-γ and TNF-α) cytokines or 1 (IFN-γ) cytokine at day 60 following Ad5 or Ad26 vaccination. Data are representative of at least three independent experiments, with n=4-5 mice per group per experiment. Error bars represent standard errors of the means (SEM). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.001$

2.4.3 Figure 3: Lowering the priming dose of Ad5 vector partially improves the phenotype and the recall responses after Ad35 boosting.



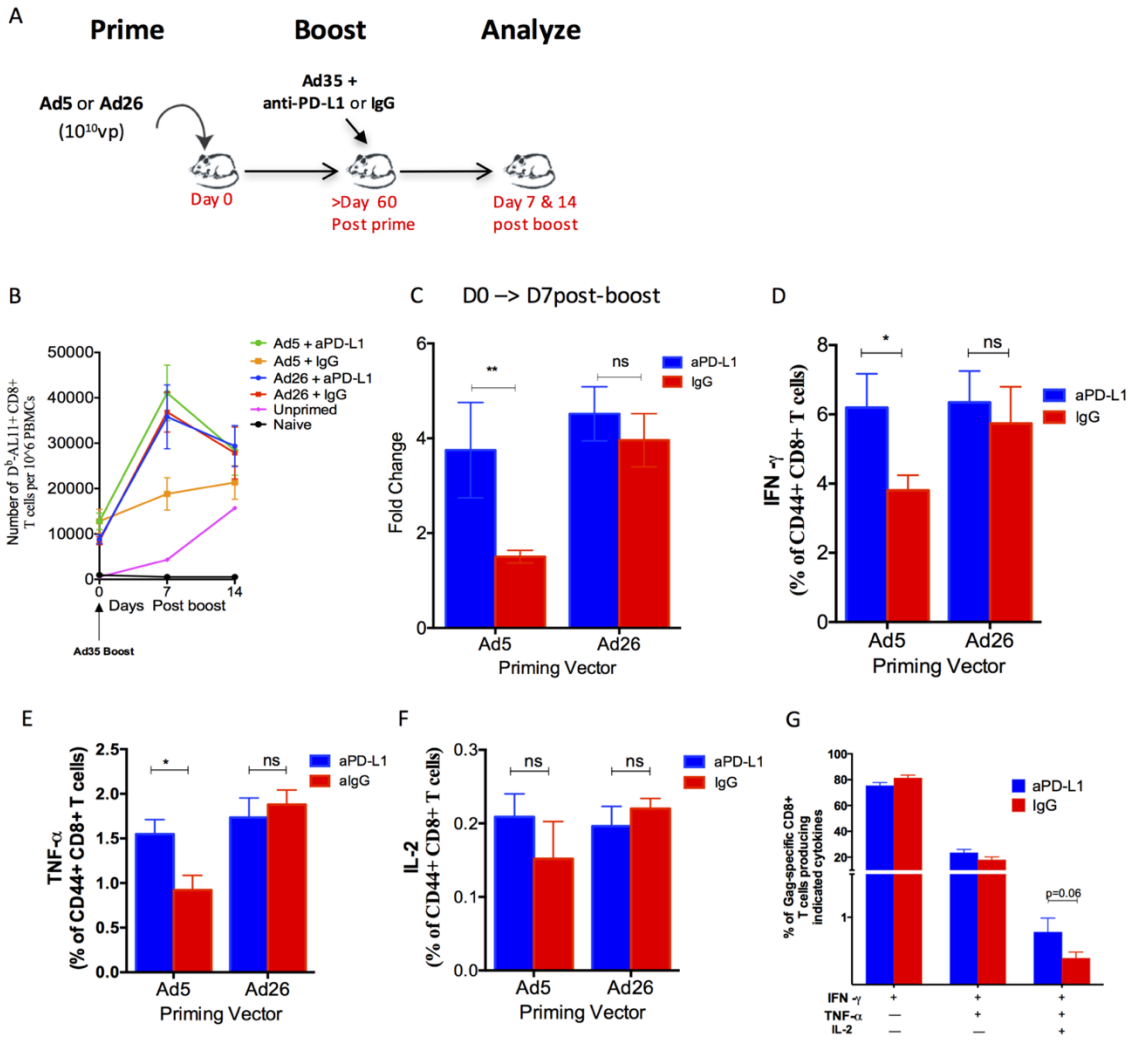
(A) Experimental outline**. (B)** Representative FACS plots showing the percentages of Gag-specific CD8 T cells in blood pre and post Ad35 boost. **(C)** Number of Gag-specific CD8 T cells in blood. **(D)** Summary showing the fold-increase of Gag-specific CD8 T cells in blood after Ad35 boost. **(E)** MFI of Ki-67 on Gag-specific CD8 T cells in blood on Day 7 post Ad35 Boost. **(F)** MFI of Granzyme B on Gag-specific CD8 T cells in blood on Day 7 post Ad35 Boost. **(G)** MFI of PD-1 on Gag-specific CD8 T cells in blood on Day 7 post boost. Data are representative of two independent experiments, with n=4-5 mice per group per experiment. Error bars represent standard errors of the means (SEM). *, $P < 0.05$; **, $P < 0.01$; ns, not significant; vp, viral particle.

2.4.4 Figure 4: Priming with low dose Ad5 in a Ad5-Ad35 heterologous prime-boost regimen produce more polyfunctional CD8 T cells.



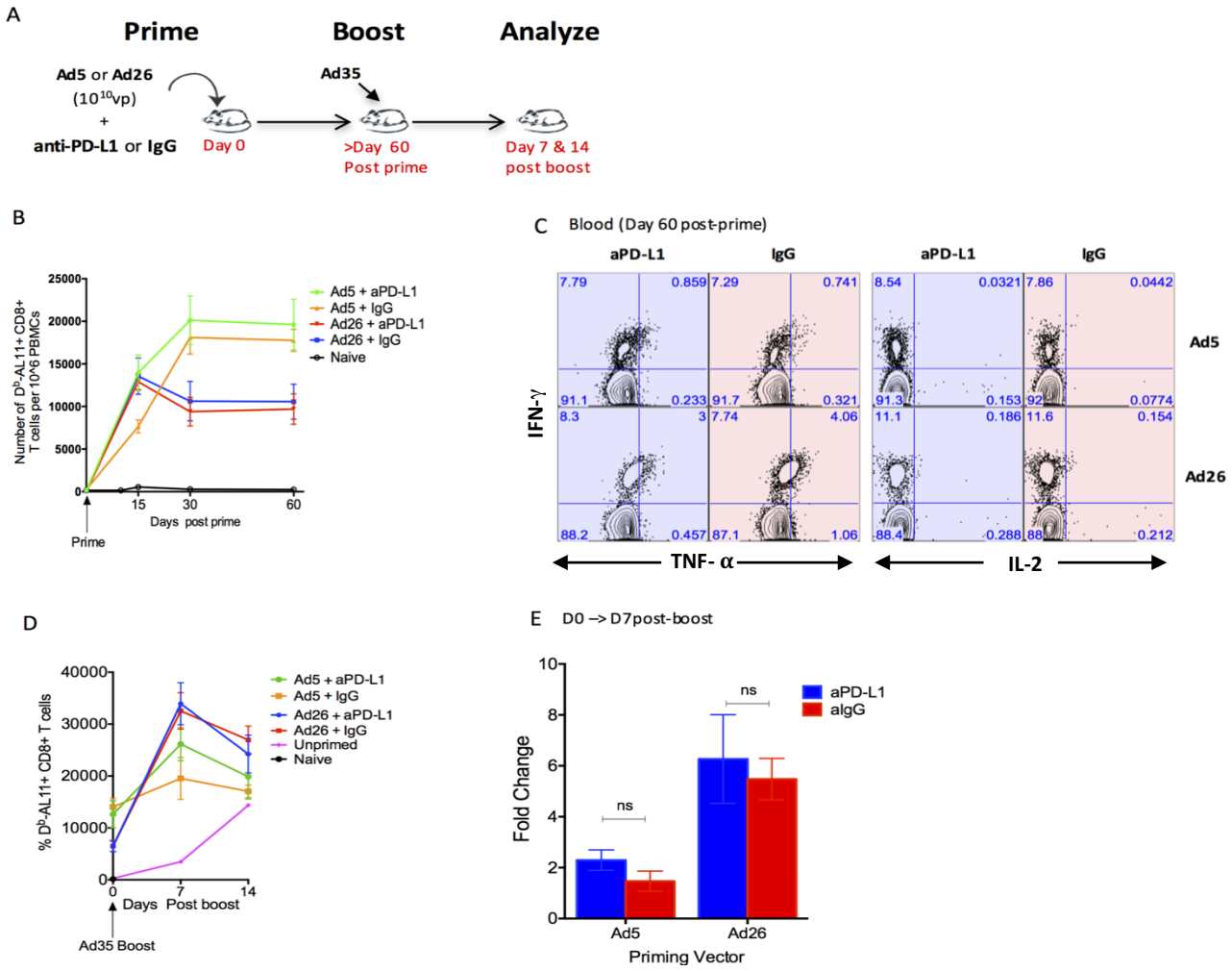
(A-C) Number of splenic Gag-specific CD8 T cells expressing (A) IFN-γ, (B) TNF-α, and (C) IL-2 on day 30 following Ad35 boost **(D)** Representative FACS plots showing the percentages of Gag-specific CD8 T cells in spleen co-expressing IFN-γ and TNF-α, or IFN-γ and IL-2 following Ad35 boost. **(E)** Proportion (%) of total Gag-specific CD8 T cells in spleen co-expressing 3 (IFN-γ, TNF-α and IL-2) cytokines, 2 (IFN-γ and TNF-α) cytokines or 1 (IFN-γ) cytokine at day 30 following Ad35 boost. Data are representative of two independent experiments, with n=4-5 mice per group per experiment. Error bars represent standard errors of the means (SEM). *, $P < 0.05$; **, $P < 0.01$; ns, not significant; vp, viral particle.

2.4.5 Figure 5: Excessive up-regulation of PD-1 on Ad5-induced memory CD8 T cells impairs their recall response.



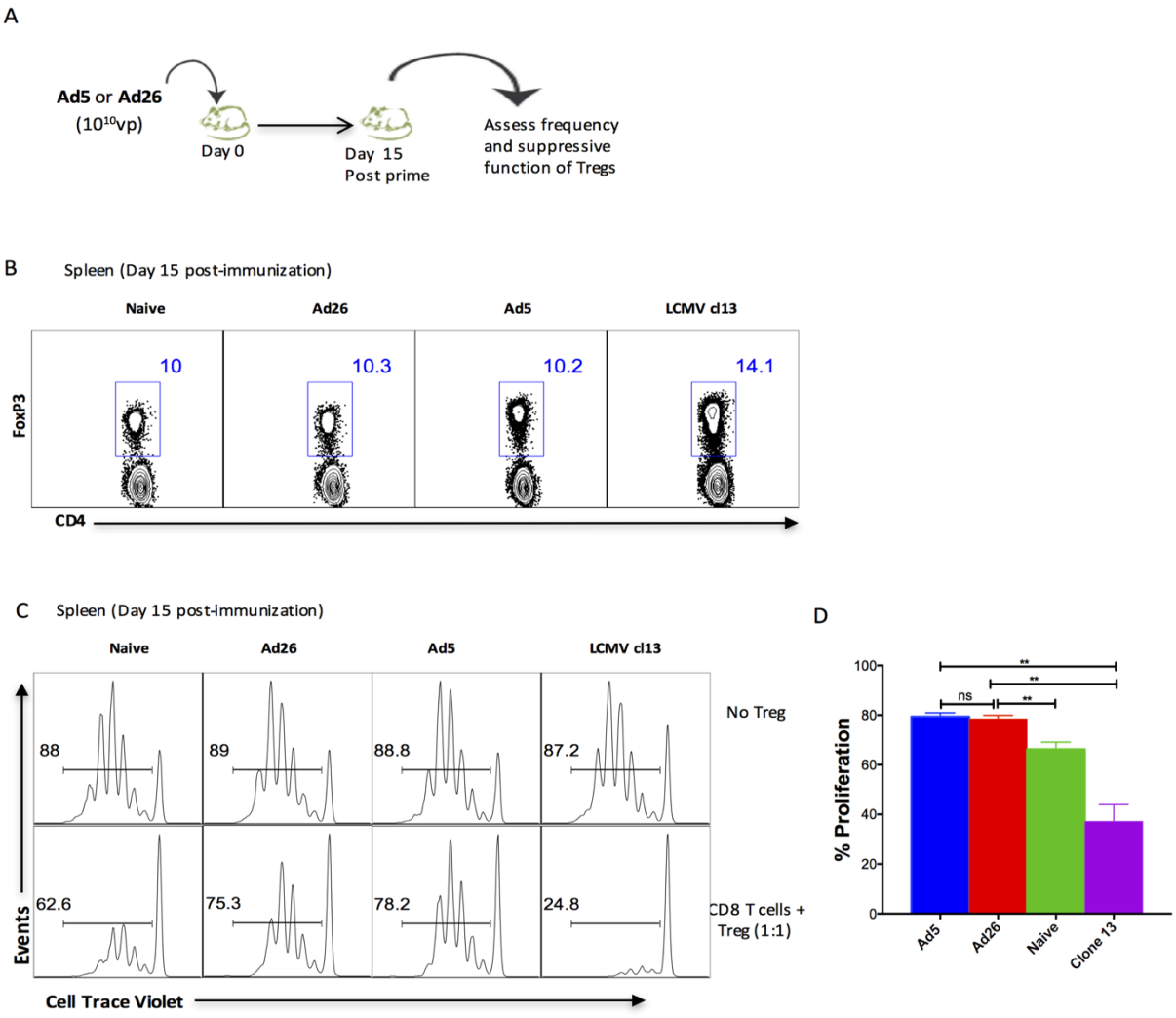
(A) Experimental outline**. (B)** Number of Gag-specific CD8 T cells in blood following Ad35 boost +/- aPD-L1 or IgG treatment. **(C)** Summary showing the fold-increase of Gag-specific CD8 T cells in blood after Ad35 boost +/- aPD-L1 or IgG treatment. **(D-F)** Percentage of splenic Gag-specific CD8 T cells expressing (D) IFN-γ, (E) TNF-α, and (F) IL-2 on day 14 following Ad35 boost +/- aPD-L1 or IgG treatment. **(G)** Proportion (%) of total Gag-specific CD8 T cells in spleen co-expressing 3 (IFN-γ, TNF-α and IL-2) cytokines, 2 (IFN-γ and TNF-α) cytokines or 1 (IFN-γ) cytokine at day 30 following Ad35 boost in Ad5-primed mice. Data are representative of two independent experiments, with n=4-5 mice per group per experiment. Error bars represent standard errors of the means (SEM). *, $P < 0.05$; **, $P < 0.01$; ns, not significant; vp, viral particle.

2.4.6 Figure 6: PD-L1 blockade prior to priming does not affect the kinetics, memory conversion or anamnestic response of Ad5-induced CD8 T cells



(A) Experimental outline**. (B)** Number of Gag-specific CD8 T cells in blood following Ad5 or Ad26 immunization +/- aPD-L1 or IgG treatment. **(C)** Representative FACS plots showing the percentages of Gag-specific CD8 T cells in spleen (D60 post immunization) co-expressing IFN-γ and TNF-α, or IFN-γ and IL-2 following Ad5 or Ad26 immunization +/- aPD-L1 or control IgG treatment. **(D)** Number of Gag-specific CD8 T cells in blood following Ad35 boost **(E)** Summary showing the fold-increase of Gag-specific CD8 T cells in blood after Ad35 boost. Data are representative of two independent experiments, with n=4-5 mice per group per experiment. Error bars represent standard errors of the means (SEM). ns, not significant; vp, viral particle.

2.4.7 Figure 7: Similar Treg frequencies and suppressive functions in Ad5- and Ad26-immunized mice.



(A) Experimental outline**. (B)** Representative FACS plots showing the percentages of foxP3^{+} CD4 T cells in spleen on day 15 following vaccination with Ad5 10^{10}vp and Ad26 10^{10}vp. Control are naïve mice and LCMV clone 13 infected mice. **(C)** Suppression of CD8 T cell proliferation by Tregs isolated at day 15 following vaccination with Ad5 10^{10}vp and Ad26 10^{10}vp. Numbers represent percentage of proliferating cells. (D) Summary showing percentage proliferation of CD8 T cells at day 15 following vaccination with Ad5 10^{10}vp and Ad26 10^{10}vp Data are representative of two independent experiments, with n=4-5 mice per group. Suppression assay was done in triplicates. Error bars represent standard errors of the means (SEM).; **, $P < 0.01$; ns, not significant; vp, viral particle.

3. Chapter 3: Discussion and perspectives

The induction of high magnitude CD8 T cell responses has been shown to correlate with vaccine-mediated protection against ebola, malaria and SIV[3, 4, 127]. Although a single administration of an Ad vector can elicit detectable CD8 T cell responses, heterologous prime-boost regimens are typical used to generate higher magnitude responses in conventional vaccination regimes. As a stand alone vaccine, Ad5 elicits high magnitude CD8 T cell response that confer some protection in preclinical studies[78]. However, following a heterologous boost, these primary CD8 T cells exhibit an impaired proliferative capacity, which subsequently impacts protective efficacy[23]. Further evaluation has revealed that the primary CD8 T cells induced by Ad5 vaccination display a partially exhausted phenotypic profile characterized by high expression of immunoinhibitory receptors, poor polyfunctionality, and an impaired memory conversion, comparable with chronic LCMV infection in mice[23, 83]. In this study, we evaluated the mechanism underlying Ad5-induced impaired immune responses by assessing the effect of reducing vector dose, blocking PD-1 signaling pathway, and evaluating treg–mediated suppression following Ad5 immunization.

First, we corroborated earlier findings that the reduction of vector dose significantly improves the phenotypic and qualitative profiles of transgene-specific CD8 T cells without affecting the magnitude of the primary immune response. We then extended this observation by showing that vector dose reduction and PD-1 signaling blockade result in an improvement in Ad5-induced immune dysfunction as evidenced by the improved memory recall following boost. Finally, we showed that, compared to alternative serotype adenovirus, Ad5

immunization does not differentially affect the magnitude or suppressive function of Tregs. Taken together, these data provide some mechanistic understanding of Ad5-induced immune dysfunction.

In broad terms, the factors influencing immune response elicited by Ad vectors may be categorized into virological and immunological factors (Figure A). Possible virological factors include the serotype of the Ad vector, its cellular receptor specificity, intracellular trafficking route employed by the vector, the vector dose or route of administration. In terms of immunological factors influencing Ad5-induced immune dysfunction, these may either be CD8 T cell intrinsic or CD8 T cell extrinsic. Likely intrinsic factors are immunoinhibitory receptors expressed on CD8 T cells, transcription factors (e.g. T-bet and Eomes) or epigenetic factors, such as DNA expression regulatory enzymes (e.g. histone deacetylases (HDAC)), similar to what has been observed in chronic LCMV infection[128, 129]. Extrinsic CD8 T cell factors include the innate immune system, such as innate cytokine and chemokine profiles or the subset of dendritic cells transduced. Other extrinsic factors include the induction of co-stimulatory or co-inhibitory molecules e.g. PD-L1 on transduced antigen presenting cells, or the effect of other cells of the adaptive immune system such as conventional CD4 T cells or regulatory T cells. All these factors are known to play important roles in the development and maintenance of T cell exhaustion in classical models of immune exhaustion.

It is known that the dose and duration of antigenic stimulation profoundly affect the phenotype, differentiation, functionality, and proliferative capacity of effector T cells produced[130, 131, 132, 133]. The typical primary CD8 T cell response following an exposure to a

rapidly-cleared antigen as seen in acute infection or vaccination can be divided into three phases: (1) an expansion phase, characterized by activation and rapid expansion of antigen-specific naïve cells into effector T cells, that mediate antigen clearance; followed by (2) a contraction phase, in which approximately 90-95% of the effector CD8 T-cell population undergo apoptotic cell death, leaving a small subset of cell that go through the (3) memory differentiation and maintenance phase, in which stable, polyfunctional memory CD8 T cells are maintained via homeostatic proliferation[134]. In contrast, persistent antigen stimulation as seen in chronic infection in mice usually leads to a much delayed CD8 T cell contraction phase due to antigen persistence[135]. It has been shown that Ad5 immunization leads to a protracted CD8 T cell expansion phase, with little or no immune contraction[23, 81, 83, 84, 136]. In our study, we showed that reducing the dose of Ad5 leads to a more pronounced CD8 T cell contraction, similar to what is observed following immunization with alternative serotype Ad vectors[23, 81], MVA[136], or following an acute LCMV infection[137]. It has also been shown that the duration and persistence of antigenic stimulation is dependent on the Ad vector type and vector dosage[138]. Although, our study did not directly assess the persistence of antigen stimulation following administration of different doses of Ad5 vector, we hypothesize that the improved contraction we observed is likely due to a reduction in amount and duration of antigen expression following low dose Ad5 immunization.

Using the LCMV mice infection model, Joshi et al. showed that effector CD8 T cells can be differentiated into SLEC ($CD127^{-}KLRG1^{+}$) and MPEC ($CD127^{+}KLRG1^{-}$) populations based on expression of both CD127 and KLRG1 on CD8+ T cells[114]. SLECs, which are short lived,

predominate in situations of antigen persistence but undergo significant apoptosis following antigen clearance, while MPECs developed into long-lived, self-renewing memory CD8 T cells. In addition, compared to SLECs, MPECs are more "functionally mature" with an increased capacity to proliferate and produce IL-2 in addition to IFNγ and TNF-α, i.e. greater polyfunctionality. We observed that a low dose Ad5 immunization leads to a higher proportion of CD8 T cells differentiating into the relatively more stable MPECs with improved polyfunctionality (IFN-γ^{+}TNF-α^{+}IL-2^{+}). This improved memory differentiation probably resulted in the enhanced recall expansion we observed following heterologous boosting with Ad35 (Fig, 2C-D) or MVA (data not shown). We also showed that there is a lower per cell PD-1 expression following low dose Ad5 immunization compared to high dose Ad5. Since the duration of antigenic stimulation is also known to affect the expression of these markers[139, 140, 141, 142], we speculate that the overall improvement seen with lower dose of Ad5 is a result of reduced antigen persistence.

The route of administration of Ad5 vector immunization may also influence the immune response elicited, such that intravenous administration leads to a more dysfunctional immune response compared to other modes of administration. Intravenous injection of adenovirus 5 leads to a rapid accumulation of the virus within the liver due to its hepatic tropism[143]. High viral load in the liver leads to T cell exhaustion and depletion of antigen-specific cytotoxic T lymphocytes[144, 145]. Therefore, another plausible explanation for Ad5-immune dysfunction is that high dose Ad5 immunization results in a significantly higher number of viral particles migrating to the liver, where they can contribute to the development of dysfunctional T cells. This possibility needs to be further explored.

Exhausted T cells seen during chronic infections such as HIV, malaria, HCV, and HBV in humans, or chronic LCMV infection in mice are known to up-regulate immunoinhibitory receptors such as PD-1. The PD-1/PD-L1 signaling pathway is involved in normal immune responses, and several regulatory processes ranging from tolerance to T cell exhaustion. The significant reduction in PD-1 expression following low dose Ad5 immunization, coupled with the improved recall response suggests that the higher PD-1 expression may be mediating the impaired recall response associated with high-dose Ad5. Indeed, Fuse et el. showed that "helpless" CD8 T cells (without CD4 T cells help) highly express PD-1 and exhibit improve recall response following PD-1 blockade[102]. Similarly, we observed that blocking PD-1 signaling via an *in vivo* PD-L1 blockade led to an improved memory recall following secondary antigen encounter. Interestingly, this observation was seen only when the blockade was done at the time of boosting, with no effect seen with PD-L1 blockade at the time of priming with Ad5. The failure of PD-L1 blockade at prime could be due to the need for an "exhausted" state (upregulation of PD-1) to be established before the effect of blocking PD-L1:PD-1 interaction can be seen. PD-L1 is expressed on T cells, B cells, DCs, and is up-regulated following IFN-γ treatment. However, there was no differential up-regulation of PD-L1 on transduced DC in the draining lymph node following Ad5 immunization compared to Ad26 immunization (Penaloza-MacMaster, P, et al,unpublished data). Our data therefore suggest that the intrinsic PD-1 signaling may be partly mediating Ad-5 induced immune dysfunction.

Tregs play a major role in maintaining peripheral tolerance, in addition to controlling immune response to infections. Their role in maintaining T cells in an exhausted state was

demonstrated by the striking rescue of exhausted T cells following Tregs ablation. In a cancer vaccine model, vaccination with alphavirus vector expressing human papillomavirus (HPV) type 16 peptides did not result in changes in the number and/or activity of Tregs, and the depletion of Tregs did not improve the efficacy of this vaccine against tumours[146]. However, alphavirus vector elicited immune responses are not known to exhibit immune exhaustion. With CD8 T cells induced by Ad5 exhibiting a partial exhausted phenotype and the significant role Tregs play in maintaining exhausted T cells, it is possible that regulatory T cells may be contributing to the impaired T cells response seen following Ad5 immunization. Our study did not find any difference in the magnitude and suppressive function of Tregs following Ad5 or Ad26 immunization. We chose Ad26 as control because it does not elicit a dysfunctional immune response like Ad5[23]. Although further studies are needed to completely exclude a role for Tregs, our data provide a hint that the regulatory T cell may not play a significant role in modulating Ad5-induced immune dysfunction.

In summary, our data suggest that antigenic stimulation and the immunoinhibitory receptor PD-1 plays a significant role in modulating Ad5-induced immune dysfunction. The absence of an effect of Ad5 immunization on Tregs and the expression of PD-L1 on antigen presenting cells also suggest that the immune dysfunction seen with Ad5 is primarily a CD8 intrinsic phenomenon. Although pre-existing vector immunity may impede the use of Ad5 in future clinical vaccine trials, particularly in regions of high seroprevalence, the mechanistic implication of the data shown here will be useful in selecting and testing other similar viral vectors for future vaccine development.

3.1 Limitations

The immunogenicity data presented in this study were acquired by assessing immune responses longitudinally in blood, with very few evaluation of the responses in tissues. It is possible that the immune response in blood may slightly differ from those of tissues - both lymphoid and non lymphoid tissues. The decision to focus on blood was due to the need to evaluate memory recall. The assessment of tissue immune response prior of boost will require sacrificing the animal which will preclude an internally controlled evaluation of the magnitude of immune expansion. In addition, the experiments reported here were all carried out in mice. Whether similar findings will be seen in non-human primates and humans is yet to be determined.

3.2 Future directions

The improved immune response seen following immunization with lower dose Ad5 may be due to an accelerated antigen clearance. Future experiments may utilize *in vivo* imaging studies, transgene transcript PCR and measurement of *in vivo* proliferation of adoptively transfer antigen-specific T cells to compare the duration of transgene expression following low dose and high dose Ad5 immunization, and comparing Ad5 to other serotype adenovirus such as Ad26. In our study, the magnitude of transgene-specific memory CD8 T cells was significantly higher in high dose Ad5-primed mice compared to low dose Ad5-primed mice prior to heterologous boost because of the profound immune contraction seen with low dose Ad5 immunization. Future adoptive transfer experiments will normalize for the number of antigen specific memory cells transferred into congenically distinct mice before heterologous boost. Future experiments

will also help to determine if Ad5-induced dysfunction is truly a CD8 T cell intrinsic phenomenon or whether it is due to differences in the immune environment. In addition, an assessment of the amount of vector reaching the liver following intramuscular immunization with high dose Ad5, and the degree of hepatotoxicity, if any, will help to determine if liver sequestration of Ad5 vector contributes to the impaired response.

We show here that PD-L1 blockade leads to an improvement in immune recall following heterologous boost even though PD-L1 expression is not differentially up-regulated on antigen presenting cells following Ad5 compared to Ad26 immunization. It is therefore important to determine if treatment with PD-1 blocking antibody will lead to a similar improvement in immune expansion. In addition, future studies should also assess if a combination of dose reduction and PD-1 signaling blockade will lead to a synergistic improvement in immune recall. The effect of low dose Ad5 immunization on the expression of other immmunoinhibitory molecules such as Tim-3 and CTLA-4, and whether blockade of these receptors will similarly lead to immune rescue should also be determined.

Our preliminary findings also suggest that Tregs may not play a critical role in modulating Ad5-induced immune dysfunction. Future experiment should determine the effect of immunization on Treg magnitude and function at later (memory) time points. It is also important to determine more conclusively the role of Tregs by assessing the effect of Treg depletion using the FoxP3^{DTR} mouse model. Finally, and most importantly, future research should assess the protective efficacy of the improved immune response observed following low dose Ad5 immunization and PD-1 signaling blockade as the ultimate goal of vaccines is to

protect against pathogens or significantly lessen disease burden.

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