Vaccination with Adenovirus Serotypes 35, 26, and 48 Elicits Greater Innate Cytokine Responses Than Adenovirus Serotype 5 in Rhesus Monkeys

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Running Title: Innate Cytokine Induction by Alternative Serotype Adenoviral Vectors

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ABSTRACT

Adenovirus (Ad) vaccine vectors have proven highly immunogenic in multiple experimental models, but the innate immune responses induced by these vectors remain poorly characterized. Here we report innate cytokine responses to 5 different Ad vectors in 26 rhesus monkeys. Vaccination with Ad35, Ad26, and Ad48 induced substantially higher levels of antiviral (IFN-γ, IP-10) and proinflammatory (IL-1RA, IL-6) cytokines as compared with Ad5 on day 1 following immunization. In vitro studies with capsid chimeric vectors and receptor blocking monoclonal antibodies suggested that fiber-receptor interactions as well as other capsid components were critical for triggering these innate responses. Moreover, multiple cell populations, including dendritic cells, monocytes/macrophages, and T lymphocytes, contributed to these innate cytokine profiles. These data demonstrate that Ad35, Ad26, and Ad48 that utilize CD46 as their primary cellular receptor induce significantly greater innate cytokine responses than Ad5, which uses the coxsackievirus and adenovirus receptor (CAR). These differences in innate triggering result in markedly different immunologic milieus for the subsequent generation of adaptive immune responses by these vaccine vectors.
Adenovirus (Ad) vectors are widely used for vaccination due to their immunogenicity, relatively large transgene coding capacity, and multiple available serotypes with diverse biological properties. While considerable data have been generated regarding adaptive immune responses elicited by Ad vectors, much less is known about innate immune responses induced by these vectors. As innate immune induction is critical for understanding both reactogenicity and adaptive immunity, it is important to define the innate pathways triggered by Ad vectors from various serotypes. Accumulating evidence suggests that different serotype Ad vectors induce qualitatively different adaptive immune response phenotypes (1)(21)(26)(43). In particular, vaccine studies using the simian immunodeficiency virus (SIV) infection model in rhesus monkeys have shown qualitative differences in adaptive immune responses elicited by various serotype Ad vectors (26), which translated into different levels of protective efficacy against SIV challenges (27)(7)(6). However, innate immune profiles of different alternative serotype Ad vectors have not previously been studied in nonhuman primates. Adenoviruses are a diverse group of double stranded DNA viruses with at least 65 known human serotypes, which are subdivided into species A-G based upon sequence homology (10)(49)(25)(29). Vectors constructed using these viruses have been shown to differ significantly in terms of primary receptor usage (13)(9)(41)(1)(50), intracellular trafficking patterns (14)(30)(32)(23), transduction and activation of dendritic cells (2)(19)(11)(53)(36)(28), utilization of secondary receptors (15)(48), cellular tropism...
(31)(33)(3)(44)(47)(46), and interaction with pattern recognition receptors (PRR) (17)(37)(35). The species C adenovirus serotype 5 (Ad5), the species B2 adenovirus serotype 35 (Ad35), and the species D adenovirus serotype 26 (Ad26) are currently being evaluated as vaccine candidates in clinical trials, yet relatively little is known about the possible differences in innate immunity induced by these vectors. Notably, Ad5 utilizes CAR as its primary cellular receptor, whereas Ad35, Ad26, and Ad48 utilize CD46 (22).

In this study, we describe the innate cytokine profiles induced in vivo by Ad vectors from 5 serotypes in 26 rhesus monkeys. We then assessed the mechanism of differential viral triggering of these innate responses using capsid chimeric vectors and receptor blocking monoclonal antibodies in vitro in human PBMC. Our studies demonstrate that Ad35, Ad26, and Ad48 vectors that utilize CD46 as their primary cellular receptor trigger profoundly different innate cytokine profiles characterized by higher levels of antiviral and proinflammatory cytokines and chemokines compared with Ad5 vectors that utilize CAR.
RESULTS

Ad35, Ad26, and Ad48 induce more potent antiviral and proinflammatory cytokines and chemokines compared with Ad5 following vaccination of rhesus monkeys. We initiated studies by assessing serum cytokine levels in rhesus monkeys following vaccination with 5 different serotype Ad vectors in 26 rhesus monkeys.

Rhesus monkeys (n=4-8/group) were immunized i.m. with $3 \times 10^{10}$ vp Ad5, Ad35, Ad26, Ad48, or chimeric Ad5 with the hexon hypervariable regions (HVR) replaced with those of Ad48 (Ad5HVR48) expressing SIV Env/Gag/Pol (40)(1). All vectors were replication-incompetent E1/E3-deleted Ad vectors that were prepared similarly and exhibited comparable characteristics, specific infectivity, and purity (1). Sera were collected on days 0, 1, 3, 7, 14 and 28 following vaccination, and cytokine levels were assessed by luminex assays.

Longitudinal analysis of cytokine responses following vaccination with Ad5 revealed only low levels of antiviral and proinflammatory cytokine and chemokine induction on day 1 following vaccination (Fig. 1A, 1B). In contrast, animals vaccinated with Ad35, Ad26, and Ad48 displayed greater induction of multiple cytokines and chemokines on day 1 following vaccination. Increased induction by Ad35, Ad26, and Ad48 as compared with Ad5 was observed for the inflammatory markers IL-1RA (17.7-, 8.2-, 26.2-fold greater induction than Ad5, respectively; p=0.006, p=0.009, p=0.02, respectively, Mann-Whitney U Test) and IL-6 (8.3-, 4.0-, and 3.4-fold greater induction than Ad5, respectively; p=NS, p=0.04, and p=0.03, respectively). Additionally, significant induction of IFN-γ (1.4-, 1.7-, and 1.5-fold greater induction than Ad5; p=NS, p=NS, p=0.02, respectively), and its downstream-signaled chemokines IP-10 (8.3-, 6.0-, and...
4.9-fold greater induction than Ad5; p=0.03, p=0.009, p=0.03, respectively) and I-TAC
(2.8-, 1.1-, and 3.6-fold greater induction than Ad5; p=0.03, p=NS, p=0.03, respectively)
was observed in animals that received Ad35, Ad26, or Ad48. These data demonstrate
that Ad35, Ad26, and Ad48 that utilize CD46 as their cellular receptor trigger
substantially greater antiviral and proinflammatory cytokine responses than does Ad5
that utilizes CAR as its cellular receptor following vaccination of rhesus monkeys.

Interestingly, on day 7 following vaccination, animals that received Ad48 or the
chimeric Ad5HVR48 vectors also displayed a temporally distinct peak of cytokine
induction characterized by the proinflammatory cytokines IL-1β, IL-6, IL-1β and TNF-α
(Fig. 1A, 1B). This cytokine peak was not observed with Ad5, Ad35, or Ad26 vectors.
Since Ad48 and Ad5HVR48 only share common hexon HVR sequences, we speculate
that this day 7 cytokine peak may be triggered by hexon rather than fiber-receptor
interactions. These data suggest that both fiber and hexon may contribute to triggering
innate immune responses.

**Cytokine induction following heterologous boost immunization.** We next assessed
whether innate cytokine responses following an Ad26 boost immunization would be
impacted by previous priming with a heterologous Ad vector. Systemic cytokine levels
were assessed in rhesus monkeys (n=4/group) that were previously primed with Ad35,
Ad48, or Ad5HVR48 vectors as described above and then boosted 24 weeks later i.m.
with 3x10^{10} vp Ad26 expressing SIV Env/Gag/Pol. Sera were collected and analyzed as
described above. Longitudinal analysis of animals revealed cytokine profiles on day 1
following the boost immunization (Fig. 2) that appeared similar to the cytokine profile
induced following priming with Ad26 (Fig. 1A), including induction of IFN-γ, IL-6, IP-10, and IL-1RA (Fig. 2). These data suggest that the innate cytokine profiles following a boost immunization are dictated primarily by the vector utilized for the boost and are not substantially imprinted by the heterologous vector utilized for priming.

Ad35, Ad26, and Ad48 induce higher levels of antiviral and proinflammatory cytokines and chemokines compared with Ad5 in vitro. We next sought to probe the mechanism by which different serotype Ad vectors elicit differential innate cytokine profiles. We therefore assessed the capacity of Ad5, Ad35, Ad26, and Ad48 to trigger secretion of innate cytokines in vitro in freshly isolated human PBMC. Human PBMC (n=8-13/group) were stimulated with Ad5, Ad35, Ad26, or Ad48 at a multiplicity of infection (MOI) of $10^3$ viral particles (vp) per cell. Cytokine and chemokine responses were measured in culture supernatant 24 hours post-infection by luminex assays.

Transduction of PBMC with Ad35 and Ad26 induced substantially higher levels of IFN-α2 (128- and 96-fold higher levels, p<0.001 and p<0.01, respectively, Kruskal-Wallis test with Dunn’s correction for multiple comparisons), IFN-γ (38- and 24-fold higher levels, respectively, p<0.001), IL-1β (10- and 5-fold higher levels, p<0.001 and p<0.01, respectively), and TNF-α (10- and 4-fold higher levels, p<0.001 and p<0.01, respectively) as compared with transduction of PBMC with Ad5 (Fig. 3A). Ad35 and Ad26 also induced higher levels of MIP-1α (18- and 8-fold, respectively, p<0.001), MIP-1β (16- and 9-fold, respectively, p<0.001), IL-6 (2.4- and 1.4-fold higher levels, p<0.001 and p=NS, respectively) and IL-1RA (3- and 3-fold higher levels, respectively, p<0.001) (Fig. 3A). The species D vector Ad48 displayed an intermediate phenotype of cytokine
and chemokine induction relative to Ad5 and those induced by Ad35 and Ad26. A complete analysis of all cytokines and chemokines measured in the luminex assays demonstrated that Ad35, Ad26, and Ad48 induced higher levels of multiple antiviral and proinflammatory cytokines, as well as chemokines including MCP-1 and IP-10 as compared with Ad5 (Fig. 3B). These in vitro data are largely consistent with the in vivo data from vaccinated rhesus monkeys with a few notable exceptions, such as the high levels of IFN-α2 induced in vitro compared with in vivo.

Both fiber and capsid components contribute to innate immune stimulation by Ad vectors. We next explored the role of the key structural proteins of the adenovirus capsid on innate immune stimulation. We utilized a panel of chimeric Ad5/Ad35 (34) vectors and stimulated human PBMC (n=8/group) as described above. Chimeric Ad vectors included Ad5 with the Ad35 fiber (Ad5f35) or Ad35 fiber and penton (Ad5p35f35), and Ad35 with the Ad5 fiber (Ad35f5) or Ad5 fiber knob (Ad35k5). Replacement of the Ad5 fiber with the Ad35 fiber, or Ad35 fiber and penton, resulted in an increased induction of multiple cytokines relative to Ad5, including IFN-α2 (126- and 184-fold higher levels, p<0.01 and p<0.001, respectively), IFN-γ (16- and 9-fold higher levels, p<0.05 and p<0.01, respectively), IL-1β (2.5- and 1.6-fold higher levels, respectively p=NS), TNF-α (12- and 11-fold higher levels, respectively, p<0.001), MIP-1α (52- and 40-fold higher levels, p<0.01 and p<0.001, respectively), MIP-1β (30- and 25-fold higher levels, respectively, p<0.001), IL-6 (7- and 7-fold higher levels, respectively, p<0.001) and IL-1RA (4.4- and 3.5-fold higher levels, p<0.05 and p<0.01, respectively) (Fig. 4).
A corresponding decrease in stimulatory capacity was observed with Ad35 vectors containing the Ad5 fiber or fiber knob as compared to Ad35, suggesting the importance of the Ad35 fiber for innate cytokine stimulation. For example, Ad35f5 and Ad35k5 induced lower levels relative to Ad35 of IFN-γ (4.7- and 7.1-fold lower levels, p<0.05 and p<0.01, respectively), IL-1β (2.7- and 10.6-fold lower levels, p<0.05 and 0<0.001, respectively), TNF-α (5.5- and 7.1-fold lower levels, respectively, p<0.01), MIP-1α (13.0- and 23.6-fold lower levels, p<0.01 and p<0.001, respectively), MIP-1β (6.0- and 9.4-fold lower levels, respectively, p<0.05) and IL-6 (3.4- and 5.2-fold lower levels, p<0.05 and 0<0.01, respectively) (Fig. 4). However Ad35f5 and Ad35k5 still induced most cytokines to a greater level than did Ad5, indicating that other capsid components in addition to fiber also likely contribute to innate cytokine stimulation. Taken together, these results are consistent with the model in which both fiber and hexon contribute to innate immune triggering.

**Fiber-receptor interactions are required for Ad35 and Ad26 stimulation of human PBMC.** We next assessed whether fiber interactions with the CD46 receptor were critical for the robust innate immune stimulation by Ad35 and Ad26 vectors. Human PBMC (n=4-8/group) were pre-incubated with 10 μg/mL of the anti-CAR monoclonal antibody RmcB (16) or the anti-CD46 monoclonal antibodies 13/42 and M177(12)(45) and were then stimulated with Ad35 and Ad26 as described above. As expected, IFN-α2 and IFN-γ levels induced by Ad35 and Ad26 vectors were not affected by RmcB. In contrast, Ad35 induced lower levels of IFN-α2 and IFN-γ in the presence of 13/42 (p<0.05), and Ad26 elicited dramatically lower levels of IFN-α2 and IFN-γ following
preincubation of cells with either 13/42 (p<0.05) or M177 (p<0.05) (Fig. 5). No cytokine responses were observed in control experiments following incubation of cells with the monoclonal antibodies alone (data not shown). These results support previous reports from our laboratory and others showing that CD46 is a primary cellular receptor for Ad35 and Ad26 (13)(1), and these data demonstrate that fiber-receptor binding contributes substantially to the innate immune stimulation by these vectors. The greater effect of CD46 blockade on innate responses triggered by Ad26 as compared with Ad35 may reflect subtle differences in primary receptor interactions and/or secondary receptor usage by these vectors.

Multiple cellular subsets are stimulated by Ad35 and Ad26. To explore the contributions of various cellular subsets to innate cytokine secretion following Ad35 and Ad26 stimulation, we depleted specific cell subsets from healthy human PBMC (n=6/group) and repeated the cytokine assays as shown in Figure 3. T cells, B cells, NK cells, monocytes/macrophages (MonoMac), myeloid dendritic cells (mDC), or plasmacytoid dendritic cells (pDC) were depleted by magnetic bead separation, and effective depletion was confirmed by flow cytometry (data not shown). PBMC depleted of these various cell subsets were then incubated with Ad35 or Ad26, and cytokine and chemokine responses were assessed. Depletion of T cells resulted in complete abrogation of IFN-γ induction and partial reduction of IL-1RA, whereas depletion of pDC led to substantial reduction of IFN-α2, TNF-α and IL-1RA (Fig. 6). Depletion of monocytes and macrophages resulted in a pronounced reduction of the proinflammatory markers IL-1RA, MIP-1α and MIP-1β (Fig. 6). These data suggest that
multiple cellular subsets contribute to the overall milieu of innate cytokines triggered by Ad vectors.
DISCUSSION

The innate immune profiles induced by various serotype Ad vaccine vectors remain poorly understood and have not previously been characterized in nonhuman primates. Here we demonstrate that the alternative serotype vectors Ad35, Ad26, and Ad48 induce substantially greater innate cytokine responses than Ad5 following vaccination of rhesus monkeys. In particular, Ad35, Ad26, and Ad48 induce greater antiviral and proinflammatory cytokines than Ad5, characterized by higher levels of IFN-γ, IP-10, I-TAC, IL-1RA, and IL-6.

Previous reports have characterized innate cytokine profiles of Ad vectors in mice (3)(4)(53) and in vitro (19)(52)(28)(2)(17)(18)(36). For example, several studies have reported that Ad35 induces higher levels of the co-stimulatory markers CD80 and CD40 and interferons than does Ad5 in vitro in human DCs (28), as well as higher levels of IL-10 and reduced proliferation in T cells co-cultured with DCs (2). Studies in mice have suggested increased proinflammatory cytokine induction by Ad5 relative to CD46-utilizing vectors (11), which contrasts with our data from in rhesus monkeys. Moreover, a recent study demonstrated that Ad35 and Ad28 induce high levels of IFN-α following immunization of mice (19). In contrast, we observed only minimal IFN-α secretion by all Ad vectors, including Ad35 and Ad26, in rhesus monkeys. Taken together, these results demonstrate the importance of studying innate immune profiles of Ad vectors in primates rather than mice, which lack the functional cellular receptor CD46 that is utilized by the alternative serotype Ad vectors (22). This is particularly important since our data suggests that the innate cytokine responses elicited by these vectors are largely triggered by receptor binding. Indeed, both our in vitro and in vivo studies
suggest that Ad fiber binding to CD46 strongly influences elicited cytokine profiles (Figs. 1, 4, 5), and thus the absence of functional CD46 in mice may account for the differences observed between the murine and primate experimental models.

Fiber-receptor interactions appear to be critical but do not fully explain the observed differences in innate immune triggering by the various serotype Ad vectors. In addition to its role as a receptor to certain species B and D adenoviruses, CD46 has a role in the binding complement components C3b and C4b (24)(5), and due to differential splicing may be immunosuppressive or immunostimulatory upon ligand binding (39)(38). Additionally, the degree of CD46 binding may influence ligand endocytic trafficking fate, as well as the induction of autophagy (42)(20). As such, Ad35, Ad26, and Ad48 binding to CD46 may target them to endosomal compartments rich in pattern recognition receptors that could then influence innate immune responses differently from those induced by Ad5. The mechanism by which CD46 binding influences Ad vector innate stimulation thus warrants further exploration. Our results also suggest that Ad capsid components such as hexon contribute to innate stimulation. In particular, we observed a distinct day 7 peak of cytokine secretion in monkeys that received Ad48 and Ad5HVR48, which only share common hexon HVRs. The ability of the hexon HVRs to influence innate immune stimulation may reflect their role in binding serum factors (48) or endocytic localization (51).

In summary, our data demonstrate for the first time that the innate cytokine responses triggered by Ad35, Ad26, and Ad48 are substantially greater than those induced by Ad5 in rhesus monkeys. Specifically, the alternative serotype Ad vectors that utilize CD46 as their primary cellular receptor induce more potent antiviral and
proinflammatory cytokine responses following vaccination than Ad5 that utilizes CAR. The in vivo characterization of these innate cytokine profiles contributes to our understanding of these Ad vectors that are currently being evaluated as candidate vaccine vectors in human clinical trials. Further studies assessing the impact of these profoundly different innate immunologic milieus on the subsequent generation of adaptive immune responses are therefore warranted.
MATERIALS AND METHODS

Viruses: E1/E3 deleted vectors Ad5, Ad35, Ad26, Ad48, and Ad5HVR48 vectors expressing SIV antigens no transgene were produced as previously described (1)(34). Briefly, vectors were produced by recombination in E1-complementing PER.55K cells and were purified by CsCl density centrifugation.

Cells: Normal human blood was collected in the presence of sodium heparin and processed by Ficoll-hypaque gradient method as previously described (8). Cells were resuspended in R10 medium (RPMI, 10%FCS, 50 μ/mL Penicillin, 50 μg/mL Streptomycin) at a concentration of 1x10^6 cells/mL and utilized in in vitro assays. All studies involving human subjects were approved by the Beth Israel Deaconess Medical Center Institutional Review Board (IRB).

Antibodies: Anti-CAR mAb RmcB (Millipore, Billerica, MA) and anti-CD46 mAbs 13/42 (LifeSpan Biosciences, Seattle, WA) and M177 (Hycult Biotechnology, Plymouth Meeting, PA), as well as anti-KLH Mouse IgG isotype control (R&D Systems, Minneapolis, MN) were washed 3x with 1 mL unsupplemented DPBS and concentrated to 1 μg/μL by centrifugation at 3,000 rpm in Amicon Ultra-4 Centrifugal Filters (30kD MW) (Millipore, Billerica, MA) and stored at 4°C for immediate use. Flow cytometry antibody panels included CD3-allophycocyanin (UCHT1), CD16-fluorscein isothiocyanate (3G8), CD123-peridinin chlorophyll protein-Cy5.5 (9F5), CD11c-phycoerythrin (B-ly6), CD56-phycoerythrin-Cy7 (B159), CD19-V450 (HIB19), CD14-allophycocyanin-Cy7 (MφP9) (BD Biosciences, San Diego, CA) and HLA-DR-AlexaFluor700 (LN3) (eBioscience, San Diego, CA).
In Vitro Cytokine Stimulation Assay: 1x10^6 PBMC were incubated with 1,000 vp/cell of the indicated adenovirus vector. In certain experiments, cells were pre-incubated with mAbs for 1 hr prior to infection. Cells were cultured at 37°C and supernatants were harvested 24 h following infection by centrifugation at 1,400 rpm for 5 minutes. Supernatants were analyzed utilizing Millipore Milliplex Map Human Cytokine/Chemokine magnetic luminex (Millipore, Billerica, MA) and Life Technologies Cytokine Human 30-Plex Panel (Life Technologies, Grand Island, NY) as per the manufacturer’s protocols. Luminex data were analyzed on BioPlex 200 instrument running Bioplex Manager 4.1 (Bio-Rad, Hercules, CA) with 80%-120% standard acceptance range. Data were graphed using Graph Pad Prism v5.0 (GraphPad Software, Inc., La Jolla, CA). Means between groups were compared using Kruskal-Wallis tests with Dunn’s correction for multiple comparisons and plotted as mean ± SEM.

Magnetic Cell Separation: PBMC were depleted of CD3, CD14, CD19, CD56, CD1c, or BDCA-1 positive cell fractions by use of Magnetic MicroBeads according to manufacturer’s protocol (Miltenyi Biotec, Auburn, CA). Depleted populations were confirmed by flow cytometry on LSRII and BD FACSDiva v.6.1.1 (BD Biosciences). Results were analyzed using FlowJo v.8.8.6 (Tree Star, Inc., Ashland, OR).

Non-Human Primate Ad Vector Vaccination: Rhesus monkeys (n=4-8/group) were immunized with 3x10^10 vp Ad5, Ad35, Ad26, Ad48, or Ad5HVR48 expressing SIV Gag/Pol/Env antigens. Serum was collected on days 0, 1, 3, 7, 14 and 28 following vaccination, thawed on ice, and inactivated with 0.05% Tween-20 for 15min at room temperature. Samples were then run on Milliplex Non-Human Primate 23plex Premix.
(Millipore) or Invitrogen Monkey Cytokine 28plex (Life Technologies), VeriKine Cynomolgus/Rhesus Interferon-Alpha Serum ELISA (PBL InterferonSource, Piscataway, NJ), and Human CXCL10/IP-10 Quantikine ELISA (R&D Systems, Minneapolis, MN) according to manufacturer’s protocols, analyzed and graphed as described above. All studies involving rhesus monkeys were approved by the Harvard Medical School Institutional Animal Care and Use Committee (IACUC).

**Data Analysis and Statistical Methods:** Concentrations of cytokines and chemokines were obtained from luminex assays using a 5 parameter logistic model. Results of in vitro PBMC studies were compared by Kruskal-Wallis tests with Dunn’s correction for multiple comparisons. Total cytokine induction in vitro was assessed by group average fold induction versus media controls. Cytokine induction in vivo was assessed as fold change of cytokine over average baseline level of all monkeys. Group fold change over averaged baseline was compared to monkeys vaccinated with Ad5 by Mann-Whitney U tests.

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REFERENCES


Figure 1. Serum concentrations of cytokines and chemokines in rhesus monkeys following vaccination with Ad vectors. Rhesus monkeys (n=4-8/group) were injected IM with 3x10^{10} vp Ad5, Ad35, Ad26, Ad48 or Ad5HVR48 vectors. Sera were collected on days 0, 1, 3, 7, 14 and 28 following vaccination, and systemic levels of cytokines and chemokines were measured by luminex and ELISA assays. (A) Systemic levels of selected cytokines are shown in colored lines with means in black lines. Groups are compared by fold changes over baseline compared with Ad5 *p<0.05, **p<0.01. (B) Mean fold induction of all cytokine responses relative to baseline by the various Ad vectors in rhesus monkeys one day (left) and seven days (right) following immunization. with the indicated adenoviral vector. Data are represented as fold change over averaged baseline.

Figure 2. Serum concentrations of cytokines and chemokines in rhesus monkeys following boost vaccination with Ad26 vector. Rhesus monkeys (n=4/group) were boosted IM with 3x10^{10} vp Ad26 vector 24 weeks after priming with 3x10^{10} vp of Ad35, Ad48, or Ad5HVR48 vectors. Sera were collected on days 0, 1, 3, 7, 14, and 28 following vaccination and systemic levels of cytokines and chemokines were measured by luminex assays. Systemic levels of selected cytokines are shown in colored lines with group means in black lines.

Figure 3. Cytokine and chemokine responses induced in vitro in human PBMC following Ad vector stimulation. (A) Individual PBMC (n=8-13/group) were
stimulated with $10^3$ vp/cell of Ad5, Ad35, Ad26, or Ad48 vectors and cytokine responses measured after 24h by luminex assays. LPS (1 ng/mL) was used as the positive control. Data are represented as individual measurements with mean responses indicated by a solid line ± SEM. Bars indicate p<0.05 (dotted line), p<0.01 (dashed line), and p<0.001 (solid line) using Kruskal-Wallis tests with Dunn’s correction for multiple comparisons. (B) Mean group fold induction of all cytokine responses relative to media by various Ad vectors in human PBMC.

**Figure 4.** Cytokine and chemokine responses elicited by chimeric Ad5/Ad35 vectors in human PBMC. Normal human PBMC (n=8/group) were stimulated for 24h in the presence of chimeric Ad5/Ad35 vectors, and cytokine and chemokine levels were measured by luminex assays. LPS (1 ng/mL) was included as the positive control. Data are represented as individual measurements with mean responses ± SEM. Bars indicate p<0.05 (dotted line), p<0.01 (dashed line), and p<0.001 (solid line) using Kruskal-Wallis tests with Dunn’s correction for multiple comparisons.

**Figure 5.** Interferon induction by Ad35 and Ad26 in the presence of anti-CAR or anti-CD46 blocking mAbs. Normal human PBMC (n=4-6/group) were preincubated with 10 μg/mL anti-CAR (RmcB) or anti-CD46 (13/42, M177) for 1 h and then stimulated with $10^3$ vp/cell Ad35 or Ad26 vectors. IFN-α2 and IFN-γ levels were measured 24h following stimulation by luminex assays. LPS (1 ng/mL) was included the positive control. Data are represented as mean responses ± SEM. Bars indicate p<0.05 (dotted line), p<0.01 (dashed line), and p<0.001 (solid line) using Kruskal-Wallis tests with Dunn’s correction for multiple comparisons.
Figure 6. Cytokine and chemokine responses elicited by Ad vectors in normal human PBMC depleted of various cell populations. Normal human PBMC (n=6/group) were depleted of the indicated cell subsets by magnetic bead separation, and depletion was confirmed by flow cytometry (data not shown). PBMCs were then stimulated with 10^3 vp/cell of Ad35 or Ad26 vectors and cytokine and chemokine responses were measured after 24 h stimulation by luminex assays. LPS (1 ng/mL) was included as positive control. Data are represented as means ± SEM, and unseparated versus depleted cell populations were compared. Bars indicate p<0.05 (dotted line), p<0.01 (dashed line), and p<0.001 (solid line) Kruskal-Wallis tests with Dunn’s correction for multiple comparisons.
Figure 1A
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Figure 2
Figure 5
Figure 6