The immunological impact of adenovirus early genes on vaccine-induced responses in mice and nonhuman primates

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Running Head: HIV-specific immune responses induced by E4-deleted Ad
Abstract

Adenovirus (Ad) is being explored for use in the prevention and treatment of a variety of infectious diseases and cancers. Ad with a deletion in early region 3 (ΔE3) provokes a stronger immune response than Ad with deletions in early regions 1 and E3 (ΔE1/ΔE3). The ΔE1/ΔE3 Ads are more popular because they can carry a larger transgene and because of the deleted E1 (E1A and E1B), are perceived safer for clinical use. Ad with a deletion in E1B55K (ΔE1B55K) has been in phase III clinical trials for use in cancer therapy in the US and has been approved for use in head and neck tumor therapy in China, demonstrating that Ad containing E1A are safe for clinical use. We have shown previously that ΔE1B55K Ad, even while promoting lower levels of an inserted transgene, promoted similar levels of transgene-specific immune responses as a ΔE3 Ad. Products of the Ad early region 4 (E4) limit the ability of cells to mount an innate immune response. Using this knowledge, we deleted the Ad E4 open reading frames 1-4 (E4orf1-4) from the ΔE1B55K Ad. Here, we show that innate cytokine network genes are elevated in the ΔE4 Ad-infected cells beyond that of ΔE3 Ad-infected cells. Further, in immunized mice the IgG2a subclass was favored as was the IgG1 subclass in immunized nonhuman primates. Thus, Ad E4 impacts immune responses in cells, in immunized mice, and immunized nonhuman primates. These Ad may offer advantages that are beneficial for clinical use.

Importance: Adenovirus (Ad) is being explored for use in the prevention and treatment of a variety of infectious diseases and cancers. Here we provide evidence in cells, mice, and nonhuman primates supporting the notion that Ad early gene-products limit specific immune responses. Ad constructed with deletions in early genes and expressing HIV envelope protein was shown to induce greater HIV-specific cellular immune responses and higher titer antibodies.
compared to the parental Ad with the early genes. In addition to eliciting enhanced immunity, the
deleted Ad possesses more space for insertion of additional or larger transgenes needed for
targeting other infectious agents or cancers.

Keywords: Adenovirus, HIV, Vaccine, Nonhuman primates, Early region 1B55K (E1B55K),
Early region 4 (E4), Immune responses, Antibody, Cytokine genes, Cytokine producing cells,
rhFLSC
Introduction

Adenovirus (Ad) is being developed as a component of vaccine strategies for HIV (1-3), influenza (4-6), *Mycobacterium tuberculosis* (7, 8) and many other infectious diseases (9-14) as well as for cancers (15, 16) because of its ability to prime immune responses (2, 17). In spite of this widespread use, the current understanding of the effects of Ad genes on the immune responses induced against the Ad vector and/or the transgene expressed, remain somewhat incomplete.

Ad is a linear double-stranded DNA-containing virus. After the genomic DNA is deposited into the nucleus, transcription of the viral genes begins with the immediate early region 1A (E1A). E1A induces the activation of the other early genes (E1B, E2, E3, and E4) (18). Even without *E1A*, (*E1*-deleted) Ad vectors are able to induce strong immune responses against Ad itself and products of an inserted transgene (19).

The E1 products transform rodent cells (20, 21). Therefore, for safety reasons, in addition to its approximate 5 kilobase (kb) transgene carrying capacity, *E1*-deleted Ad (*ΔE1* Ad) are the most common form of Ad currently being explored for clinical use (22). However, evidence including the fact that Ad with deletion in *E1B55K* (*ΔE1B55K* Ad) have made it to phase I, II and III clinical trials in the US and are used in China for the treatment of head and neck cancer (23), makes the case that E1A-containing Ad are safe for clinical use.

Previously, to expand the use of *ΔE1B55K* Ad, we replaced the Ad *E3* with full-length single-chain HIV*BaL* gp120 linked to rhesus macaque CD4 (rhFLSC) (24-26) and showed that even while producing a lesser amount of the HIV transgene in infected cells, mice immunized with this construct produced similar levels of cytokine producing T-cells and binding antibodies as mice immunized with the *ΔE3* Ad (24).
Products of the Ad early region 4 (E4) are reported to limit the ability of Ad-infected cells to mount an innate immune response (27-29). Based on this knowledge we hypothesized that deleting the E4 genes presented a way to enhance the immune priming ability of the ΔE1B55K Ad while simultaneously expanding the transgene carrying capacity from ~3.8kb to ~5.2kb. For some time we have used the Ad type 5 host-range mutant (Ad5hr) (30) which can replicate in rhesus macaques as a model for development of HIV vaccine strategies (31-33). Here, using this Ad5hr, we deleted the DNA sequences that code for the E1B55K and E4orf1-4 genes and replaced the E3 genes with rhFLSC. For simplicity we call this construct ΔE4 Ad. Cells infected with the ΔE4 Ad expressed higher levels of innate cytokine network genes than cells infected with the ΔE3 Ad. Mice inoculated with the ΔE4 Ad exhibited higher levels of HIV-specific IgG2a binding antibodies than the ΔE3 Ad-immunized mice. What is more, nonhuman primates (NHPs) inoculated with the ΔE4 Ad displayed higher levels of HIV-specific cytokine-producing mucosal T-cells and HIV-specific IgG1 antibodies than ΔE3 Ad-immunized NHPs. Thus, we created Ad vaccine candidates with expanded transgene-carrying capacity and show here in cultured cells, mice, and nonhuman primates, evidence that Ad early genes impact the immune responses generated.

Results

Ad early genes modulate levels of viral late proteins, and viral progeny production: Ad with deletions in E1B55K and the different E4 genes have been previously described (34-36). However, because they lacked a transgene, whether the E4 products play a role in the Ad-induced transgene-specific immune responses has remained somewhat unclear. In Fig 1A we provide schematics of the genomes of the Ads used in this study. The ΔE3 (24, 37, 38) and ΔE1B55K Ad (24) were previously described. In Fig 1 we characterize the
ΔE1B55K/ΔE3/ΔE4orf1-4 Ad (termed ΔE4 from this point on) in A549 cells. Previously described primers (24, 37) were used in PCR experiments to reveal the deletion of the Ad E4orf1, 1-2, 1-3 and E4orf4 genes as well as E1B55K (Fig 1B). At high multiplicity of infection (MOI), the lack of differences in levels of Ad fiber DNA (Fig 1C) and RNA (data not shown) in the ΔE4 Ad-infected cells do not predict the differences in the progeny yield shown in Fig 1D. There, compared to the ΔE3 Ad, the ΔE4 Ad promotes significantly lower amounts of progeny (Fig 1D). No discernable differences in levels of Ad DNA-binding protein (DBP) encoded by Ad early region 2A, were observed at high MOI (Fig. 1E). This was in stark contrast to the diminished levels of Ad late proteins, for example hexon, penton, and fiber, observed over time. In the ΔE4 Ad-infected cells levels of Ad late proteins remained lower than those observed in ΔE3-infected cells even at 72 hours post infection (hpi) (Fig 1E). The rhFLSC (data not shown) and gp120 (Fig 1F) are expressed around the same time as the Ad late proteins as reported for ΔE1B55K Ad (24) and were also diminished in ΔE4 Ad-infected cells compared to ΔE3 Ad-infected cells. Similar results were observed in HeLa, TC1, and CV1 cells (data not shown). We did not measure any later times. However, from these results it appears that levels of viral progeny measured in ΔE4 Ad-infected cells (Fig. 1D) closely parallel the diminished levels of Ad late proteins (Fig 1E) as previously reported (36, 39).

Ad early genes modulate levels and types of cytokines produced during an infection:

Cytokines are an important part of the initial cascade of molecules that communicate the presence of an ongoing infection. The E1B55K (24, 40, 41), and E4 gene-products (27-29, 42) are reported to limit the ability of Ad-infected cells to mount an innate immune response. Yet the effects of the deletion of these genes on levels of innate cytokine genes during an Ad-infection were never cataloged. A comparison of 28 innate cytokine network genes evaluated in A549
cells revealed significant differences in the ability of the two vaccine candidates to control the
levels of interferon beta (IFNB), interleukin 12 (IL-12), interleukin 6 (IL-6), interleukin 1 (IL-1),
and Lymphotoxin-alpha (LTA) (Fig 2A). The bars with values below 0 represent genes that are
suppressed. Of these 13 were from ΔE3 Ad-infections but only 5 from the ΔE4 Ad-infection (Fig
2A) for an overall significant difference of p < 0.0001 (Fig 2B). The bars with values above zero
include the innate cytokine network genes that were induced. Significantly more of these were
from cells infected with the ΔE4 Ad (Fig 2C, p < 0.0003). From Fig 2 it is clear that infections
with the ΔE4 Ad allow for the expression of higher numbers as well as in some cases higher
levels of innate cytokine network genes compared to infections with ΔE3 Ad. These findings
suggest that E1B55K and E4 gene-products may act to suppress cytokine gene expression in Ad-
infected cells.

**Ad early genes modulate levels of transgene-specific antibodies produced in immunized
mice:** Ad5hr vaccine candidates that express rhFLSC promote high levels of HIV-specific
antibodies in mice (24, 37) and NHPs (38). The antibodies elicited by the rhFLSC immunogen
are capable of neutralizing HIV (25, 38). Thus, an enhancement of levels of rhFLSC antibodies
induced by vaccines may prove important. To determine the effects of deleting Ad E1B55K and
E4orf1-4 on antibody induction, mice were inoculated intraperitoneally with either ΔE3 Ad or
the ΔE4 Ad as shown in Fig 3A. The titers of Ad-specific and rhFLSC-specific serum antibodies
induced were compared (Fig 3B). Mice immunized with the ΔE4 Ad produced similar levels of
Ad-specific IgG antibodies as those immunized with ΔE3 Ad. In contrast, mice inoculated with
the ΔE4 Ad produced a greater than 10-fold increase in IgG antibody titer specific for rhFLSC
compared to mice inoculated with ΔE3 Ad (p = 0.011; Fig 3B).
The rhFLSC construct is designed to present epitopes that are exposed by the binding of CD4 to the HIV envelope (CD4-induced or CD4i) \(^\text{29}\). These conserved epitopes are important targets of the adaptive humoral immune response and induce broadly cross-reactive antibodies against the HIV-1 envelope glycoprotein \((38, 43)\). However, as the rhesus CD4 component of the rhFLSC immunogen is foreign to mice, it serves as an additional antibody target, leading to higher IgG titers in both the ΔE3 and ΔE4-immunized mice compared to IgG titers against Ad and gp120 itself (Fig 3B). Nevertheless, the gp120-specific IgG titers retained the same pattern as that seen for the rhFLSC-specific IgG antibodies, with higher titers observed following immunization with the ΔE4 Ad (Fig 3B).

In mice, of the five IgG subclasses, IgG2a is reported to clear viral pathogens \((44, 45)\). Therefore, we assessed the levels of Ad- and HIV-specific IgG2a antibodies produced in the immunized mice. Mice immunized with the ΔE4 Ad promoted higher levels of rhFLSC-specific IgG2a approaching significance \((p = 0.059)\) when compared to ΔE3 Ad-immunized mice (Fig. 3C). IgG2a antibodies specific for gp120 exhibited a similar pattern. Thus, based on the rhFLSC data, products of the E4 genes appear to suppress transgene-specific antibody production, including the IgG2a subclass reported to be important for viral clearance in mice \((44, 45)\).

**Ad early genes modulate levels of transgene-specific cytokine producing cells in the mucosa of immunized NHP:** Vaccine-induced cellular immunity is also important for protective efficacy. We wanted to assess cellular immunity induced by the mucosal Ad immunizations at a mucosal site. In order to obtain sufficient mucosal cells and also because the replication of human Ad is restricted in mice \((46)\), we assessed levels of HIV\(_{BAL}\)gp120-specific cytokine-producing T-cells in rectal cells of rhesus macaques permissive for Ad5hr replication \((30)\). In this pilot study, groups of 3 rhesus macaques each were primed mucosally at weeks 0 and 12 either
with ΔE3 Ad, a ΔE1B55K Ad, or the ΔE4 Ad and boosted intramuscularly at weeks 24 and 36
with rhFLSC protein in alum adjuvant (Fig 4A). This mucosal immunization strategy has been
shown to prime both systemic and mucosal responses (38, 43). A control macaque received the
empty ΔE3 Ad-vector and alum only. Using intracellular cytokine staining (ICS) and flow
cytometry we quantified the percentage of mucosal cytokine-producing central memory (CM)
and effector memory (EM) CD4+ T-cells specific for HIVBAL gp120 (Fig 4B). Two weeks after
the second priming (wk14) the levels of cytokine-producing CD4 CM T-cells increased in the
NHP that were inoculated with the ΔE1B55K and ΔE4 Ad when compared to pre-immunization
levels (Fig 4B; p = 0.004 for both). The response patterns of both CM and EM CD8+ T-cells
were also significantly increased at week 14 in macaques immunized with the ΔE4 Ad (p =
0.014 and p = 0.022, respectively (Fig 4C). At week 14 levels of CD4+ CM T-cells producing the
three cytokines measured were significantly higher in NHP immunized with the ΔE4 Ad
compared to levels in NHP immunized with ΔE3 Ad (Fig 4D; p = 0.0025). Both CD8+ CM (Fig
4F; p = 0.013) and EM (Fig 4G; p = 0.051) T cells from macaques immunized with the ΔE4 Ad
expressed greater levels of cytokines compared to ΔE3 Ad-immunized NHP. While the numbers
of NHP used were low, these results suggest that products of the Ad E1B55K and E4 genes may
act to suppress levels of mucosal cytokine producing memory CD4+ and CD8+ T-cells in
immunized NHP.

Ad early genes modulate levels of transgene-specific antibodies produced in immunized

NHP: To assess the ability of the Ad vaccine candidates to promote a humoral response in NHP,
we titered the levels of rhFLSC and gp120-specific serum binding antibodies produced over the
course of the immunization by ELISA. In Fig 5, consistent with the T-cell data in Fig 4,
macaques immunized with ΔE1B55K or the ΔE4 Ad exhibited significant enhancement
(p=0.0015 and p<0.0001, respectively) in rhFLSC-specific antibodies after the first priming inoculation compared to levels elicited by $\Delta E3$ Ad (Fig 5A). Two weeks after the second inoculation the differences in antibody levels induced by the $\Delta E4$ Ad compared to $\Delta E3$ Ad remained significant (p=0.0035) and were also higher compared to the $\Delta E1B55K$ Ad-immunized macaques (p=0.033). This pattern continued into the boosting phase of the vaccine regimen where the antibody levels induced by the $\Delta E4$ Ad remained elevated compared to the $\Delta E3$ Ad (post 2nd boost, p=0.055). Throughout the study, the area under the curve (AUC) of rhFLSC-specific antibody induced by the $\Delta E4$ Ad remained significantly higher (p=0.029) than that induced by $\Delta E3$Ad (Fig 5A).

We measured and compared the levels of HIV$_{BAL}$gp120-specific antibodies induced by each of the vaccine candidates (Fig 5B). As in the above mouse study, levels of gp120-specific antibodies were lower than those induced against the rhFLSC immunogen. However, levels of gp120-specific IgG induced in $\Delta E4$ Ad-immunized NHP remained higher than those observed for $\Delta E3$ Ad-immunized NHP (post 1st priming p=0.098, post 2nd priming p<0.016) (Fig 5B).

Binding antibodies to variable regions 1 and 2 of the HIV envelope were among the only correlates of protection in the RV144 phase 3 HIV vaccine trial (47) where 31% vaccine efficacy was shown (48). So far, our results suggest that deleting Ad $E1B55K$ and the $E4$ genes represents a means to enhance transgene-specific antibody responses in immunized NHP. Among the functions that binding antibodies participate in, antibody-dependent cell mediated cytotoxicity (ADCC) may have beneficial impact on HIV infection (49, 50). ADCC occurs when the Fc of the antibody forms a bridge between a target cell (bearing the HIV antigens on its surface) and an effector cell expressing the Fc receptor (51). Ligation to the Fc receptor initiates a cascade of signals that results in the lysis of the target cell. We have previously shown that antibodies
induced by the ΔE3 Ad are capable of promoting cell killing through ADCC (38). Thus, to determine if the E4 genes had any impact on this ability we performed ADCC as summarized in the method section and described elsewhere (52). Here, even though the ADCC values from the ΔE4 Ad-immunized NHPs were higher than those of the other Ad-immunized NHPs, no significant differences were observed either against gp120 or rhFLSC targets (Fig 5C).

Some of the binding antibodies produced by the Ad-immunized NHPs may have the ability to neutralize and otherwise inactivate HIV. To assess whether the E4 genes impacted this ability we performed neutralization assays as before (38). As with the ADCC results, the geometric mean neutralizing serum titer from the ΔE4 Ad-immunized NHPs was slightly higher than that of the other Ad-immunized NHPs but not significantly (Fig 5D).

**Ad E4 genes modulate IgG1-specific antibodies produced in immunized NHP:** Because IgG2a, reported to be important for clearing viral pathogens (44, 45), was induced to greater levels by ΔE4 Ad compared to ΔE3 Ad, the ΔE4 Ad may have also promoted the equivalent antibody subtype in NHPs. Similar to the IgG2a of mice the IgG1 subclass antibodies of NHP exhibit the broadest function (53). In Fig 6A and B we show NHP inoculated with the ΔE4 Ad continued to produce high levels of IgG1 against the HIV-transgene for weeks longer than ΔE3 Ad. For example, two weeks following the 2nd boost, rhFLSC-specific (Fig 6A) and BaLgp120-specific IgG1 titers (Fig. 6B) induced by the ΔE4 Ad were significantly higher than those against both the ΔE3 Ad (p<0.001 and p = 0.0038, respectively) and the ΔE1B55K Ad (p<0.0001 for both). Unlike the levels of HIV-specific IgG shown in Fig 5, in Fig. 6A the levels of rhFLSC-specific IgG1 elicited by the ΔE1B55K Ad-immunized NHP were consistently lower than those elicited in NHP inoculated with ΔE3 Ad (p =
Analysis of BaLgp120-specific IgG1 antibodies (Fig. 6B) confirmed the induction of higher titers by the ΔE4 Ad compared to the ΔE3 Ad (p = 0.001). Thus, in Ad that contain the same surface proteins, products of the E4 genes seem to act to suppress subtype-specific antibody responses.

**Discussion**

Ad vectors are being developed as part of a vaccine strategy for a variety of different maladies because they evoke strong transgene-focused immune responses. Here, we provide evidence across cells, mice and nonhuman primates showing that Ad early genes (namely, E1B55K and E4orf1-4) impact the nature of the induced transgene-specific immune responses. Our findings follow an earlier study in mice that showed that deletion of the Ad E4 genes blunt host immune responses induced by ΔE1 Ad (54). One difference between the studies may be that all our vectors contain E1A. E1A-containing Ad are being explored for use in vaccine delivery (5, 6, 55-58) and exhibit potent immunogenicity. In one study a ΔE3 Ad-HIV recombinant was shown to induce a higher frequency of HIV Env-specific interferon gamma-secreting lymphocytes and T-cell proliferative responses, higher anti-Env binding and neutralizing antibody titers, and better induction of antibody-dependent cellular cytotoxicity than a ΔE1/ΔE3 Ad (59). The two vectors, with and without E1 respectively, contained the same E4 suggesting that activities associated with the Ad E1 (E1A and/or E1B) provoked transgene-specific cellular and adaptive immune responses. Also, in the ΔE4 Ad we retained E4orf6. E4orf6 has been reported to cooperate with the E1A proteins (60). So E4orf6 may act to enhance the activity of E1A while E1B55K along with E4orf1-4, act to counter the effects (Figures 2-6).

We’ve shown that cells infected with Ad with deletions in E1B55K produced higher levels of cytokines, and displayed higher levels of NK cell activating markers on their surfaces than...
cells infected with the ΔE3 Ad (24). This, as well as the enhanced cellular (Figure 4) and early 
antibody responses elicited by the ΔE1B55K Ad (Figure 5A-B), suggests that E1B55K acts to 
inhibit Ad-induced immune activation. As we show in Fig 2, many of the cytokine genes that 
exhibited elevated expression in ΔE1B55K Ad-infected cells (such as IFN, IL-12, TNFα and IL-
6) (24), also exhibited enhanced expression during infection with ΔE4 Ad. If produced by 
susceptible cells in mouse or NHP, these may mediate innate immune cell attraction and 
activation (61). Interferon Beta 1 (IFNB1) activates Janus kinases, leading to the phosphorylation 
and activation of signal transducer and activator of transcription (STAT) 1 (STAT1), STAT2 and 
STAT3, resulting in the expression of hundreds of IFN responsive genes (62). Interleukin 12 (IL-
12) is a heterodimeric cytokine of 70 kDa comprising two covalently linked subunits, p35 and 
p40 (IL-12A, and IL-12B). IL-12 receptors are located mainly on T cells and NK cells, and 
stimulate the Th1 phenotype. The tumor necrosis factor (TNF) superfamily is involved in the 
regulation of multiple biological processes including cell proliferation, differentiation, and cell 
death. Like TNF, IL-6 is produced by many cell types and attracts innate immune cells. Similar 
to IL-12, IL-6 is a class I cytokine which uses gp130 in their receptor complex (63, 64) and plays 
a critical role in immune responses (65, 66).

In a previous publication (24), we compared the immune responses engendered by ΔE3 Ad 
to those induced by a ΔE1B55K Ad. In that study the induced HIV-specific immune responses 
elicited by both Ads were equally high and indistinguishable (24). Here in Fig 3 however, the 
results are very different, showing that the ΔE4 vaccine candidate promoted elevated levels of 
binding antibodies that were distinguishable from those induced by ΔE3 vaccine candidate. 
Binding antibodies are among the only correlates of vaccine-induced HIV protection shown in 
the phase 3 RV144 HIV vaccine trial (47). In an earlier study we noted that sera from mice
vaccinated with Ad with deletions of E4orf1-4 differentially recognize Ad antigens and the rhFLSC transgene. Here we show that in the context of the E1B55K deletion, further deletion of E4orf1-4 results in an Ad that preferentially stimulates IgG2a in mice (and Fig 3) and IgG1 in NHPs (Fig 6). Our assessments of ADCC and neutralizing antibody activity in sera of macaques immunized with the various Ad constructs did not reveal significant differences. However, in view of the differences seen among the groups in IgG1 binding titers, it would have been informative to have isolated the IgG1 induced for evaluation of functionality including other antibody functions, such as antibody-dependent cell-mediated virus inhibition (67), antibody-dependent phagocytic activity (68), or inhibition of transcytosis (69). This remains for future studies. Nevertheless, the data we provide demonstrate that Ad early genes can impact the levels and quality of the induced immune responses.

One of the greatest advantages of the $\Delta E1/\Delta E3$ Ad has been its transgene-carrying capacity of approximately 5 kb. This allows the $\Delta E1/\Delta E3$ Ad to be used in instances where large transgenes are to be expressed. The deletion of E1B55K (~0.89kb), E3 (~3.0kb) and E4orf1-4 (~1.2kb) erased the transgene size advantage of the $\Delta E1/\Delta E3$ Ad. Moreover, since the $\Delta E3$ Ad was shown to induce a more robust transgene-specific immune response than the $\Delta E1/\Delta E3$ Ad, and as we show here that in many cases the transgene-specific immune responses elicited by the $\Delta E4$ Ad eclipse those induced by $\Delta E3$ Ad, we believe the $\Delta E4$ vector may offer immunological advantages not provided by Ad vectors currently being explored in the clinic for use in vaccine delivery.

Lastly, the Ad used here stimulated both T-cell and antibody responses. Both are considered essential for protection from HIV. Our pilot study in rhesus macaques did not include enough animals for evaluation of protective efficacy. Nevertheless, our results represent the first
demonstration across cells, mice and nonhuman primates of a role for Ad early gene-products encoded by E1B55K and E4orf1-4 in modulating the quality and magnitude of transgene-specific immune responses.

Materials and methods

Ethics Statement

All experiments were approved by the Institutional Biosafety Committee at both Howard University and the National Cancer Institute (NCI). All animal experiments were approved by the NCI Animal Care and Use Committee prior to study initiation under protocols VB-016 and VB-017. The NCI animal facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The standard practices closely follow recommendations made in the Guide for the Care and Use of Laboratory Animals of the United States – National Institutes of Health.

Immunogens

The rhFLSC transgene consists of full length single-chain HIV_BaL gp120, a flexible linker, and the D1 and D2 domains of rhesus macaque CD4 (25).

Antibodies

Anti-actin (Sigma); anti-HIV-1 gp120 (Meridian Life Sciences); anti-hCD4 (R&D Systems); and anti-Ad type 5 (Abcam), Anti-DBP (Gift from David Ornelles). HRP conjugated secondary antibody: anti-mouse IgG, anti-human IgG, anti-rabbit IgG, or anti-goat IgG (Invitrogen) were used as dictated by the primary isotype. Mouse-anti-rhesus IgG2 (3C10), Mouse-anti-rhesus IgG1 (7h11) (AIDS reagent resource), Anti-CD4 horseradish peroxidase (HRP)-conjugated anti-mouse total IgG, anti-IgG2a, and CD28PE-Cy7 (ebioscience), CD3-Alexa 700, gamma interferon (IFN-γ)-FITC, interleukin-2 (IL-2)–APC, CD4-PE, IFN-γ-FITC CD95-PE- cy5 (BD
Biosciences), and tumor necrosis factor alpha (TNF-α)-Brilliant Violet 785™ and CD8a-
APC/Cy7 (Biolegend).

Viruses

The dl1520 Ad contains an 827-bp deletion in the region encoding the 55kDa protein (70). The
ΔE3 virus, previously termed MAD5rhFLSC, was described elsewhere (37). The
ΔE1B55K/ΔE3rhFLSC (ΔE1B55K Ad), and ΔE1B55K/ΔE3/ΔE4orf1-4rhFLSC Ad (ΔE4 Ad)
were created by recombining Spe1 digest dl1520 DNA with BamHI digested pBRAd5hrΔE3
(TPL-rhFLSC-pA) plasmid in which we deleted the coding regions for E4orf1-4. The resulting
viral DNAs were screened by PCR for the presence or absence of the E1B55K and the E4orf1-4
genes (Fig 1B). All the viruses were amplified and the concentrations determined at ViraQuest,
Inc. (Iowa) using plaque assays.

Cells

We used the human cervical carcinoma-derived HeLa cell line (ATCC® CCL-2™) because most
of what is known about Ad is in the context of this cell line. We used the human lung A549 cell
line (ATCC® CCL-185) as Ad is known to target the upper respiratory air way. We used the
murine lung epithelial cell line TC1 (Gift from Masaki Terabe, NCI) to simulate infection of
mouse cells by Ad5. We used the green monkey kidney-derived CV-1 cell line (ATCC® CCL-
70) to simulate infection of nonhuman primate cells by Ad5. We used the Ad transformed human
embryonic kidney-derived 293 cells (ATCC® CRL-1573) as they support the growth of E1
deleted viruses. All the cells were maintained as previously described (37).

Mice

Six- to eight-week-old female BALB/c mice were housed and maintained in a pathogen-free
environment according to the standards of the American Association for Accreditation of
Laboratory Animal Care at the National Institutes of Health (NIH Bethesda, MD). The protocol was reviewed and approved by the Animal Care and Use Committee prior to implementation (VB-016). Mice were randomly assigned to 5 experimental groups and inoculated intraperitoneally at weeks 0 and 4 with 5.0x10^8 cell-determined plaque forming units (PFU) of ΔE3 Ad or ΔE4 Ad per mouse. Five naïve mice served as controls. Spleens and blood were collected at week 6 and treated as previously described (37).

NHP
10 male rhesus macaques (Macaca mulatta), approximately 2.5 years old and weighing between 3.5 and 4.4 kg at the time of study initiation, were housed and maintained at the NCI Animal Facility, National Institutes of Health, (NIH, Bethesda, MD) according to the standards of the American Association for Accreditation of Laboratory Animal Care. All were negative for SIV, simian retrovirus type D and STLV. The protocol was reviewed and approved by the Animal Care and Use Committee prior to implementation (VB-017). Three macaques per group were immunized at week 0 intranasally and orally, and at week 12 intratracheally, with ΔE3 Ad, ΔE1B55K Ad, or ΔE4 Ad at a dose of 5x10^8 cell-determined PFU/recombinant/route. The macaques were boosted intramuscularly at two sites with recombinant rhFLSC protein (Profectus BioSciences, Inc.), a total of 100 μg/macaque, in Alum adjuvant (Aluminum hydroxide gel) at weeks 24 and 36. One control macaque (C) received empty Ad5hr vector (5x10^8 cell-determined PFU/macaque) and adjuvant alone.

Sample collection
Peripheral blood mononuclear cells (PBMC) obtained throughout the immunization course were purified from whole blood by Ficoll gradient centrifugation and used immediately for intracellular cytokine staining assays or viably frozen for later use. The rectal biopsies were...
processed and the lymphocytes isolated using a percoll gradient of 35% and 65% layered solutions. Serum samples were collected, aliquoted, and stored at −70°C until use as before (71).

**Intracellular cytokines**

Freshly isolated PBMC (2x10⁶) were stimulated with pools of HIV BaL gp120 peptides (Advanced BioScience Laboratories, Inc., Rockville, MD) and stained as described previously (72) except fluorochromes for CD4 and gamma interferon (IFN-γ) antibodies were changed to CD4-FITC and IFN-γ-PE. A singlet, followed by live/dead and then lymphocytic gates, were first applied. CD3⁺ T-cells were divided into CD4⁺ and CD8⁺ populations, and each population was further subdivided into CD28⁺ CD95⁺ central memory (CM) and CD28⁻CD95⁺ effector memory (EM) cells. The percentage of cytokine-secreting (IFN-γ, TNF-α and IL2) cells in each memory cell subset was then determined following subtraction of the values obtained with nonstimulated samples and the values were summed. Data were analyzed using FlowJo software (TreeStar Inc.).

**Antibody analyses**

Antibody binding titers were assayed by enzyme-linked immunosorbent assay (ELISA) as described previously (37). The plates were read at 450nm within 30 min on a Biotek ELISA reader (ELx803) and analyzed with Gen5 3.02 software. Endpoint titer was defined as the reciprocal of the serum dilution at which the OD of the test serum was twice that of the background OD of the plate.

ADCC activity was assessed as previously described using EGFP-CEMNKr-CCR5-SNAP cells that constitutively express GFP as targets (52). Briefly, one million target cells were incubated with 50 μg of BAL gp120 or rhFLSC recombinant protein for 2 h at 37°C, washed, and labeled with SNAP-Surface Alexa Fluor 647 (New England Biolabs, Ipswich, MA; cat.# S9136S) as
recommended by the manufacturer for 30 min at RT. Heat inactivated plasma samples were serially diluted (7 ten-fold dilutions starting at 1:10) and 100 μl were added to a 96-well V-bottom plate (Millipore Sigma). Following this, 5000 gp120- or rhFLSC-coated target cells (50 μl) and 250,000 human PBMCs (50 μl) as effectors were added to each well to give an effector/target (E/T) ratio of 50:1. The plate was incubated at 37°C for 2 h followed by two PBS washes. The cells were resuspended in 200 μl of a 2% PBS–paraformaldehyde solution and acquired on an LSRII equipped with a high throughput system (BD Biosciences, San Jose, CA). Specific killing was measured by loss of GFP from the Alexa647+ target cells. Target and effector cells cultured in the presence of medium were used as negative controls. The ADCC endpoint titer (data not shown) is defined as the reciprocal dilution at which the percent ADCC killing was greater than the mean percent killing of the negative control wells containing medium, target and effector cells, plus three standard deviations. Neutralizing antibody titers against SHIVBaL-P4 were assayed in TZM-Bi cells as described (73) using MLV-pseudotyped virus as a negative control for non-HIV-specific inhibition of signal. Titers were defined as the reciprocal serum dilution at which there was a 50% reduction in relative luminescence units compared to virus control wells. Western Blot Gel electrophoresis and western blotting were performed as described previously (37). DNA extraction and PCR DNA was extracted using the Qiagen DNA blood Mini kit. The purity and quantity of all extracted DNA was determined with a Nano Drop spectrophotometer (ThermoFisher). The PCR assays were performed as described (37) with minor modifications. Briefly, test sample DNA was added to the Dream Taq Green master mix (ThermoFisher). Reverse and forward primers
for Ad fiber and PCR-quality water (ThermoFisher) were added to reach a final volume of 25 μL. A negative control (no template DNA) was included in each run. PCR amplification was performed using a DNA Thermocycler (Life Technologies). The PCR product was loaded on a 1% agarose gel, stained, and photographed with a LI-COR imaging system (LI-COR). The molecular weight of the PCR products was determined by comparison to a 1 kb DNA ladder (ThermoFisher).

**Real time Quantitative PCR**

Cells were not infected (mock) or infected with ΔE3 Ad or the ΔE4 Ad at a MOI of 50 PFU/cell for 48 hours. Total RNA was isolated with the RNAzol® RT (Sigma). The RNA concentrations were determined using a Nano Drop spectrophotometer (ThermoFisher). The RNA was reverse transcribed to cDNA with a Quantitect® reverse Transcription Kit (QIAGEN). Briefly, samples were treated with 10 U DNase (Pharmacia) for 2 min at 42 °C, followed by 30 min cDNA synthesis and inactivation of the Quantiscript Reverse Transcriptase.

Gene expression was assayed using a TaqMan® Array Human Cytokine Network 96-well FAST Plate (ThermoFisher). The TaqMan® Array Plate contains 28 assays for genes associated with pro- and anti-inflammatory cytokines and four assays for candidate endogenous control genes. The quantitative real-time PCR amplification was performed using QuantStudio™ 6 (Applied Biosystems®). The PCR program used consisted of sample holding at 50°C for 2 min and then 95°C for 10 min, followed by 40 cycles, each consisting of 95°C for 15s, 56°C for 15, and 62°C for 30s. All assays were plated in triplicate. The highest cycle number 40 was used for all undetermined values. The ∆∆ct fold number for each experiment was determined and converted into Log2.

**Statistical analysis**
Differences between groups or between times within groups were assessed using one-way, two-way and repeated measures analysis of variance and multiple T tests, combining panels with common outcomes for appropriate degrees of freedom. Logarithmic transformation of titers and arcsine transformation of percentage data were applied before analysis to reduce skewness in raw data. Corrections for multiple comparisons were made where noted. Wilcoxon-Mann-Whitney and Wilcoxon signed rank tests were used for selected comparisons of groups of adequate size. Analyses were performed using Prism (GraphPad Software, Inc.) and SAS/STAT software version 9.4 (SAS Institute Inc.).

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Fig legends

Fig 1: Characterization of Ad5 HIV-vaccine candidates in infected human lung epithelial cells. (A) Adenovirus genome with “X” indicating deleted genes. (B-F) A549 cells were infected at a MOI of 5 or 50 for 24, 48, and 72 hours. (B) Previously described E4orf1, 1-2, 1-3, E4orf4 and E1B55K primers (24, 37) were used in PCR experiments to reveal the presence or absence of the indicated genes. (C) PCR for a segment of the Ad Fiber gene was performed. (D) Plaques were counted and mean values ± SEM were plotted. (E) The static levels of Ad DNA binding protein (DBP), actin, Ad late proteins (hexon, penton and fiber) and (F) levels of gp120 were assessed by western blot. All experiments were repeated at least 3 times. Representative examples are shown in B,C and E,F. P values were obtained by ANOVA after Bonferroni correction.

Fig 2: Expression levels of innate cytokine network genes in Ad-infected human epithelial cells. (A, B, C) A549 cells were infected at a MOI of 50 with ΔE3 or ΔE4 Ad for 48 hours. Total RNA was isolated and reverse transcribed to cDNA. Fold change was determined using the 2^-ΔΔCt method. (A) P values were obtained using multiple t tests. (B) The mean value of all the innate cytokines measured from ΔE4- and ΔE3-infected A549 cells were compared, (C) as well as those above zero from both vectors. P values were obtained using Wilcoxon matched-pairs signed rank test. For each, 3 biological replicates were performed and analyzed. * = p < 0.05, ** = p < 0.01.

Fig 3: Levels of Env-specific serum binding antibodies in immunized mice. (A-C) Randomly assigned experimental groups of 5 mice were inoculated intraperitoneally (IP) at weeks 0 and 4
with 5.0x10^8 PFU ΔE3 or ΔE4 Ad per mouse. Five naïve mice served as controls. Blood was
collected at week 6. (B) Titters of Ad-, rhFLSC-, and gp120-specific IgG antibodies were
measured by ELISA. (C) Titters of Ad-, rhFLSC-, and gp120-specific serum IgG2a antibodies
were measured by ELISA. The values for the control mice were below the background and are
not shown. Results are presented as mean +/- SEM. P values were obtained using 2way
ANOVA.

Fig 4: Levels of Env-specific cytokine-producing mucosal memory CD4 and CD8 cells in
immunized Rhesus macaques. (A-E) Three macaques per group were immunized at week 0
intranasally (IN) and orally (O), and at week 12 intratracheally (IT), with ΔE3, ΔE1B55K or ΔE4
Ad as described in the methods section. One control macaque, C, received empty Ad5hr. The
rhFLSC boost in alum was administered intramuscularly (IM). (B,C) The mean percentages ±
SEM of cytokine (TNFα, IL2, IFNγ) producing rectal CM and EM CD4 and CD8 T cells prior to
immunization (pre) and at wk 14 for each immunization group are shown. (D-G) The mean
percentages ± SEM of cytokine (TNFα, IL2, IFNγ) producing T cells on week 14 are compared
across immunization groups.

Fig 5: Levels of Env-specific IgG antibody in immunized Rhesus macaques. Serum levels of
(A) rhFLSC- and (B) gp120-specific IgG antibodies were measured by ELISA. Results are
displayed as log 10 geometric mean. (C) Mean value of week 38 ADCC for gp120 and rhFLSC
coated target cells are shown. (D) Geometric mean value of SHIV_BAL p4 week 38 serum
neutralization titers are shown. P values were obtained using repeated measures ANOVA and the
Wilcoxon-Mann-Whitney test.

Fig 6: Levels of Env-specific IgG1 antibody in immunized Rhesus macaques. Serum levels
of (A) rhFLSC- and (B) gp120-specific IgG1 antibodies were measured by ELISA. Results are
displayed as log 10 geometric mean. P values were obtained using repeated measures ANOVA
and the Wilcoxon-Mann-Whitney test.
A diagram showing the Adenoviral genome with different deletions, including \( \Delta E3 \) Ad, \( \Delta E1B55K \) Ad, and \( \Delta E4 \) Ad. There are also experiments showing the effect of these deletions on different viral markers such as \( \Delta E4orf1 \), \( \Delta E4orf1-2 \), \( \Delta E4orf1-3 \), and \( \Delta E4orf4 \). Additionally, there are bar graphs showing the MOI50 and PFU/mL for different MOIs and time points. These graphs indicate a significant difference at p<0.0001, p<0.0012, and p<0.0003. There are also Coomassie stained gels showing the expression of different viral proteins such as Hexon, Penton, and Fiber at different time points (12hpi, 24hpi, 48hpi, and 72hpi) and MOIs (5, 50). The bands are labeled with arrows pointing to gp120.
Pre wk 14

0.0 0.5 1.0 1.5 2.0 2.5
% Cytokine producing cells
Rectal mucosa: CD4CM - EnvBAL

ΔE3
ΔE1B55K
ΔE4
Pre wk 14

p=0.004

Rectal mucosa: CD4EM - EnvBAL

ΔE3
ΔE1B55K
ΔE4
C

p=0.004

p=0.0025
CD4CM

Rectal mucosa: CD8CM - EnvBAL

ΔE3
ΔE1B55K
ΔE4
C

p=0.014

Rectal mucosa: CD8EM - EnvBAL

ΔE3
ΔE1B55K
ΔE4
C

p=0.022

p=0.051
p=0.010
p=0.042
CD8EM
Figure A: **rhFLSC IgG titers**

- AUC: ΔE3 vs ΔE4, p = 0.029

Figure B: **BaLgp120 IgG titers**

- p < 0.016

Figure C: **gp120**

- % ADCC Killing Wk 38: ΔE3, ΔE1B55K, ΔE4

- rhFLSC

- Geomean Serum Neutralization titers: SHIV-Bal P4 Wk38
