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Adenovirus Specific T-Cell Responses in Humans Following Natural Infection and Vaccination

Natalie A. Hutnick
University of Pennsylvania, hutnick@mail.med.upenn.edu

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Abstract

The adenovirus (Ad) vector is an attractive candidate for vaccines designed to elicit cellular immunity as studies in animals and humans have proven Ad vectors are capable of inducing large transgene-specific T-cell responses. However, given that natural infection by Ad is prevalent globally, pre-existing Ad immunity is a major impediment to the use of recombinant Ad-based vaccines. Though the prevalence of pre-existing neutralizing antibodies has been well characterized, there is a lack of information on the functionality and phenotype of Ad-specific T-cell responses among heterogeneous human cohorts. The lack of protection and possible increased risk of HIV infection in the Merck Ad5 HIV vaccine STEP trial further highlights the need to understand vector-specific immunity in order to produce safe, effective Ad-based vaccines. We aimed to characterize Ad-specific T-cell responses in humans following natural infection and vaccination. Ad-specific T-cell responses were measured by stimulating peripheral blood mononuclear cells (PBMCs) with whole Ad vector or overlapping Ad hexon peptide pools. PBMCs were obtained from 17 healthy adults to study natural infection and longitudinally from 40 participants in Merck phase I Ad5 HIV vaccine studies, 10 of which were enrolled in the STEP trial precursor study using the same vector, dosing, and schedule used for the STEP study. T-cell phenotype and functionality were measured by polychromatic flow cytometry. We found that both CD4+ and CD8+ Ad5-specific T-cells were universally present in subjects independent of their serostatus. Ad5-specific CD8+ T-cells exhibited an effector phenotype and produced the effector functions MIP1α and perforin whereas Ad5-specific CD4+ T-cells had an effector memory phenotype producing IL-2, IFN-γ and TNFα. Ad5-specific T-cells recognized both conserved and variable hexon epitopes making them highly cross-reactive with chimpanzee serotypes. Upon Ad5-based vaccination, Ad5-specific CD4+ T-cells were only transiently expanded and there were no differences in activation or mucosal homing marker expression between Ad5-seronegative and Ad5-seropositive subjects. These data suggest the increased risk of HIV infection observed in the STEP trial was not a result of Ad5-specific CD4+ T-cells. Ad5-specific CD8+ T-cells were also transiently expanded by Ad5-based vaccination, however, there were no changes in functionality. Together, these data suggest though pre-existing Ad-specific T-cells may reduce vaccine efficacy, they should not represent a safety concern for the use of Ad-based vaccines.

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Natalie A. Hutnick

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Supervisor of Dissertation

Signature_____

Dr. Michael R. Betts Ph.D.

Assistant Professor, Department of Microbiology

Graduate Group Chairperson

Signature_____

Dr. Daniel S. Kessler Ph.D.

Associate Professor, Cell and Developmental Biology

Dissertation Committee

Dr. Hildegund CJ Ertl Ph.D., Professor, Department of Immunology, Wistar Institute

Dr. Katherine High M.D., Professor, Department of Pediatrics, Childrens Hopital of Philadelphia

Dr. Guido Silvestri M.D., Associate Professor, Department of Pathology and Laboratory Medicine

Dr. David Weiner Ph.D., Professor, Department of Pathology and Laboratory Medicine

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Chapter 1: Introduction

Vaccine History

The first vaccine was invented over 200 years ago in 1796 by Edward Jenner [1]. Prior to Jenner's small pox vaccine, it was known that infection with small pox resulted in immunity from re-infection. As early as 430 BC women whom survived small pox infection would nurse children in order to transfer protection [2]. The practice of inoculation, or "to graft", was also often practiced. To inoculate, a pustule from an infected individual was sliced and the material subcutaneously introduced into an uninfected individual to induce immunity. This practice, however, had the risk of the inoculated subject developing systemic small pox or contracting other blood born diseases.

Jenner developed his small pox vaccine after considering the stories of cow maids. Women believed that if they were infected with cow pox they would not become infected with small pox. Therefore, Jenner reasoned that the infecting agents must be similar enough so that infection with cow pox, which caused only mild local lesions, would protect from infection with the more devastating small pox. On May 14, 1796 Jenner inoculated 8-year-old James Phillips with material from a cow pox lesion on the hand of Sarah Nelms. When challenged with fresh small pox material a month later, Philips showed no signs of disease [1].

It wasn't until the late eighteen hundreds that a second vaccine was developed by Louis Pasteur to prevent rabies. The field of vaccinology was initially slow to develop but exploded in the 20th century. Since 1900, 21 vaccines have been developed and there has been a 100% reduction in the morbidity of common childhood diseases including smallpox, diphtheria, polio and measles in the US [3]. Despite these great advances there are still several areas of infectious disease with an unmet need.

The World Health Organization (WHO) has identified tuberculosis, malaria, and HIV as diseases which should be targeted for accelerated vaccine development programs [4]. According to the WHO, approximately 33 million people worldwide are infected with HIV/AIDS and 25 million people have died since the epidemic began in 1981 [5]. Although antiretroviral therapies (ART) are available to control HIV, only approximately 25% of HIV infected individuals in lower middle income countries who need ART qualify for it [6]. Additionally, only approximately 20% of individuals in sub-Sahara Africa who are HIV positive know they are infected [6]. With no cure for HIV, eliminating infection with the use of an effective HIV vaccine would have the largest impact on the global HIV pandemic.

T-cell Vaccines

Traditional vaccine approaches utilized a killed or attenuated virus, protein, or toxin to induce neutralizing antibodies (nAb) that target the native

pathogen. The first HIV vaccine was designed as a traditional subunit vaccine utilizing a portion of the HIV envelope, gp160 [7]. Its study in humans began in 1987 and involved immunizing 138 subjects. After *ex-vivo* studies showed a lack of broadly neutralizing HIV specific Abs, the HIV vaccine field turned to producing vaccines that induce cytotoxic T-cells (CTL) [8]. Researchers were further pushed towards a T-cell vaccine In 2003 when a Phase 3 trial of AIDSVAX, an HIV protein subunit vaccine designed to elicit neutralizing antibodies, proved ineffective at preventing HIV infection or lowering viremia in infected subjects [9,10].

T-cell vaccines for HIV have also been pursued because a number of studies have suggested CD8$^+$ CTL are capable of controlling HIV infection. First, the generation of HIV specific CD8$^+$ T-cells in infected subjects occurs at the same time as a decrease in acute viremia [11]. Second, depletion of CD8$^+$ T-cells in SIV infected rhesus macaques resulted in an increase in viremia [12]. Third, several human class I MHC alleles are associated with control of virus and a slower progression to AIDS [13,14]. Finally, the immunological pressure exerted on HIV epitopes by CTL results in viral escape [15,16]. These findings indicate HIV specific CD8$^+$ T-cells exert some control over viremia following infection. Therefore, a vaccine designed to elicit HIV-specific CTL given prior to HIV exposure may prevent the establishment of infection or more likely lower set point viremia, thus improving patient outcome and the transmission rates.
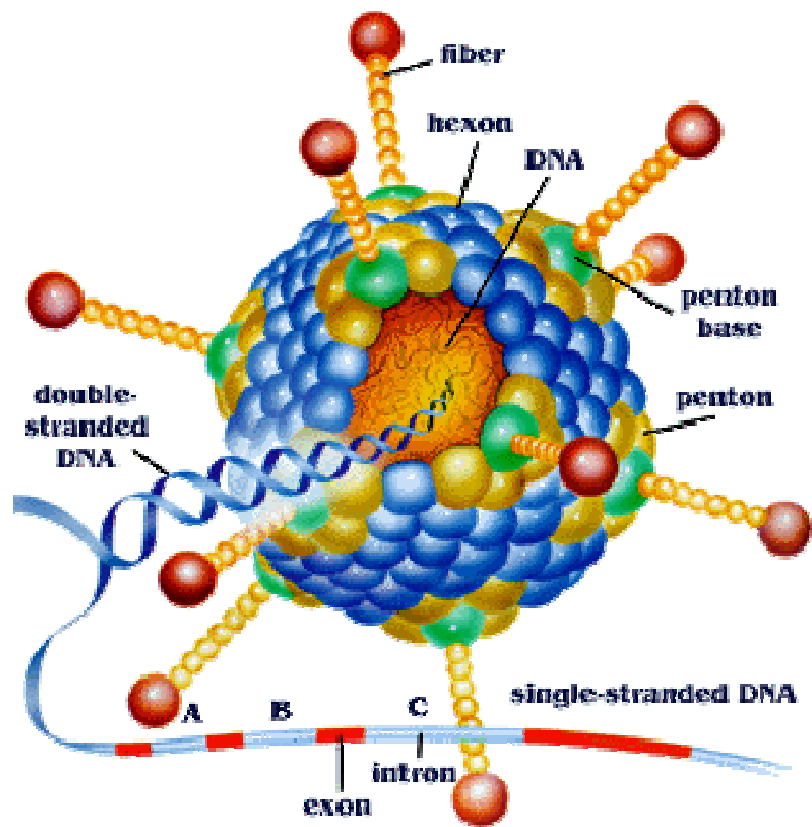
Figure 1: Adenovirus structure. www.nobelprize.org
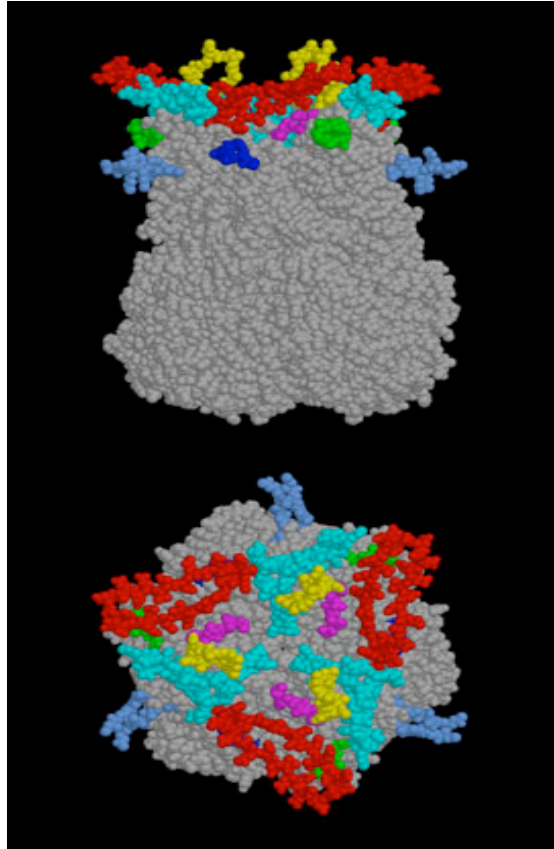
Figure 2: Adenovirus hexon protein trimmer. Grey areas represent protein sequences conserved between diverse serotypes. Colored sequences represent the 7 loops that are hypervariable and define the 51 human serotypes. [17]

Adenovirus

The primary platforms in development for generating CTL responses utilize viral vectors, the most potent among these appears to be recombinant adenoviruses (Ad). Ads are a group of icosahedral, non-enveloped viruses (Figure 1) [18]. There are currently 52 identified human Ad serotypes classified into 6 subgroups, A-F [19,20]. Subgroups are associated with specific tissue tropism in humans that are largely a result of individual subgroups utilizing unique cellular receptors differentially expressed throughout the body which are required for virus binding and entry [21]. The prevalent Ad5 serotype binds to the coxsackie adenovirus receptor (CAR) that is responsible for tight junctions between polarized epithelial cells [22]. The CAR has been detected in human tissues including the heart, brain, pancreas, intestine, lung, liver, testis and prostate [22,23,24]. Virus binding requires the CAR to interact with the Ad fiber, one of the three major Ad capsid proteins [25]. The fiber protein is a long filament anchored by penton proteins [26,27]. The remainder and majority of the Ad capsid consists of trimmers of 240 hexon proteins [28,29]. Within the hexon protein there are seven hypervariable regions (Figure 2) [30,31]. The remaining ~80% of the hexon sequence are conserved between Ad serotypes (Figure 3) [32].

The Ad capsid contains a double stranded, linear, DNA genome of approximately 34-43 kb (Figure 4). The genome contains two inverted

Pan 7   (1)MATPSMLPQWAYMHIAGQDASEYLSPGLVQFARATDTYFSLGNKFRNPTVAPTHDVTTDRSQRLTLRFVPVDREDNTYSYKVRYTLAVGDNRVLDMASTYFDIRGVLDRGPSFKPYSGTAYNSLAPKGAPNTCQWTYKAG
Pan-6   (1)MATPSMLPQWAYMHIAGQDASEYLSPGLVQFARATDTYFSLGNKFRNPTVAPTHDVTTDRSQRLTLRFVPVDREDNTYSYKVRYTLAVGDNRVLDMASTYFDIRGVLDRGPSFKPYSGTAYNSLAPKGAPNSSQWEQAKT
Ad5   (1)MATFSMMPQWSYMHISGQDASEYLSPGLVQFARATEDTYFSLGNKFRNPTVAPTHDVTTDRSQRLTLRFIPVDREDTAYSYKARFTLAVGDNRVLDMASTYFDIRGVLDRGPTFKPYSGTAYNALAPKGAPNPCEWDEAAT

Pan 7(141)-DTD------------------TEKTYTYGNAPVQGIS---ITKDGIQLGTDSDG---QAIYADETYQPEPQVGDAEWHDITGTDEKYGGRALKPDTKMKPCYGSFAKPTNKEGGQANVKTETG--GTKEYDIDMAFFDN
Pan 6(141)GNGG------------------TMETHTYGVAFMGGEN---ITKDGLQIGTDVTANQNKPIYADKTFQPEPQVGEENWQETEN---FYGGRALKKDTNMKPCYGSYARPTNEKGGQAKLKVGDDGVPTKEEDIDLAFFDT
Ad5   (141)ALEINLEEEDDDNEDEVDEQAEQQKTHVFGQAPYSGIN---ITKEGIQIGVEGQT----PKYADKTFQPEPQIGESQWYETEIN--HAAGRVLKKTTPMKPCYGSYAKPTNENGGQGILVKQQN--GKLESQVEMQFFST
                    HVR 1   HVR 2   HVR 3   HVR 4

Pan 7(254)RSAAAAG---LAPEIVLYTENVDLETPDTHIVYKAGTDDSSESINLGQQSMPNRPNYIGFRDNFIGLMYYNSTGNMGVLAGQASQLNAVVDLQDRNTELSYQLLLDSLGDRTRYFSMWNQAVDSYDPDVRIIENHGVEDE
Pan 6(257)PGGTVNGQDEYKADIVMYTENTYLETPDTHVVYKPGKDDASSEINLVQQSMPNRPNYIGFRDNFIGLMYYNSTGNMGVLAGQASQLNAVVDLQDRNTELSYQLLLDSLGDRTRYFSMWNQAVDSYDPDVRIIENHGVEDE
Ad5   (270)TEATAGNGDNLTPKVVLYSEDVDIETPDTHISYMPTIKEGNSRELMGQQSMPNRPNYIARDNFIGLMYYNSTGNMGVLAGQASQLNAVVDLQDRNTELSYQLLLDSIGDRTRYFSMWNQAVDSYDPDVRIIENHGTEDE
         HVR 5   HVR 6

Pan 7(391)LPNYCFPLDAVGRTDTYQGIKAN----GDNQTTWTKDDTVNDANELGKGNPFAMEINIQANLWRNFLYANVALYLPDSYKYTPANITLPTNTNTYDYMNGRVVAPSLVDAYINIGARWSLDPMDNVNPFNHHRNAGLRYR
Pan 6(397)LPNYCFPLDGSGTNAAYQGVKVKDGQDGDVESEWENDDTVAARNQLCKGNIFAMEINLQANLWRSFLYSNVALYLPDSYKYTPTNVTLPTNTNTYDYMNGRVTPPSLVDAYLNIGARWSLDPMDNVNPFNHHRNAGLRYR
Ad5   (410)LPNYCFPLGGVINTETLTKVKPKTG---QENGWEKDATEFSDKNEIRVCNNFAMEINLNANLWRNFLYSNIALYLPDKLKYSPSNVKISDNPNTYDYMNKRVVAPGLVDCYINLGARWSLDYMDNVNPFNHHRNAGLRYR
                    HVR 7

Pan 7(527)SMLLGNGRYVPFHIQVPQKFFAIKSLLLLPGSYTYEWNFRKDVNMILQSSLGNDLRTDGASIAFTSINLYATFFPMAHNTASTLEAMLRNDTNDQSFNDYLSAANMLYPIPANATNVPISIPSRNWAAFRGWSFTRLKTR
Pan 6(537)SMLLGNGRYVPFHIQVPQKFFAIKSLLLLPGSYTYEWNFRKDVNMILQSSLGNDLRTDGASIAFTSINLYATFFPMAHNTASTLEAMLRNDTNDQSFNDYLSAANMLYPIPANATNVPISIPSRNWAAFRGWSFTRLKTR
Ad5   (547)SMLLGNGRYVPFHIQVPQKFFAIKNLLLLPGSYTYEWNFRKDVNMVLQSSLGNDLRVDGASIKFDSICLYATFFPMAHNTASTLEAMLRNDTNDQSFNDYLSAANMLYPIPANATNVPISIPSRNWAAFRGWAFTRLKTK

Pan 7(667)ETPSLGSGFDPYFVYSGSIPYLDGTFYLNHTFKKVSITFDSSVSWPGNDRLLTPNEFEIKRTVDGEGYNVAQCNMTKDWFLVQMLAHYNIGYQGFYVPEGYKDRMYSFFRNFQPMSRQVVDEVNYKDYQAVTLAYQHNNS
Pan 6(677)ETPSLGSGFDPYFVYSGSIPYLDGTFYLNHTFKKVSITFDSSVSWPGNDRLLTPNEFEIKRTVDGEGYNVAQCNMTKDWFLVQMLAHYNIGYQGFYVPEGYKDRMYSFFRNFQPMSRQVVDEVNYKDYQAVTLAYQHNNS
Ad5   (687)ETPSLGSGYDPYYTYSGSIPYLDGTFYLNHTFKKVAITFDSSVSWPGNDRLLTPNEFEIKRSVDGEGYNVAQCNMTKDWFLVQMLANYNIGYGFYIPESYKDRMYSFFRNFOPMSROVVDDTKYKDYQOVGILHQHNNS

Pan 7(807)GFVGYLAPTMRQGQPYPANYPYPLIGKSAVASVTQKKFLCDRVMWRIPFSSNFMSMGALTDLGQNMLYANSAHALDMNFEVDPMDESTLLYVVFEVFDVVRVHQPHRGVIEAVYLRTPFSAGNATT
Pan 6(817)GFVGYLAPTMRQGQPYPANYPYPLIGKSAVASVTQKKFLCDRVMWRIPFSSNFMSMGALTDLGQNMLYANSAHALDMNFEVDPMDESTLLYVVFEVFDVVRVHQPHRGVIEAVYLRTPFSAGNATT
Ad5   (827)GFVGYLAPTMREGQAYPANFPYPLIGKTAVDSITQKKFLCDRTLNRIPFSSNFMSMGALTELGVGRELYANSAHALDMTFEVDPMDEFNGRVAELPFDVVRVHRPHRGVIETYYLRTPFSAGNATT

Figure 3: Adenovirus hexon sequence alignment. The Ad hexon amino acid sequence for human Ad5, chimpanzee Ad7 (Pan-7) and chimpanzee Ad6 (Pan-6). Hypervariable regions (HVR) are underlined. Sequences highlighted in grey are conserved among the three Ads[38].
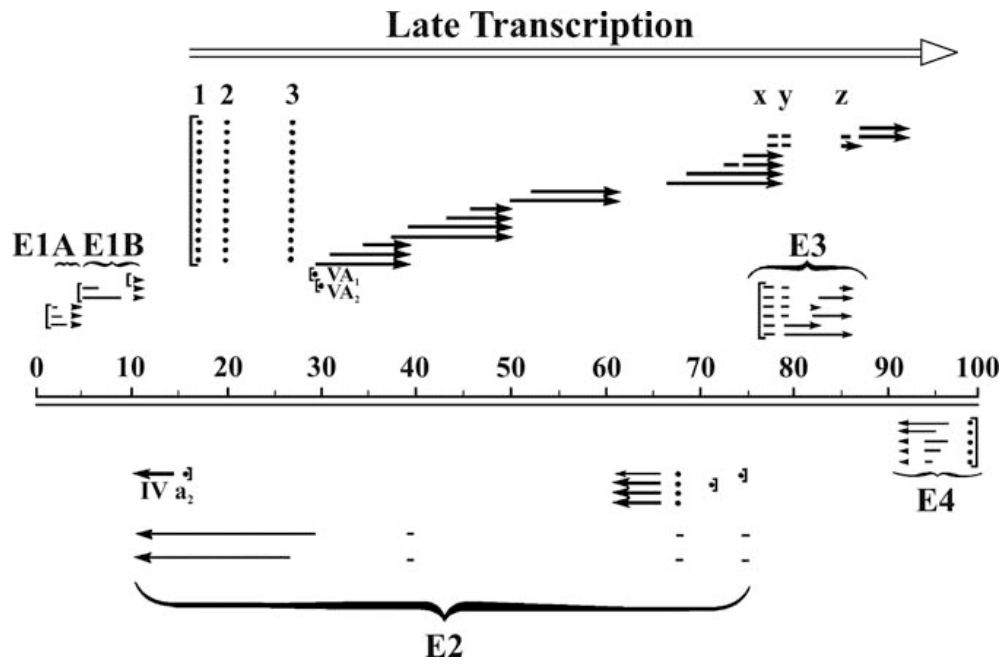
Figure 4: Wild type adenovirus genome [39].

terminal repeats (ITRs), and genes are grouped by early (E1A, E1B, E2, E3, E4, and E5) and late transcription (L1-L5) [33]. Upon infection, the early E1a gene is expressed first and acts as a promoter for the expression of the remaining early genes [34,35]. Therefore, deletion of the E1a gene results in a replication-incompetent virus [36,37]. The E1a gene is also responsible for promoting apoptosis, immune evasion, inducing host cell proliferation and blocking differentiation [40,41,42,43,44].

Another critical Ad gene product is the E3 gene. E3 helps the host cell evade immune recognition through a number of mechanisms. First, E3 allows the virus to evade host immunity by preventing MHC class I transport to the cell surface where it can activate CD8+ T-cells [45,46,47]. Second, The E3 protein also interacts with the transporter associated with antigen processing (TAP) to prevent transport of viral peptides into the ER where they can bind MHC class I [48]. Third, E3 causes surface expression of the receptor internalization and degradation (RID) complex that blocks death receptor-induced apoptosis [49,50,51]. These critical immunomodulatory roles help Ad avoid recognition by CD8^{+} CTL and Ad-infected cell death, thereby ensuring production of new Ad virions.

Ad infections generally occur during childhood and result in a range of acute symptoms depending on the infecting serotype [52]. For example, Ad5 and other group C serotypes generally cause an acute respiratory infection resulting in symptoms such as a fever, headache and runny nose, whereas

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infection with a group A or F virus results in gastrointestinal illness [53,54]. Rarely, Ad infection results in severe disease such as meningitis, though immunocompromised subjects are more susceptible to serious outcomes [55]. The prevalence of Ad infection varies by serotype and geographical region, with approximately 50% of US adults and 90% of African adults testing seropositive for Ad5 nAbs [56]. In contrast, only 5-15% of adults test seropositive for the rare serotype Ad35 [57]. Despite only acute symptoms, Ad maintains its genome in an episomal state in lymphoid tissues and has been detected in healthy adults years after infection [58,59,60].

Adenovirus Vector Design

The potential of replication-defective Ad vectors for gene therapy and vaccine was recognized for several reasons. First, Ads have the ability to infect and persist in a wide variety of cells, including those that are not dividing [71,72]. Second, the transgene is expressed at a high level despite the observation that the viral genome does not integrate into the host genome. This eliminates a major regulatory concern as integration could result in insertional mutagenesis, increasing the patient's risk of cancer [72,73]. Third, the virus is easily engineered replication-defective and grows well in large-scale cell culture. Finally, and perhaps most importantly, Ad vectors have been shown to induce potent and protective T-cell responses [74,75,76].

In order to design an appropriate and safe Ad vector for gene therapy or vaccines, several important considerations need to be made regarding vector serotype, Ad genome deletions and production. First generation Ad vectors based on Ad5 had the E1 gene deleted in order to render the vector replication-defective [96,97]. Molecular cloning is currently used to construct an E1-deleted vector by ligating the desired gene into a bacterial plasmid containing the entire Ad genome [98]. Successful recombinants can be selected by expression of herpes simplex virus thymidine kinase, green fluorescent protein or b-galactosidase [99,100,101]. After removing all bacterial sequences, vector can be grown in an appropriate production cell line [102,103,104]. There are a number of advantages to molecular cloning including the availability of commercially available kits for Ad5 vector production, ease in designing alternative serotype vectors, and the elimination of possible contaminants within primary Ad isolates used for vector construction through homologous recombination.

For production of E1-deleted vector the E1 gene needs to be provided in trans. This can be accomplished by co-transfecting a vector which provides E1 or producing vector in human HEK 293 cells that contain the human Ad5 E1 gene [102]. One regulatory concern with either method was the possibility for homologous recombination and the production of replication-competent Ad particles in a clinical product [105]. This can be overcome by using newer cells lines with truncated E1 sequences such as PER.C6 and N52/E6 or by

using an Ad vector based on non-human serotypes such as chimpanzee Ad [103,104]. Human Ad E1 will allow the production of non-human Ad vectors and the sequences differ enough to prevent homologous recombination in HEK 293 cells [103]. Importantly, some human Ad vectors such as Ad35 are not transcomplemented by Ad5 E1 and therefore require the modification of existing cell lines.

Deleting the E1 gene allows for insertion of approximately 3.5 kb of transgene sequence [106,107,108]. In order to accommodate larger transgenes of up to 7.5 kb, E3 may also be removed [106,107]. Because E3 is not required for Ad replication, E1- and E3-deleted vectors can be grown in cell lines containing E1. Further deletions include E2, E4 and a gutted vector containing only the Ad ITRs [109,110]. In addition to increasing the size of insert that can be used, deleting the E4 gene may reduce immunogenicity by eliminating expression of late gene products. To produce vectors with additional deletions in E2 or E4, cell lines must be created to provide the genes in trans [109,110]. Gutted vectors require use of a helper plasmid to produce vector in cell culture which increases the risk for homologous recombination and contamination with replication competent particles [111]. The vector serotype, deletions, and production method used should be carefully chosen to optimize gene therapy or vaccine efficacy and safety.

Adenoviruses in Gene Therapy

Ads were first studied as a delivery vehicle for gene transfer starting in the early 1990s. Studies with several Ad constructs and inserts proved effective in animal models and were further studied in humans [61,62]. Among the most advanced testing of an Ad vector for gene therapy was for the correction of ornithine transcarbamylase (OTC) deficiency [63,64]. OTC disease is a single x-linked mutation that results in deficient urea synthesis [65]. Treatments for this disease are currently limited and there is a high morbidity and mortality associated with the disease, especially in homozygous males [66,67]. These characteristics make OTC deficiency an ideal candidate for gene therapy.

Following successful and safe OTC gene therapy in mice and monkeys with an E1- and E4-deleted Ad vector, a pilot human study was initiated in 1997 [63,68,69,70]. Seventeen subject with OTC mutations received 2×10^9 viral particles (vp)/kg to 6×10^{11} vp/kg E1- and E4-deleted Ad5 expressing human OTC mRNA delivered via the hepatic artery [63]. Following gene transfer, less than 1% of hepatocytes were transduced and no significant improvements in ureagenesis were observed. Side effects included fever, fatigue, thrombocytopenia, anemia, decreased blood phosphate levels and acute biochemical liver damage.

The outcome of the 18th subject was drastically different [64]. After receiving 6×10^{11} vp/kg vector he progressed to acute respiratory distress and

organ failure that ultimately lead to his death four days after treatment. It was later determined that Ad vector infusion had resulted in systemic immune activation which ultimately lead to the patients death. The demonstrated immunogenicity of Ad vectors made them a less desirable vector for gene therapy and the field has predominantly switched to the use of other vectors for gene correction purposes.

Adenovirus Vaccines

Although Ad were largely ineffective as vectors for gene therapy, their utility as vectors for CTL vaccines was quickly realized. Ad vaccine vectors have been tested most extensively in the setting of HIV. The validity of the Ad5 vector for an HIV-1 vaccine was supported by a large primate study comparing several prominent vaccine vectors that encoded SIV gag inserts. These were DNA, a modified vaccinia ankara virus vector, and a replication-incompetent Ad5 alone or in combination with the DNA vector [74]. Ad5 alone or Ad5 with a DNA prime produced the largest percentage of gag-specific CD8$^+$ T-cells following vaccination. Following challenge with SHIV 89.6P, the DNA prime-Ad5 boost and Ad5 prime- boosted animals more effectively controlled set-point viral load reducing viruses to less than 10^3 vp/ml.

In retrospect, this study was not stringently designed to test the efficacy of an Ad5 HIV-1 vaccine. First, only three monkeys were used in each vaccine group making it difficult to determine what was the average

response and what would be considered an outlier. Second, all Ad5-vaccinated monkeys had the Mamu*A01 MHC class I allele, which is naturally protective from SIV and SHIV infection [77,78]. Lastly, animals were challenged with SHIV 89.6P which appears to be more sensitive to T-cell based vaccine approaches [79].

Following these results with Ad5-based vaccines in Rhesus Macaques; a Phase 1 human study was conducted. Starting May 1 2003, 259 subjects were enrolled in a dose-escalation safety and immunogenicity trial designated as the Merck 016 trial [80]. Subjects received $3x10^6$ vp to $1x10^{11}$ vp Ad5 expressing HIV-1 clade B gag, pol or nef at weeks 0, 4 and 26. There were no serious adverse events associated with vaccination. Subjects receiving $3x10^{10}$ and $1x10^{11}$ vp had a similar immunogenicity with approximately 70% of subjects responding to an HIV insert 4 weeks after the last injection, and lasting up to week 78. One concern raised by animal studies was that pre-existing Ad5 nAb would limit vaccine efficacy, however this was not observed in seropositive subjects (nAb titer >200) in the $3x10^{10}$ vp dose group. Therefore, based on the immunogenicity and safety of a recombinant Ad5-HIV-1 vaccine vector delivered by the IM route, it was decided to further test the vaccine in the phase IIb STEP trial with subjects at a high risk for HIV-1 infection.

STEP trial

The Phase IIb trial was name STEP. It was a multi-center, randomized, double-blind and placebo-controlled trial aimed at establishing efficacy of the Ad5 HIV-1 gag/pol/nef vaccine. All subjects were HIV-1 negative but at a high risk for HIV infection. Half of the three-thousand enrolled subjects were Ad5 seronegative at baseline and half were seropositive. Subjects received three doses of $3x10^{10}$ vp at weeks 0, 4 and 30. The trial was ended at a planned interim analysis by the data safety monitoring board on September 18th, 2007 because no efficacy was shown with the endpoints of either reduced rates of infection or reduced viral load in those infected as well as a possible increased risk of infection in vaccinated subjects.

Analysis revealed the vaccine was immunogenic with 77% of subjects eliciting a T-cell response against at least one of the HIV insert antigens [81]. Immunogenicity, as measured by response rate, magnitude, or functionality of HIV-specific T-cells, was similar in cases (HIV infected during the trial) and non-cases (HIV-). If the vaccine was immunogenic why was it not effective at lowering viral load or preventing infection? One explanation might be that T-cell responses were relatively modest with less than 1.0% of $CD8^+$ T-cells responding to the vaccine antigens [82]. The quality of responding T-cells may also have been different than that required to prevent or modify HIV-1 infection. Additionally, subjects recognized only three HIV antigens and it is

possible that the vaccine epitopes differed from the infecting virus sequence [81]. The final reason for why the Ad5 vaccine may not have been effective is CTL responses alone may not be sufficient to prevent HIV-1 infection.

Subsequent analysis confirmed that there was an increased risk of infection in vaccinated subjects, however this was only seen in subjects who were Ad5 seropositive at baseline. In participants with no Ad5 pre-existing immunity (nAb titer <200 units) there was no difference in the level of HIV infection between placebos and vaccinees [82,83]. However, in participants with a pre-existing Ad5 nAb titer greater than 200 units, 21 cases of HIV infection were reported in those who received the vaccine versus only 9 cases in those who received the placebo. These results suggest that pre-existing immunity to Ad5 as measured by nAb titers increased the risk of HIV infection following Ad5- based vaccination.

One theory for the increased risk of infection in subjects with pre-existing Ad5 immunity is that Ad5-specific CD4$^+$ T-cells became activated upon vaccination. Activated CD4$^+$ T-cells may then up-regulate the HIV-1 receptor CCR5 or traffic to the gut mucosal tissue where they would be the primary targets for HIV infection and replication [84,85,86]. However, post-hoc analysis suggests Ad5-specific T-cell responses in Ad5-seropositive subjects were not the cause of the possible increased risk of HIV infection. The magnitude of Ad-specific CD4$^+$ and CD8$^+$ T-cell responses were actually lower in cases compared with non-cases and there were no differences in the

level of activation as measured by Ki67, Bcl-2 and CCR5. Unfortunately, samples from the STEP trial are limited by sparse peripheral blood mononuclear cells (PBMC) sampling and the lack of a baseline sample. The possibility that a vaccine may increase the risk of infection with the disease it was aimed at preventing highlights the need to understand T-cell specific responses not only to the transgene but also to the vector itself.

Alternative Serotype Vaccines

The efficacy of Ad-based vaccines is reduced by pre-existing Ad-specific immunity. To avoid pre-existing immunity, vectors with a low seroprevalence are in development. These include human Ad26, 35 and 48, as well as chimpanzee AdC6, C7 and C68. There are a number of differences between Ad5 and Ad35 that may affect vaccine efficacy. The worldwide seroprevalence of Ad5 is 40-90%, whereas Ad35 has a seroprevalence of only 2-16% [87]. However, due to a hexon sequence homology of close to 90% between Ad5 and Ad35, it is likely that pre-existing cross-reactive T-cells will recognize Ad35 even in seronegative subjects [38]. Unlike Ad5, Ad35 utilizes CD46 as a receptor for cellular entry [88]. CD46 is a complement regulatory protein expressed on all cell types including hematopoietic and dendritic cells that lack CAR expression. Despite different receptor usage, Ad35 and Ad5 both transduced muscle cells, showed limited biodistribution, were rapidly cleared, and showed similar toxicities when given

as an intramuscular injection [89]. This is in contrast to intravenous delivery in which Ad35 showed limited organ transduction and reduced toxicity compared with Ad5 [90].

Another approach to avoiding pre-existing immunity is the development of Ad vectors based on chimpanzee viruses such as AdC6 and AdC7. The seroprevalence of AdCs is less than 10%, which is even lower than the rare human serotypes [91]. AdCs are easily constructed by molecular cloning and can be grown in cell lines expressing Ad5 E1 without the risk of homologous recombination and the production of replication-competent vectors [92]. Based on E1 sequence homology, AdC6 and AdC7 are most closely related to the human Ad4, though sequence homology with Ad5 still high at approximately 90% [56,92]. Similar to Ad5, the chimp AdC6 and AdC7 utilize the CAR receptor, can be produced at high titers, and have similar levels of *in-vivo* infectivity [92,93]. Animal studies with AdCs have shown induction of potent insert-specific nabs and CD8^{+} T-cell responses prompting further development as vaccine vectors [94,95].

Adenovirus Immunity

Pre-existing Ad-specific immunity represents a major obstacle to the use of Ad vectors not only for gene therapy but also for Ad-based vaccines. Vaccination is less effective in animal models and humans if pre-existing Ad-

specific nAbs are present [112,113]. In addition, nAbs can pose a problem for an Ad-based vaccine even in individuals without pre-existing immunity since an effective CTL vaccine will likely require at least one boost to generate sufficient levels of memory T-cells. The efficacy of a subsequent boosting injection may be limited by nAbs generated during the priming injection. It has been shown that significant levels of nAb result after a single Ad5 injection and can reduced transgene expression following a second injection [114]. Therefore, vaccination with an Ad-based vaccine for one infectious disease would also inhibit the efficacy of a subsequent Ad-based vaccine against heterologous pathogens.

Ad-specific T-cells have also been shown to reduce the transgene-specific response to an Ad5-based vaccine [115]. Ad epitopes have been identified in humans for both CD4^{+} and CD8^{+} T-cells [116,117,118]. The conservation of these epitopes between the 52 serotypes suggests Ad-specific T-cells will be highly cross-reactive [116,117,119,120]. This was partially confirmed in experiments in which human CTL cell lines created against an Ad5 vector were shown to kill target cells infected with diverse Ad serotypes [116,119]. CTL responses were targeted primarily against the hexon protein, which is the most abundant capsid protein delivered upon Ad vector administration [120].

Though nAb are serotype-specific, the cross-reactivity of Ad-specific T-cells suggests they will be universally present in humans, as infection with at

least one Ad serotype occurs in most individuals. Indeed, several studies found Ad5-specific CD4$^+$ and CD8$^+$ T-cell responses irrelevant of seropositivity [59,82,121]. Additionally, T-cells that recognize simian Ad24 were identified in 14 of 14 healthy subjects despite a seroprevalence of only 10% in subjects from the same region [59]. Together, these data support some level of cross-reactivity in Ad-specific T-cells due to Ad sequence conservation among diverse serotypes.

T-cells

An important aspect of studying T-cells is characterizing their functionality and phenotype. The T-cell response to an infectious agent will generate a unique response consisting of cells making a variety of cytokines and expressing an assortment of surface receptors. By utilizing polychromatic flow cytometry, multiple T-cell functions can be measured including macrophage inflammatory protein 1 alpha (MIP1α), interferon gamma (IFN-γ), tumor necrosis factor alpha (TNFα), interleukin-2 (IL-2), perforin, and CD107a. These six functions are reliably assessed by flow cytometry and have unique immunological effects. MIP1α acts as a chemoattractant, recruiting inflammatory cells to the site of infection and mediating a T$_H$1 response [122,123]. IFN-γ and TNFα are both pro-inflammatory cytokines with several immunomodulatory and antiviral effects [124]. IL-2 is required for T-cell survival and the generation of memory responses [125]. Perforin is a

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protein release by cytotoxic cells to mediate target cell killing [126]. CD107a is expressed in the membrane of secretory granules and is exposed upon degranulation. Staining for CD107a, therefore, serves as a surrogate for the release of cytotoxic granules from T-cells [127].

To assess the magnitude of a T-cell response, IFN-γ as measured by ELISpot or flow cytometry is the gold standard assay often used [128,129,130]. However, by measuring IFN-γ alone, the total magnitude of the response may be underestimated, as not all responding cells produce IFN-γ, and IFN-γ alone is often not a correlate of the efficacy of a T-cell response [131,132]. A higher proportion of T-cells making both IFN-γ and IL-2 during HIV, hepatitis C virus, and *M. tuberculosis* infection correlated with better disease control than IFN-γ alone [133,134,135]. Furthermore, a more polyfunctional T-cell response consisting of IFN-γ, IL-2, TNFα, MIP1-β, and CD107a was associated with a slower progression to AIDS [136,137]. Lastly, a larger percentage of polyfunctional T-cells were observed following infection with viruses that are cleared or persist at a low level such as influenza, vaccinia virus, and cytomegalovirus, in contrast to chronic HIV infected patients where there is a high level of persistent antigen [138]. Though a highly functional T-cell response appears to be induced during natural viral infection, a highly functional Ad-specific T-cell response against a vaccine vector may be detrimental, since virally infected cells would be cleared before

transgene expression reaches a high enough level to stimulate a protective

immune response.

Memory Phenotype

In addition to studying the functionality of T-cells, it is also critical to

understand the memory phenotype of antigen-specific cells in order to better

tailor an effective vaccine. T-cells newly differentiated from hematopoietic

stem cells in the bone marrow travel to the thymus where they undergo

positive and negative selection [139,140,141]. This process ensures cells are

not reactive to self-proteins, are able to recognize peptides on self-MHC, and

determines whether T-cells will be a CD4^{+} helper T-cell or CD8^{+} cytolytic T-

cell. Cells emerging from the thymus are considered naïve because they have

yet to see their cognate antigen. Naïve T-cells can be distinguished because

they express costimulatory receptors (CD27, CD28, CD45RA) which need to

be engaged along with the T-cell receptor in order for cells to proliferate and

differentiate into effector T-cells [142,143,144]. Naïve cells also express

adhesion molecules such as CD62L and CCR7 that allow them to traffic

through the lymph nodes where they may encounter their antigen [145,146].

Once T-cells encounter their antigen they become activated,

proliferate, and can differentiate into an effector or memory cell. There are

two theories for how memory cells develop [147]. One theory proposes that

after an infection is cleared a subset of effector cells remain and mature into

memory cells, whereas the other theory proposes that when a naïve cell encounters antigen it proliferates and some progeny become effectors and some memory. Either way, memory and effector cells have unique characteristics and are both critical for an effective vaccine.

Effector cells generated by vaccination are necessary to effectively fight off an infection by producing cytokines to activate innate immunity, providing B-cell help, and directly killing infected target cells. These functions are mediated by the expression of cytokines such as IFN-γ, IL-17, and IL-2; chemokines such as MIP1α and rantes; and cytolytic proteins such as granzymes and perforin [148,149]. During an acute infection, effector cells proliferate to fight the pathogen and then undergo apoptosis once the infection is cleared [150,151]. In chronic infection they often become exhausted from constant antigen stimulation and gradually lose their effector functions [152,153]. Prolonged antigenic stimulation can also result in terminally differentiated cells that lose the ability to proliferate (CD57+) [154]. Once activated, cells down-regulate costimulatory surface proteins (CD27, CD62L) and up-regulate homing proteins (CCR5, $\alpha 4\beta 7$) that allow them to enter the periphery and combat pathogens at the site of infection [155].

In order for a vaccine to remain effective, long-lived memory cells must also be generated. Memory cells have a lower threshold for activation, create a larger pool of antigen specific T-cells, and produce different effector functions compared with naïve cells [156,157]. These characteristics allow for

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the rapid production of effector cells and a quicker secondary response [158]. There are two subsets of memory cells which can be defined by the expression of CCR7 [156]. Central memory cells express CCR7 allowing them to circulate through the secondary lymphoid organs where they have a higher chance of encountering antigens and initiating an immune response. Effector memory cells lacking CCR7 patrol areas of inflamed tissue and have immediate effector capabilities to eliminate pathogens.

Though the phenotype of a vaccine-induced T-cell response is critical for efficacy and long-term protection, the quality of the response may be just as important. Studies of an Ad-based *Leishmania major* vaccine in mice showed a milder disease upon infection with *L. major* when a lower dose of the Ad vaccine was administered [159]. The degree of protection did not correlate with the magnitude of insert-specific IFNγ^+ CD4$^+$ T-cells but did correlate with the quality of the response. Mice given the low Ad dose had more polyfunctional CD4$^+$ T-cells expressing IL-2, TNFα, and IFN-γ compared with mice given the high dose. Additionally, triple positive CD4$^+$ T-cells produced more IFN-γ and TNFα on a per-cell basis compared with single and double positive cells, which may explain why having more triple positive cells correlated with better control. Though this model defined polyfunctional T_H1 CD4$^+$ T-cells as a correlate of *L. major* control, no correlates of protection have been defined for vaccine induced CD8$^+$ T-cell responses.

Thesis Goals

The failure of Ad vectors in gene therapy and the STEP trial highlights the need to understand how pre-existing Ad-specific immunity affects the efficacy and safety of Ad vector-based gene delivery. We aimed to characterize the magnitude, phenotype, functionality, and cross reactivity of Ad-specific T-cells following natural infection and vaccination. We hypothesized that Ad-specific T-cells would be present in most donors and cross-react with diverse serotypes based on sequence homology between Ad serotypes. Ad-specific T-cells would have an effector and effector memory like phenotype due to continual stimulation by persistent Ad virus and repeat infection with any of the 52 human serotypes. Likewise, vaccination with Ad5 vector would stimulate pre-existing Ad-specific CD4$^+$ and CD8$^+$ T-cells to a similar extent in baseline Ad5-seropositive and Ad5-seronegative subjects.

There are several facets of this study that are critical to our understanding of Ad T-cell immunity. First, studying a heterologous human population is essential for understanding Ad-specific T-cells as animal models do not replicate the continual Ad re-stimulation human T-cells receive from persistent virus and periodic infection. Second, by using the most advanced flow cytometry techniques we can precisely characterize the detailed phenotype and functionality of Ad-specific T-cells. Third, we developed a novel stimulation assay that allows us to detect T-cell responses to the entire Ad vector directly *ex-vivo* with minimal cell manipulation, thereby reducing the

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chance for responses that are an assay artifact. Lastly, we have access to

very limited and extensive samples from the Merck phase I safety trial using

the same vector, dose and schedule as used for the STEP trial. These

samples are critical for understanding the possible increased risk of infection

in Ad5-seropositive patients as samples from the STEP trial are limited and

lack a baseline time point. This work represents the most extensive

characterization of human Ad-specific T-cell responses performed to date.

The result of this analysis suggests important considerations for future T-cell

based vaccine design.

Chapter 2: Materials and Methods

Subjects

Peripheral blood mononuclear cells (PBMCs) were obtained by aphaeresis of HIV and HCV negative adult healthy donors by the University of Pennsylvania Center for AIDS Research Immunology Core, under the institutional guidelines required for conduct of experiments on human samples.

For studies involving Ad5 vaccination we obtained frozen PBMCs from various Merck phase I Ad5 HIV vaccine trials (Table 3). Written informed consent was obtained from participants. The vaccination dose and schedule for the seropositive and seronegative groups were identical to that used in the phase II STEP trial. PBMCs were obtained from study weeks 0, 4, 8, 18, 26, 30, 42, 52 and 78.

Vector

Human adenovirus 5 (Ad5), chimpanzee adenovirus 6 (AdC6) and chimpanzee adenovirus (AdC7) vectors were prepared using previously described methods [160]. For cell stimulation we used an E1-deleted Ad5 vector that expressed the rabies virus glycoprotein [32,161]. The Ad5 vector was grown on HEK293 cells in DMEM supplemented with 10% fetal calf serum, antibiotics and glutamine [162]. Vectors were purified by CsCl

gradients and quality controlled (infection unit to vp ratios, lack of replication competent Ad5 virus, genome integrity, lack of LPS contamination, sterility)

Neutralizing Antibody Titer

Ad5 neutralizing antibody titers were measured by Lisa Kierstead at Merck Research Laboratories as previously described [163]. Briefly, $2x10^4$ HEK293 cells per well in a 96 well plate were seeded for 2 days. Ad-secreted alkaline phosphatase (SEAP) was incubated for 1 hour at 37 °C either alone or with serial dilutions of serum then added to the 95-100% confluent 293 cells and incubated for 1 hr at 37 °C. Supernatant was then removed and replaced with 10% fetal bovine serum (FBS) in Dulbecco's Modified Eagle Medium (DMEM). SEAP expression was measured 24 ± 2 hrs later with the chemiluminescent substrate from the Phospha-Light™ kit. (Applied Biosystems).

Antibodies

We obtained directly conjugated antibodies from the following: BD Biosciences: TNFα (Pe-Cy7), IFN-γ (Alexa700); Caltag: CD14 (APC-Alexa750), CD19 (APC-Alexa750), β_7 Integrin (PeCy5), CD49d α_4 (APC), Ki67 (Fitc), CD103 (FITC) and CD4 (Pe-Cy5.5); Beckman Coulter: CD8

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(ECD), CD27 (Pe-Cy5); eBioscience CCR7 (APC-Alexa750) and R&D systems.

Whole Vector Stimulation

To measure responses to the Ad5, AdC6 and AdC7 vectors, 2×10^6 PBMCs were incubated overnight with 1×10^{11} vp and costimulatory antibodies (αCD28 and 49d, 1 μg/ml; αCD28 alone for mucosal marker staining, BD Biosciences) at 37 $^\circ$C and 5% CO_2 in 1 ml R10 media (RMPI 1640 with 10% heat inactivated FBS, 100 U/ml Penicillin, 100 μg/ml streptomycin sulfate and 1.7 mM sodium glutamate) in 5 ml BD Facs tubes. We stimulated a positive control with *Staphylococcus* enterotoxin B (SEB, 1 mg/ml; Sigma-Aldrich) and a negative control received only costimulatory antibodies. The following morning we added Monensin (Golgi Stop, 0.7 μg/ml; BD Biosciences) and Brefeldin A (1 μg/ml; Sigma-Aldrich) to each sample and incubated the cells for six hr at 37 $^\circ$C and 5% CO_2 before staining.

Peptide Stimulation

Peptide libraries were stimulated for Ad5, AdC6 and AdC7 hexon sequence consisting of 15 amino acids peptides that overlap by 11 amino acids. Peptides were pooled into five groups of approximately 40 peptides each. One pool consisted of sequences in the variable regions of the hexon

and the other four pools were linear pools of the conserved regions (Appendix).

To stimulate cells, PBMCs were thawed or obtained from fresh aphaeresis and rested overnight at 2×10^6 cells/ml in R10 media (RMPI 1640 with 10% heat inactivated FBS, 100 U/ml Penicillin, 100 mg/ml streptomycin sulfate and 1.7 mM sodium glutamate) in filter top T-flasks. The following morning, cells were counted and resuspended at $1\text{-}2\times10^6$ cells/ml in R10 media. Costimulatory antibodies (αCD28 and 49d, 1 μg/ml) were added to cells and 1 ml cells were aliquoted to BD Facs Tubes containing 5 μl (2 μg/ml each peptide in DMSO) of a given peptide pool. A negative control received only co-stimulatory antibodies and a positive control was stimulated with *Staphylococcus* enterotoxin B (SEB, 1 μg/ml; Sigma-Aldrich).

Staining

Following vector or peptide stimulation samples were washed in phosphate buffered saline (PBS) and stained for viability (Aqua live/dead amine reactive dye; Invitrogen). To determine viability the aqua dye tube was reconstituted in 12.5 μl DMSO and stored at -20°C. This stock was then diluted 1:60 in PBS. PBS was decanted following the wash and cells in facs tubes were stained with 5 μl for 10 min at room temperature in the dark. Following live dead staining surface antibodies were added for 20 min at room temperature in the dark (Table 2-4). Surface stain mixes were made up

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Target	Color	Clone	Manufacturer	Catalog Number	Stain
Aqua Live Dead	Aqua	NA	Invitrogen	L34597	Pre-stain
CD3	QD585	OKT3	In Lab	American Type Culture Collection	intracellular
CD4	PeCy5.5	S3.5	Caltag	MHCD0418	surface
CD8	Texas Red PE	3B5	Coulter	6604728	surface
CD27	PeCy5	1A4LDG5	Coulter	6607107	surface
CD45RO	QD 705	UCHL1	In Lab	AbD Serotech	surface
CD57	QD565	TB01	In Lab	AbD Serotech	surface
CD14	APCAlexa750	TüK4	Caltag	MHCD1427	surface
CD19	APCAlexa750	SJ25-C1	Caltag	MHCD1927	surface
IL-2	APC	MQ1-17H12	R&D	554567	intracellular
IFN-g	Alexa700	L11370	BD	557995	intracellular
TNFa	PeCy7	MAb11	BD	557647	intracellular
Perforin	PacBlue	B-D48	In Lab	Diaclone	intracellular
MIP1a	PE	93342	R&D	IC2701P	intracellular
CD107a	FITC	H4A3	BD	555800	Pre-stain

Table 1: Normal Donor Functionality Staining

Target	Color	Clone	Manufacturer	Catalog Number	Stain
Aqua Live Dead	Aqua	NA	Invitrogen	L34597	Pre-stain
CD3	QD585	OKT3	In Lab	American Type Culture Collection	intracellular
CD4	PeCy5.5	S3.5	Caltag	MHCD0418	surface
CD8	Texas Red PE	3B5	Coulter	6604728	surface
CD27	PeCy5	1A4LDG5	Coulter	6607107	surface
CD45RO	QD 705	UCHL1	In Lab	AbD Serotech	surface
CD57	QD565	TB01	In Lab	AbD Serotech	surface
CD14	APCAlexa750	TuK4	Caltag	MHCD1427	surface
CD19	APCAlexa750	SJ25-C1	Caltag	MHCD1927	surface
IL-2	APC	MQ1-17H12	R&D	554567	intracellular
IFN-g	Alexa700	L11370	BD	557995	intracellular
TNFa	PeCy7	MAb11	BD	557647	intracellular
Perforin	PacBlue	B-D48	In Lab	Diaclone	intracellular
MIP1a	PE	93342	R&D	IC2701P	intracellular
Ki67	FITC	35	BD	612472	intracellular

Table 2: Vaccine Functionality Staining

to 50 μl per tube in Facs Buffer (PBS with 1% fetal bovine serum and 0.1%

sodium azide). Following surface staining cells were washed once in facs

buffer then permeabilized and fixed by adding 250 μl Cytofix/Cytoperm (BD

Biosciences) for seventeen min at room temperature in the dark followed by a

wash with perm wash buffer (BD Biosciences). Cells were then stained with

intracellular fluorochrome-labeled antibodies for 1 hour at room temperature

in the dark. Intracellular staining antibodies were made up to a final volume of

50 μL per tube in perm wash buffer. Following staining cells were washed

with perm wash buffer, fixed (2% paraformaldehyde in PBS) and stored at

4°C in the dark until analysis. All antibodies were titrated to determine the

optimal staining.

Flow Cytometry

We analyzed cells on a modified LSR II flow cytometer (BD

Immunocytometry Systems) with 200,000 to 1,000,000 events collected per

sample. Data was analyzed using FlowJo 8.7 (TreeStar). Cells were initially

gated to remove doublets followed by a lymphocytes gate on forward scatter

area versus side scatter area. We removed dead cells by gating CD3 versus

Aqua blue removing cells that are Aqua blue bright. Contaminating CD14$^+$

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and CD19$^+$ cells were also removed before gating sequentially on CD3$^+$, and CD8$^+$/CD4$^+$ and CD4$^-$/CD8$^-$ versus IFN-γ to account for receptor down-regulation. We then made a gate for each respective function and the Boolean gating platform was used to create the array of possible functional combinations. Data are reported after background subtraction of the no stimulation condition.

Statistics

For normal donor samples, variability in the assay was tested using Levene's robust variance test with Brown and Forsythe's 10% trimmed mean alternative. This method is robust to non-normality in the data. Analyses were conducted using Stata MP 10.0. The T-cell response to different vectors and peptide pools were compared using a Friedman Statistic. This method is similar to an ANOVA for non-parametric matched data. When two groups were compared, a Wilcoxon's sum rank test, which is similar to a t-test for non-parametric data, was used. These statistics were performed in Graphpad Prism.

For vaccine samples mixed effects models were performed to test for group differences over time. Mixed effects models were also used for comparisons between baseline and subsequent time points within each group. Time was considered to be a discrete variable, lessening the power of

these tests compared to tests where time is a continuous variable. Spearman correlations were used to test the relationship between Ad5 nAb titers and T-cell functions at baseline. Correlations over the entire time period were computed using partial correlation coefficients controlling for individual subject effects in the repeated measurements. All data was log transformed using base e. Vaccine statistics were performed by Sarah Ratcliffe from the University of Pennsylvania Center for Clinical Epidemiology and Biostatistics.

CFSE Staining

Fresh PBMCs obtained from aphaeresis were centrifuged at 1500 RPM for six minutes and resuspended at 5×10^6 cells/ml in sterile PBS. A stock vial of CFSE was prepared by reconstituting a CFSE dye vial (Invitrogen) with 18 ml DMSO. CFSE stock (0.5 μl/ml) was added to cells for 10 min at 37°C. Cells were then quenched with 10 ml ice-cold R10 media and incubated on ice for 10 min before washing twice in R10 media. Cells were counted and resuspended at 2×10^6 cells/ml in R10 media and stimulated with 1×10^{11} vp Ad5 or 2 μl Ad5 lysate for 6 days. On the 6th day cells were restimulated with Ad5 vp or lysate and 3 μl/ml αCD49/CD28d. After two hours, 1 μl/ml brefeldin A and 0.7 μl/ml monensin were added to each tube and the cells incubated at 37°C for an additional two hours before staining as indicated above.

Chapter 3: Adenovirus-Specific Human T-cells are Pervasive, Polyfunctional, and Cross Reactive

Abstract

Pre-existing immunity to adenovirus (Ad) reduces the efficacy of Ad-based vaccines. The goal of this study was to define the prevalence, magnitude, functionality and phenotype of Ad-specific human T-cells directly *ex vivo*. To study the magnitude of T-cell responses to Ad, we developed a highly reproducible whole Ad vector stimulation assay for use with polychromatic flow cytometry. Ad-specific CD4$^+$ and CD8$^+$ T-cells were detected in all 17 human subjects tested and were capable of proliferating upon restimulation. Ad-specific CD4$^+$ T-cells were primarily monofunctional CD4$^+$ T-cells that produced IL-2, IFN-γ or TNFα and expressed the memory markers CD27 and CD45RO. In contrast, Ad5-specific CD8$^+$ T-cells were more polyfunctional, expressing effector-like combinations of IFN-γ, MIP1α and perforin, and generally lacked CD27 and CD45RO expression. Ad-specific CD4$^+$ and CD8$^+$ T-cell responses against chimpanzee-derived AdC6 and AdC7 were found in all subjects, indicating the commonality of cross-serotype reactivity of Ad-specific T-cells. This cross-reactivity is due in part to extensive CD4$^+$ and CD8$^+$ T-cell recognition of hexon regions conserved between multiple Ad serotypes. The prevalence, cross-reactivity and effector-

like functions of Ad-specific T-cells in humans may affect the efficacy of Ad

vector-based vaccines by eliminating vector infected cells even when rare

serotype Ad vectors are employed.

Introduction

Adenovirus (Ad) vectors are commonly used as vaccine carriers because of their ability to induce insert-specific CD8^{+} T-cell responses. However, pre-existing Ad-specific immunity represents a major obstacle for Ad-based vaccines [76,113]. In animal models and humans, vaccination is less effective in the presence of neutralizing antibodies (nAb) [80,112,113]. It has also been shown that significant levels of nAb are generated after a single Ad5 injection, thereby reducing the efficacy of a homologous vaccine boost [114]. The prevalence of nAb to the commonly used Ad5 varies worldwide, and was shown to be as high as 90% in Africa. Seroprevalence of the other 52 identified human Ads also fluctuate globally with the occurrence of natural infection. To avoid the potential limitations imposed by pre-existing immunity, vectors based on alternative Ad serotypes are in development, including Ad26, 35, 48, and the chimpanzee-derived AdC6, C7, and C68. Neutralizing Ab titers to these various rare Ad serotypes are typically low in humans, with seroprevalence to AdC6 and AdC7 less than 5% of adults in the United States and less than 10% seropositive in equatorial Africa, the natural habitat for chimpanzees [91].

Although the prevalence and effects of Ad-specific nAb on vaccine efficacy have been studied, little work has been done to characterize the naturally occurring T-cell response to Ad, or the potential of Ad-specific

T-cells to influence Ad-based vaccine efficacy. Ad-specific CD8^{+} T-cell responses can limit the effectiveness of Ad-vectored vaccines in animal models [31,114], presumably due to the direct elimination of vector-transduced antigen presenting cells. Such studies, however, have not been performed in the setting of natural Ad infection in humans. Ad-specific T-cells have been detected ex vivo in humans, both before and after Ad vector vaccination, in peripheral blood and mucosal tissues [59,82,116,117,164]. Several MHC class II-restricted CD4^{+} T-cell epitopes have been identified in the Ad5 hexon, residing primarily in regions conserved between disparate Ad serotypes, such as the HLA-DP4 restricted CD4^{+} T-cell epitope (hexon 910-924) [117,118,165]. MHC class I restricted CD8^{+} T-cell epitopes have also been identified in the Ad hexon, penton, and fiber [117,120]. Responses to Ad appear to be almost ubiquitous in the human population [59,82]; however, beyond simple quantification, little is known regarding the functionality and phenotype of Ad-specific CD4^{+} and CD8^{+} T-cells in humans. Moreover, while serotype cross-reactivity has been noted for both Ad-specific CD4^{+} and CD8^{+} T-cells, it is unclear whether Ad-specific T-cells cross-reacting with a disparate Ad serotype will function in a similar manner.

To address these issues, we have developed a highly reproducible polyfunctional flow cytometry-based assay to quantify and characterize Ad-specific CD4^{+} and CD8^{+} T-cells directly *ex vivo* from human peripheral blood lymphocytes. Herein, we describe the functional and phenotypic properties of

human Ad-specific T-cells against human Ad5 and cross-reactive responses against chimpanzee-derived AdC6 and AdC7.

Results

Optimization of Ad particle stimulation for Ad-specific T cell responses by intracellular cytokine staining

Previous studies of Ad-specific T-cell responses have focused on examining Ad hexon protein-specific T-cell responses using ELISpot assays. However, by using only hexon peptides, T-cells specific for other Ad proteins are missed and the magnitude of Ad-specific T-cells are underestimated. To better quantify and characterize human Ad-specific T-cell responses, we developed a stimulation procedure using intact Ad particles, followed by a T-cell flow analysis using standard intracellular cytokine-staining (ICS) assay. To begin, we determined the optimal concentration of intact Ad particles to maximize expression within peripheral blood mononuclear cells (PBMC) for presentation to T-cells. We incubated an Ad5 vector expressing green fluorescent protein (GFP) at various concentrations ranging from 1×10^9 vp to 1×10^{11} vp with PBMC for 18 hrs and assessed GFP expression in B cells (CD19$^+$), monocytes (CD14$^+$),
T-cells (CD3$^+$/CD4$^+$/CD8$^+$), or the remaining PBMCs negative for these markers. After an overnight incubation, GFP was detected in CD14$^+$ monocytes and CD19$^+$ B cells in a dose dependent manner (Figure 5A). No
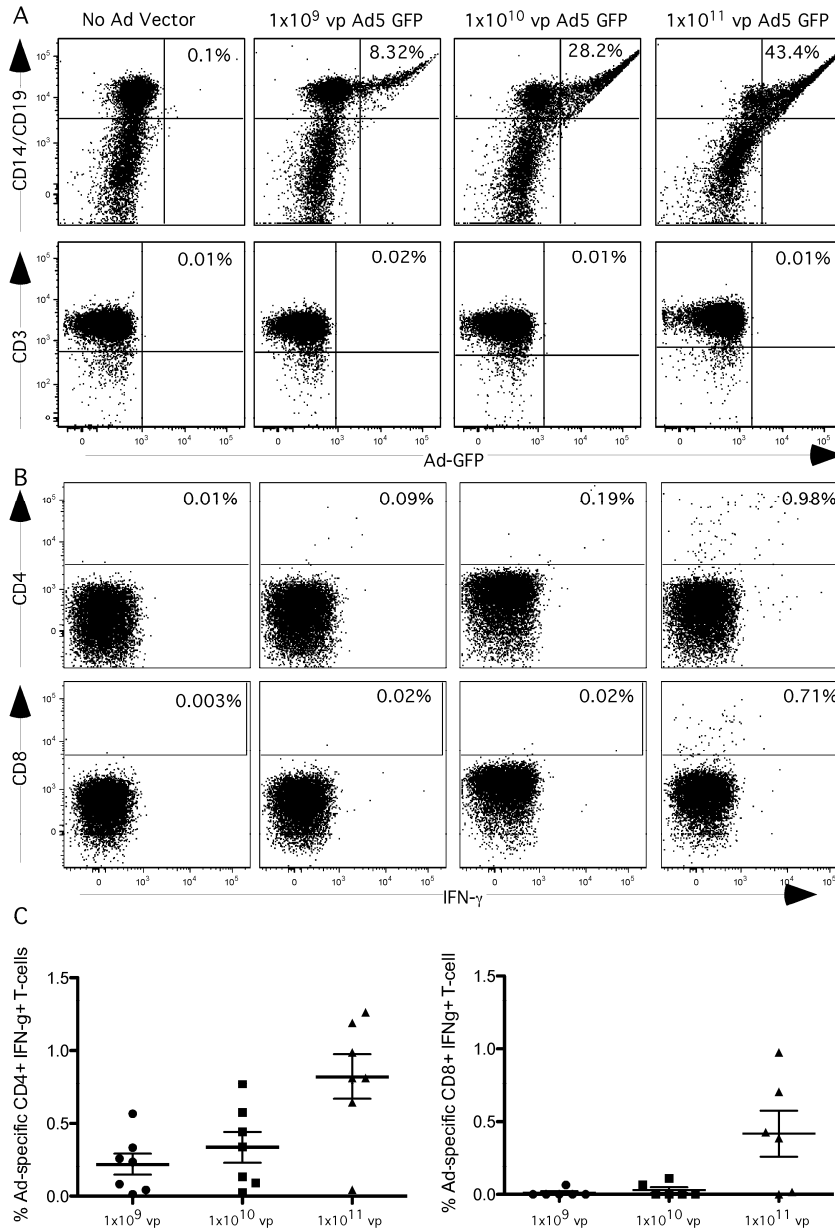
Figure 5: Measuring adenovirus specific T-cell responses following whole vector stimulation. PBMCs from healthy adults were cultured overnight at 37°C in 5% CO_2 with Ad5 vector and costimulatory antibodies αCD28 and αCD49d. The following morning golgi secretion inhibitors brefeldin A and monensin were added for 6 hours before standard intracellular cytokine staining. A) PBMCs were incubated with $1x10^9$-$1x10^{11}$ vp Ad5 expressing green fluorescent protein (GFP). The upper graphs show GFP expression in CD14^{+} monocytes and CD19^{+} B-cells, and the lower graph shows expression in CD3^{+} T-cells. B) PBMCs were stimulated with $1x10^9$-$1x10^{11}$ vp Ad5 vector and CD4^{+} and CD8^{+} T-cell responses were measured by IFN-γ production. C) Ad-specific IFN-γ^{+} CD4^{+} and CD8[1] T-cell responses were measured in seven donors following stimulation with $1x10^9$-$1x10^{11}$ vp Ad5.

GFP was expressed in other cell types. Along with increasing vector

concentration we also detected an increase in Ad-specific CD4$^+$ and CD8$^+$

T-cell responses as measured by IFN-γ production (Figure 5B). To confirm

the optimal vector dose for T-cell stimulation the vector titration was repeated

using PBMC from 6 normal donors. Optimal IFN-γ expression was observed

at the maximal dose of 1 x 10^{11} Ad particles/million PBMC (Figure 5C).

Polyfunctional analysis of Ad-specific T-cell responses

Having established the optimal conditions to detect IFN-γ producing

Ad-specific T-cells using whole Ad particles, we next adapted the procedure

to a polychromatic flow cytometry panel that simultaneously detects T-cell

memory phenotype and 5 unique effector functions, i.e. IL-2, IFN-γ, TNFα,

MIP1α, and perforin, along with standard T-cell lineage markers, and

exclusion markers. To ensure that we were detecting only Ad-specific T-cells

and minimizing background, we followed a strict gating strategy that removes

dead cells as well as CD14$^+$ monocytes and CD19$^+$ B cells. We were able to

detect Ad-specific CD4$^+$ and CD8$^+$ T-cells capable of eliciting multiple

functions after stimulation (Figure 6). We next re-evaluated our Ad vector

titration to determine if there were differential functional responses based on

vector dose. The Ad-specific T-cell response was similar at all vector

concentrations for CD8$^+$ T-cells, which produced a combination of IFN-γ,
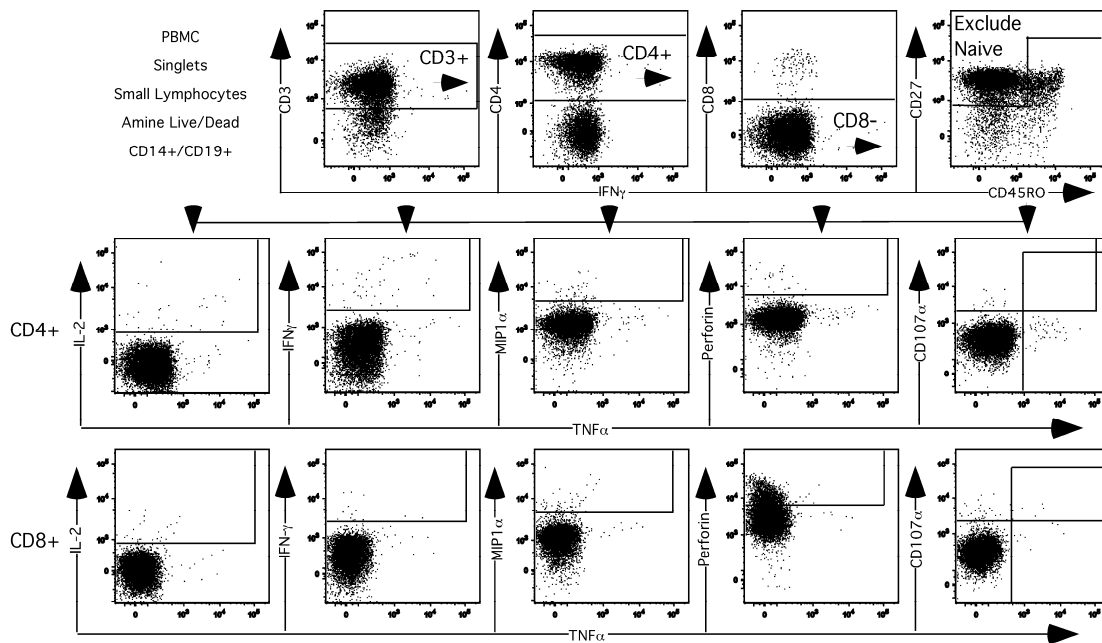
Figure 6: Gating strategy for measuring Ad-specific T-cell responses.
Gating strategy for determining Ad-specific CD4^{+} and CD8^{+} T-cells producing IL-2 IFN-γ MIP1α, Perforin and TNF α
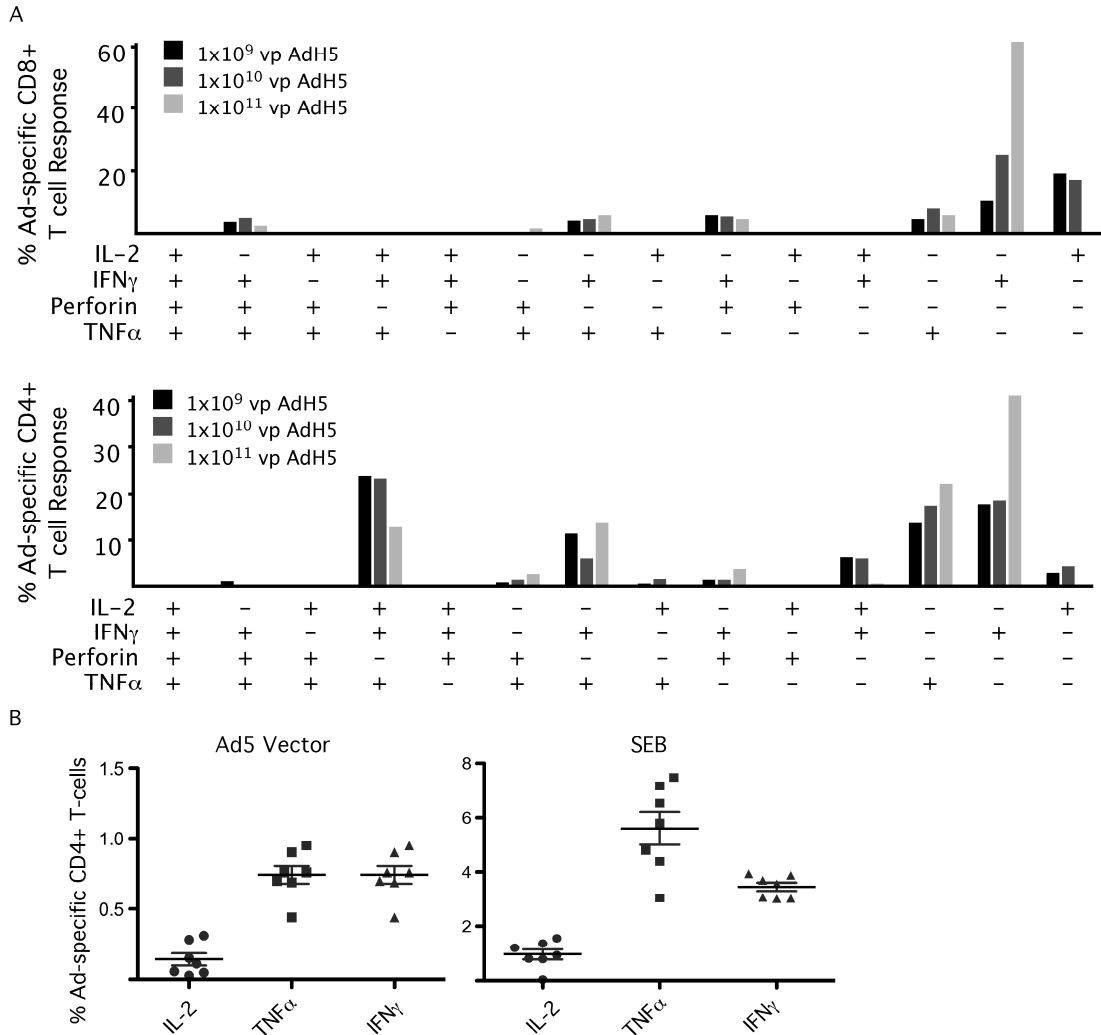
Figure 7: Adenovirus particle stimulation for measuring polyfunctional Ad-specific T-cell responses. A) Polyfunctional CD4^{+} and CD8^{+} T-cell responses following stimulation with 1×10^9-1×10^{11} vp Ad5. B) PBMCs from a single donor were stimulated with 1×10^{11} vp Ad5 on 7 different days to test the reproducibility of the whole vector stimulation assay. The percentage of CD4^{+} T-cells producing IL-2, IFN-γ and TNF α was compared following Ad vector and positive control *streptococcus* enterotoxin B (SEB) stimulation. The degree of variability was significantly less (p<0.05) following Ad stimulation compared with SEB.

TNFα and perforin with small amounts of IL-2. CD4$^+$ T-cells predominantly

produced IFN-γ, IL-2, and TNFα (Figure 7A, average of six subjects). At

1×10^{11} vp Ad5 the percentage of CD4$^+$ and CD8$^+$ T-cell producing only IFN-γ

was appreciably higher than the other functional combinations. Finally, we

examined the reproducibility of the assay system in the setting of

simultaneous IL-2, IFN-γ, and TNFα production (Figure 7B). Using

cryopreserved samples, we measured the Ad-specific T-cell response at the

1×10^{11} Ad particle dose with the same batch of PBMC obtained from a single

donor on seven separate days. The variability in Ad-specific CD4$^+$ and CD8$^+$

cell frequency producing IL-2, TNFα, and IFN-γ was low, and

significantly lower than the range of variability observed for the superantigen

positive control (SEB, $p < 0.05$).

Ad5-specific CD4$^+$ and CD8$^+$ T cells are common in humans

We next assessed the functionality and phenotype of Ad5-reactive

T-cells in 17 healthy adults. CD4$^+$ and CD8$^+$ T-cell responses to Ad5 were

detected in all subjects. Most subjects had primarily monofunctional CD4$^+$

T-cells that produced IL-2, IFN-γ or TNFα (Figures 8A, C). In contrast, Ad5-

specific CD8$^+$ T-cells were more polyfunctional, expressing effector-like

combinations of IFN-γ, MIP1α and perforin (Figures 8B, D). With the

46

% Ad-specific CD4+ T-cells

4

3

1

IL-2 IFN-γ MIP1α Perforin TNFα

B

% CD8+ Ad-specific T-cells

10

8

6

4

2

0

IL-2 IFN-γ MIP1α Perforin TNFα

C

% Ad-specific CD4+ T cells

30

20

10

IL–2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
IFNγ	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−
MIP1α	+	+	+	+	−	−	−	−	+	+	+	+	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	−	−	−
Perforin	+	+	−	−	+	+	−	−	+	+	−	−	+	+	−	+	+	−	−	+	+	−	−	+	+	−	−	+	+	−
TNFα	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+

D

% Ad-specific CD8+ T-cells

40

30

20

10

IL–2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
IFNγ	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−
MIP1α	+	+	+	+	−	−	−	−	+	+	+	+	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	−	−	−
Perforin	+	+	−	−	+	+	−	−	+	+	−	−	+	+	−	+	+	−	−	+	+	−	−	+	+	−	−	+	+	−
TNFα	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+

Figure 8: Adenovirus 5 specific T-cell responses are common in humans. We assessed the functionality and phenotype of Ad5-specific T-cells in 17 healthy adults by stimulating PBMCs with whole Ad5 vector and performing intracellular cytokine staining. A) The percentage of Ad5-specific CD4^{+} T-cells producing IL-2, IFN-γ MIP1α, Perforin and TNFα. B) The percentage of Ad5-specific CD8^{+} T-cells producing IL-2, IFN- γ, MIP1α, perforin and TNFα. C) The average polyfunctional Ad5-reactive CD4^{+} T-cell response from 17 donors. D) The average polyfunctional Ad5-reactive CD8^{+} T-cell response from 17 donors

Figure 9: Memory phenotype and proliferation of Ad5-specific T-cells. We assessed the phenotype, functionality and proliferation of Ad5-specific T-cells by stimulating PBMC with replication defective Ad5 vector. Proliferation was accessed by CFSE dilution after 6 days. A) Ad5-reactive CD4$^+$ and CD8$^+$ T-cells proliferate in response to Ad5 stimulation. The percentage of CFSE low dividing cells is shown following no stimulation or stimulation with Ad5 vector. B) Memory phenotype of Ad5-specific CD4$^+$ and CD8$^+$ T-cells. Memory phenotype of all CD4$^+$ and CD8$^+$ T-cells is shown in grey. Red dots represent Ad-specific cells positive for IL-2, IFN-γ, MIP1α, Perforin and or TNFα. Ad5-specific CD4$^+$ cells are primarily central memory-like (CD27$^+$CD45RO$^+$) and effector memory (CD27$^-$CD45RO$^+$) phenotypes. Ad5-specific CD8$^+$ T-cells are primarily effector (CD27$^-$CD45RO$^-$) and central memory like (CD27$^+$CD45RO$^+$) phenotypes. C) Memory phenotype of proliferating Ad-specific cells. Memory phenotype of all T-cells after a 5-day stimulation with Ad5 is shown in grey. CFSE low T-cells are shown in red. CD4$^+$ T-cells are on the left and CD8$^+$ T-cells are on the right.

exception of monofunctional responses, nearly all Ad5-specific CD8$^+$ T-cells produced perforin together with at least one other function (Figure 8D).

Ad5-specific CD4$^+$ and CD8$^+$ T-cells were also capable of proliferating upon stimulation (Figure 9A). Ad-specific CD4$^+$ T-cells generally exhibited a central memory-like phenotype (CD27$^+$CD45RO$^+$) with a small contribution of effector memory (CD27$^-$CD45RO$^+$) cells (Figure 9B, left panel). In marked contrast, the majority of Ad-specific CD8$^+$ T-cells displayed an effector-like phenotype (CD27$^-$CD45RO$^-$), with a smaller contribution of central memory-like cells (Figure 9B, right panel). In contrast, proliferating Ad-specific CD4+ and CD8$^+$ T-cells had primarily a central memory-like phenotype after 6 days of stimulation (Figure 9C).

Ad5 hexon-specific responses are commonly observed in humans and are directed against both conserved and variable regions

Next, we determined whether Ad5 hexon-specific responses were directed against conserved or variable hexon regions. We also assessed the functionality of responses to the different regions of hexon. The latter analysis was conducted to elucidate differences in responses to the constant regions that had presumably been recalled repeatedly due to infections with different Ad serotypes and those to the variable loops that presumably have a different stimulation history. Starting with overlapping 15-mer peptides spanning the hexon amino acid sequence, we divided the conserved regions

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Figure 10: Ad5-specific T-cells recognize variable and conserved regions of the hexon. We assessed the functionality and phenotype of Ad5-specific T-cells in 17 healthy adults by stimulating PBMCs with overlapping 15mer Ad5 hexon peptides. Approximately 44 peptides were combined into each of 5 pools: 1 for all sequences in the variable regions of the hexon, and 4 containing linear sequences within the conserved regions of the hexon. The percentage of CD4^{+} and CD8^{+} T-cells responding to the conserved 1 (C1), conserved 2 (C2), conserved 3 (C3), conserved 4 (C4), and variable (V) hexon pools are shown. Cells staining positive for IL-2, IFN-γ, MIP1α, Perforin and or TNF α were summed to compute the total percentage of responding cells. CD4^{+} responses are depicted in white bars and CD8^{+} responses in grey. Center line represents the mean with whiskers depicting the standard error.

into 4 linear pools of ~ 44 peptides each (C1-C4), and made a single pool

consisting of peptides from variable (V) regions. We could readily detect

CD4^{+} T-cell responses to all 5 pools (Figure 10, open bars), indicating that

CD4^{+} T-cells can recognize epitopes spread throughout the hexon, with the

largest responses in the C2, C3, and V pools. There was no significant

difference in the summation of the total response between the C2 and C3

pools compared with the V pool, while responses to the C1 (p<0.001) and C4

(p<0.02) pools were significantly lower. CD8^{+} T-cells also responded potently

to Ad hexon (Figure 10, grey bars). The magnitude of responding CD8^{+} T-

cells was higher than that of CD4^{+} T-cells with the largest responses against

the C3 and V pool.

Both CD4^{+} and CD8^{+} T-cells had similar functionality following

stimulation with conserved and variable Ad hexon pools (Figure 11). Ad-

specific CD4^{+} T-cells produced little perforin, while IL-2, IFN-γ, MIP1α, and

TNFα dominated the responses. While there were multifunctional responses

(two or more functions simultaneously), the bulk of Ad-specific CD4^{+} T-cells

were monofunctional (Figure 11A, top), producing either IFN-γ, IL-2, TNFα, or

MIP1α only (Figure 11B, top). The CD4^{+} T cell responses against the hexon

peptide pools were similar in functionality compared to the whole Ad vector,

with the exception that whole Ad vector responses were dominated by IFN-γ

production, while responses elicited by hexon peptides tended to skew to
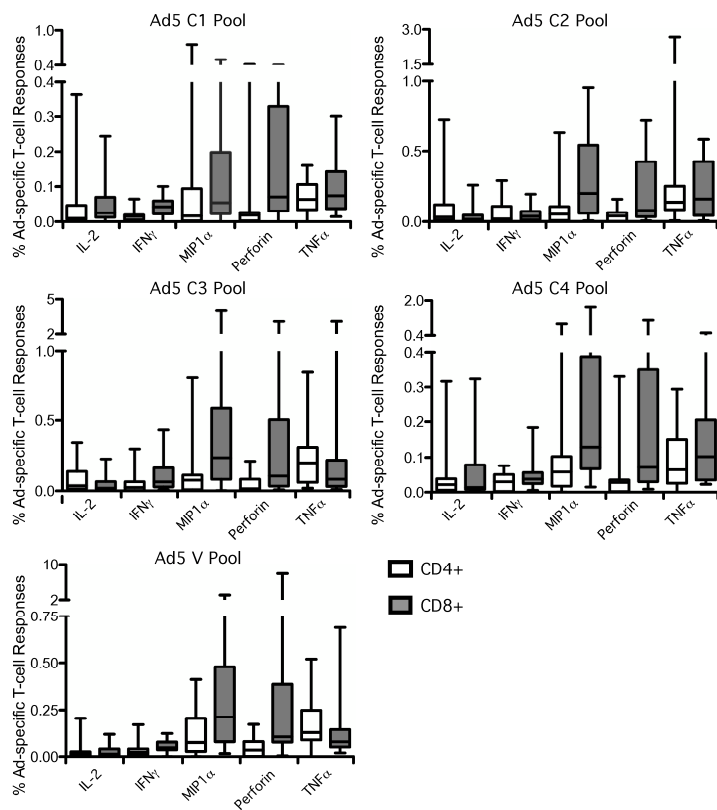
Figure 11: Polyfunctionality of Ad5-specific T-cells is similar to variable and conserved regions of the hexon. We assessed the functionality and phenotype of Ad5-specific T-cells in 17 healthy adults by stimulating PBMCs with overlapping 15mer Ad5 hexon peptides. Approximately 44 peptides were combined into 5 pools: 1 for all sequences in the variable regions (V) of the hexon, and 4 containing linear sequences (C1-C4) within the conserved regions of the hexon A) Percentage of Ad-specific cells with a polyfunctional response. Pies represent all responding Ad-specific cells making IL-2, IFN- γ, MIP1a, Perforin and or TNFα following stimulation with Ad5 vector or Ad5 hexon pools C1, C2, C3, C4 or V. Each slice represents the proportion of the cells producing four of five cytokines (blue), three of five (green), two of five (yellow) and one of five (orange). B) Percentage of Ad-specific cells with a polyfunctional response. Bars represent the percentage of CD4^{+} (top) and CD8^{+} (bottom) T-cells making each combination of IL-2, IFN-γ, MIP1α, Perforin and or TNFα following stimulation with Ad5 vector (blue) or Ad5 hexon pools C1 (red), C2 (neon green), C3 (orange), C4 (pink) or V (dark green). Plus signs represent cells staining positive (+) for each cytokine.

TNFα production (Figure 11B, top). This difference may be attributed to the different assay conditions used for testing whole vector (overnight) vs. hexon peptides (6 hrs), or could reflect different levels of antigenic stimulation.

In contrast to hexon-specific CD4$^+$ T-cells, perforin, TNFα, and MIP1α dominated the hexon-specific CD8$^+$ T-cell responses, and IL-2 and IFN-γ tended to be lower. The overall level of functionality in the hexon-specific CD8$^+$ T-cell response tended to be higher than the CD4$^+$ hexon-specific response, with the majority of CD8$^+$ T-cells responding with at least two functions (Figure 11A, bottom). Similar to whole Ad vector responses, hexon-specific CD8$^+$ T-cells were highly skewed towards effector like activity, with perforin clearly dominating the entire response. With the exception of monofunctional responses, perforin was present in combination with another function in nearly every hexon-specific CD8$^+$ T-cell (Figure 11B, bottom). There was no apparent difference in functionality between hexon-specific CD8+ T-cell responses directed against conserved or variable regions.

Cross-serotype reactivity of Ad-specific T cells in humans

To test the ability of Ad-specific T-cells to cross-react with disparate Ad serotypes, we examined T-cell responses against the chimpanzee-derived adenoviruses AdC6 and AdC7, which humans are rarely seropositive for. In all subjects examined CD4$^+$ T-cells responded to Ad5, AdC,6 and AdC7, demonstrating a high level of cross-serotype reactivity. The predominant
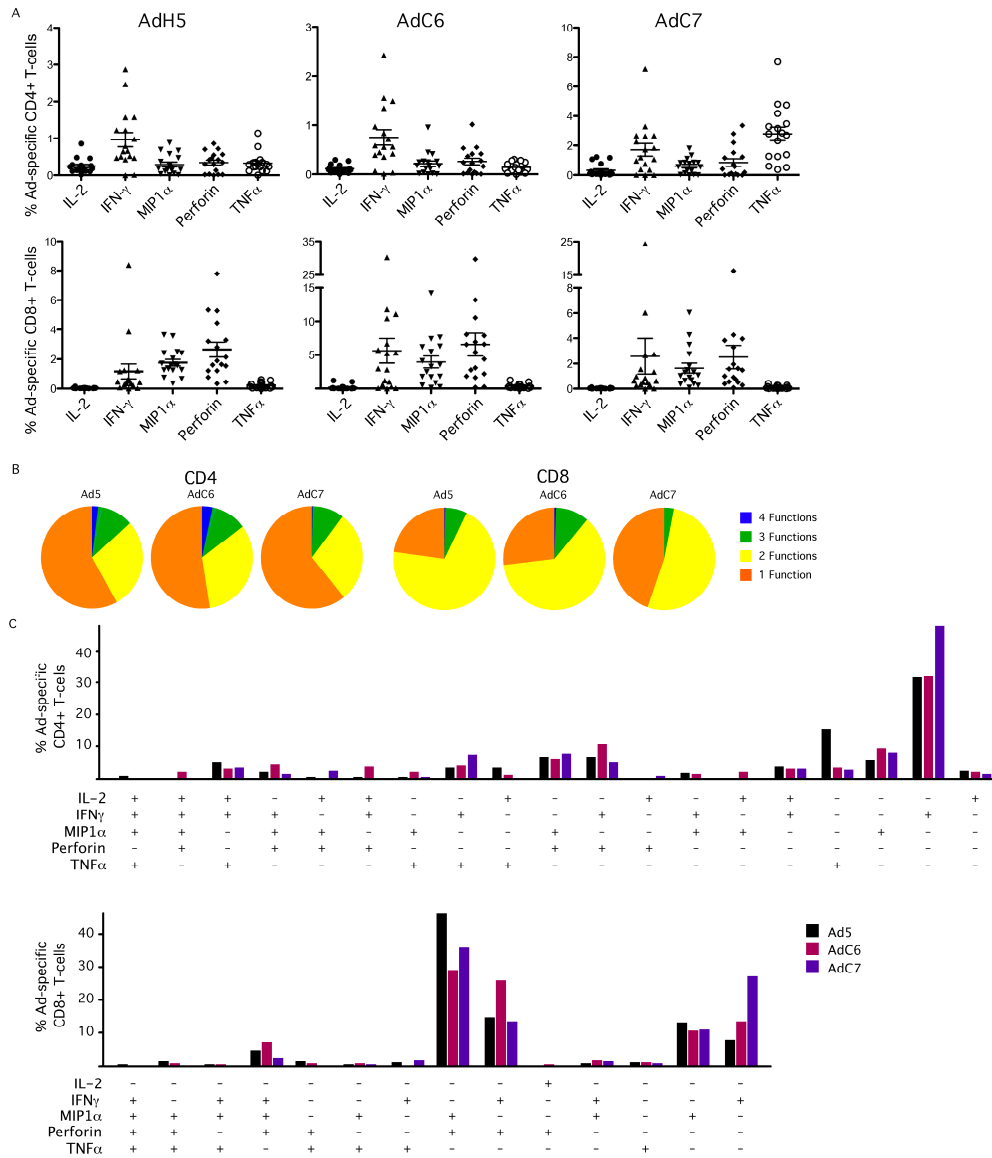
Figure 12: Ad-specific T-cells are cross reactive. Cross reactivity of Ad-specific T-cells was measured in seventeen healthy donors by stimulating PBMCs overnight with human adenovirus 5 (Ad5), chimpanzee 6 (AdC6) and chimpanzee 7 (AdC7) followed by intracellular cytokine staining. A) The total percentage of Ad5, AdC6 and AdC7 CD4^{+} (top row) and CD8^{+} (bottom row) T-cells. Ad-specific cells stained positive for IL-2, IFN-γ, MIP1α, Perforin and or TNF-α. B) Percentage of Ad-specific cells with a polyfunctional response. Pies represent all responding Ad-specific cells making IL-2, IFN-γ, MIP1α, Perforin and or TNFα following stimulation with Ad5, AdC6, or AdC7 vector. Each slice represents the proportion of the cells producing four of five cytokines (blue), three of five (green), two of five (yellow) and one of five (orange). C) Percentage of Ad-specific cells with a polyfunctional response. Bars represent the percentage of CD4^{+} (top) and CD8^{+} (bottom) T-cells making each combination of IL-2, IFN- γ, MIP1α, Perforin and or TNFα following stimulation with Ad5 (black) AdC6 (pink) and AdC7 (purple) vector. Plus signs represent cells staining positive (+) for each cytokine.

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functional response differed between the vectors, with Ad5 and AdC7

inducing mainly IFN-γ, while AdC6 induced primarily TNFα (Figure 12A).

Despite these differences, the overall functionality of the Ad-specific CD4$^+$

T-cell response was quite similar between all three vectors (Figure 12B, C).

The CD4$^+$ T cell response to AdC6 was significantly larger than to Ad5

($p<0.01$). Cross-serotype reactive Ad-specific CD8$^+$ T-cells were also present

in every donor, with no differences in magnitude and a similar degree of

functionality (Figure 12B, C). There was no significant difference in the total

magnitude of the CD8$^+$ T-cell response to Ad5, AdC6 and AdC7, and the

functional profiles of cross-reactive Ad-specific CD8$^+$ T-cells were also similar.

Discussion

Ad vectors are commonly used to deliver transgenes in gene therapy

and vaccination. It is well known that Ad-specific neutralizing antibodies can

limit the effectiveness of Ad-based vectors; however the potential role of Ad-

specific T-cells to further curtail Ad vector efficacy is unclear. Here we

provide a minimal estimate of the level of Ad-specific T-cell responses in

humans. We find that Ad-specific T-cell responses are universal, as every

subject we tested had a detectable CD4$^+$ and CD8$^+$ T-cell response against

Ad5, despite a seroprevalence of only 40% in the United States.

Our findings indicate that Ad-specific T-cells are readily detectable

using replication defective Ad particles or Ad hexon peptides. Although the

response magnitude against each particular stimulant may vary between

subjects, both CD4$^+$ and CD8$^+$ T-cells responded in a similar fashion. Interestingly, Ad-specific T-cells appear to be maintained continually in an effector-like state, particularly Ad-specific CD8$^+$ T-cells. Unlike our observations for common human viral pathogens, including cytomegalovirus, Epstein-Barr virus, and Influenza, Ad-specific CD8$^+$ T-cells are highly prone to effector function upon stimulation [166]. This is further manifested in the effector-like memory phenotype maintained by a substantial proportion of the Ad-specific CD8$^+$ T-cell response. Due to extensive sequence homology between various human Ad serotypes, Ad-specific T-cells in part cross-react and are likely repeatedly stimulated by periodic infections with different Ad serotypes [116,118,161]. As a result, human Ad-specific CD8$^+$ T-cells are unlikely to be restricted specifically to a single Ad serotype, and able to recognize not only targets infected with virus homologous to the vector used for expansion but also heterologous virus from diverse serotypes. This high level of cross-reactivity very likely leads to the continual maintenance of Ad-specific CD8$^+$ T-cells in an effector-like state, as humans are expected to be repeatedly infected with different serotypes of Ad. Furthermore, Ad viruses persist in lymphatic tissues and if they remain transcriptionally active this would further maintain T-cells at an activated state [71].

One reason for the divergence between Ad serostatus and Ad responsive T-cells is the presence of common T-cell epitopes in conserved regions of the Ad hexon protein, which accounts for ~80% of the entire hexon

sequence. Similar to previous findings obtained with CD4$^+$ and CD8$^+$ T-cell lines, we find that both CD4$^+$ and CD8$^+$ T-cell responses can be detected against the conserved and variable hexon peptide pools. Conservation of T-cell epitopes in hexon leads to cross-reactivity even among divergent serotypes from chimpanzees, AdC6 and AdC7. Because of this high degree of cross-reactivity, it is impossible to know whether, for example, Ad5-reactive T-cells are truly Ad5-specific and generated from natural Ad5 infection, or simply cross-reacting with Ad5 following infection with another Ad serotype. Finally, due to this extensive cross-reactivity, it is likely that transgene product-specific immune responses induced by Ad vectors derived from rare human serotypes, which are currently under development, may still be affected by Ad-specific cytotoxic T-cells capable of recognizing Ad vector transduced cells.

Material and Methods

T-cell Responses

Peripheral blood mononuclear cells (PBMCs) were obtained from aphaeresis of adult healthy donors by the University of Pennsylvania Center for AIDS Research Immunology Core. T-cell responses to Ad5, AdC6 and AdC7 were measured by flow cytometry following whole vector and peptide stimulation. Samples were analyzed on a modified LSR II flow cytometer (BD

Immunocytometry Systems, San Jose, CA). Data analysis was performed using FlowJo 8.7 (TreeStar, San Carlos, CA).

Statistics

Variability in the assays was tested using Levene's robust variance test with Brown and Forsythe's 10% trimmed mean alternative.. Analyses were conducted using Stata MP 10.0. The T-cell response to different vectors and peptide pools were compared using a Friedman Statistic. When two groups were compared a Wilcoxon's sum rank test, a non-parametric t-test, was used.

Portions of this work have been previously published: Hutnick NA, Carnathan DG, Demers K, Makedonas G, Ertl HC, Betts MR. "Adenovirus-Specific Human T cells are Pervasive, Polyfunctional, and Cross Reactive." Vaccine. 2010 Feb 23; 28(8):1932-41

Chapter 4: Baseline Ad5 serostatus does not predict Ad5-HIV vaccine-induced expansion of Ad-specific CD4$^+$ T-cells

Abstract

The mechanisms underlying possible increased HIV-1 acquisition in adenovirus 5 (Ad5)-seropositive subjects vaccinated with Ad5-HIV-1 vectors in the Merck STEP trial remain unclear. One hypothesis for these results is pre-existing Ad5-specific CD4$^+$ T-cells in Ad5-seropositive subjects became activated upon vaccination, making them the optimal targets for HIV infection and replication. To examine this hypothesis, we studied Ad5-specific CD4$^+$ T-cell responses in the Merck phase I Ad5 gag/pol/nef safety study testing the same vector, dosing and schedule used during the STEP study. We find Ad5 serostatus does not predict Ad5-specific CD4$^+$ T-cell frequency, and Ad5-specific CD4+ T-cells were present in over 90% of subjects. No significant differences in the magnitude or functionality of Ad5-specific CD4$^+$ T-cells between Ad5-seropositive and Ad5-seronegative subjects were observed following vaccination. Vaccination did not affect the activation or mucosal homing receptor expression of Ad5-specific CD4$^+$ T-cells in either serogroups.

These findings indicate no causative role for Ad5-specific CD4$^+$ T-cells in increasing HIV-1 susceptibility in Ad5-seropositive STEP trial subjects.

Introduction

Given the urgent need for an effective HIV vaccine, it is of paramount importance to gain further insight into the reasons underlying the failure of the Merck STEP HIV vaccine trial. Post-hoc analysis of the STEP trial showed vaccination with an adenovirus 5 (Ad5) vector based HIV-1 vaccine increased HIV-1 acquisition rates in volunteers with an adenovirus 5 neutralizing antibody (nAb) titer greater than 200 [83]. It has been proposed that the Ad5 vector caused activation and expansion of pre-existing Ad5-specific CD4$^+$ T-cells in vaccinees with high neutralizing antibody titers to Ad5 prior to vaccination. Activated Ad5-specific CD4$^+$ T-cells then served as targets for HIV infection [167]. However, neither the prevalence of Ad5-specific CD4$^+$ T-cells in humans, nor their relationship with Ad5 neutralizing antibody titer has been characterized. Moreover, it is unknown to what degree vaccination with Ad5 vectors stimulates pre-existing Ad5-specific CD4$^+$ T cells *in vivo* . It is not possible to adequately addressing these questions for the STEP trial as blood samples were not collected before vaccination and at only two time points, weeks 8 and 30, after vaccination [81].

To test if Ad5-specific CD4$^+$ T-cells may have increased the vaccinee's susceptibility to HIV-1 infection, we analyzed samples from subjects enrolled in Merck phase I HIV vaccine trials that utilized similar or identical Ad5

vectors to those used in the STEP trial at various dosing regimens. As pre-vaccination samples were available for each subject, we could precisely determine relationships between pre-existing Ad5-specific CD4$^+$ T-cell responses and Ad5 nAb titers, and measure the longitudinal effect of vaccination on frequencies, phenotypes, and functionalities of Ad5-specific CD4$^+$ T-cells.   Our results show that Ad5-specific CD4$^+$ T-cells are nearly universally present in subjects and do not correlate with Ad5 nAb titer. Ad5-specific CD4$^+$ T-cells are only expanded following vaccination in subjects receiving the highest Ad5 vector dose, $3x10^{10}$ vp, the same dose used for the STEP trial. However, it is unlikely Ad5-specific CD4$^+$ T-cells were responsible for the increased acquisition of HIV observed in the STEP trial, as there were no significant differences in the magnitude, activation, or mucosal homing of Ad5-specific CD4$^+$ T-cells in Ad5-seronegative and Ad5-seropositive subjects.

Results

Ad5 nAb titers and T-cell responses were tested in samples from seven cohorts that received various Ad5 HIV vaccines in Merck phase I trials (Table 3).   To test for Ad-specific T-cell responses we developed a sensitive and reproducible polychromatic intracellular cytokine-staining assay using replication-defective Ad5 particles for stimulation (Chapter 3, Figure 1A, 1C). Stimulating cells with the entire Ad5 vector allows for the detection of T-cells

Table 3: Merck Ad5 Vaccine Subjects			
Group	Number of Subjects	Vaccine Schedule	Baseline Neutralizing Antibody titer
Placebo	5	Placebo (AIPO4) Weeks 0, 4, 26	18-2598
A	6	Placebo (AIPO4) weeks 0,4,8, $1x10^{10}$Ad5gag week 26	≤18-58
B	5	5mg gag DNA/AIPO4 weeks 0, 4, 8 $1x10^{10}$vp Ad5 gag week 26	≤18-278
C	5	Ad5 gag $1x10^{10}$, weeks 0, 4, 26	≤18-1047
D	5	HIV-1 gag MRK Ad5 $1x10^9$ vp at weeks 0, 4, 26	≤18-1146
Seronegative (016 trial)	5	MRKAd5HIV-1gag/pol/nef, 3 $x10^{10}$, at weeks 0 followed weeks 0-78	≤18
Seronegative (016 trial)	5	MRKAd5HIV-1gag/pol/nef, 3 $x10^{10}$, at WKS 0,4,26	≤18
Seropositive (016 trial)	5	MRKAd5HIV-1gag/pol/nef, 3 $x10^{10}$, at weeks 0, 4, 26	203-442

Table 3: Merck Ad5 HIV vaccine subject groups.

recognizing Ad5 epitopes from the entire vector as opposed to using overlapping peptides for a single protein such as hexon only.

To characterize the relationship between Ad5 nAb titers and Ad5-specific CD4$^+$ T-cell responses, we analyzed samples from 40 subjects with varying Ad5 nAb titers who were enrolled in various Ad5 HIV vaccine trials. Of these subjects, 15 (five seronegative weeks 0-4, five seronegative weeks 0-78, and five seropositive weeks 0-78) were enrolled in the Merck 016 phase I HIV-1 vaccine safety trial and received Ad5 vectors used in the STEP trial at weeks 0, 4, and 26 [80]. All subjects independent of vaccination group were combined at baseline based on Ad5 serostatus (seronegative Ad5 nAb ≤18, seropositive Ad5 nAb >18). We detected similar frequencies of Ad5-specific CD4$^+$ T-cells with a frequency of 0.05% to 2.0% in > 80% of Ad5-seropositive and Ad5-seronegative subjects at baseline (Figure 13A). Within Ad5-seropositive subjects, Ad5-specific CD4$^+$ T-cell frequencies did not correlate with Ad5 nAb titers (Figure 13B). Though nAbs are serogroup-specific, Ad -reactive CD4$^+$ T-cells cross-react due to extensive sequence homology between the 51 human serotypes, accounting for the universal presence of Ad5-reactive CD4$^+$ T-cell despite a seroprevalence of only 40% [32,116,117,118,161].

Following vaccination, there were no increases in the percentage of Ad5-specific CD4$^+$ T-cells in groups A-D. Subjects in these groups received a lower dose of Ad5 vector, and in some cases only one vector injection in
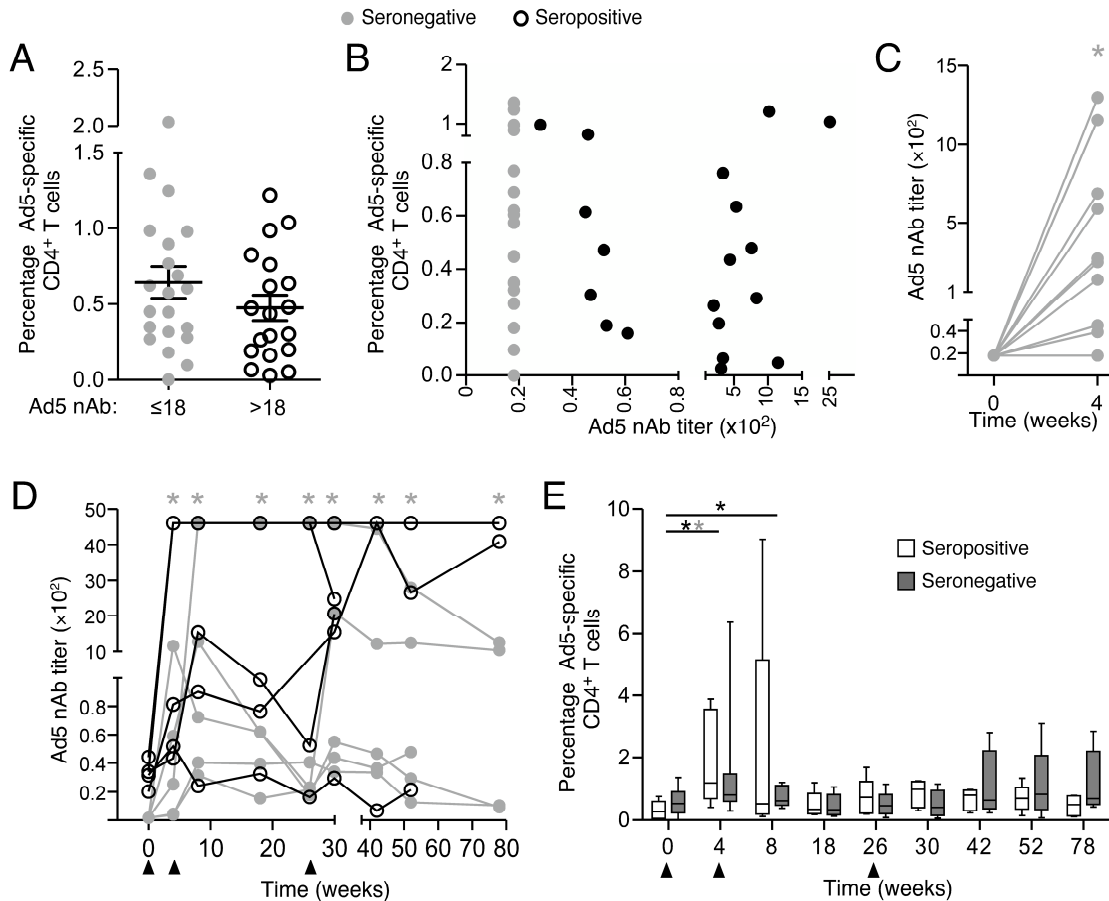
Figure 13: Ad5-specific CD4+ T-cell frequency does not correlate with Ad5 neutralizing antibody titer. Forty total subjects with a range of Ad5 nAb titers were analyzed (A,B). Ten Ad5-seronegative (five assessed weeks 0-4, five assessed weeks 0-78, gray symbols) and five Ad5-seropositive subjects (black symbols) received 3x10^10 vp Merck Ad5 gag/pol/nef at weeks 0, 4 and 26 (C, D, E). Ad-specific CD4+ T-cells represents the percentage of cells producing IFN-γ, IL-2, MIP1α, TNFα, and/or perforin production in response to Ad5 virus particle stimulation. A) Similar Ad5-specific CD4+ T-cell magnitude regardless of baseline Ad5 serostatus. B) No correlation between total Ad5-specific CD4+ T-cell magnitude and Ad5 nAb titer. C) Ad5 nAbs titers increase in Ad5-seronegative subjects after one vaccination (P < 0.05). D) Ad5 nAb titers remain elevated in baseline Ad5-seronegative subjects throughout the vaccine course (gray asterisk, P < 0.05, weeks 4-78). E) Ad5-specific CD4+ T-cell frequency increases after vaccination in Ad5-seropositive subjects (open boxes, black asterisk) at weeks 4 (P < 0.002) and 8 (P < 0.03) and Ad5-seronegative subjects (grey boxes, gray asterisk) at week 4 (P < 0.02). Plots depict the median, 25th and 75th percentile (box plots) and the minimum and maximum values (whiskers). Triangles indicate vaccination time points.

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contrast to the three doses of $3x10^{10}$ vp used in the seropositive and seronegative 016 subjects and STEP trial. Though Ad5-specific CD4$^+$ T-cells were prevalent, this data suggests they require a high dose of vector to become activated by vaccination. Because there were no significant differences in Ad5-specific CD4$^+$ T-cells observed in groups A-D or the placebos, the remainder of our analysis will focus on the seronegative and seropositive 016 trial subjects only.

Four weeks after the first Ad5-HIV-1 vector administration in the fifteen 016 trial vaccinated subjects, Ad5 nAb titers in baseline Ad5-seronegative subjects (n = ten) increased ($P < 0.05$), becoming comparable to those seen in baseline Ad5-seropositive subjects (n = five) in all but one individual (Figure 13c) who seroconverted by week 8 (Figure 13D). Ad5-specific CD4$^+$ T-cells increased in both groups ($P < 0.002$, baseline seropositive; $P < 0.03$, baseline seronegative) after the initial vector dose (Figure 13E, Figure 14). Successive vaccinations further expanded Ad5-specific T-cells in some subjects, but these responses were transient in most individuals (Figure 13E, Figure 15). At no point was there a statistical difference between the serogroups.

Having found no differences between the serogroups in the magnitude or expansion of total Ad5-specific CD4$^+$ T-cells, we next examined the relationship between Ad5 serostatus and potential functional differences in Ad5-specific CD4$^+$ T-cells before and after vaccination. Ad5-specific CD4$^+$ T-
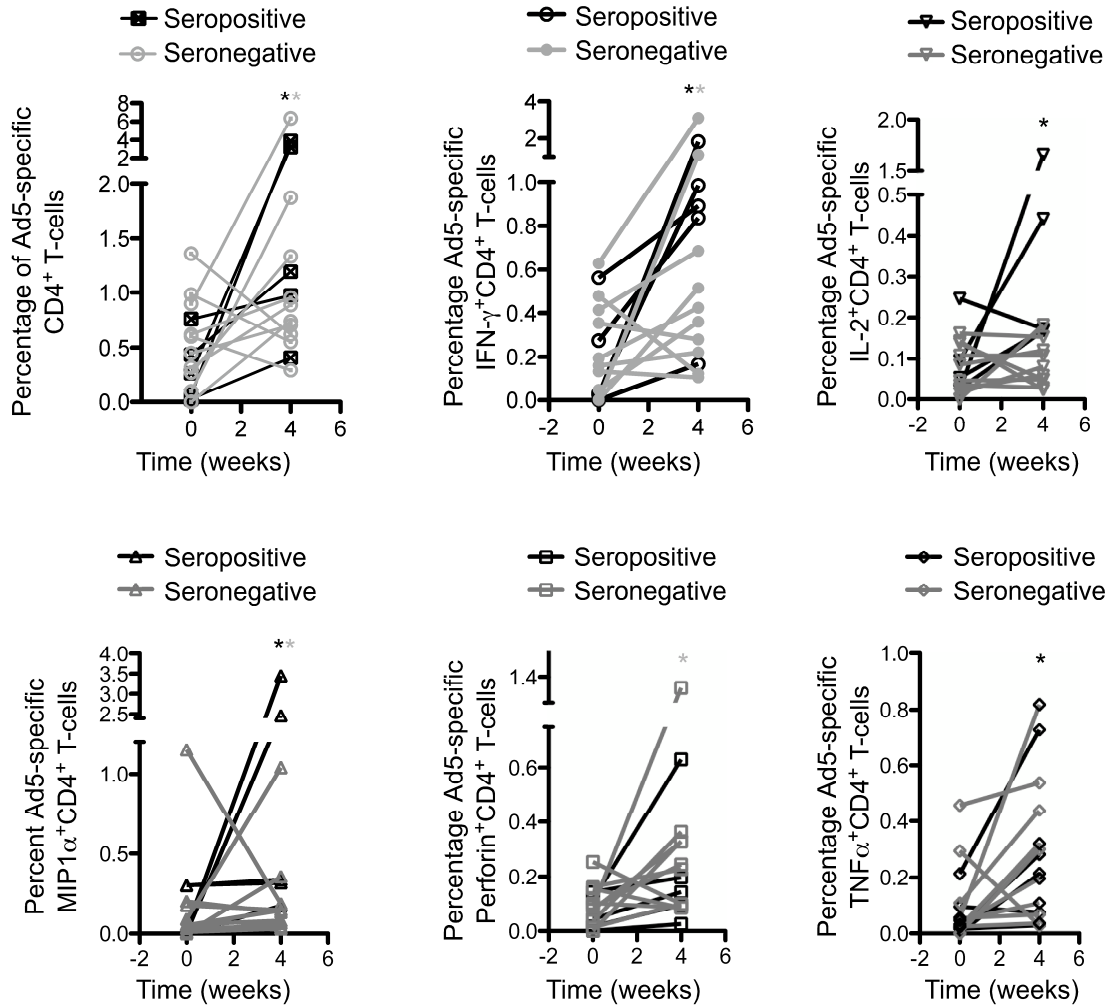
Figure 14: Ad5-specific T-cell responses following a single vaccination.
Ad5-seronegative subjects (n = ten, grey) and Ad5-seropositive subjects (n = five, black) received $3x10^{10}$ vp Merck Ad5 gag/pol/nef at week 0. We measured the percentage of cytokine$^+$ CD4$^+$ T-cells by intracellular flow cytometry and all results are background subtracted. In both groups, total responses were elevated at week 4 compared to baseline (P < 0.002, baseline seropositive; P < 0.03, baseline seronegative). In Ad5-seropositive subjects the percentage of Ad-specific CD4$^+$ T-cells expressing IFN-γ (P < 0.002), IL-2 (P < 0.002), MIP1α (P < 0.03), and TNFα (P < 0.0001) were significantly increased at week 4, while the percentage of Ad-specific CD4$^+$ T-cells expressing IFN-γ (P < 0.03), MIP1α (P < 0.01), and perforin (P < 0.001) were significantly increased above baseline in Ad5-seronegative subjects at week 4.
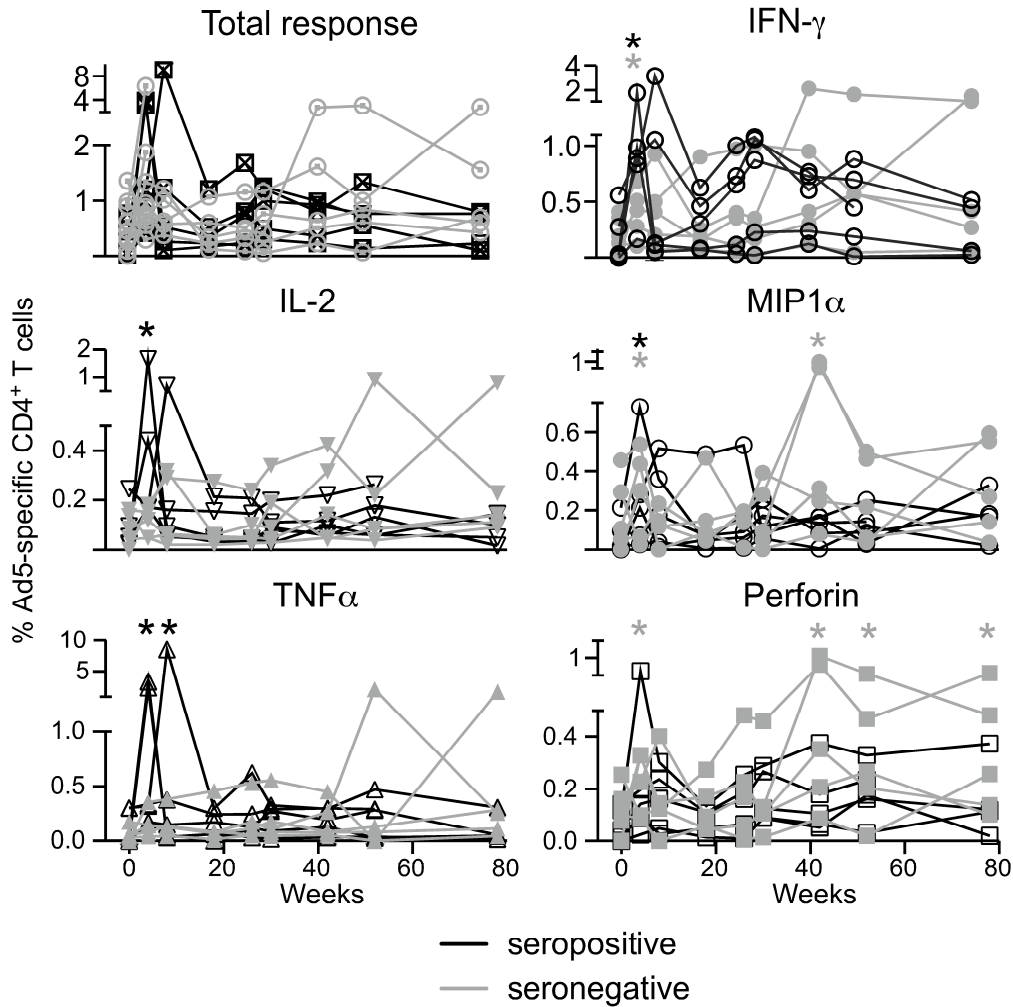
Figure 15: Change in the percentage of Ad5-specific CD4$^+$ T-cells. Ten Ad5-seronegative (Ad5 nAb titer ≤18, five subjects weeks 0-4 and five subjects weeks 0-78, grey) and five Ad5-seropositive subjects (Ad5 nAb titer > 18, weeks 0-78, black) received 3x10^{10} vp Merck Ad5 gag/pol/nef at weeks 0, 4 and 26 with PBMCs collected at weeks 0, 4, 8, 18, 26, 30, 42, 52 and 78. We measured the percentage of CD4$^+$ IFN-γ IL-2, MIP1α, TNFα and perforin positive T-cells by intracellular flow cytometry. Ad5-specific CD4$^+$IFN-γ T-cell frequency increases after vaccination in Ad5-seropositive subjects at weeks 4 (black asterisks, P < 0.005), 8 (P < 0.05) and 30 (P < 0.5) and Ad5-seronegative subjects at week 4 (grey asterisks, P < 0.03). In Ad5-seropositive subjects the percent of Ad-specific CD4$^+$ T-cells producing IL-2 increased above baseline at week 4 (P < 0.03), MIP1α at week 4 (P < 0.03), and TNFα at weeks 4 (P < 0.0001) and 8 (P < 0.005); in Ad5-seronegative subjects the percent of Ad-specific CD4$^+$ T-cells producing MIP1α increased above baseline at weeks 4 (P < 0.005) and 42 (P < 0.001), and perforin at weeks 4 (P < 0.001), 42 (P < 0.0001), 52 (P < 0.05) and 78 (P < 0.05).

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cells that produced IFN-γ, IL-2, MIP-1α, TNF-α, and/or perforin were present

at baseline in most individuals at similar frequency regardless of Ad5

serostatus (Figure 16A). There was no correlations between Ad5 nAb titer

and the percentage of Ad5-specific CD4$^+$ T-cells that produced any one or

more functions (data not shown). IFN-γ dominated the response in both

serogroups, but accounted for only ~50% of the total response (Figure 16B).

Measuring IFN-γ alone by intracellular staining or ELISpot would

underestimate the magnitude of the response. Approximately 20% of

responding CD4$^+$ cells also produced the effector functions MIP1α and

perforin regardless of serostatus.

After the first vaccination Ad5-specific CD4$^+$IFN-γ^+ and MIP1α^+ T-cells

increased in both groups ($P < 0.05$), with no differences in the fold change

between serogroups (Figure 14, Figure 16C). Seropositive subjects had an

early expansion of memory-like cells. The frequency of Ad5-specific CD4$^+$ T-

cell producing IL-2 ($P < 0.03$) and TNFα ($P < 0.001$) increased in Ad5-

seropositive subjects only and accounted for a higher proportion of the total

response ($P < 0.05$) compared with seronegative subjects (Figure 14, Figure

15, Figure 16C-D). In contrast to these early responses, Ad5-seronegative

subjects had a later more effector-like expansion of Ad5-specific CD4$^+$ T-

cells. In seronegative subjects, the effector functions perforin and MIP1α were

increased above baseline following the third vector dose. (Figure 15, Figure

16D). Despite these transient increases in CD4$^+$ T-cell functions within
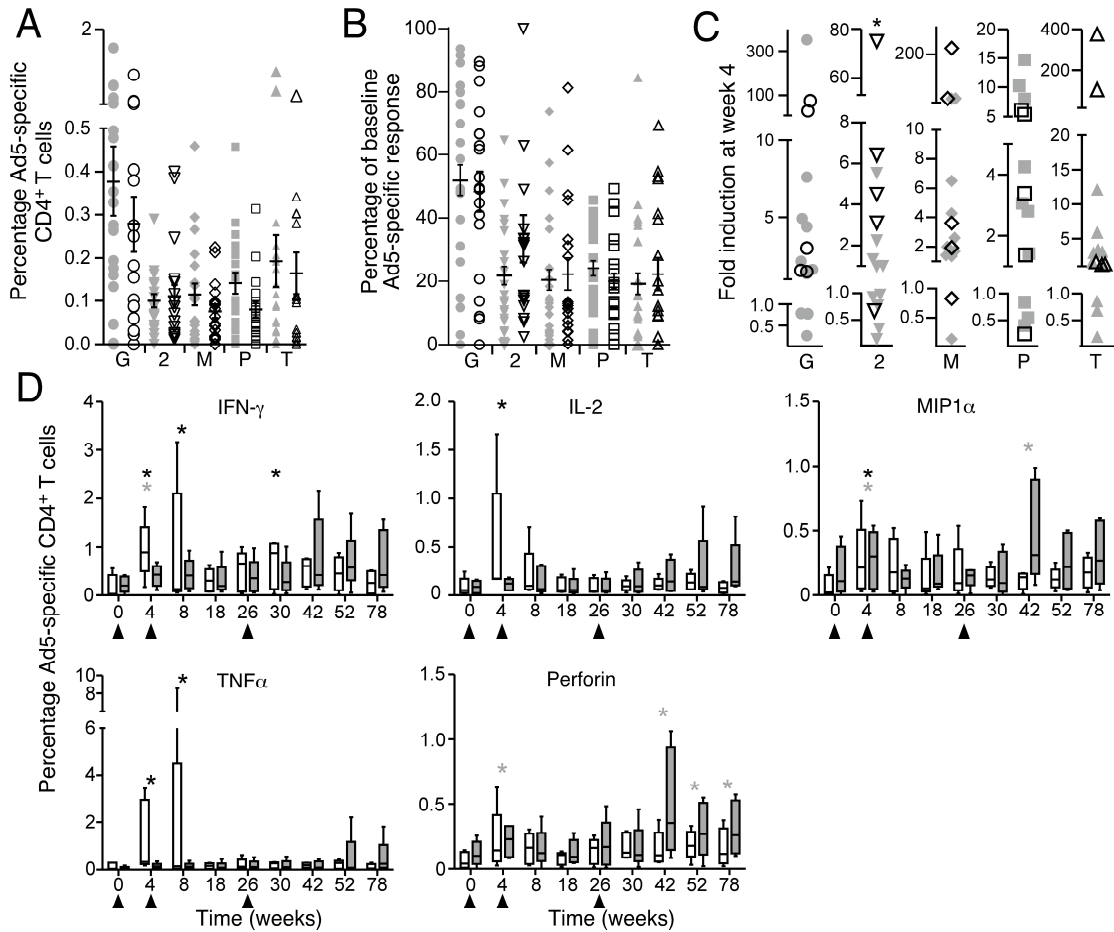
Figure 16: CD4⁺ functionality does not differ with baseline serostatus. IL-2 (2, downward triangle), IFN-γ (G, circle), MIP1α (M, diamond), perforin (P, square) and TNFα (T, upward triangle) production in response to Ad5 virus were measured by intracellular cytokine staining. For all panels, gray symbols, lines, or box plots depict baseline Ad5-seronegative subjects, and open black symbols, lines, or box plots depict baseline Ad5-seropositive subjects. A) Percentage of baseline Ad5-specific CD4⁺ T-cells producing various responses separated by Ad5 serostatus. Bars represent the mean ± SEM. B) Percent contribution of Ad5-specific CD4⁺ T-cells making each respective function to the total Ad5-specific CD4⁺ T-cell response at baseline. C) The fold change in each Ad-specific CD4⁺ T-cell function after a single vaccination. The fold change in IL-2 was significantly higher in Ad5-seropositive subjects at week 4 (P < 0.02). D) Transient changes in Ad-specific CD4⁺ T-cell function after vaccination. In Ad5-seropositive subjects IFN-γ increased from baseline (black asterisk) at week 4 (P < 0.005), 8 (P < 0.05) and 30 (P < 0.5), IL-2 at week 4 (P < 0.03), MIP1α at week 4 (P < 0.03), and TNFα at weeks 4 (P < 0.0001) and 8 (P < 0.005); in Ad5-seronegative subjects, IFN-γ increased (gray asterisk) above baseline at week 4 (P < 0.03), MIP1a at weeks 4 (P < 0.005) and 42 (P < 0.001), and perforin at weeks 4 (P < 0.001), 42 (P < 0.0001), 52 (P < 0.05) and 78 (P < 0.05). Plots depict the median, 25th and 75th percentile (boxes) and the minimum and maximum values (whiskers). Triangles indicate vaccination time points.
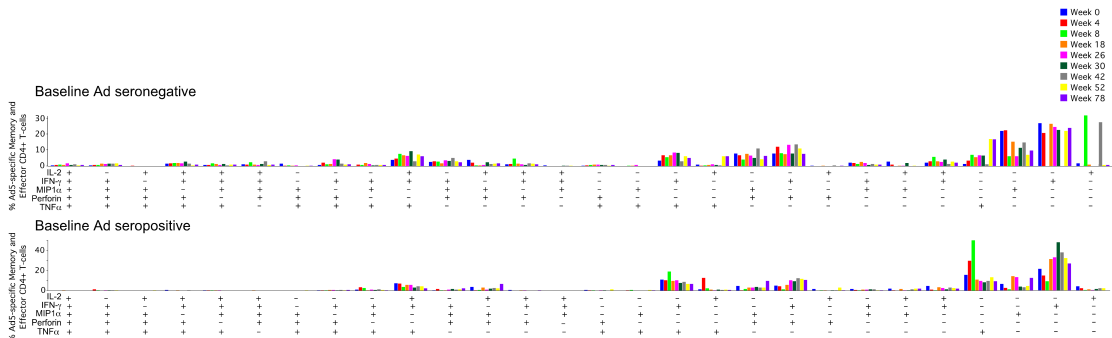
Figure 17: Polyfunctionality of Ad5-specific CD4⁺ responses in vaccinated subjects. We stimulated PBMCs with whole Ad5 vector and cytokine responses were measured by intracellular cytokine staining. The percentage of cells making each possible combination of cytokines was calculated using the Boolean function in FlowJo. Bars represent the average percentage of responding Ad5-specific CD4⁺ T-cells making each cytokine combination at each time point. Ad5-Seronegative subjects demonstrated similar polyfunctionality at all time points compared with Ad5-seropositive subjects.

serogroups, there was never a significant difference between the groups for the percentage of Ad5-specific CD4$^+$ T-cells producing IFN-γ, IL-2, MIP1α, TNFα, or perforin. The degree of polyfunctionality of Ad5-specific CD4$^+$ T-cells remained comparable between baseline Ad5-seronegative and Ad5-seropositive subjects and vaccination did not alter the polyfunctional profile of the CD4$^+$ T-cell response (Figure 17). No difference was found in Ki-67 expression for total (data not shown) or Ad5-specific CD4$^+$ T-cells (Figure 18A-B) between the serogroups or compared to baseline.

Expression of the mucosal trafficking-associated markers α_4 and β_7 did not differ significantly from baseline within either serogroup on total memory (T$_M$: all CD45RO$^+$ and CCR7$^-$CD45RO$^-$) and effector memory (T$_{EM}$: CCR7$^-$ CD45RO$^+$) CD4$^+$ T-cells (Figure 18D first graph) or Ad5-specific T$_M$ or T$_{EM}$ CD4$^+$ T-cells (Figure 18C, Figure 18D second graph) after vaccination. Moreover, Ad5-specific CD4$^+$ T$_M$ and T$_{EM}$ cells represented a small fraction of total circulating $\alpha_4{}^+$ $\beta_7{}^+$CD4$^+$ T-cells, and did not change significantly after vaccination (Figure 18D third graph). Thus, while transient changes in the phenotype and magnitude of Ad5-specific CD4$^+$ T-cell responses were detected within groups after vaccination, no significant differences between groups were observed.

Ad-specific CD4$^+$ T cells at baseline are capable of expressing markers indicative of central memory-like (CD27$^+$CD45RO$^+$CD57$^-$), effector memory (CD27$^-$CD45RO$^+$CD57$^-$), and effector cells (CD27$^+$CD45RO$^+$CD57$^{+/-}$)

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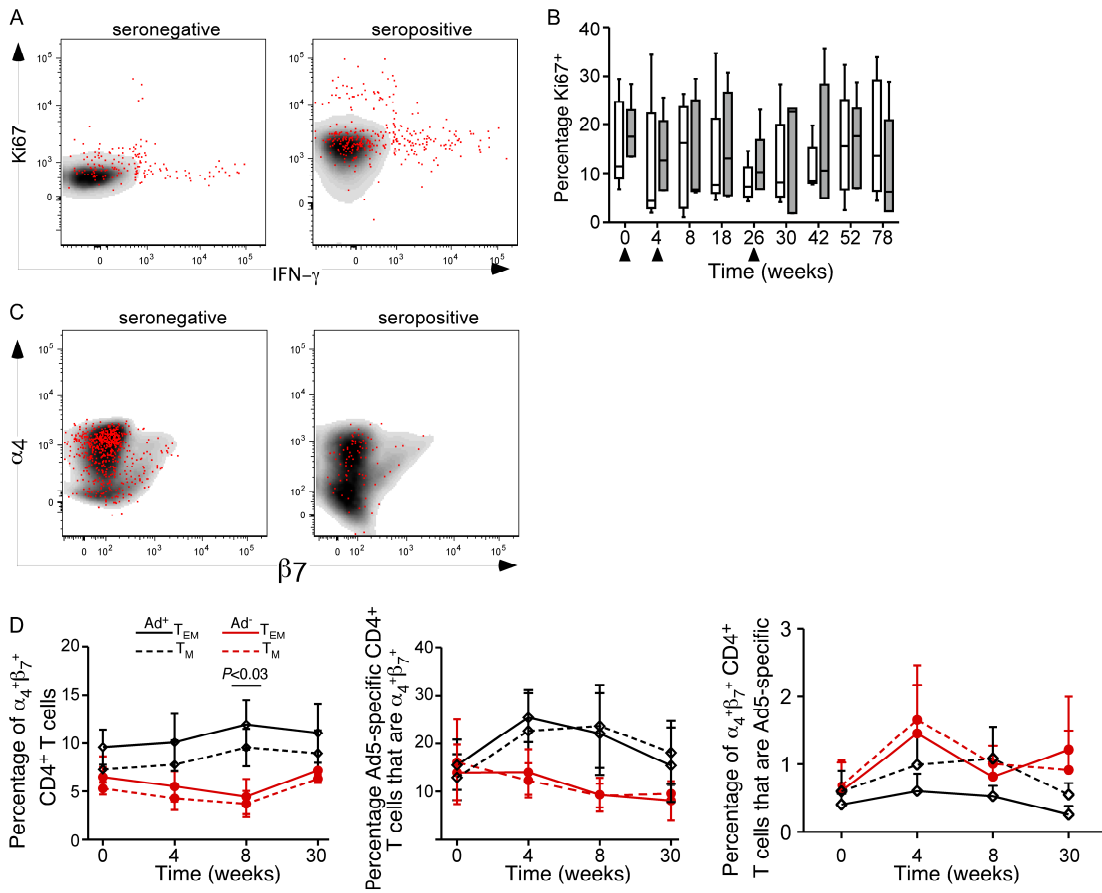
Figure 18: Representative flow plots of Ki67 and α_4/β_7 staining. Data shown have been gated on CD3$^+$ CD4$^+$ T-cells. Grey density plots represent total memory CD4$^+$ T-cells. Red dot overlays represent Ad-specific CD4$^+$ T-cells as defined by production of one or more cytokine A) Ki67 staining in total (grey) and Ad-specific (red) CD4$^+$ T-cells in a representative donor. B) Average percentage of Ad-specific Ki67$^+$ CD4$^+$ T-cells. C) representative staining on α_4 and β_7 expression on total (grey) and Ad-specific (red) CD4$^+$ T-cells. D) Percentage of total (left) and Ad5-specific (center) $\alpha_4^+\beta_7^+$CD4$^+$T-cells. Percentage of $\alpha_4^+\beta_7^+$CD4$^+$T-cells that are Ad5-specific (right).
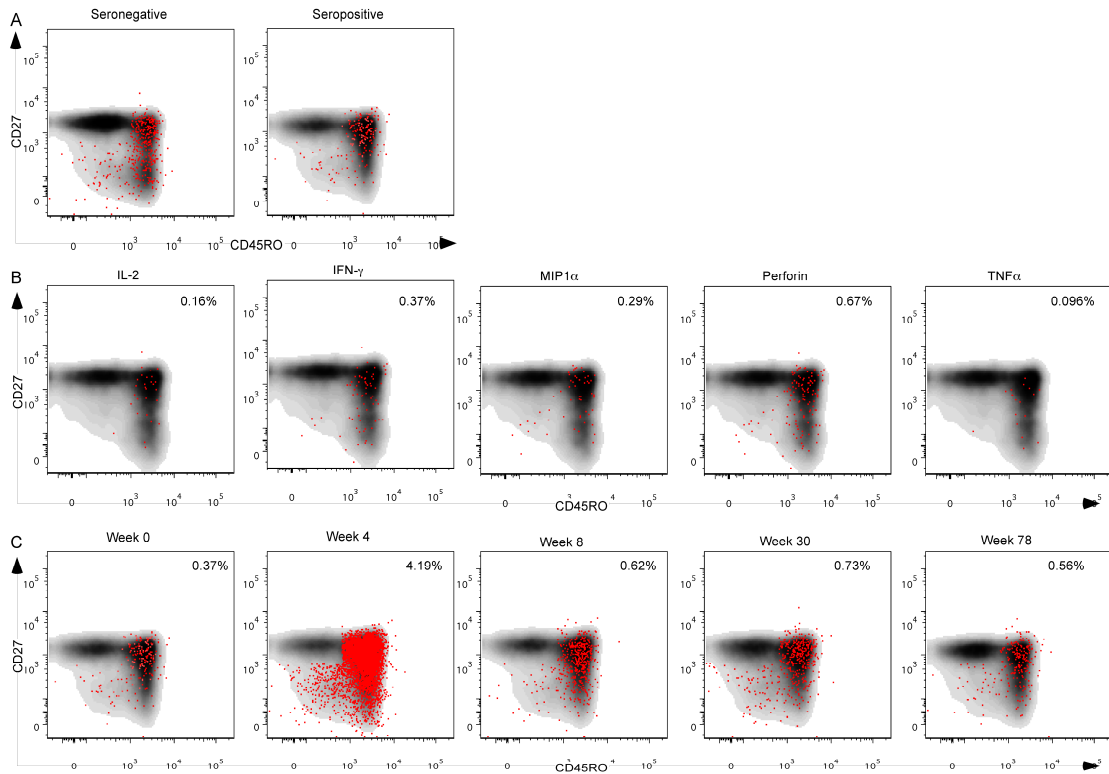
Figure 19: Ad-specific T-cells expanded by vaccination have an effector and central memory-like phenotype. Surface staining and analysis by flow cytometry was used to determine the phenotype of cytokine producing Ad-specific CD4+ T-cells in previously described subjects. Effector cells are defined as CD27-CD45RO-, effector memory as CD27-CD45RO+ and central memory-like as CD27+CD45RO+. Cells making IFN-γ, IL-2, TNFα, MIP1α and or perforin following vaccination are in red and all CD4+ T cells are in grey. A) The phenotype of Ad5-specific CD4+ T-cells at baseline in representative subjects. B) The phenotype of cytokine producing cells for a representative subject at week 4. C) The phenotype of ad-specific cells following vaccination in a representative subject.

(Figure 19A; CD57 data not shown).   The presence of each memory

phenotype varies between subjects likely based on the frequency, time, and

serotype of natural Ad exposure. Despite variations in the magnitude of a

given phenotype, the functionality of a given phenotype remained relatively

constant. For example, in the representative donor shown, week 4 effector

cells made predominantly perforin, whereas central memory-like cells

produced predominantly IL-2 and TNFa and effector memory cells produced

all 5 cytokines (Figure 19B). Following vaccination, there was an expansion of

Ad-specific cells with all phenotypes but no alterations in the percentage of

the response with a given phenotype in either Ad5-seropositive or Ad5-

seronegative subjects (Figure 19C).

Discussion

Here, we examined Ad-specific CD4^{+} T-cell responses before and after

vaccination to determine their potential role in enhancing susceptibility to

HIV-1 acquisition. We tested subjects from several Merck phase I trials that

received Ad5 vectors, including subjects from the 016 trial, that used the

same product, dosage, and regimen as was used in the STEP trial. Most

importantly, our studies include baseline samples for each subject, thus

permitting precise characterization of changes in the Ad5-specific CD4^{+}

T-cell response to vaccination. Such samples were not collected in the STEP

trial; which made it impossible to assess the actual effect of Ad5 vector

administration on Ad5-specific CD4$^+$ T-cells and their potential role in enhancing susceptibility to HIV-1 acquisition.

One hypothesis for why an increased risk of HIV infection occurred in vaccinated seropositive subjects was pre-existing Ad5-specific CD4$^+$ T-cells were activated by vaccination making them the optimal targets for HIV infection. Our results indicate that Ad5-specific CD4$^+$ T-cell responses induced by Ad5 vector administration likely did not play a role in increasing susceptibility to HIV infection.   Three findings support this conclusion: first, we found no correlation between Ad5 nAb titers and the presence or magnitude of Ad5-specific CD4$^+$ T-cell responses prior to vaccination.   Thus, high Ad5 nAb titers do not predict frequencies of Ad5-specific CD4$^+$ T-cells. Second, Ad5-specific T-cells within subjects who are Ad5 nAb seronegative at baseline expand similarly to Ad5 nAb seropositive subjects in response to Ad5 vector administration. There were also no differences between the serogroups in the level of activation as measured by Ki67, or mucosal homing as measured by $\alpha_4\beta_7$. However, enhanced susceptibility to infection was not found within STEP trial participants with low or absent Ad5 nAb titers at baseline. Third, after vaccination, Ad5 nAb seronegative subjects uniformly become Ad5 nAb seropositive, yet again no enhanced susceptibility was noted in baseline Ad5-seronegative STEP participants after receiving multiple doses of the Ad5 vector.

Taken together, these data suggest that any linkage between Ad5-

serostatus and Ad5-specific T-cell-related increase in HIV-1 acquisition should only have been observed early after the first vaccine dose, for afterwards Ad5-specific T-cell responses in baseline Ad5-seronegative subjects appear immunologically equivalent to baseline Ad5-seropositive subjects. With the caveat that our analyses are restricted to circulating CD4$^+$ T-cells and did not address potential differences in activated Ad5-specific CD4$^+$ T-cells within mucosal tissues after vaccination, our results do not support the hypothesis that Ad5-specific CD4$^+$ T-cells contributed to the potential increased HIV-1 acquisition in the STEP trial.

Materials and Methods

T-cell Responses

Frozen PBMCs were obtained from participants in 5 Merck phase I HIV Ad5 trials (Table 3). The vaccination dose and schedule used for the seropositive and seronegative subjects was identical to that used in the phase II STEP trial. T-cell responses were by stimulating PBMCs over night with whole replication-defective Ad5 vector. Cells were analyzed on a modified LSR II flow cytometer (BD Immunocytometry Systems, San Jose, CA). Data analysis was performed using FlowJo 8.7 (TreeStar, San Carlos, CA). Ad5 neutralizing antibody titers were measured as previously described [163].

Statistics

        To test for group differences over time we performed mixed effects models. Mixed effects models are similar to regression models except they account for the inherent correlation between measurements from the same subject and allow us to use all available measurements over time. For comparisons between baseline and subsequent time points within each group we also used mixed effects models. Time was considered to be a discrete variable, lessening the power of these tests compared to tests where time is a continuous variable. Correlations between Ad5 nAb titers and T-cell functions were computed at baseline using Spearman correlations. Correlations over the entire time period were computed using partial correlation coefficients controlling for individual subject effects in the repeated measurements. All data was log-transformed using base e.

Portions of this work have been previously published: Hutnick NA, Carnathan DG, Dubey SA, Makedonas G, Cox KS, Kierstead L, Ratcliffe SJ, Robertson MN, Casimiro DR, Ertl HC, Betts MR. "Baseline Ad5 serostatus does not predict Ad5 HIV vaccine-induced expansion of adenovirus-specific CD4+ T cells." Nature Medicine. 2009. 15(8), 876.

Chapter 5: Vaccination with Adenovirus 5 HIV-1 vector differentially expands Ad5-specific CD8+ T-cells in seropositive and seronegative subjects

Abstract

Adenoviral (Ad) vaccine vectors represent both a vehicle to present a novel antigen to the immune system as well as restimulate immune responses against the Ad vector itself. To what degree Ad-specific CD8+ T-cells are restimulated by Ad-vector vaccination is unclear, although such knowledge would be important as vector-specific CD8+ T-cell expansion could potentially limit Ad vaccine efficacy further beyond Ad-specific nAb alone. Here we address this issue by measuring human Ad5-specific CD8+ T-cells in recipients of the Merck Ad5 HIV-1 vaccine vector before, during, and after vaccination by multicolor flow cytometry. Ad5-specific CD8+ T-cells were detectable in 95% of subjects prior to vaccination, and displayed primarily an effector-type functional profile and phenotype. Peripheral blood Ad5-specific CD8+ T-cell numbers expanded after Ad5-HIV vaccination in all subjects, but differential expansion kinetics were noted in some baseline Ad5 nAb

seronegative subjects compared to baseline Ad5 nAb seropositive subjects. However, in neither group did vaccination alter polyfunctionality, mucosal targeting marker expression, or memory phenotype of Ad5-specific CD8$^+$ T-cells. These data indicate that repeat Ad5-vector administration in humans expands Ad5-specific CD8$^+$ T-cells without overtly affecting their functional capacity or phenotypic properties.

Introduction

Vectors based on the human Ad5 are currently leading candidates for vaccines designed to elicit cellular immunity. Studies both in animals and humans have demonstrated that Ad5 vectors are capable of inducing potent and sustained transgene product-specific CD4$^+$ and CD8$^+$ T-cell responses [74,76,113]. Additionally, these vectors have been generally safe and well tolerated [80,170,171]. However, one major hurdle to Ad-vector based vaccines is the presence of pre-existing Ad-specific immunity.

Most studies of pre-existing Ad-specific immunity have focused on neutralizing antibodies (nAb). In animals and humans, Ad5 vaccination is less effective if there are pre-existing Ad5-specific nAbs [112,113]. Similarly, pre-exposure to Ad5 vector reduces the efficacy of subsequent booster vaccinations, thereby limiting the ability for homologous vector boosting [115]. The prevalence of nAbs to Ad5 varies worldwide, with up to 50% of adults in the United States and as many as 90% of adults in Africa testing seropositive [31]. To overcome this limitation, rare Ad serotypes with low seroprevalence have been developed as vaccine vectors [17,56,172].

Ad-specific CD4$^+$ and CD8$^+$ T-cell responses have also been detected in humans [59,117]. However, their magnitude, functional properties, and phenotypic characterization directly *ex vivo* are not well described. Ad-specific T-cells are likely more prevalent in humans than Ad serotype-specific nAbs due to cross-reactivity of T-cells against conserved viral sequences, unlike

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nAbs that are predominantly directed to the hypervariable outer loops of the Ad hexon. Indeed it has been demonstrated that *ex vivo* generated human CTL lines are capable of killing target cells infected with Ad serotypes from multiple subgroups [116]. Though Ad-specific T-cells have been identified following natural infection, it is unclear whether Ad-specific T-cells stimulated by vaccination are similar to those induced by natural infection. Moreover, the effect of repeat homologous E1-deleted Ad5 vector administration upon pre-existing Ad5-specific CD8$^+$ T cells has not been assessed in human vaccine recipients.

To assess the effect of Ad vector administration on the Ad-specific CD8$^+$ T-cell response in humans, we analyzed peripheral blood mononuclear cells (PBMCs) from subjects enrolled in a Phase 1 Ad5-vector HIV vaccine trial. Using a whole Ad5 vector stimulation together with polyfunctional flow cytometry, we defined the prevalence, magnitude, functionality and phenotype of Ad5-specific CD8$^+$ T-cells before and after Ad5 vector administration. Our results demonstrate that while Ad5-specific CD8$^+$ T-cells are present in most humans and transiently expand after vaccination, they do not change in either phenotype or function.

Results

To assess the total magnitude, functionality, and phenotypes of Ad5-specific CD8$^+$ T-cells, we stimulated human PBMCs with Ad5 vector and

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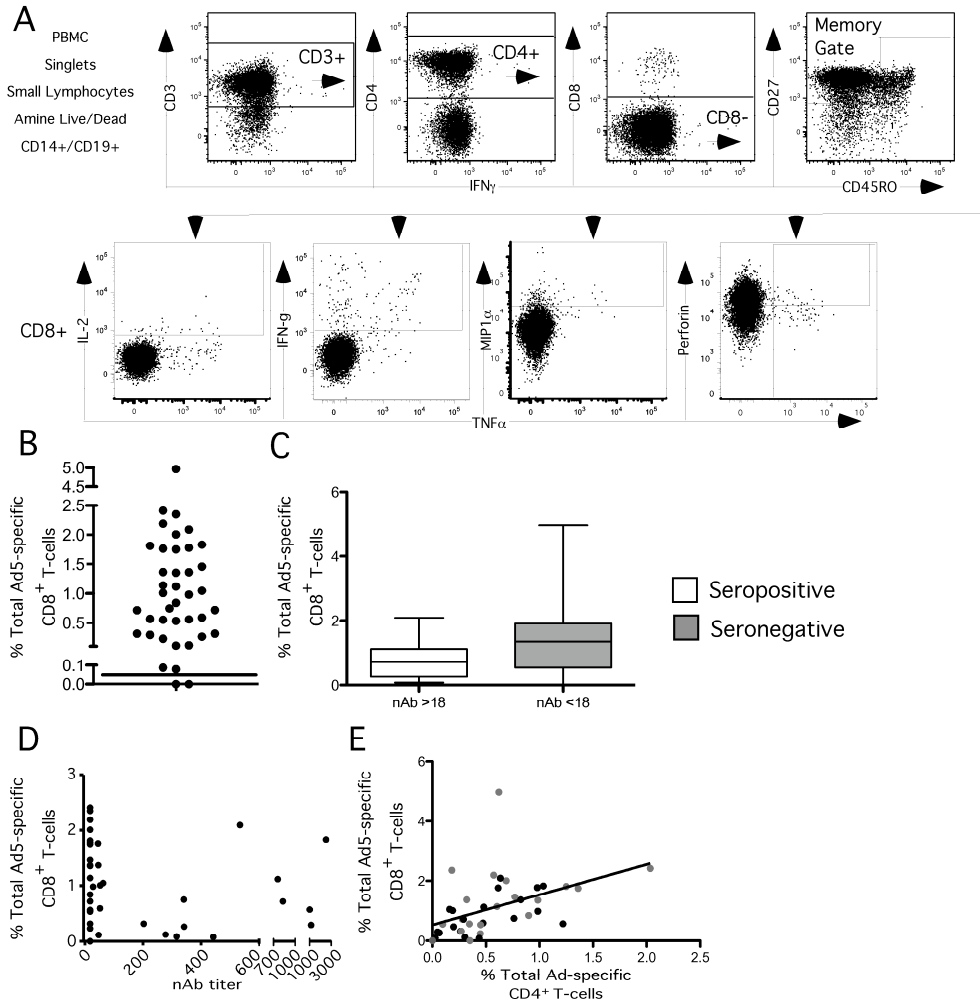
Figure 20: Baseline CD8⁺ T-cell responses. Forty total subjects with a range of Ad5 nAb titers were analyzed. Five Ad5-seronegative (Ad5 nAb titer ≤18, gray symbols) and five Ad5-seropositive subjects (Ad5 nAb titer >18, black symbols and white boxes) received Merck Ad5 gag/pol/nef as described in Methods. Box plots represent 25th-75th percentile with lines being the 10-90% range. outliers are shown as dots. CD8⁺ T-cell responses were measured by flow cytometry following whole Ad5 vector stimulation. A) Gating strategy for measuring Ad5-specific T-cells by intracellular cytokine staining. At least 100,000 PBMCs were collected on a modified LSR II. Singlets were selected with a FSC-A and FSC-H, followed by a lymphocytes gate, dead cell exclusion, and exclusion of contaminating CD14⁺ monocytes and CD19⁺ B-cells. CD3⁺ T-cells were selected and then CD8⁺ cells by CD8⁺CD4⁺. Central memory, effector memory and effector CD8⁺ T cells were selected before gating on each cytokine. Because cells can store perforin and these appear perforin⁺, Ad5-specific CD8⁺perforin⁺ T cells must also be positive for another function to be considered as a responding event. B) Total Ad5-specific CD8⁺ response. The total Ad5-specific CD8⁺ response was computed by summing cells making at least IL-2, IFN-γ, MIP1α, or TNFα as measured by flow cytometry. C) There was no difference in the magnitude of the Ad-specific CD8⁺ T-cell response between serogroups at baseline. D) There was no correlation between the magnitude of Ad5-specific CD8⁺ T-cell responses and nAb titer at baseline. E) There was a correlation between the magnitude of Ad5-specific CD4⁺ and Ad5-specific CD8⁺ T-cell responses at baseline.

measured CD8$^+$ T-cell responses by polychromatic flow cytometry (Figure 20A). We detected Ad5-specific CD8$^+$ T-cell responses in 95% of all donors at the week 0 baseline (Figure 20B). There was no difference in frequencies of Ad5-specific CD8$^+$ T-cells between baseline seropositive and seronegative subjects (Figure 20C, p>0.05). Likewise, there was no correlation between frequencies of Ad5-specific CD8$^+$ T-cells and the Ad5 nAb titer at baseline (Figure 20D, p>0.05). There was however a positive correlation between frequencies of baseline Ad5-specific CD4$^+$ and CD8$^+$ T-cells (Figure 20E).

Vector induced CD8$^+$ T-cell expansion

We next examined the effect of Ad5 vector administration on the pre-existing Ad5-specific CD8$^+$ T-cell response. After the initial vaccine administration, frequencies of Ad5-specific CD8$^+$ T-cells in the blood increased significantly above pre-vaccination frequencies in baseline Ad5-seropositive (p<0.0001) but not in baseline Ad5-seronegative subjects as a group (Figure 21A). On an individual basis, frequencies of Ad5-specific CD8$^+$ T-cells increased in three of five seronegative subjects following the initial dose (data not shown). The remaining two seronegative subjects without Ad5-specific CD8$^+$ T-cell expansion had large baseline responses.

Four weeks after the first homologous Ad5 vector boost at week 4, Ad5-specific CD8$^+$ T-cell frequencies were higher than baseline in both subject groups (p<0.03). Following this first boost, Ad5-specific CD8$^+$ T-cell

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Figure 21: Ad-specific CD8⁺ T-cells magnitude following vaccination. Five Ad5-seronegative (Ad5 nAb titer ≤18, gray boxes) and five Ad5-seropositive subjects (Ad5 nAb titer >18, white boxes) received Merck Ad5 gag/pol/nef as described in Methods. CD8⁺ T-cell responses were measured by flow cytometry following whole Ad5 vector stimulation. Box plots represent 25th-75th percentile with lines being the 10-90% range. Outliers are shown as dots. Grey asterisk represent a significant increase from baseline in seronegative subjects and black asterisk represent a significant difference from baseline in seropositive subjects. Black bars represent a significant difference between the serogroups at that time point. A) Percentage of Ad5-specific CD8⁺ T-cells. Seronegative subjects were significantly increased above baseline at week 8 (p<0.03), 26 (p<0.03), 42 (p<0.001), 52 (p<0.01), and 78 (p<0.001). Total Ad-specific CD8⁺ T-cells were increased above baseline in seropositive subjects at week 4 (p<0.0001), 8 (p<0.03), 30 (p<0.02), and 42 (p<0.04). Serogroups significantly differed at week 42 (p<0.02), 52 (p<0.01), and 78 (p<0.003). B) Percentage of Ad5-specific Ki67⁺ CD8⁺ T-cells. C) Percentage of Ki67⁺ total CD8⁺ T-cells. There was a significant increase above baseline in seronegative subjects at week 4 (p<0.01) and seropositive subjects at week 52 (p<0.12).

responses returned to pre-vaccination levels in baseline Ad5 seropositive subjects, and were only briefly expanded again at weeks 30 and 42 following the 2nd boost at week 26 (p<0.04). In contrast, Ad5-specific CD8$^+$ T-cell responses remained elevated above baseline in the seronegative cohort (p<0.03). The only time we observed a difference in Ad5-specific CD8$^+$ T-cell expansion between the seronegative and seropositive groups was following the third vaccination (weeks 42, 52 and 78; p<0.03)(Figure 21A).

The observed increase in Ad5-specific CD8$^+$ T-cell frequencies was not reflected by an increase in Ki67 on Ad5-specific CD8$^+$ T-cells after either the primary vaccination or subsequent boosts in either serogroup (Figure 21B, p=N.S.). Furthermore, we detected only transient differences in global Ki67 levels on total CD8$^+$ T-cells (Figure 21C). Thus, while increases in Ad5-specific CD8$^+$ T-cell frequencies were observed in both baseline Ad5-seronegative and Ad5-seropositive subjects following vaccination, sustained changes or global effects on the proliferative capacity of CD8$^+$ T-cells were not found.

Vector-induced changes in Ad5-specific CD8$^+$ T-cell functionality

At baseline, the majority of Ad5-specific CD8$^+$ T-cells produced predominantly the effector functions MIP1α and perforin in both seropositive and seronegative subjects (Figure 22A). The percentage of Ad5-specific CD8$^+$ T-cells producing TNFα was significantly higher in baseline seropositive
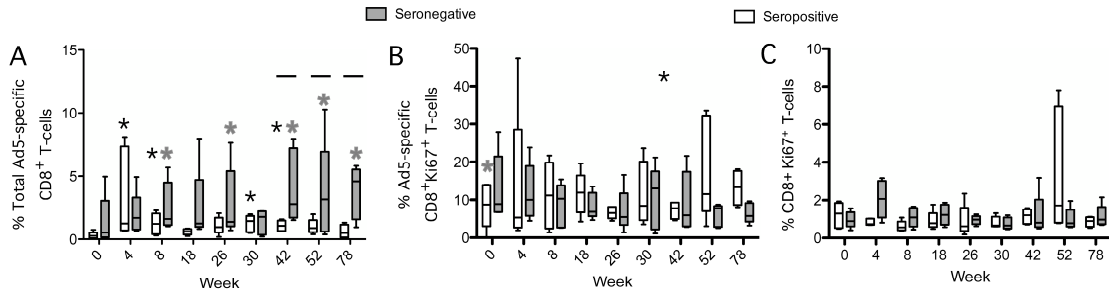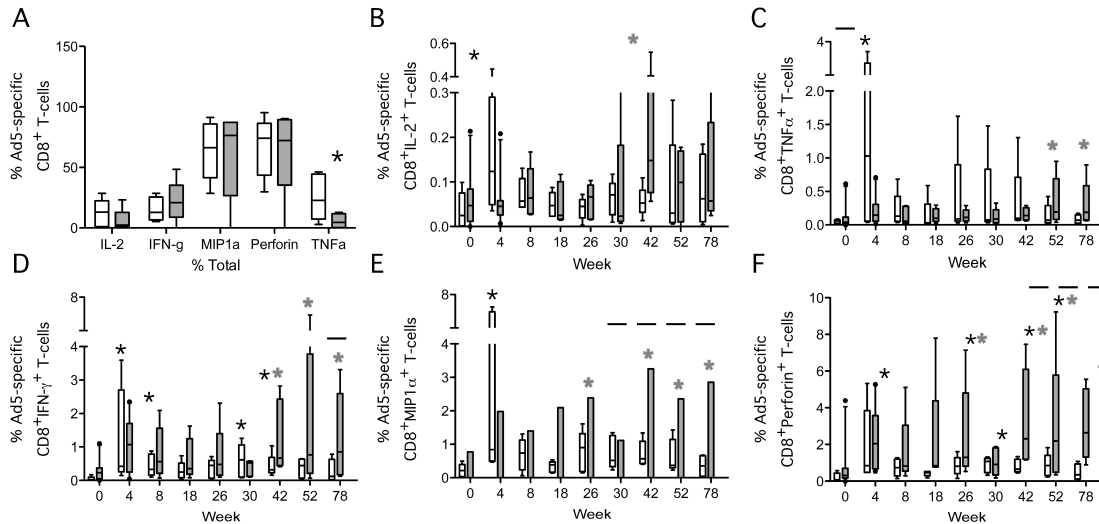
Figure 22: Ad5-specific CD8+ T-cell functionality following vaccination. . Five Ad5-seronegative (Ad5 nAb titer ≤18, gray boxes) and five Ad5-seropositive subjects (Ad5 nAb titer >18, white boxes) received Merck Ad5 gag/pol/nef as described in Methods. CD8+ T-cell responses were measured by flow cytometry following whole Ad5 vector stimulation. Box plots represent 25th-75th percentile with lines being the 10-90% range. Outliers are shown as dots. Grey asterisk represent a significant increase from baseline in seronegative subjects and black asterisk represent a significant difference from baseline in seropositive subjects. Black bars represent a significant difference between the serogroups at that time point. A) Percent of the total Ad-specific response producing each cytokine. The total Ad-specific CD8+ response was computed by summing cells making at least IL-2, IFN-g, MIP1a, or TNFa as measured by flow cytometry. The percentage of the total Ad-specific response was then computed for each cytokine. The percentage of the total response consisting of TNFa was significantly higher in seropositive subjects (p<0.0005) at baseline. B) Percentage of IL-2+ Ad-specific CD8+ T-cells. There was a significant increase above baseline in seronegative subjects at week 42 (p<0.01) and seropositive subjects at week 4(p=0.015). C) Percentage of TNFa+ Ad-specific CD8+ T-cells. There was a significant increase above baseline in seropositive subjects at week 52 (p<0.01) and 78 (p<0.02) and seropositive subjects at week 4 (p<0.01). There was a significant difference at baseline in the percentage of TNFa+ CD8+ T-cells between serogroups (p<0.05). D) Percentage of IFN-g Ad-specific CD8+ T-cells. The percentage of IFN-g+ CD8+T-cells was significantly increased above baseline in seronegative subjects at weeks 42 (p<0.004), 52 (p<0.003), and 78 (0.009) and in seropositive subjects at weeks (4 (p<0.0001), 8 (p<0.04), 30 (p<0.01) and 42 (p<0.04). There was a significant difference in the percentage of CD8+IFN-g+ CD8+ T-cells at week 78 between the serogroups (p<0.05). E) Percentage of MIP1a+ Ad-specific CD8+ T-cells. Seronegative subjects had a significantly increased percentage of MIP1a+ CD8+ T-cells above baseline at weeks 26 (p<0.03), 42 (p<0.001), 52 (p<0.05) and 78 (p<0.004). Seropositive subjects had a significantly increased percentage of MIP1a+ CD8+ T-cells at week 4 (p<0.0005) compared with baseline There was a significant difference in the percentage of MIP1a+CD8+ T-cells between the serogroups at weeks 30 (p<0.04), 42 (p<0.012), 52 (p<0.005), and 78 (p<0.001). F) Percentage of perforin+ Ad-specific CD8+ T-cells. The percentage of perforin+CD8+ T-cells was significantly increased above baseline at weeks 26 (p<0.02), 42 (p<0.001) 52 (p<0.05) and 78 (p<0.004) in seronegative subjects and week 4 (p<0.0005) in seropositive subject. There was a significant difference in the percentage of MIP1a+CD8+ T-cells at weeks 30 (p<0.04), 42 (p<0.012), 52 (p<0.005) and 78 (p<0.0013).

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subjects prior to vaccination (p<0.001) but there were no differences between the groups for IL-2, IFN-γ, MIP1α or perforin.

Having observed increases in the total percentage of Ad5-specific CD8$^+$ T-cells following vaccination we next determined whether vaccination affected the functionality of these cells. Consistent with the increase in Ad5-specific CD8$^+$ T-cell frequencies following the initial vector vaccination, baseline seropositive subjects showed a transient increase in the percentage of Ad5-specific CD8$^+$ T-cells producing IL-2 (Figure 22B, p=0.015), TNFα (Figure 22C, p<0.01), and IFN-γ (Figure 22D, p<0.001) as well as the MIP1α (Figure 22E, p<0.0005), and perforin (Figure 22F, p< 0.0002).

The functionality of Ad5-specific CD8$^+$ T-cells was similar in baseline seronegative and seropositive subjects, however in baseline seronegative subjects the expansion of Ad5-specific CD8$^+$ T cells was delayed. Following the third vector dose baseline seronegative subjects had transiently elevated IL-2 producing CD8$^+$ T-cells at week 42 (Figure 22B) and TNFα from weeks 52-78 (Figure 22C). CD8$^+$ T cells that produced the effector functions IFN-γ, MIP1α and perforin were expanded for a more prolonged period of time (weeks 42-78) following the third vector dose. This delayed expansion resulted in a higher percentage of Ad5-specific CD8$^+$ T-cells in baseline seronegative subjects compared with baseline seropositive subjects following the third vector dose with MIP1α significantly elevated at weeks 30-78 and perforin at weeks 42-78.
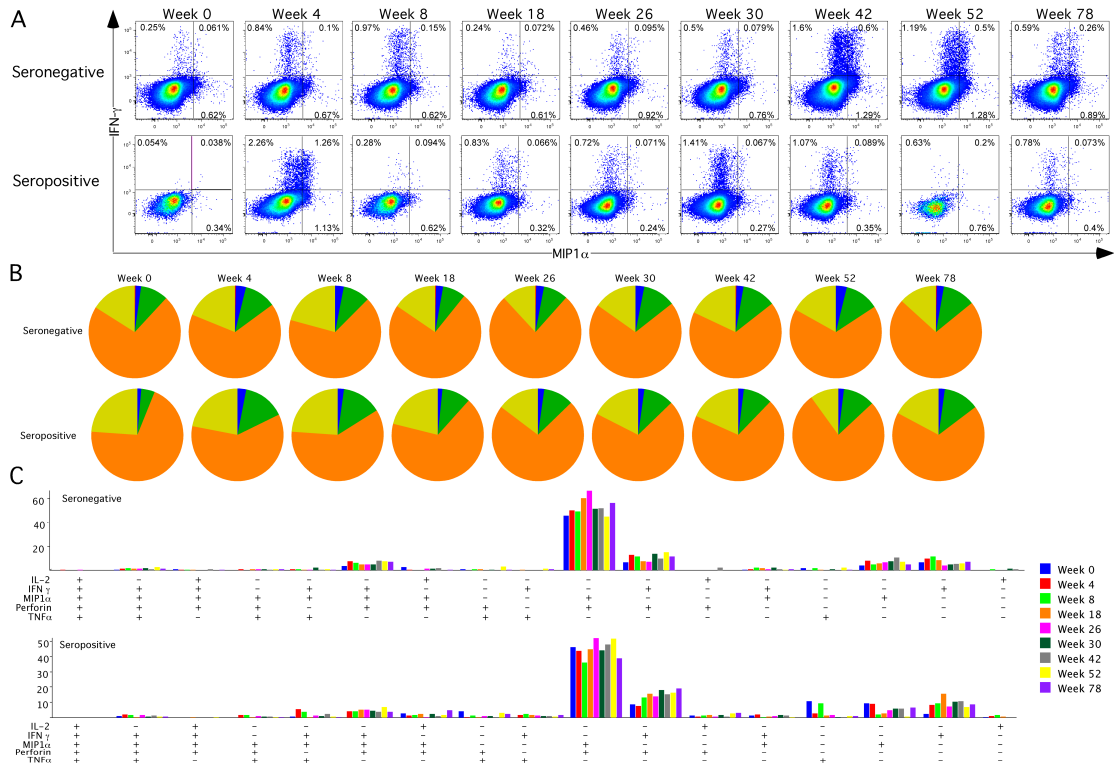
Figure 23: Polyfunctional Ad5-specific CD8⁺ T-cell Responses. Five
seronegative (Ad5 nAb titer ≤18) and five seropositive subjects (Ad5 nAb titer >18) received
Merck Ad5 gag/pol/nef as described in Methods. CD8⁺ T-cell responses were measured by
flow cytometry following whole Ad5 vector stimulation. A) Percentage of CD8⁺ T-cells
expressing IFN-γand MIP1α. B) Percentage of Ad-specific CD8⁺ T-cells producing all five
(red) functions: IL-2, MIP1α, TNFα, IFN-γ and Perforin, four (blue), three (green), two
(orange), or one (yellow) of the five functions at each time point. Pies represent an average of
the two groups. C) Bars represent the percentage of Ad-specific CD8+ T-cells making a
combination of IL-2, TNFα, MIP1α, Perforin and IFN- γ at each week. Positive symbols
represent cells staining positive for a function, and minus symbols represent cells staining
negative for a function.

Although we observed increases in the frequencies of Ad5-specific CD8$^+$ T-cells producing various functions, the overall polyfunctionality of Ad5-specific CD8$^+$ T-cells remained similar to baseline after vaccination (Figure 23A, B) in both groups. Furthermore, there was no substantial difference between the groups in the functional combinations produced (Figure 23C). In both groups the major response consisted of cells producing MIP1α with perforin and MIP1α with IFN-γ.

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Ad5-specific CD8$^+$ Phenotype

To investigate whether the effector-like functionality of Ad5-specific CD8$^+$ T-cells corresponded to an effector phenotype, we assessed CD45RO and CD27 expression on Ad5-specific CD8$^+$ T-cells. Ad5-specific cells that produced MIP1α, and perforin were primarily of an effector-like phenotype (CD27$^-$CD45RO$^-$) (Figure 24A), whereas more diverse memory subsets produced IFN-γ, TNFα, and IL-2. Approximately half of all Ad5-specific CD8$^+$ T-cells had an effector-like phenotype at baseline in both seronegative and seropositive subjects (Figure 24B-C). In baseline seropositive subjects, vaccination induced transient decreases in the percentage of Ad5-specific effector CD8$^+$ T-cells (Figure 24C, p<0.05) that corresponded with an increase in the percentage of Ad5-specific effector memory cells (CD27$^-$CD45RO$^+$; Figure 24D, p<0.05). In baseline seronegative subjects the effector phenotype observed at baseline remained stable following

vaccination with only a transient increase in the percentage of Ad5-specific memory cells observed at week 26 (Figure 24E). In the total CD8$^+$ T-cell pool, the effector phenotype dominated in both serogroups at baseline. Following vaccination the percentage of total effector CD8$^+$ T-cell (Figure 24F) in seropositive subjects decreased, coinciding with an increase in total central memory-like CD8$^+$ T-cells (CD27$^+$CD45RO$^+$; Figure 24H). In seronegative subjects only transient changes in total CD8$^+$ phenotype occurred (Figure 24F-H). Thus, while transient changes in memory phenotype were observed after vaccination, there were no sustained alterations in the memory phenotype of total or Ad5-specific CD8$^+$ T-cells in either of the cohorts. These data suggest that Ad5-based vaccination does not induce global bystander CD8$^+$ activation.
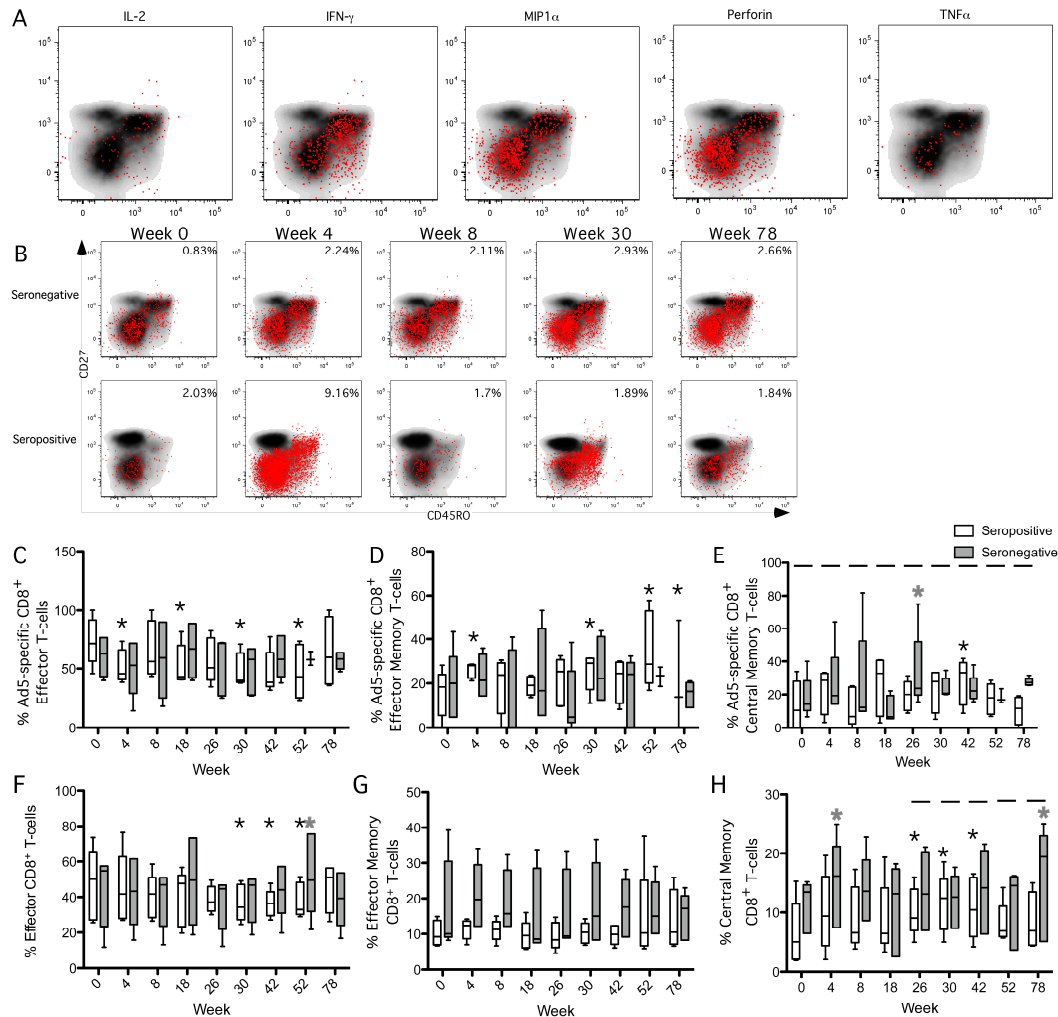
Figure 24: Phenotype of Ad5-specific CD8$^+$ T-cells. Five seronegative (Ad5 nAb titer ≤18, gray boxes) and five seropositive subjects (Ad5 nAb titer >18, white boxes) received Merck Ad5 gag/pol/nef as described in Methods. Ad-specific CD8+ T-cell responses were measured by flow cytometry. Box plots represent 25th-75th percentile with lines being the 10-90% range. Outliers are shown as dots. Grey asterisk represent a significant increase from baseline in seronegative subjects (p<0.05) and black asterisk represent a significant difference from baseline in seropositive subjects (p<0.05). Black bars represent a significant difference between the serogroups at that time point. A) The phenotype of Ad-specific CD8$^+$ T-cells in a representative donor. Black areas represent total CD8$^+$ T-cells and red dots represent Ad-specific CD8$^+$ T-cells expressing IFN-γ, IL-2, MIP1α, Perforin or TNFα. B) Phenotype of Ad-specific CD8$^+$ T-cells following vaccination. Black areas represent total CD8$^+$ T-cells and red dots represent total Ad-specific CD8$^+$ T-cells expressing IFN-γ, IL-2, MIP1α, Perforin or TNFα. C) Percentage of Ad-specific CD8$^+$ T-cells with an effector phenotype (CD27$^-$CD45RO$^-$). D) Percentage of Ad-specific CD8$^+$ T-cells with an effector memory phenotype (CD27$^-$CD45RO$^+$). E) Percentage of Ad-specific CD8$^+$ T-cells with a central memory-like phenotype (CD27$^+$CD45RO$^+$). F) Percentage of total CD8$^+$ T-cells with an effector phenotype (CD27$^-$CD45RO$^-$). G) Percentage of total CD8$^+$ T-cells with an effector memory phenotype (CD27$^-$CD45RO$^+$). H) Percentage of total CD8$^+$ T-cells with a central memory-like phenotype (CD27$^+$CD45RO$^{+)}$.

Discussion

Recombinant Ad vectors are one of the primary vaccine platforms that are being tested for a wide range of human pathogens including HIV, malaria, and tuberculosis [171,173,174]. While these studies primarily focus on the generation of immune responses against the recombinant insert, an often-overlooked issue is the induction of vector-specific immunity. Here we have examined Ad5-specific CD8$^+$ T-cell responses in recipients of an Ad5 HIV-1 vaccine candidate. We find that regardless of baseline Ad5 nAb serostatus, Ad5 vector administration results in a potent restimulation and expansion of pre-existing Ad5-specific CD8$^+$ T-cells. This finding by itself is curious. One would have expected that E1-deleted Ad vectors only produce trace amounts of structural Ad proteins, as the transcription of late genes is under the control of a gene product of the deleted E1 domain. Nevertheless, as has been shown by others, CD8$^+$ T cell responses to Ad vector particle proteins can be induced efficiently by cross priming. This mechanism would circumvent the need for de novo synthesis of Ad structural proteins for induction or recall of Ad-specific CD8$^+$ T cells [175].

At baseline, Ad5-specific CD8$^+$ T-cells were detectable in 38 of 40 subjects, despite a seroprevalence in the US of up to only 50%. This magnitude of Ad5-reactive CD8$^+$ T-cells is consistent with smaller studies showing Ad5-specific CD8$^+$ T-cell responses in greater than 80% of subjects [59,82,121]. The prevalence of Ad5-specific CD8$^+$ T-cells is likely the result of

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cross-reactive CD8+ T-cells generated from infection with alternate serotypes. Many Ad proteins have highly conserved regions between various Ad serotypes, likely resulting in conservation of T-cell epitopes and the generation of cross-serotype reactive Ad5-specific CD8+ T-cells. Although we were able to observe a high frequency of responders after stimulation with an Ad5 vector *in vitro*, it is unclear whether detected responses were induced by a natural infection with an Ad5 virus.

Although vaccination increased frequencies of Ad5-specific effector CD8+ T-cells in both serogroups, there were no significant changes either within or between the groups for the cell cycle marker Ki67. Though the PBMC sampling in this study was intensive, there was a four-week period between vaccination and PBMC collection. It is possible that expansion of Ad5-specific T-cells and expression of Ki67 occurred transiently during this period. Alternatively, Ki67+ Ad5-specific CD8+ T-cells may have trafficked out of the peripheral blood by this time.

Interestingly, we observed no changes in polyfunctionality of Ad5-specific CD8+ T-cells compared to baseline following vaccination in either serogroup. As we have previously observed (Chapter 3), Ad5-specific CD8+ T-cells are continuously maintained with both an effector-like phenotype and functionality. This likely reflects continued or intermittent exposure to Ad viruses from repeat infection with different serotypes or virus persistence. Ad5 vector administration did not alter the baseline functional response, but

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instead further expanded it. The expansion and high frequency of Ad-specific effector-like CD8+ T-cells could reduce the efficacy of Ad-vaccine boosting with both homologous and heterologous vector by direct lysis through perforin release of vector-transduced cells.

Materials and Methods

T-cell Responses

Frozen peripheral blood mononuclear cells (PBMCs) were obtained from unvaccinated subjects at week 0 baseline ($n = 25$), seronegative subjects receiving three doses $3x10^{10}$ vp Mrk Ad5 gag/pol/nef at weeks 0, 4 and 26 ($n = 5$, Ad5 neutralizing antibody titer ≤18) and seropositive ($n = 5$, Ad5 neutralizing antibody titer > 18) subjects receiving three doses $3x10^{10}$ vp Mrk Ad5 gag/pol/nef at weeks 0, 4 and 26 ($n = $ five) as part of the Merck phase I 016 trial. Ad5-specific T-cell responses were measured by stimulating PBMCs overnight with whole E1-deleted Ad5 vector. Cells were analyzed on a modified LSR II flow cytometer (BD Immunocytometry Systems). Data was analyzed using FlowJo 8.7.1 (TreeStar). Adenovirus 5 neutralizing antibody titers were measured as previously described[163].

Statistics

Linear mixed effects models were performed to test for group differences over time as well as comparisons between baseline and subsequent time points within each group. Time was considered to be a discrete variable, lessening the power of these tests compared to tests where time is a continuous variable. Spearman correlations were used to test the relationship between Ad5 nAb titers and T-cell functions at baseline. Correlations over the entire time period were computed using partial correlation coefficients controlling for individual subject effects in the repeated measurements. All data was transformed using base e. Analyses was performed using SAS 9.1.

Portions of this work have been submitted for publication: Hutnick NA, Carnathan DG, Dubey S, Cox K, Kierstead L, Makedonas G, Ratcliffe S, Robertson MR, Casimiro D, Ertl HC, Betts MR. "Vaccination with Ad5 vectors expands Ad5-specific CD8+ T cells without altering memory phenotype or functionality" In submission.

Chapter 6: Discussion

Modern vaccinology has had one of the largest impacts on human health with the elimination of smallpox and 100% reduction in the occurrence of several devastating childhood diseases including polio, measles and pertussis in the United States. However, there are still areas of unmet need that result in the suffering and death of millions of people worldwide. Ad-based vaccines are currently being researched as a means of inducing CD8$^+$ CTL to prevent or modify infection. Though insert-specific responses are often analyzed, until recently, little work has been done to characterize Ad-specific immune responses. As highlighted by the STEP trial, it is important to understand vector-specific immunity to ensure a safe, efficacious vaccine. Here, we have presented the most thorough characterization of human Ad-specific T-cell immunity following both natural infection and immunization. We find that Ad-specific T-cells are universally present in humans with an effector and effector memory like phenotype and functionality. Following Ad5-based vaccination both CD4$^+$ and CD8$^+$ Ad-specific T-cells transiently expanded, however, there were no durable changes in polyfunctionality, phenotype or homing marker expression. Ad5-seropositive and Ad5-seronegative subjects had similar baseline and post-vaccination Ad5-specific CD4$^+$ T-cell responses, suggesting Ad5-specific CD4$^+$ T-cells were not the cause of an increased risk of HIV infection in Ad5-seropositive STEP trial subjects.

Adenovirus Immunity from Natural Infection

Ads have been developed as vectors for use in both gene therapy and vaccines. It is known that pre-existing Ad nAbs limit vaccine efficacy in humans, and animal studies suggest that CD8$^+$ T-cells may also play a role. Seroprevalence has been used as a surrogate for pre-existing T-cell immunity, however, recent studies suggest that this may not be an accurate assumption. Only 40% of US adults are seropositive to Ad5 yet studies have found T-cell responses to Ad5 to be almost universal [59,121]. To clarify Ad-specific T-cell immunity we sought to further define the prevalence, functionality, phenotype and cross-reactivity of pre-existing Ad-specific T-cells in naturally infected humans.

We studied Ad-specific T-cell responses in 17 healthy subjects by flow cytometry and found both CD4$^+$ and CD8$^+$ T-cell responses to replication-defective Ad5 vector in all subjects. This result is critical for vaccine design as seroprevalence cannot be used as a measure for pre-existing T-cell immunity. The universality of Ad5-specific T-cells despite a seroprevalence of only 40% suggests that Ad-specific T-cells are highly cross-reactive. To measure cross-reactivity we stimulated PBMCs from the same 17 subjects with chimpanzee vectors (AdC) which have an exceptionally low seroprevalence in the United States [91]. One weakness of this study is the absence of nAb titers. Because PBMCs were obtained from aphaeresis, there was no plasma to determine nAb titers to Ad5, AdC6, and AdC7. It is unlikely that subjects had been

exposed to AdC6 or AdC7, yet 16 of 17 subjects had both CD4+ and CD8+ T-cells that responded to both AdC. Sequence homology within conserved regions of the major capsid protein, hexon, is over 90% between Ad5, AdC6 and AdC7, and it is likely that T-cell epitopes are conserved between diverse Ad serotypes [176]. Indeed, we found that Ad reactive T-cells recognized epitopes within both the conserved and variable regions of Ad5, AdC6 and AdC7. The cross-reactivity of Ad-specific T-cells makes it difficult to define a truly Ad5-specific T-cell response.

The cross-reactivity of Ad-specific T-cells may have important implications for Ad vector vaccines. To avoid the limitations of pre-existing immunity as measured by nAb, many researchers have developed vaccines based on rare serotypes with low seropositivity. However, these vectors will not avoid a reduction in vaccine efficacy by universally present Ad-specific CD8+ T-cells that may eliminate vector-transduced cells due to an at least 90% homology between human serotypes. Likewise, using heterologous vectors in a prime boost schedule will also not avoid pre-existing Ad-reactive T-cells. The impact on efficacy from pre-existing Ad-specific CTLs in humans is not known, but based on the universality of Ad-reactive T-cells it will be a hurtle that needs to be overcome by all serotype vectors in order for an Ad-based vaccine to succeed in the clinic.

The cross reactivity of Ad-specific T-cells also suggests they may be continually restimulated when an individual is infected with any of the 52

serotypes throughout their life. In addition to constant restimulation by repeat infection, Ad has been shown to persist in the lymphatic tissue and could produce continual antigen presentation and T-cell stimulation [59,71]. Indeed we found that Ad-reactive T-cells display an effector and effector memory-like phenotype and produce predominantly effector functions such as MIP1α and perforin. The effector functionality of Ad-specific CTL may make them efficient at eliminating vector-infected cells and reducing vaccine efficacy compared with other viral platforms.

The effector-like characteristics of Ad-specific T-cells appears to be functionally unique, even among other human viruses. A recent study accessed the functionality of virus specific CD8^{+} T-cells to Epstein-Barr virus (EBV), cytomegalovirus (CMV), Ad, and influenza (Flu) [166]. Both CMV and EBV establish latency whereas Flu is an acute infection. Approximately 75% of Ad-specific CD8^{+} T-cells expressed perforin whereas only 25% of CMV - specific and less than 5% of EBV- and Flu-specific CD8^{+} T-cells expressed perforin [166]. In contrast to the results for perforin, IL-2 production was high in EBV- and Flu-specific CD8^{+} T-cells, low in CMV-specific CD8^{+} T-cells and virtually undetectable in Ad-specific CD8^{+} T-cells. Perforin positive CD8^{+} T-cells had a higher level of the T-cell transcription factor, T-bet. A large portion of Ad-specific T-cells express perforin, therefore it is possible Ad-specific T-cell also express a high level of T-bet. However, the transcriptional profile of human Ad-specific T-cells has yet to be assessed.

Though Ad infection is considered acute clinically, the continual presentation of antigen may make it immunologically more similar to a chronic non-latent infection such as HIV or HCV. One hallmark of chronic infection is the eventual exhaustion of T-cells from continual antigen stimulation. T-cell exhaustion can be measured by the expression of surface inhibitory receptors such as PD-1, CTLA-4 and Lag3 [152]. Exhausted T-cells undergo a hierarchical loss of functions such as IL-2 and TNFα with IFN-γ being the last function lost [177,178]. The effector-like functionality with perforin and MIP1α production in our cohort is not consistent with an exhausted phenotype. We also observed Ad-specific CD4$^+$ and CD8$^+$ T-cells were capable of proliferating upon restimulation. Following the 5-day Ad stimulation CFSE low cells that had undergone proliferation had a central memory-like CD27$^+$CD45RO$^+$ phenotype. Together, these data suggest that although Ad antigens may be continually present, it does not appear that Ad-specific T-cells undergo functional exhaustion. However, the expression of surface receptors associated with exhaustion has not been performed.

Effect of Ad vector vaccination on Ad-specific CD4$^+$ T-cells

The Merck Ad5 HIV-1 vaccine trial showed a possible increased risk of infection in baseline Ad5-seropositive subjects. One hypothesis to explain this result was Ad5-seropositive subjects would have higher levels of baseline Ad5-specific CD4$^+$ memory T-cells. Upon vaccination, these cells would

become activated, proliferate, and up regulate expression of the HIV-1 entry co-receptor CCR5. It is also proposed that activated Ad5-specific memory CD4^{+} T-cells may traffic to the initial site of Ad infection in mucosal tissues such as the gut, which is also a preliminary site for HIV-1 replication. Therefore, vaccinating an Ad5-seropositive subject would result in the enrichment of CD4^{+} T-cells that are optimal targets for HIV-1 infection.

Our study of Ad5-specific CD4^{+} T-cells in subjects from the phase I STEP precursor study indicates that the pre-existing Ad5-specific CD4^{+} T-cell hypothesis does not explain the STEP trial results. First, prior to vaccination Ad5-seropositive and Ad5-seronegative subjects had similar levels of Ad5-specific CD4^{+} T-cells. Therefore, pre-existing CD4^{+} T-cells were not present in Ad5-seropositive subjects only. Similar results were observed by other groups [121,179,180] with only one study suggesting a correlation between Ad-specific T-cell responses and Ad5 nAb [181]. Second, following vaccination, Ad5-specific CD4^{+} T-cells were transiently expanded to an equal degree in both Ad5-seropositive and Ad5-seronegative subjects. This suggests Ad5-seropositive subjects do not generate more Ad5-specific CD4^{+} T-cell following vaccination. Additionally, the expansion in both groups was only transient and the increased infection rate in Ad5-seropositive subjects occurred up to 52 weeks following vaccination.

In addition to there being no difference in the presence or expansion of Ad5-specific CD4^{+} T-cells between Ad5-seronegative and Ad5-seropositive

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subjects, we also saw no increase or difference between serogroups in the activation of Ad5-specific CD4$^+$ T-cells as measured by Ki67. One shortcoming of our study was not measuring the expression level of CCR5 on Ad5-specific CD4$^+$ T-cells. Whether activated Ad5-specific CD4$^+$ T-cells express more CCR5 and are more susceptible to HIV-1 infection has recently been debated. In STEP study participants there was no difference in the level of CCR5 expression in HIV-1 infected cases and non-cases [82]. Also, the level of CCR5 expression in other studies was found to be similar between baseline Ad5-seropositive and Ad5-seronegative subjects and did not increase with vaccination [121,182]. However, two opposing studies suggest that culturing Ad5-specific CD4$^+$ T-cells with Ad5 vector-infected dendritic cells resulted in proliferation, CCR5 upregulation, and increased HIV-1 infection [181,183]. It is possible the differences in results between the various studies were due to the use of a short-term versus long-term stimulation of PBMCs. We are planning on further studying the effect of the two stimulation assays on activation and homing marker expression.

One caveat to our findings is that all responses were measured only in T-cells from the blood. It is hypothesized that Ad5-specific CD4$^+$ memory T-cells may traffic to the gut mucosal upon activation by vaccination. Currently, there have been no reported gut biopsies performed on Ad5 vaccinated humans however studies are planned by the HIV vaccine trials network (HVTN). As a surrogate for measuring the magnitude and phenotype of Ad5

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specific CD4$^+$ T cells in the gut mucosa, we measured the expression of gut homing markers on Ad5-specific peripheral blood T-cells. The $\alpha_4\beta_7$ integrin heterodimer is expressed on T-cells and binds to MAdCAM-1 on venule epithelial cells allowing T-cells to migrate into gut mucosal tissue. The $\alpha_4\beta_7$ heterodimer may also be a co-receptor for HIV-1 entry, therefore an increase in heterodimer receptor expression on Ad5-specific CD4$^+$ T-cells could also make them more susceptible to HIV-1 infection [184,185]. It is unlikely this hypothesis is correct as T-cells appear to down regular $\alpha_4\beta_7$ upon entering gut mucosal tissue. Preliminary data of gut biopsies from non-vaccinated humans shows little total or Ad5-specific $\alpha_4\beta_7$ expression on CD4$^+$ T-cells.

We observed no increase in the expression of the mucosal homing integrins α_4 and β_7 on peripheral blood CD4$^+$ T-cells following vaccination in either serogroup. However, recent studies have seen contradictory results [181,183]. The study by Benlahrech *et al.* found that memory CD4$^+$ T-cells from Ad5-seropositive subjects cultured for 5 days with Ad5 pulsed dendritic cells proliferated and upregulated the expression of CCR5 and $\alpha_4\beta_7$. The stimulation method used and functionality results differed from our study and may account for the differences in $\alpha_4\beta_7$ expression observed. There were also a number of caveats that may impact the interpretation of data. First, the study only included normal subjects and did not examine whether post vaccination responses differed from baseline or were altered in baseline Ad5-seronegative subjects after the first dose when they became Ad5-

seropositive. Second, T-cells stimulated with tetanus toxoid as a control had a similar level of proliferation and $\alpha_4\beta_7$ upregulation as Ad5 stimulated CD4$^+$ T-cells. The correlation for tetanus toxoid-specific CD4$^+$ T-cell responses and tetanus toxoid nAb titer was not shown and would be a more appropriate control to determine if Ad5-specific CD4$^+$ T-cell responses are unique in Ad5-seropositive subjects or if the responses observed are simply a characteristic of a virus-specific memory T-cell response.

The study by Chakupurakal *et al.* also saw an increase in $\alpha_4\beta_7$ when CD4$^+$ T-cell were cultured with replication defective E1- and E3- deleted Ad5 vector for 7 days. The group observed an approximately 40% increase in the percentage of CD4$^+$ $\alpha_4\beta_7^+$ T-cells compared with our results. This is likely a result of the extended *in vitro* assay but could also suggest a large degree of variability in study populations, or a difference in cytometry procedures. We have plans to perform a similar assay on Merck phase I vaccinated subjects to determine how $\alpha_4\beta_7$ differs in a 6 hour Ad stimulation compared with a 6 day stimulation and the effect vaccination has on $\alpha_4\beta_7$ expression.

T-cell trafficking in humans is not well understood but is important for the field of vaccinology. We measured gut mucosal homing markers in the blood as a surrogate for the gut population of Ad5-specific CD4$^+$ T-cells. However, the origin and fate of Ad-specific CD4$^+$ T-cells in the blood is unclear. 90% of all CD4$^+$ T-cells reside in the gut mucosa. It is unclear whether cells in the blood expressing gut homing markers are recently

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activated naïve cells trafficking to the mucosa where they will stay, or whether effector and memory cells in the mucosa can traffic between the gut and the blood. Another question is what induce a naïve cell to express $\alpha_4\beta_7$ and traffic into the gut, and does stimulating naïve Ad-specific CD4$^+$ T-cells result in the same homing patterns as stimulating effector or memory Ad-specific CD4$^+$ T-cells? Being able to manipulate the homing properties of antigen specific T-cells would increase the efficacy of vaccines designed to prevent mucosal infections such as HIV. Studying T-cell responses to replicating Ad infection in animal models may help us to better understand what shapes the natural T-cell response to Ad and how Ad-based vaccines alter this immunity.

It has been two years since the STEP trial was halted and the debate over whether Ad5 vaccination increased the risk of HIV-1 infection in Ad5-seropositive subjects continues. Our results suggest that Ad5-specific CD4$^+$ T-cells were not the cause of an increased risk. However, it is possible that there are differences in the magnitude or activation of Ad5-specific CD4$^+$ T-cells in the mucosal tissue of Ad5-seropositive vs. Ad5-seronegative subjects that were not observed in the blood. Indeed, Ad-specific CD4$^+$ T-cells have been readily detected in the gut mucosal tissue of healthy adults [59].

Another limitation of our study is the timeline of subject sampling. One weakness of analyzing STEP trial samples was the absence of baseline PBMCs collected prior to vaccination. Though our study consisted of extensive sampling every four weeks after vaccination as well as long-term

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follow-up, it is possible that Ad5-reactive CD4$^+$ T-cells were activated or expanded in seropositive subjects following vaccination but this was not observed by 4 weeks after vaccination. For example, Ki67 is expressed when a cell enters the cell cycle but not when a cell is in a resting state [186]. Another possibility is that there was an increase in activated cells, but by 4 weeks after vaccination they had contracted to baseline levels or left the blood to enter the lymphatic system or peripheral tissues. Following vaccination with the yellow fever 17D virus or vaccinia virus, the magnitude of activated (Ki67$^+$, Bcl-2^{low}; CD38$^+$, HLA-DR$^+$) CD8$^+$ T-cells peaked at day 15 and had returned to baseline by day 30 [187].

One debate in the area of HIV vaccines is what is the best animal model to replicate human infection. The same question needs to be asked for Ad vaccine candidates. Pre-exposing mice or monkeys to a replication-defective Ad vector does not mimic pre-existing immunity to natural infection in humans where there is repeat exposure to multiple Ad serotypes over the lifetime of an individual and persistence in lymphatic tissue. Simian Ad have been identified, and Rhesus macaques infected with replication competent simian Ad may provide an effective model for human pre-existing immunity [188]. However, the persistence of Rhesus Ad will need to be studied as well as the phenotype and functionality of the T-cell response. If the natural Ad-specific T-cell response resembles humans, Rhesus macaques infected with Rhesus Ad may provide a good model to study T-cell activation and trafficking

following Ad vaccination and Ad vaccine efficacy. An accurate animal model may also clarify the debate about whether Ad-specific CD4$^+$ T-cells were responsible for the increased susceptibility observed in the STEP trial.

Effect of Vaccination on Ad-specific CTL

Both Ad-specific nAb and CTL may limit vaccine efficacy by reducing vector-transduced cells before the insert gene is expressed. We found that Ad5-specific CD8$^+$ T-cells were detectable at baseline in 38 of 40 subjects despite an Ad5 seroprevalence of only 40% in the US. This CD8$^+$ T-cell prevalence is similar to that detected for Ad5-specific CD4$^+$ T-cells, and is likely due to cross-reactivity between multiple serotypes. Also similar to Ad5-specific CD4$^+$ T-cells, Ad5-specific CD8$^+$ T-cells were effector-like even at baseline. Periodic restimulation by infection with multiple serotypes may be the reason why Ad5-specific CD8$^+$ T-cells are maintained in an effector state.

Vector induced expansion of Ad5-specific CD8$^+$ T-cells occurred following the first vaccine dose in Ad5-seropositive subjects and the third vaccine dose in Ad5-seronegative subjects. The reason behind this differential CD8$^+$ T-cell expansion between the serogroups is unclear, but may have been caused by Ad5-specific nAb. Ad5 nAb levels increased in baseline Ad5-seronegative and Ad5-seropositive subjects following vaccination but remained higher in Ad5-seropositive subjects throughout the study. Ad5 nAb binding to vector could facilitate vector uptake by antigen-

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presenting cells, cross presentation and expansion of Ad-specific CD8^{+} T cells [189]. Alternatively, late responses in Ad5-seropositive subjects could have been dampened by high nAb titers binding to vector and preventing infection, thereby reducing the effective vector dose and CD8^{+} T-cell response.

The high percentage of perforin expressing Ad5-specific effector CD8^{+} T-cells should efficiently eliminate vector-transduced cells as perforin expressing CD8^{+} T-cells have been shown to effectively lyse target cells [190]. Studies in mice have shown transgene expression decreases by 98% 20 days after vaccination in immune competent mice but persists for over 110 days at post-vaccination levels in immune compromised nude mice. This data suggests the reduction in transgene expression is likely due to immune destruction of transduced cells [191]. The destruction of transduced cells and limited insert expression may result in the need for booster vaccinations to maintain effective lifelong immunity.

Repeat homologous vaccination resulted in the expansion of Ad-specific CD8^{+} T-cells in both Ad5-serogroups, however, we observed no changes in the polyfunctionality of Ad5-specific CD8^{+} T-cells compared to baseline in either serogroup. This is a classic example of original antigenic sin, where the quality of the response induced by the primary antigen exposure is not altered by subsequent antigen exposure, even if the antigen is slightly altered as may occur by infection with a heterologous serotype. Our

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findings have important implication for the field of therapeutic vaccination. The goal of a therapeutic vaccine is to boost anti-viral immunity after infection to control viremia and disease progression. Though therapeutic vaccines for HIV have succeeded in boosting anti-HIV CD8^{+} T-cell responses, they have been ineffective at controlling viremia [192]. Without therapy, HIV-specific CD8^{+} T-cell responses are unable to control and clear the virus following natural infection. Our data suggests the ineffective naturally induced HIV-specific CD8^{+} response would be expanded by therapeutic vaccination with HIV antigens but the ineffective functionality would not be altered. In order for therapeutic vaccination to be effective, we need to understand what an effective HIV-specific CD8^{+} T-cell response is and how we can modify the naturally induced response to one that is capable of controlling or clearing virus. Unpublished work from our lab suggests a CD8^{+} HIV-specific response with the ability to upregulate perforin may be protective. Further work is being performed to identify transcription factors associated with perforin production and may provide insights into how the immune system could be manipulated to produce and effective anti-HIV CD8^{+} T-cell response.

Future directions

This work was initiated as part of a larger project to develop rare serotype Ad vaccines to prevent HIV infection. By studying human Ad-specific T-cell responses this work has provided some important insights and

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questions about the efficacy and outcome of Ad vaccines. Our results have also raise some critical questions for further research.

By studying Ad-specific T-cell immunity to human and chimpanzee serotypes we confirmed that Ad-specific T-cells are highly pervasive and cross-reactive. One limitation of our study is the assumption that subjects were seronegative to the chimpanzee vectors based on studies showing low seropositivity in healthy US adults. To truly demonstrate cross-reactivity future studies will perform the same studies outlined in Chapter 3 using subjects from South Africa, Uganda and Botswana. Serum from these subjects will be available so the nAb titer to AdC6, AdC7 and Ad5 can be measured in addition to T-cell responses.

Studying Ad-specific immunity in African subjects will also help to define the utility of rare serotype vaccines. Though an Ad serotype may be rare in developed nations, prevalence is often high in third world countries where water, sewage, and sanitation systems are not as consistent, allowing for the spread of Ad. There have been few studies demonstrating pre-existing T-cells to rare Ad serotypes. Future studies will be examining the T-cell response and nAb levels to one rare human serotype in development, Ad26, in both US and African subjects [193]. Preliminary results suggest the magnitude and prevalence of Ad26-reactive T-cells is similar to Ad5-reactive T-cell in the US. This study will help define the utility of Ad26 as a vector compared with the extensively tested Ad5.

Another important question is whether vector-specific T-cell responses influence insert-specific response. The effector-like qualities of Ad-specific CD8^{+} T-cells would be desirable as insert-specific responses for a T-cell vaccine. CMV infection is similar to Ad as both viruses persist and are known to induce potent effector responses in humans [166]. Recent work has shown induction of effector memory cells and protection from SIV infection in CMV vaccinated monkeys [194], however the risks for a CMV vector are higher than other viral vectors, limiting their clinical development. Therefore, being able to induce effector like immunity through adjuvants and cytokines to alter the functionality of T-cell responses in other viral vectors may be critical to the development of an effective CTL vaccine.

Future studies are also planned to examine the transcriptional profile of Ad-specific CD8^{+} T-cells. By understanding the molecular mechanism behind effector CTL development and perforin production we may be better prepared to design successful vaccines capable of eliciting insert specific CTL. Studies in our lab suggest the transcription factor T-bet is expressed at a higher level in perforin producing CTL. By understanding what induces T-bet expression we may be able to increase perforin production in activated T-cells and viral elimination.

Large Ad specific effector CD8^{+} T-cell responses may alternatively result in a diminished CD8^{+} T-cell response to the insert. The average response to HIV insert proteins in the STEP trial was 0.4-1.0% whereas the

average response to Ad in our studies following vaccination was 2.7% and as high as 8% [180]. There are a number of reasons why this may occur. The response to Ad may be larger because Ad transfected cells are eliminated before the insert is expressed, cells are transduced and survive but expression of the insert is low, or Ad epitopes more efficiently bind MHC class I, thereby skewing the immune response towards Ad. A possible strategy for reducing Ad vector immunity would be to delete more of the vector genome to reduce wild type Ad gene expression. Another method would be to improve the efficacy of transgene expression so a lower dose of Ad-vector could be delivered, thereby reducing Ad capsid protein levels.

To address whether insert-specific responses positively or negatively correlate with vector-specific responses, we plan on comparing the phenotype and functionality of Ad-specific and HIV-specific T-cell responses in Merck phase 1 016 trial participants. We have obtained PBMCs from over 50 subjects with a range of Ad5 nAb titers allowing for the comparison of how nAbs and Ad-specific CD8$^+$ T-cells may affect insert specific responses. This would be a novel study in humans as vector- specific immunity including nAbs and T-cells before and after vaccination has not be extensively studied.

In addition to increasing efficacy of Ad-based vaccines, a major concern following the STEP trial is whether Ad-based vaccines can be safely used in high-risk HIV populations. To further address whether Ad-specific T-cells may have been responsible for the increased risk of infection observed

in Ad5-seropositive STEP trial participants we are planning an extensive analysis of phase I Merck Ad5 trial participants. In addition to examining $\alpha_4\beta_7$ expression, future studies will measure the gut associated homing receptors CD161, and CCR10 to better define the trafficking and phenotype of Ad-specific CD4$^+$ T-cells before and after Ad5 vaccination.

This thesis comprises the largest body of work to date studying human Ad-specific T-cell responses following natural infection and vaccination. I have shown that Ad-specific T-cells are prevalent, effector-like, and cross-reactive. Vaccination only transiently expanded Ad-specific T-cells irrespective of Ad5-serostatus and was unlikely to have been the cause of the increased risk of HIV infection in seropositive STEP trial participants. These studies have provided us with a good understanding of Ad-specific T-cell responses, as well as highlighted areas of further study where we may be able to improve Ad-based vaccine efficacy and safety.

Appendix

peptide number	sequence	pool	peptide number	sequence	pool	peptide number	sequence	pool
1	MATPSMMPQWSYMHI	C1	73	LMGQQSMPNRPN'	C2	145	TNDQSFNDYLSAANM	C3
2	MMPQWSYMHISGQDA	C1	74	SMPNRPNYIAFRD	C2	146	FNDYLSAANMLYPI	C3
3	SYMHISGQDASEYL	C1	75	PNYIAFRDNFIGLM	C2	147	LSAANMLYPIPANA	C3
4	ISGQDASEYLSPGLV	C1	76	FRDNFIGLMYYNS'	C2	148	NMLYPIPANATNVPI	C3
5	ASEYLSPGLVQFARA	C1	77	LMYYNSTGNMGVL	C2	149	IPANATNVPISIPSR	C3
6	SPGLVQFARATETYF	C1	78	NSTGNMGVLAGQ/	C2	150	TNVPISIPSRNWAAF	C3
7	VQFARATETYFSL	C1	79	GNMGVLAGQASQ	C2	151	SIPSRNWAAFRGWAF	C3
8	ARATETYFSLNNKFR	C1	80	LAGQASQLNAVVD	C2	152	NWAAFRGWAFTRLK	C3
9	TYFSLNNKFRNPTVA	C1	81	ASQLNAVVDLQDR	C2	153	FRGWAFTRLKTK	C3
10	NNKFRNPTVAPTHDV	C1	82	LNAVVDLQDRNTE	C2	154	GWAFTRLKTKETPSL	C3
11	NPTVAPTHDVTTDR	C1	83	VDLQDRNTELSYQ	C2	155	RLKTKETPSLGSGY	C3
12	APTHDVTTDRSQRL	C1	84	RNTELSYQLLLDSI	C2	156	KETPSLGSGYDPYY	C3
13	DVTTDRSQRLTLRFI	C1	85	LSYQLLLDSIGDRT	C2	157	SLGSGYDPYYTY	C3
14	RSQRLTLRFIPVDR	C1	86	LLDSIGDRTRYFSN	C2	158	GSGYDPYYTYSGSI	C3
15	LTLRFIPVDREDTAY	C1	87	GDRTRYFSMWNQ	C2	159	DPYYTYSGSIPYL	C3
16	IPVDREDTAYSYKAR	C1	88	RYFSMWNQAVDS'	C2	160	YTYSGSIPYLDGTFY	C3
17	EDTAYSYKARFTLAV (15)	C1	89	SMWNQAVDSYDP	C2	161	SIPYLDGTFYLNHTF	C3
18	SYKARFTLAVGDNRV (15)	C1	90	AVDSYDPDVRIIEN	C2	162	DGTFYLNHTFKKVAI	C3
19	FTLAVGDNRVLDMA (14)	C1	91	DPDVRIIENHGTEI	C2	163	LNHTFKKVAITF	C3
20	VGDNRVLDMASTYF (14)	C1	92	IENHGTEDELPNY(	C2	164	HTFKKVAITFDSSV	C3
21	RVLDMASTYFDIRGV (15)	C1	93	TEDELPNYCFPLG(	C2	165	KVAITFDSSVSW	C3
22	ASTYFDIRGVLDR (13)	C1	94	PNYCFPLGGVINTE	C2	166	AITFDSSVSWPGNDR	C3
23	YFDIRGVLDRGPTFK (15)	C1	95	LGGVINTETLTKVK	V	167	SSVSWPGNDRLL	C3
24	GVLDRGPTFKPY (12)	C1	96	INTETLTKVKPK	V	168	VSWPGNDRLLTPNEF	C3
25	LDRGPTFKPYSGTAY (15)	C1	97	TETLTKVKPKTGQE	V	169	NDRLLTPNEFEIKR	C3
26	TFKPYSGTAYNALA (14)	C1	98	KPKTGQENGWEKI	V	170	LTPNEFEIKRSV	C4
27	YSGTAYNALAPKGA (14)	C1	99	GQENGWEKDATEI	V	171	PNEFEIKRSVDGEGY	C4
28	AYNALAPKGAPNPCEW (16)	C1	100	NGWEKDATEFSDI	V	172	IKRSVDGEGYNVA	C4
29	PKGAPNPCEWDEAA (14)	C1	101	EKDATEFSDKNEIF	V	173	SVDGEGYNVAQCNM	C4
30	PNPCEWDEAATALEI (15)	V	102	EFSDKNEIRVGNN	V	174	EGYNVAQCNMTKDWF	C4
31	WDEAATALEINL (12)	V	103	NEIRVGNNFAMEII	C2	175	AQCNMTKDWFLVQML	C4
32	EAATALEINLEEEDD (15)	V	104	GNNFAMEINLNAN	C2	176	TKDWFLVQMLANYNI	C4
33	LEINLEEEDDDNEDEV (16)	V	105	MEINLNANLWRNF	C2	177	LVQMLANYNIGYQGF	C4
34	EEDDDNEDEVDEQA (14)	V	106	NANLWRNFLYSNI/	C2	178	ANYNIGYQGFYI	C4
35	DNEDEVDEQAEQQK	V	107	RNFLYSNIALYLPDI	C2	179	YNIGYQGFYIPESYK	C4
36	EVDEQAEQQKTHVF	V	108	SNIALYLPDKLKY	C2	180	YQGFYIPESYKDRMY	C4
37	EQAEQQKTHVFGQA	V	109	ALYLPDKLKYSPSN	C2	181	IPESYKDRMYSFFR	C4
38	EQQKTHVFGQAPY	V	110	DKLKYSPSNVKI	C2	182	YKDRMYSFFRNFQPM	C4
39	KTHVFGQAPYSGINI	V	111	LKYSPSNVKISDNI	C2	183	YSFFRNFQPMSRQVV	C4
40	GQAPYSGINITKEGI	V	112	VKISDNPNTYDYM	C2	184	NFQPMSRQVVDDTKY	C4
41	SGINITKEGIQIGV	V	113	NPNTYDYMNKRVV	C2	185	SRQVVDDTKYKDY	C4
42	ITKEGIQIGVEGQTPK	V	114	YDYMNKRVVAPGL	C2	186	VVDDTKYKDYQQVGI	C4
43	IQIGVEGQTPKYADK	V	115	NKRVVAPGLVDCY	C2	187	KYKDYQQVGILHQH	C4
44	EGQTPKYADKTF	V	116	VAPGLVDCYINLG/	C2	188	YQQVGILHQHNNSGF	C4
45	GQTPKYADKTFQPEPQI	C1	117	VDCYINLGARWSL	C2	189	ILHQHNNSGFVGYLA	C4
46	DKTFQPEPQIGESQW	C1	118	NLGARWSLDYMDI	C2	190	NNSGFVGYLAPTMR	C4
47	PEPQIGESQWYETEI	C1	119	RWSLDYMDNVNPI	C2	191	FVGYLAPTMREGQAY	C4
48	GESQWYETEINHAA	V	120	YMDNVNPFNHHRI	V	192	APTMREGQAYPANF	C4
49	WYETEINHAAGRVLK	V	121	VNPFNHHRNAGLR	C3	193	REGQAYPANFPYPLI	C4
50	INHAAGRVLKKTTPM	C1	122	HHRNAGLRYRSML	C3	194	YPANFPYPLIGKTAV	C4
51	GRVLKKTTPMKPCY	C1	123	AGLRYRSMLLGNG	C3	195	PYPLIGKTAVDSI	C4
52	KKTTPMKPCYGSYAK	C1	124	RSMLLGNGRYVPF	C3	196	LIGKTAVDSITQKKF	C4
53	MKPCYGSYAKPTNEN	C1	125	GNGRYVPFHIQVP	C3	197	AVDSITQKKFLCDR	C4
54	GSYAKPTNENGGQAI	C1	126	VPFHIQVPQKFFAI	C3	198	ITQKKFLCDRTLWRI	C4
55	PTNENGGQGILVK	C1	127	IQVPQKFFAIKNLL	C3	199	FLCDRTLWRIPF	C4
56	ENGGQGILVKQQNGK	V	128	KFFAIKNLLLLPGS'	C3	200	CDRTLWRIPFSSNFM	C4
57	GILVKQQNGKLESQV	V	129	KNLLLLPGSYTYEW	C3	201	WRIPFSSNFMSMGAL	C4
58	KQQNGKLESQVEMQF	V	130	LLPGSYTYEWNFRI	C3	202	SSNFMSMGALTDL	C4
59	KLESQVEMQFFSTTEA	V	131	SYTYEWNFRKDVN	C3	203	FMSMGALTDLGQNLL	C4
60	EMQFFSTTEATA	V	132	WNFRKDVNMVLQ	C3	204	ALTDLGQNLLYANSA	C4
61	MQFFSTTEATAGNGDNL	V	133	DVNMVLQSSLGNI	C3	205	GQNLLYANSAHALDM	C4
62	EATAGNGDNLTPKVV	V	134	LQSSLGNDLRVDG	C3	206	YANSAHALDMTFEV	C4
63	NGDNLTPKVVLY	V	135	LGNDLRVDGASIK	C3	207	AHALDMTFEVDPM	C4
64	DNLTPKVVLYSEDV	C1	136	LRVDGASIKFDSI(	C3	208	LDMTFEVDPMDEPTL	C4
65	PKVVLYSEDVDI	C1	137	ASIKFDSICLYATFF	C3	209	EVDPMDEPTLLYVLF	C4
66	VVLYSEDVDIETPDTH	C1	138	DSICLYATFFPMAH	C3	210	DEPTLLYVLFEVFDV	C4
67	DVDIETPDTHISYM	C1	139	LYATFFPMAHNTA	C3	211	LYVLFEVFDVVRVHR	C4
68	ETPDTHISYMPTIK	C1	140	TFFPMAHNTASTL	C3	212	EVFDVVRVHRPHRGV	C4
69	THISYMPTIKEGNSR	V	141	AHNTASTLEAMLR	C3	213	VRVHRPHRGVIETVY	C4
70	MPTIKEGNSRELM	V	142	TASTLEAMLRNDTI	C3	214	PHRGVIETVYLRTPF	C4
71	IKEGNSRELMGQQSM	V	143	EAMLRNDTNDQSI	C3	215	IETVYLRTPFSAGNA	C4
72	SRELMGQQSMPNR	C2	144	LRNDTNDQSFNDY	C3	216	LRTPFSAGNATT	C4

Table 4: Ad5 hexon peptide pools.

peptide number	sequence	pool	peptide number	sequence	pool	peptide number	sequence	pool
1	MATPSMLPQWAYMHI	C1	73	NYIGFRDNFIGLMYY	C1	145	TNDQSFNDYLSAANM	C3
2	MLPQWAYMHIAGQDA	C1	74	RDNFIGLMYYNSTGNM	C1	146	FNDYLSAANMLYPI	C3
3	AYMHIAGQDASEYL	C1	75	LMYYNSTGNMGVLA	C1	147	LSAANMLYPIPANA	C3
4	IAGQDASEYLSPGLV	C1	76	NSTGNMGVLAGQA	C2	148	NMLYPIPANATNVPI	C3
5	ASEYLSPGLVQFARA	C1	77	GNMGVLAGQASQLNA	C2	149	IPANATNVPISIPSR	C3
6	SPGLVQFARATDTYF	C1	78	LAGQASQLNAVVDL	C2	150	TNVPISIPSRNWAAF	C3
7	VQFARATDTYFSL	C1	79	ASQLNAVVDLQDR	C2	151	SIPSRNWAAFRGWSF	C3
8	ARATDTYFSLGNKFR	C1	80	LNAVVDLQDRNTEL	C2	152	NWAAFRGWSFTRLK	C3
9	TYFSLGNKFRNPTVA	C1	81	VDLQDRNTELSYQLL	C2	153	FRGWSFTRLKTR	C3
10	GNKFRNPTVAPTHDV	C1	82	RNTELSYQLLLDSL	C2	154	GWSFTRLKTRETPSL	C3
11	NPTVAPTHDVTTDR	C1	83	LSYQLLLDSLGDRTR	C2	155	RLKTRETPSLGSGF	C3
12	APTHDVTTDRSQRL	C1	84	LLDSLGDRTRYFSMW	C2	156	RETPSLGSGFDPYFV	C3
13	DVTTDRSQRLTLRFV	C1	85	GDRTRYFSMWNQAV	C2	157	LGSGFDPYFVYSGSI	C3
14	RSQRLTLRFVPVDR	C1	86	RYFSMWNQAVDSY	C2	158	DPYFVYSGSIPYL	C3
15	LTLRFVPVDREDNTY	C1	87	SMWNQAVDSYDPDVR	C2	159	FVYSGSIPYLDGTFY	C3
16	VPVDREDNTYSYKVR	C1	88	AVDSYDPDVRIIENH	C2	160	SIPYLDGTFYLNHTF	C3
17	EDNTYSYKVRYTLAV	C1	89	DPDVRIIENHGV	C2	161	DGTFYLNHTFKKVSI	C3
18	SYKVRYTLAVGDNRV	C1	90	DVRIIENHGVEDEL	C2	162	LNHTFKKVSITF	C3
19	YTLAVGDNRVLDMA	C1	91	IENHGVEDELPNYCF	C2	163	HTFKKVSITFDSSV	C3
20	VGDNRVLDMASTYF	C1	92	VEDELPNYCFPL	C2	164	KVSITFDSSVSW	C3
21	RVLDMASTYFDIRGV	C1	93	DELPNYCFPLDGSGTNA	C2	165	SITFDSSVSWPGNDR	C3
22	ASTYFDIRGVLDR	C1	94	FPLDGSGTNAAYQGV	V	166	SSVSWPGNDRLL	C3
23	YFDIRGVLDRGPSFK	C1	95	SGTNAAYQGVKVK	V	167	VSWPGNDRLLTPNEF	C3
24	GVLDRGPSFKPY	C1	96	NAAYQGVKVKDGQDGD	V	168	NDRLLTPNEFEIKR	C3
25	LDRGPSFKPYSGTAY	C1	97	KVKDGQDGDVESEW	V	169	LTPNEFEIKRTV	C3
26	SFKPYSGTAYNSLA	C1	98	GQDGDVESEWENDDTV	V	170	PNEFEIKRTVDGEGY	C3
27	YSGTAYNSLAPKGA	C1	99	ESEWENDDTVAAR	V	171	IKRTVDGEGYNVA	C3
28	AYNSLAPKGAPNSSQW	C1	100	WENDDTVAARNQLCK	V	172	TVDGEGYNVAQCNM	C3
29	PKGAPNSSQWEQAK	V	101	TVAARNQLCKGNIFA	C2	173	EGYNVAQCNMTKDWF	C3
30	PNSSQWEQAKTGNGG	V	102	NQLCKGNIFAMEINL	C2	174	AQCNMTKDWFLVQML	C3
31	EQAKTGNGGTMETH	V	103	GNIFAMEINLQANLW	C2	175	TKDWFLVQMLAHYNI	C3
32	TGNGGTMETHTYGVA	V	104	MEINLQANLWRSFLY	C2	176	LVQMLAHYNIGYQGF	C4
33	TMETHTYGVAPM	V	105	LQANLWRSFLYSNVA	C2	177	AHYNIGYQGFYV	C4
34	ETHTYGVAPMGGENI	V	106	WRSFLYSNVALYL	C2	178	YNIGYQGFYVPEGYK	C4
35	GVAPMGGENITKDGL	V	107	FLYSNVALYLPDSYK	C2	179	YQGFYVPEGYKDRMY	C4
36	GGENITKDGLQI	V	108	VALYLPDSYKYTPTNV	C2	180	VPEGYKDRMYSFFR	C4
37	ENITKDGLQIGTDV	V	109	DSYKYTPTNVTL	C2	181	YKDRMYSFFRNFQPM	C4
38	KDGLQIGTDVTA	V	110	YKYTPTNVTLPTNTNTY	C2	182	YSFFRNFQPMSRQVV	C4
39	GLQIGTDVTANQNK	V	111	VTLPTNTNTYDYM	C2	183	NFQPMSRQVVDEVNY	C4
40	GTDVTANQNKPIYA	V	112	PTNTNTYDYMNGRV	C2	184	SRQVVDEVNYKDYQA	C4
41	TANQNKPIYADKTF	V	113	NTYDYMNGRVTPPSL	C2	185	DEVNYKDYQAVTLAY	C4
42	NKPIYADKTFQPEPQV	C1	114	MNGRVTPPSLVDAYL	C2	186	KDYQAVTLAYQH	C4
43	DKTFQPEPQVGEENW	C1	115	TPPSLVDAYLNIGAR	C2	187	YQAVTLAYQHNNSGF	C4
44	PEPQVGEENWQETENF	V	116	VDAYLNIGARWSL	C2	188	LAYQHNNSGFVGYLA	C4
45	EENWQETENFYGGRA	V	117	YLNIGARWSLDPM	C2	189	NNSGFVGYLAPTMR	C4
46	ETENFYGGRALKK	V	118	IGARWSLDPMDNV	C2	190	FVGYLAPTMRQGQPY	C4
47	NFYGGRALKKDTNMK	C1	119	RWSLDPMDNVNPFNH	C2	191	APTMRQGQPYPANY	C4
48	RALKKDTNMKPCY	C1	120	PMDNVNPFNHHRNA	C2	192	RQGQPYPANYPYPLI	C4
49	KKDTNMKPCYGSYAR	C1	121	VNPFNHHRNAGLRYR	C2	193	YPANYPYPLIGKSAV	C4
50	MKPCYGSYARPTNEK	C1	122	HHRNAGLRYRSMLL	C2	194	PYPLIGKSAVASV	C4
51	GSYARPTNEKGGQAK	C1	123	AGLRYRSMLLGNGRY	C2	195	LIGKSAVASVTQKKF	C4
52	PTNEKGGQAKLKV	V	124	RSMLLGNGRYVPFHI	C2	196	AVASVTQKKFLCDRV	C4
53	EKGGQAKLKVGDDGV	V	125	GNGRYVPFHIQVPQK	C2	197	TQKKFLCDRVMWRI	C4
54	AKLKVGDDGVPTKEF	V	126	VPFHIQVPQKFFAIK	C2	198	FLCDRVMWRIPF	C4
55	GDDGVPTKEFDIDLA	V	127	IQVPQKFFAIKSLLL	C2	199	CDRVMWRIPFSSNFM	C4
56	PTKEFDIDLAFF	V	128	KFFAIKSLLLLPGSY	C2	200	WRIPFSSNFMSMGAL	C4
57	KEFDIDLAFFDTPGGTV	V	129	KSLLLLPGSYTYEW	C2	201	SSNFMSMGALTDL	C4
58	AFFDTPGGTVNGQDEY	V	130	LLPGSYTYEWNFRK	C3	202	FMSMGALTDLGQNML	C4
59	GGTVNGQDEYKADIV	V	131	SYTYEWNFRKDVNMI	C3	203	ALTDLGQNMLYANSA	C4
60	GQDEYKADIVMY	V	132	WNFRKDVNMILQSSL	C3	204	GQNMLYANSAHALDM	C4
61	DEYKADIVMYTENTY	V	133	DVNMILQSSLGNDLR	C3	205	YANSAHALDMNFEV	C4
62	DIVMYTENTYLETPDTH	C1	134	LQSSLGNDLRTDGA	C3	206	AHALDMNFEVDPM	C4
63	NTYLETPDTHVVYK	C1	135	LGNDLRTDGASIAF	C3	207	LDMNFEVDPMDESTL	C4
64	ETPDTHVVYKPGK	C1	136	LRTDGASIAFTSINL	C3	208	EVDPMDESTLLYVVF	C4
65	DTHVVYKPGKDDA	C1	137	ASIAFTSINLYATFF	C3	209	DESTLLYVVFEVFDV	C4
66	VVYKPGKDDASSEI	V	138	TSINLYATFFPMAH	C3	210	LYVVFEVFDVVRVH	C4
67	PGKDDASSEINLV	V	139	LYATFFPMAHNTA	C3	211	FEVFDVVRVHQPHR	C4
68	DDASSEINLVQQSM	V	140	TFFPMAHNTASTLEA	C3	212	DVVRVHQPHRGVIEA	C4
69	SEINLVQQSMPNR	V	141	AHNTASTLEAMLR	C3	213	HQPHRGVIEAVYLR	C4
70	NLVQQSMPNRPNYI	C1	142	TASTLEAMLRNDTND	C3	214	RGVIEAVYLRTPFSA	C4
71	VQQSMPNRPNYIGFR	C1	143	EAMLRNDTNDQSF	C3	215	AVYLRTPFSAGNATT	C4
72	PNRPNYIGFRDNFI	C1	144	LRNDTNDQSFNDYL	C3			

Table 5: AdC6 Hexon Peptide Pools.

peptide number	sequence	pool	peptide number	sequence	pool	peptide number	sequence	pool
1	MLPQWAYMHIAGQDA	C1	72	NSTGNMGVLAGQA	C2	145	NMLYPIPANATNVPI	C3
2	AYMHIAGQDASEYL	C1	73	GNMGVLAGQASQLNA	C2	146	IPANATNVPISIPSR	C3
3	IAGQDASEYLSPGLV	C1	74	LAGQASQLNAVVDL	C2	147	TNVPISIPSRNWAAF	C3
4	ASEYLSPGLVQFARA	C1	75	ASQLNAVVDLQDR	C2	148	SIPSRNWAAFRGWSF	C3
5	SPGLVQFARATDTYF	C1	76	LNAVVDLQDRNTEL	C2	149	NWAAFRGWSFTRLK	C3
6	VQFARATDTYFSL	C1	77	VDLQDRNTELSYQLL	C2	150	FRGWSFTRLKTR	C3
7	ARATDTYFSLGNKFR	C1	78	RNTELSYQLLLDSL	C2	151	GWSFTRLKTRETPSL	C3
8	TYFSLGNKFRNPTVA	C1	79	LSYQLLLDSLGDRTR	C2	152	RLKTRETPSLGSSF	C3
9	GNKFRNPTVAPTHDV	C1	80	LLDSLGDRTRYFSMW	C2	153	RETPSLGSGFDPYFV	C3
10	NPTVAPTHDVTTDR	C1	81	GDRTRYFSMWNQAV	C2	154	LGSGFDPYFVYSGSI	C3
11	APTHDVTTDRSQRL	C1	82	RYFSMWNQAVDSY	C2	155	DPYFVYSGSIPYL	C3
12	DVTTDRSQRLTLRFV	C1	83	SMWNQAVDSYDPDVR	C2	156	FVYSGSIPYLDGTFY	C3
13	RSQRLTLRFVPVDR	C1	84	AVDSYDPDVRIIENH	C2	157	SIPYLDGTFYLNHTF	C3
14	LTLRFVPVDREDNTY	C1	85	DPDVRIIENHGV	C2	158	DGTFYLNHTFKKVSI	C3
15	VPVDREDNTYSYKVR	C1	86	DVRIIENHGVEDEL	C2	159	LNHTFKKVSITF	C3
16	EDNTYSYKVRYTLAV	C1	88	IENHGVEDELPNYCF	C2	160	HTFKKVSITFDSSV	C3
17	SYKVRYTLAVGDNRV	C1	89	VEDELPNYCFPLDAV	C2	161	KVSITFDSSVSW	C3
18	YTLAVGDNRVLDMA	C1	90	PNYCFPLDAVGR	C2	162	SITFDSSVSWPGNDR	C3
19	YTLAVGDNRVLDMA	C1	91	YCFPLDAVGRTDTY	C2	163	SSVSWPGNDRLL	C3
20	RVLDMASTYFDIRGV	C1	92	LDAVGRTDTYQGIKA	V	164	VSWPGNDRLLTPNEF	C3
21	ASTYFDIRGVLDR	C1	93	RTDTYQGIKANGDNQ	V	165	NDRLLTPNEFEIKR	C3
22	YFDIRGVLDRGPSFK	C1	94	YQGIKANGDNQTTW	V	166	LTPNEFEIKRTV	C4
23	GVLDRGPSFKPY	C1	95	NGDNQTTWTKDDTV	V	167	PNEFEIKRTVDGEGY	C4
24	LDRGPSFKPYSGTAY	C1	96	NQTTWTKDDTVNDA	V	168	IKRTVDGEGYNVA	C4
25	SFKPYSGTAYNSLA	C1	97	WTKDDTVNDANELGK	V	169	TVDGEGYNVAQCNM	C4
26	YSGTAYNSLAPKGA	C1	98	TVNDANELGKGNPFA	C2	170	EGYNVAQCNMTKDWF	C4
27	AYNSLAPKGAPNTCQW	C1	99	NELGKGNPFAMEINI	C2	171	AQCNMTKDWFLVQML	C4
28	PKGAPNTCQWTYKA	C1	100	GNPFAMEINIQANLW	C2	172	TKDWFLVQMLAHYNI	C4
29	PNTCQWTYKAGDTDTEK	V	101	MEINIQANLWRNFLY	C2	173	LVQMLAHYNIGYQGF	C4
30	YKAGDTDTEKTYTY	V	102	IQANLWRNFLYANVA	C2	174	AHYNIGYQGFYV	C4
31	DTDTEKTYTYGNAPV	V	103	WRNFLYANVALYL	C2	175	YNIGYQGFYVPEGYK	C4
32	KTYTYGNAPVQGISI	V	104	FLYANVALYLPDSYK	C2	176	YQGFYVPEGYKDRMY	C4
33	GNAPVQGISITKDGI	V	105	VALYLPDSYKYTPA	C2	177	VPEGYKDRMYSFFR	C4
34	VQGISITKDGIQL	V	106	LPDSYKYTPANITL	C2	178	YKDRMYSFFRNFQPM	C4
35	ISITKDGIQLGTDSD	V	107	YKYTPANITLPTNTNTY	C2	179	YSFFRNFQPMSRQVV	C4
36	DGIQLGTDSDGQAIY	V	108	ITLPTNTNTYDYM	C2	180	NFQPMSRQVVDEVNY	C4
37	GTDSDGQAIYADETY	V	109	PTNTNTYDYMNGRVV	C2	181	SRQVVDEVNYKDYQA	C4
38	GQAIYADETYQPEPQV	C1	110	TYDYMNGRVVAPSLV	C2	182	DEVNYKDYQAVTLAY	C4
39	DETYQPEPQVGDAEW	C1	111	NGRVVAPSLVDAYI	C2	183	KDYQAVTLAYQH	C4
40	PEPQVGDAEWHDI	C1	112	VAPSLVDAYINIGAR	C2	184	YQAVTLAYQHNNSGF	C4
41	PQVGDAEWHDITGTDEK	V	113	VDAYINIGARWSL	C2	185	LAYQHNNSGFVGYLA	C4
42	WHDITGTDEKYGGRA	V	114	YINIGARWSLDPM	C2	186	NNSGFVGYLAPTMR	C4
43	GTDEKYGGRALK	C1	115	IGARWSLDPMDNV	C2	187	FVGYLAPTMRQGQPY	C4
44	DEKYGGRALKPDTKM	C1	116	RWSLDPMDNVNPFNH	C2	188	APTMRQGQPYPANY	C4
45	GRALKPDTKMKPCY	C1	117	PMDNVNPFNHHRNA	C2	189	RQGQPYPANYPYPLI	C4
46	KPDTKMKPCYGSFAK	C1	118	VNPFNHHRNAGLRYR	C3	190	YPANYPYPLIGKSAV	C4
47	MKPCYGSFAKPTNK	C1	119	HHRNAGLRYRSMLL	C3	191	PYPLIGKSAVASV	C4
48	YGSFAKPTNKEGGQA	C1	120	AGLRYRSMLLGNGRY	C3	192	LIGKSAVASVTQKKF	C4
49	KPTNKEGGQANVK	C1	121	RSMLLGNGRYVPFHI	C3	193	AVASVTQKKFLCDRV	C4
50	NKEGGQANVKTETGGTK	C1	122	GNGRYVPFHIQVPQK	C3	194	TQKKFLCDRVMWRI	C4
51	NVKTETGGTKEYDI	V	123	VPFHIQVPQKFFAIK	C3	195	FLCDRVMWRIPF	C4
51	NVKTETGGTKEYDI	V	124	IQVPQKFFAIKSLLL	C3	196	CDRVMWRIPFSSNFM	C4
52	ETGGTKEYDIDMAFF	C1	125	KFFAIKSLLLLPGSY	C3	197	WRIPFSSNFMSMGAL	C4
53	KEYDIDMAFFDNRSA	V	126	KSLLLLPGSYTYEW	C3	198	SSNFMSMGALTDL	C4
54	DMAFFDNRSAAAAGL	V	127	LLPGSYTYEWNFRK	C3	199	FMSMGALTDLGQNML	C4
55	DNRSAAAAGLAPEIV	V	128	SYTYEWNFRKDVNMI	C3	200	ALTDLGQNMLYANSA	C4
56	AAAGLAPEIVLY	V	129	WNFRKDVNMILQSSL	C3	201	GQNMLYANSAHALDM	C4
57	AGLAPEIVLYTENV	C1	130	DVNMILQSSLGNDLR	C3	202	YANSAHALDMNFEV	C4
58	PEIVLYTENVDL	C1	131	LQSSLGNDLRTDGA	C3	203	AHALDMNFEVDPM	C4
59	IVLYTENVDLETPDTH	C1	132	LGNDLRTDGASIAF	C3	204	LDMNFEVDPMDESTL	C4
60	NVDLETPDTHIVYKA	C1	133	LRTDGASIAFTSINL	C3	205	EVDPMDESTLLYVVF	C4
61	TPDTHIVYKAGTDDS	C1	134	ASIAFTSINLYATFF	C3	206	DESTLLYVVFEVFDV	C4
62	IVYKAGTDDSSSSI	V	135	TSINLYATFFPMAH	C3	207	LYVVFEVFDVVRVH	C4
63	AGTDDSSSSINL	V	136	LYATFFPMAHNTA	C3	208	FEVFDVVRVHQPHR	C4
64	TDDSSSSINLGQQSM	V	137	TFFPMAHNTASTLEA	C3	209	DVVRVHQPHRGVIEA	C4
65	SSINLGQQSMPNR	C2	138	AHNTASTLEAMLR	C3	210	HQPHRGVIEAVYLR	C4
66	NLGQQSMPNRPNYI	C2	139	TASTLEAMLRNDTND	C3	211	RGVIEAVYLRTPFSA	C4
67	GQQSMPNRPNYIGFR	C2	140	EAMLRNDTNDQSF	C3	212	AVYLRTPFSAGNATT	C4
68	PNRPNYIGFRDNFI	C2	141	LRNDTNDQSFNDYL	C3	214	KANGDNQTTWTK	C2
69	NYIGFRDNFIGLMYY	C2	142	TNDQSFNDYLSAANM	C3			
70	RDNFIGLMYYNSTGNM	C2	143	FNDYLSAANMLYPI	C3			
71	LMYYNSTGNMGVLA	C2	144	LSAANMLYPIPANA	C3			

Table6: AdC7 hexon peptide pools.

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