Late Endosomal Trafficking of Alternative Serotype Adenovirus Vaccine Vectors Augments Antiviral Innate Immunity

Jeffrey E. Teigler¹, Jonathan C. Kagan², and Dan H. Barouch¹,³

¹Center for Virology and Vaccine Research, Beth Israel Deaconess Medical Center, Boston, MA, USA; ²Department of Pediatrics, Harvard Medical School, Boston, MA, USA; ³Ragon Institute of MGH, MIT, and Harvard, Boston, MA, USA

Running Title: Adenovirus Vector Trafficking and Innate Immunity

Corresponding author: Dan H. Barouch, dbarouch@bidmc.harvard.edu
Adenovirus (Ad) vaccine vectors have found widespread use as vaccine platforms against multiple infections and cancers, and multiple serotypes have been shown to differ significantly in their biologic properties and immune phenotypes. Our laboratory and others have previously described differential innate immune stimulation elicited by various Ad serotypes. Here we show that Ad5 traffics rapidly to the nucleus following infection, whereas Ad35 and Ad26 accumulate in late endosomes between 2-8 hours post-infection. Innate immune cytokine elicitation by all Ad serotypes was abrogated by blockade of endosomal acidification, Cathepsin B and Caspase-1, suggesting that virus interactions with acid-dependent sensors such as Toll-like receptor- and cathepsin-dependent inflammasome activation in late endosomes may trigger innate immunity. These data suggest a mechanism by which Ad vectors from various serotypes differentially trigger innate antiviral pathways via distinct intracellular trafficking to late endosomes.
Adenoviruses (Ad) are widely used for applications of vaccination and gene therapy. Importantly, Ad vectors have been shown to differ significantly in their innate immune profiles both *in vivo* and *in vitro*. The molecular mechanism which underlies these observed differences has important implications for the development of improved vaccines. In this study, we propose a mechanism in which the degree of late endosomal trafficking of Ad vectors results in differential stimulation of late endosomal pattern recognition receptors.
INTRODUCTION

Given the limitations that have become evident with adenovirus serotype 5 (Ad5) vectors, alternative serotype Ad vectors have been developed from serotypes against which high-level humoral responses are less common in the global population, including Ad35 and Ad26 (1–3). Interestingly, in addition to circumventing high levels of Ad5 neutralizing antibodies, alternative serotype Ad vectors differ substantially from Ad5 in the phenotypes of innate and adaptive immune responses elicited by vaccination (1, 4, 5). Moreover, several alternative serotype Ad vector-based vaccines confer increased protection relative to Ad5 against pathogenic SIVmac251 challenges in rhesus monkeys (4, 6, 7).

Innate immune stimulation is a critical determinant for establishing the magnitude and phenotype of adaptive immune responses (8–11). Previously, our group and others have shown that Ad vectors differ markedly in their innate immune stimulatory properties (12, 13). In particular, Ad35 and Ad26 induce significantly higher levels of antiviral and proinflammatory cytokines and chemokines in serum from vaccinated rhesus monkeys and in fresh human PBMC as compared with Ad5 (12). However, the mechanism underlying these observed differences has not previously been determined.

Results from several groups have suggested that Toll-like receptor 9 (TLR9), late endosomal lysis, and/or differences in tropism may contribute to innate stimulation by Ad vectors (14–17). Previous reports utilizing a variety of cell systems have also shown that Ad vectors can utilize different endosomal trafficking pathways such as clathrin-mediated endocytosis or macropinocytosis, and they may exit either early or late endosomes (18–24). We therefore hypothesized that differential Ad vector trafficking and access to late endosomal pattern recognition receptors (PRR) might account for the differential innate stimulation elicited by these vectors.

In this study, we show that Ad35 and Ad26 accumulate in the late endosomal compartment more extensively than Ad5 at 2-8 hours following infection. Innate immune
stimulation by all Ad vectors was sensitive to inhibitors of endosomal acidification, Cathepsin B, and Caspase-1. Thus, whereas Ad vectors stimulate common innate immune sensing pathways in the late endosome, marked differences in intracellular trafficking and access to this late endosomal compartment may account for the different innate immune phenotypes elicited by these Ad vectors.
MATERIALS AND METHODS

Viruses. E1/E3-deleted replication incompetent Ad5, Ad35, Ad26, Ad35k5, and Ad5f35 vectors expressing no transgene and eGFP were generated as previously described (25)(1). Briefly, viruses were produced in E1-complementing PER.55K cells and purified by CsCl gradient ultracentrifugation.

Cells. A549 cells (ATCC) were serially passaged in Dulbecco’s Eagle’s Modified Medium supplemented with 10% Fetal Calf Serum (FCS) and grown at 37°C, 5% CO₂. Normal human blood was collected in the presence of sodium heparin and PBMC isolated by the Ficoll-hypaque density gradient method (26). Cells were resuspended at a concentration of 1x10⁶ cells/mL in R10 medium (RPMI, 10% Fetal Calf Serum (FCS), 50U/mL penicillin, 50μg/mL streptomycin) and further utilized in in vitro assays.

Chemicals. The endosomal inhibitor Bafilomycin A1 (Enzo Life Sciences, Farmingdale, NY), Cathepsin inhibitors Ca074-Me, CAA0225, Cathepsin G Inhibitor (Millipore, Billerica, MA), and Z-FA (R&D Systems, Minneapolis, MN), and the Caspase inhibitors Z-LEHD, Z-WEHD, Z-YVAD, and Z-VAD (R&D Systems, Minneapolis, MN) were dissolved in sterile DMSO. Endosomal acidification inhibitors Chloroquine (Sigma-Aldrich, St. Louis, MO), and Ammonium chloride (Sigma-Aldrich, St. Louis, MO) were reconstituted in sterile deionized water.

Concentrations of inhibitors utilized were determined from prior literature or by titration for function and cellular viability.

In Vitro Stimulation Assay. For all reactions 1x10⁶ PBMC were stimulated with 1,000 vp/cell of the indicated Ad vectors. Cells were treated with various chemical inhibitors prior to or following stimulation with Ad vectors. Cells were cultured at 37°C, 10% CO₂, and culture supernatants were harvested 24 following Ad vector stimulation by centrifugation at 1,400 rpm for 5 minutes. Supernatants were analyzed using the Millipore Milliplex MAP Magnetic Human Cytokine/Chemokine Panel (Millipore, Billerica, MA) according to manufacturer’s protocol. Luminex data were acquired on a BioPlex 200 instrument running BioPlex Manager v4.1 (Bio-
Rad, Hercules, CA) with an 80% to 120% standard acceptance range. Data were analyzed using GraphPad Prism v5.0. Means were compared between groups using Kruskal-Wallis Tests and plotted as means and standard error of the mean (S.E.M.).

**Fluorescent Labeling of Adenoviral Vectors.** Adenoviral vectors were fluorescently labeled with the NHS-ester of Atto633 (Atto-tec GmbH, Siegen, Germany) as previously described (20). Briefly, Ad vectors were incubated for 10-20 minutes at RT with Atto633 and washed 3x at 4°C with ice-cold PBS containing 5% Sucrose (w/v) by centrifugation at 3,000 rpm for 30 min in Amicon 10,000kDa molecular weight centrifuge concentration vials (Millipore, Billerica, MA). Atto633:Capsomere ratio was determined using the extinction coefficient of Atto633 and Abs$_{260}$. All preparations had ratios between 0.5 and 1.8 and displayed no defect in cellular binding or trafficking.

**Infection of A549 Cells with Atto633-Labeled Ad Vectors.** 15,000 A549 cells were plated onto No.1 German Glass 15mm Coverslips (BD Biosciences, San Jose, CA) and incubated O/N at 37°C, 5% CO$_2$. Cells were placed on ice, washed 2x with ice-cold Phosphate Buffered Saline (PBS), and overlaid with 5,000-50,000 vp/cell of indicated Atto633-labeled Ad vectors diluted in unsupplemented DMEM. Cells were incubated on ice with Ad vector for 90 min, with gentle rocking every 15 min. Cells were washed 2x with ice-cold PBS and viral infection induced with overlay of 37°C pre-warmed DMEM supplemented with 10% FCS. Cells were cultured at 37°C, 10% CO$_2$. At indicated timepoints, cells were fixed by immersion into ice-cold 4% Paraformaldehyde diluted in PBS for 10 min. Cells were washed 2x with ice-cold PBS and permeabilized by immersion in 0.01%Saponin diluted in PBS supplemented with 5% FCS for 15min. Cells were washed 3x in ice-cold PBS and stored for further use.

**Immunofluorescence Histochemistry.** Cells were stained by overlay onto a drop of the indicated antibodies at the indicated concentrations diluted in PBS supplemented with 5% FCS in a humidified chamber protected from light. Primary antibodies were incubated for 1 hour and secondary antibodies were incubated for 30 min. All staining steps were followed by 3x washes.
with 1 mL ice-cold PBS supplemented with 5% FCS. Antibodies and concentrations used were Rabbit anti-LAMP1 (1:1000) (Abcam, Cambridge, MA), Mouse anti-EEA1 (1:500) (BD Biosciences, San Jose, CA), Mouse anti M6P (1:100) (Abcam, Cambridge, MA), Goat anti-Mouse IgG (2μg/mL), and Donkey anti-Rabbit IgG (2μg/mL) (Life Technologies, Grand Island, NY). Following staining, coverslips were inverted onto a drop of ProLong Gold containing DAPI mounting medium (Life Technologies, Grand Island, NY) on microscope slides, were allowed to cure overnight at room temperature, and were stored at -20°C for future use.

Confocal Microscopy. Slides were analyzed at the Beth Israel Deaconess Medical Center Imaging Core on a Zeiss LSM Meta with a 10x optical magnification and a Zeiss 63x oil-immersion lens. Images were acquired using Zeiss LSM 510(Carl Zeiss GmbH, Jena, Germany) in the Z-stack setting with optical slices taken at a width of 0.5μm and an interval of 0.3μm. For each virus, timepoint, and endosomal marker, at least 3x coverage of 2-7 independent experiments were performed.

Image Processing and Analysis. Images were processed utilizing Volocity Software (Perkin-Elmer, Waltham, MA). Virions were detected as particles of intensity >3x standard deviation of image at least 0.078 μm³. Endosomal compartments were measured as positive by signal >3x standard deviation of image. Cells were drawn manually as Regions of Interest (ROI) and virion colocalization within ROI’s was measured by Manzel correlation coefficient of 0.5 or greater. Virion colocalization with a given compartment was calculated on a per ROI basis as a percentage of total individual ROI virions. Image figures were generated using Fiji package for ImageJ. All images for a given endosomal marker or virus were adjusted identically for brightness and contrast for the purpose of image printing, and images utilized for Volocity analysis were unaltered prior to analysis. Colocalization of viruses with a given compartment was analyzed by GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA), and differences in colocalization between viruses was assessed by Student’s t tests.
Data Analysis and Statistics. Cytokine and chemokine levels were assessed from Luminex assays as determined using a 5-parameter logistic model. Levels of cytokines and chemokines for inhibitor treated and untreated cells was assessed by Kruskal-Wallis tests. Colocalized virions/cell was calculated for each endosomal marker and virus analyzed and colocalization amounts were compared between viruses by Student’s t tests.
RESULTS

Ad35 and Ad26 accumulate to higher levels than Ad5 in the late endosomal compartment at 2-8 hours following infection. To assess the intracellular trafficking pathways for Ad5, Ad35, and Ad26, vectors were covalently labeled with the NHS-ester of Atto633 and tested for viability as previously described (20). Briefly, A549 cells were infected with an MOI of 5,000-50,000 viral particles (vp)/cell of fluorescently-labeled Ad vector, and colocalization was measured over time by confocal microscopy for the early endosome marker Early Endosome Antigen 1 (EEA1) or the late endosomal markers Lysosomal-Associated Membrane Protein 1 (LAMP1) or Mannose-6-Phosphate Receptor (M6P) by immunofluorescence histochemistry, and nuclei were labeled by DAPI (27–29). Viruses were measured for colocalization in a given endosomal/nuclear compartment by calculation of Manzel correlation coefficient, and differences between vector localization at a given time point were determined by Student’s t tests.

Ad5 virions rapidly accumulated in the peri-nuclear region, with 72.6% of virions colocalizing with nuclear staining by 2 h post-infection (Figure 1A, 1B). Colocalization of Ad5 with the early endosomal marker EEA1 occurred only minimally and never exceeded 7% at any timepoint measured (Figure 1A). Ad5 virions also localized to minor levels with late endosomes, as indicated by low levels of colocalization with LAMP1 or M6P at 2 h post infection (4.0% and 2.7% colocalization, respectively) (Figure 1A, 1B). These results indicate that Ad5 rapidly trafficked to the nucleus and only minimally accumulated in early and late endosomal compartments.

Ad35 and Ad26 exhibited marked differences in intracellular trafficking pathways as compared with Ad5. Ad35 and Ad26 displayed substantial but transient colocalization with the early endosomal marker EEA1 at 30 min (18.5% and 45.5% colocalization, respectively) and at 1 h (11.9% and 19.9% colocalization, respectively) post infection (Figure 1A). The colocalization of Ad35 and Ad26 with early endosomes was greater than that observed for Ad5 at both 30 min
Student's t tests) and at 60 min (2.8x and 4.6x fold greater colocalization, respectively; p=0.0082, p=0.0048, respectively). At later time points, both Ad35 and Ad26 demonstrated substantial colocalization with the late endosomal marker LAMP1, with 12.6% and 25.7% of virions colocalizing with late endosomes at 2 h post-infection, respectively (Figure 1A, 1B). Ad35 and Ad26 also colocalized with the late endosomal marker M6P at 2 h post-infection (15.4% and 26.2% of virions, respectively) (Figure 1A, 1B). Analysis of adherent PBMC, which include multiple cellular subsets (30), showed that Ad35 and Ad26 similarly colocalized with late endosomes by 2h post-infection (data not shown). Both Ad35 and Ad26 accumulated in the late endosomal compartment to significantly higher levels than Ad5 at 2 h post infection as measured by both LAMP1 (3.1x and 6.4x fold greater colocalization, respectively; p=0.0088, p=0.0001, respectively) and M6P (5.8x and 9.8x fold greater colocalization, respectively; p<0.0001, both) colocalization. This association of Ad35 and Ad26 in the late endosomes persisted for all time points observed between 2-8 h post-infection. Taken together, these results indicate that Ad35 and Ad26 display a markedly different intracellular trafficking pattern as compared with Ad5, characterized by substantially greater association with late endosomes 2-8 hours post-infection.

Ad Innate immune stimulation is blocked by inhibitors of late endosomal acidification. Ad35 and Ad26 induce markedly higher levels of antiviral and proinflammatory cytokines and chemokines, including IFN-α, IFN-γ, IP-10, IL-6, and IL-1RA, than Ad5 in serum from vaccinated rhesus monkeys and in human PBMC (12). Interestingly, these differences in innate immune cytokines and chemokines corresponded with the observed levels of Ad vector trafficking to the late endosome. To probe the possible late endosomal innate sensing pathways the different Ad vectors may activate, we assessed the dependence of innate triggering by these vectors to late endosomal acidification. Fresh human PBMC (n=4/group) were isolated and pre-incubated with...
the endosomal acidification inhibitors Bafilomycin A1 (BafA), chloroquine, or ammonium chloride for 1 h prior to infection with 1,000 vp/cell of Ad5, Ad35, or Ad26 (31, 32). Cytokine induction was measured 24 h following infection by Luminex assays as previously described (12).

Pre-incubation of human PBMC with BafA, chloroquine, or ammonium chloride markedly decreased induction of innate cytokines and chemokines by all the Ad vectors studied (Figure 2). This inhibition was most pronounced for IFN-α2 with pre-incubation of PBMC with BafA, chloroquine, or ammonium chloride, leading to reduced levels of IFNα2 upon stimulation with Ad5, Ad35, or Ad26 (Figure 2). Activity of inhibitors on intracellular innate immune signaling was confirmed by the inhibition of intracellular Poly I:C sensing but not extracellular LPS sensing by all three endosomal acidification inhibitors. Together, these data suggest that innate stimulation by all three vectors was sensitive to inhibitors of late endosomal acidification.

Innate immune stimulation by Ad5, Ad35, and Ad26 is sensitive to Cathepsin B, Cathepsin L, and pan-Cathepsin inhibition. We next evaluated the role of specific cathepsins in Ad innate sensing. Cathepsins are a group of late endosomal papain-like proteases which serve several important roles in late endosomal cargo processing as well as autophagy and apoptosis (33). Fresh human PBMC (n=4/group) were pre-incubated for 1 h with the Cathepsin B inhibitor Ca074-Me, Cathepsin L inhibitor CAA0225, a Cathepsin G inhibitor, the pan-Caspase inhibitor Z-VAD, the pan-Cathepsin inhibitor Z-FA, or a DMSO vehicle control. Cells were then stimulated with 1,000 vp/cell of Ad5, Ad35, or Ad26. Elicited cytokine and chemokine levels were analyzed 24 h post-infection by Luminex assays as described above.

Human PBMC pre-incubated with Ca074-Me, CAA0225, or Z-FA exhibited reduced levels of multiple cytokines and chemokines produced in response to Ad vectors. In particular, levels of IFN-α2, IFN-γ, MIP-1β, and TNF-α were significantly reduced following pre-incubation of human PBMC with these cathepsin inhibitors in response to Ad35 and Ad26 relative to DMSO vehicle control, and similar trends were observed for Ad5 (Figure 3). Pre-incubation with
a Cathepsin G inhibitor, however, had minor effects on cytokines and chemokines elicited by Ad35 and Ad26 (Figure 3). These results suggest that Ad35 and Ad26, and to a lesser extent Ad5, are inhibited by Cathepsin B and pan-Cathepsin inhibition, suggesting the functional relevance of late endosomal localization to triggering innate immunity.

**Inhibition of Caspase-1 reduces innate immune stimulation elicited by Ad5, Ad35, and Ad26.** Caspases are proteases that serve as major components of pro-apoptotic and pro-inflammatory pathways (34). To assess the role of caspases in Ad innate stimulation, fresh human PBMC (n=3-4/group) were stimulated with Ad5, Ad35, or Ad26 following pre-incubation for 1 h with the Caspase-9 inhibitor Z-LEHD, the Caspase-1 inhibitor Z-WEHD, the Caspase-4 inhibitor Z-YVAD, or DMSO vehicle control. Pre-incubation of human PBMC with Z-WEHD led to a marked reduction of IFN-α2 and IFN-γ elicited by Ad35 and Ad26 (Figure 4). In contrast, pre-incubation of human PBMC with the Caspase-9 inhibitor Z-LEHD or the Caspase-4 inhibitor Z-YVAD had little to no effect on the innate cytokine stimulation by Ad5, Ad35, and Ad26. Similar to experiments performed with Cathepsin inhibitors, overall levels of cytokines and chemokines elicited by Ad5 were lower than Ad35 and Ad26, yet remained sensitive to Z-WEHD inhibition. These data suggest a role for Caspase-1 in the induction of immune responses by these Ad vectors.

**Innate stimulation by Ad5, Ad35, and Ad26 is initiated between 0-6 hours post infection.** To determine the timing of the initial triggering of the innate sensing pathways by Ad vectors, fresh human PBMC were stimulated with 1,000 vp/cell of Ad5, Ad35, or Ad26. At 0, 4, 6, 8, or 12 h post-infection, cells were treated with chloroquine, ammonium chloride, Ca074-Me, CAA0225, or Z-FA at the concentrations described above. Cytokine and chemokine levels were assessed 24 h following infection by Luminex assays and compared to infected cells with mock inhibition.
Addition of inhibitors at the time of infection (0 h) effectively inhibited induction of IFN-α2, IFN-γ, and TNF-α (Figure 5), in agreement with the previous experiments (Figures 2-4). For all three vectors, the majority of inhibition of innate sensing by chloroquine and Z-FA occurred earlier than 6 h post-infection, with the most prominent inhibition caused by Z-FA occurring up to 12 h post-infection (Figure 5). Interestingly, inhibition of cytokine responses elicited by Ad26, and to a lesser extent by Ad5, was sensitive to inhibition up to 6 h post infection, while those elicited in response to Ad35 were able to be significantly inhibited up to 4 h post infection. Taken together, these data indicate that innate immune sensing of Ad5, Ad35, and Ad26 was initiated between 0-6 h post-infection. These findings are consistent with the possibility that localization of Ad vectors to the late endosome is important for Ad innate sensing, as inhibition of innate immune sensors reduced Ad innate immune stimulation during times at which Ad vectors were observed to be present in late endosomes.

**Chemical inhibition of innate immune stimulation does not perturb transduction efficiency.** We next investigated whether chemical inhibition of Ad innate immune stimulation was simply due to blockade of Ad infectivity. To test whether chemical inhibitors had an effect on Ad35 or Ad26 transduction efficiency, human PBMC (n=4/group) were treated at the time of infection with chloroquine, ammonium chloride, Ca074Me, CAA0225, Z-FA, or DMSO vehicle control as described above. These cells were then stimulated with 1,000 vp/cell of Ad35 or Ad26 expressing eGFP. Levels of elicited cytokines and chemokines were measured 24 h post infection by Luminex assays, and levels of transduced cells were measured by flow cytometry.

Similar to our results above, chloroquine, ammonium chloride, Ca074-Me, CAA0225, and Z-FA reduced cytokine and chemokine induction in human PBMC elicited by Ad35 or Ad26 expressing eGFP at 24 h post infection (data not shown). Flow cytometry on stimulated cells showed no inhibition of transduction of PBMC by either Ad35 or Ad26 for any of the chemical inhibitors analyzed, as measured by %eGFP positive cells 24 h post-infection (Figure 6). These
results show that chemical inhibition of Ad35 and Ad26 innate sensing did not substantially reduce vector infectivity.

**Both Ad fiber and capsid components influence intracellular trafficking pathways.** Both fiber and capsid components of Ad5 and Ad35 can influence innate stimulatory properties of Ad vectors (12). In particular, both Ad35 fiber and capsid components were required to confer the increased innate stimulatory properties of Ad35 relative to Ad5. We sought to determine the influence of Ad fiber and capsid components on access to the late endosomal compartment and the associated Ad innate sensing mechanisms. To explore the influence of the Ad fiber and capsid on Ad intracellular trafficking, we utilized Ad35k5, a chimeric Ad35 with its fiber knob domain replaced with that of Ad5, and Ad5f35, a chimeric Ad5 with its fiber protein replaced with that of Ad35. A549 cells were infected with Ad35k5 and Ad5f35 vectors and subsequently processed for immunofluorescence histochemistry and confocal microscopy as described in Figure 1.

Ad5f35 and Ad35k5 both displayed an initial colocalization with the EEA1 compartment at 30 min (32.9% and 20.2%, respectively) and 60 min (21.4% and 22.4%, respectively), similar to that observed with Ad35. Furthermore, both Ad5f35 and Ad35k5 displayed increased late endosomal trafficking beginning at 2 h post infection, as indicated by higher colocalization relative to Ad5 at 2 h for both LAMP1 (2.8x and 5.9x fold greater colocalization relative to Ad5, respectively; p=0.0215 and p<0.0001, respectively; Students’ t tests) and M6P (5.6x and 8.8x higher colocalization, respectively; p=0.0003 and p<0.0001, respectively) (Figure 7). These data indicate that both fiber and capsid components of Ad vectors influence intracellular trafficking patterns, and suggest increased late endosomal association as a mechanism for the previously observed increase in innate cytokine and chemokine stimulation by both Ad5f35 and Ad35k5 relative to Ad5 (12).
In this study, we show that Ad5, Ad35, and Ad26 differ substantially in their intracellular trafficking patterns, with Ad35 and Ad26 accumulating to a greater degree than Ad5 in late endosomes, and Ad5 rapidly trafficking to the nucleus. All three vectors were sensitive to chemical inhibitors of late endosomal innate immune sensing, in particular endosomal acidification, cathepsin B, and caspase-1. These studies suggest a mechanism that could account for the differential innate immune stimulatory phenotypes of Ad vectors in which intracellular trafficking to the late endosome contributes to these observed differences in innate immunity (12, 13) (Figure 8). In the late endosome, virions stimulate stereotypical innate immune pathways that require endosomal acidification, cathepsins, and caspase-1 (Figure 8).

Taken together, these data suggest that intracellular Ad vector trafficking critically influences innate immune stimulation by various Ad serotypes. Innate immune stimulation elicited in response to all three Ad vectors was sensitive to inhibition of acidification, cathepsins, and caspase-1, suggesting that accumulation in late endosomes, rather than differential Pattern Recognition Receptor (PRR) stimulation per se, may be responsible for the observed differences in innate immune phenotypes elicited by these vectors. The requirement of endosomal acidification for innate sensing suggests that late endosomal TLRs may be the endosomal acidification-dependent portion of the sensing pathway (Figures 2, 8). For example, TLR9 has been previously implicated in Ad vector dsDNA genome sensing, and TLR9 requires endosomal acidification for its function (14, 35–38). Additionally, late endosomal lysis and Cathepsin B release into the cytosol activates the NALP3 inflammasome, and Cathepsin B activity is required for this process (39). The sensitivity of Ad vector innate stimulation to both the Cathepsin B inhibitor Ca074-Me as well as the pan-Cathepsin inhibitor Z-FA for an extended period following Ad endocytosis temporally is consistent with our observation that Ad35 and Ad26 vectors potentially begin exiting the late endosome at 2-6 hours post-infection (Figures 1A, 5). Additionally, the fact that Ad vector

sensing was sensitive to Cathepsin inhibitors for up to 6 h following infection indicates that a defect in endosomal TLR processing is unlikely to account for the reduced innate immune stimulation observed. Of note, recent studies have suggested the importance of the cytosolic DNA sensor cGAS for innate sensing of Ad5 in murine cells (40). The potential influence of cGAS on Ad innate immune sensing as well as putative differences between Ad5, Ad35, and Ad26 on cGAS stimulation will therefore be of interest for further understanding of Ad innate immune sensing. Importantly, our data confirm prior observations of the importance of acid-dependent innate immune sensors and inflammasome activation, and extend these findings by suggesting that differential trafficking of several Ad serotypes currently in pre-clinical and clinical development to late endosomal compartments contributes to their innate immune stimulatory phenotypes. Importantly, these data also suggest, in agreement with previous studies showing Ad vector innate sensing is redundant in vivo, that several pattern recognition receptors are likely important for Ad vector sensing (13, 16, 41).

Our studies utilized A549 cells and human PBMC for the analysis of Ad vector trafficking, and human PBMC for the assessment of innate immune phenotypes (12, 18, 22, 23, 42). A limitation of studying vector trafficking in A549 cells is that they are more homogeneous than the diverse cellular subsets in PBMC. We previously showed that the innate immune cytokines and chemokines elicited by Ad vectors is dependent on several PBMC subsets (12). Due to the bulk nature of the measurements of innate stimulation performed on PBMC, it is possible that inhibition of particular cellular subsets may be responsible for the results observed. As such, the chemical inhibitor data shown above cannot formally differentiate between induction of common sensing pathways in all PBMC subsets or triggering of different innate sensing pathways in different PBMC subsets. Nevertheless, our results strongly suggest that important innate signaling pathways likely involve late endosomes.

Both the fiber as well as other capsid components appear to contribute to the innate stimulatory properties of Ad vectors, suggesting roles of receptor binding proteins as well as
acid-dependent capsid protease components in determining Ad innate immune stimulatory phenotypes (12). Interestingly CD46-binding by the fiber protein appears to be sufficient to confer late endosomal trafficking on Ad vectors (22). These data suggest that Ad innate immune stimulation phenotypes observed in previous studies may be reflective of increased late endosomal trafficking conferred by the fiber rather than primary receptor usage (15, 17, 37). Furthermore, the increased innate stimulatory capacity of Ad5f35 relative to Ad35k5, despite their roughly equivalent ability to access late endosomal compartments, suggests an active role of CD46 in influencing Ad vector innate immune stimulation, a possibility which warrants further investigation.

In summary, our data suggest a potential mechanism by which Ad5, Ad35, and Ad26 vectors differentially stimulate innate immunity. These vectors trigger similar innate immune pathways, but the extent of stimulation is driven by differential intracellular trafficking to late endosomes, resulting in profound differences in innate immunity. These results have important implications for the development of vaccine and gene therapy vectors and could lead to strategies for rational vector design that specifically tune desired innate immune phenotypes.
ACKNOWLEDGEMENTS

We thank S. Blackmore, E. Borducci, A. McNally, L. Parenteau, J. Smith, and K. Stanley for technical assistance. We thank D. Cureton, L. Ang, and Y. Zheng, and the Imaging Core at BIDMC for confocal microscopy expertise and advice. We acknowledge support from the National Institutes of Health (AI078526, AI096040), the Bill and Melinda Gates Foundation (OPP1033091, OPP1040741), and the Ragon Institute of MGH, MIT, and Harvard.
REFERENCES


**FIGURE LEGENDS**

**Figure 1.** Ad35 and Ad26 accumulate more extensively than Ad5 in the late endosomal compartment. A549 cells were cold synchronized for endocytosis and infected with 5,000-50,000 vp/cell of Atto633-labeled Ad5, Ad35, or Ad26. Cells were fixed in 4% paraformaldehyde at various time points post infection and stained for Nucleus by DAPI and Early Endosomal Antigen 1 (EEA1), Lysosomal-associated membrane protein 1 (LAMP1), or Mannose-6-phosphate receptor (M6P) by immunofluorescence histochemistry. Cells were imaged at 630x magnification by confocal microscopy with z-stack slice depth of 0.5 μm and slice interval of 0.3μm. A.) Percentage Ad5, Ad35, and Ad26 virions colocalized with Nuclei, EEA1, LAMP1, and M6P per cell following synchronized infection. Z-stack images (n=2-7 replicate experiments) analyzed for colocalization utilizing Volocity image analysis software. Compartments identified as >3x image standard deviation, and virions identified as particles >0.078 μm³ with intensity >3x image standard deviation. Virions colocalization with compartments measured using Manzel correlation coefficients of >0.5. Symbols indicate timepoints where both Ad35 and Ad26 colocalization values were both significantly different than Ad5, with the less significant value indicated (*p<0.01, *p<0.001; Student’s t tests). B) Representative images of Ad5, Ad35, and Ad26 subcellular localization 2 h post-infection, with subpanel region indicated by gray outline. Indicated virus shown in green and indicated endosomal compartment or nucleus shown in red, with areas of overlap shown as yellow. Images processed using Fiji, scale bar = 20μm.

**Figure 2.** Ad innate immune stimulation is blocked by inhibitors of late endosomal acidification. Fresh human PBMC (n=4/group) were pre-incubated for 1 h with bafilomycin A1 (BafA) (1 μM), chloroquine (200 μM), or ammonium chloride (1 mM) prior to infection with 1,000 vp/cell of Ad5, Ad35, or Ad26. Induced cytokines and chemokines were measured 24 h post-infection by Luminex assays. Data shown as mean response of individual donors ± SEM. Bars indicate p=0.0286, Kruskal-Wallis Tests. High Molecular Weight (HMW) Poly I:C (Poly I:C) (10 μg/mL) and Lipopolysaccharide (LPS) (1 ng/mL) included as positive controls.
Figure 3. Ad innate immune stimulation is sensitive to Cathepsin B, Cathepsin L, and pan-Cathepsin inhibition. Fresh human PBMC (n=4/group) were isolated as described above and pre-incubated for 1 h with the Cathepsin B inhibitor Ca074-Me (10 μM), the Cathepsin L inhibitor CAA0225 (10 μM), a Cathepsin G inhibitor (10 μM), the pan-Caspase inhibitor Z-VAD (100 μM), the pan-Cathepsin inhibitor Z-FA (100 μM), or a DMSO vehicle control. Cells were stimulated with 1,000 vp/cell of Ad5, Ad35, or Ad26 and cytokines measured 24h post-infection by Luminex assays. Data shown as mean response of individual donors ± SEM. Bars indicate p=0.0286, Kruskal-Wallis Tests.

Figure 4. Ad innate immune stimulation is reduced by inhibition of Caspase-1. Fresh human PBMC (n=3-4/group) were pre-incubated for 1 h with the Caspase-9 inhibitor Z-LEHD (100 μM), the Caspase-1 inhibitor Z-WEHD (100 μM), the Caspase-4 inhibitor Z-YVAD (100μM), or DMSO vehicle control. PBMC were then stimulated with 1,000 vp/cell of Ad5, Ad35, or Ad26 and elicited cytokine and chemokine levels were measured 24 h following infection by Luminex assays. Data shown as mean response of individual donors ± SEM. Bars indicate p=0.0286, Kruskal-Wallis tests.

Figure 5. Ad innate immune stimulation occurs with similar kinetics and is sensitive to chemical inhibitors from 0-6 hours post-infection. Fresh human PBMC (n=4/group) were isolated as described above and stimulated with 1,000 vp/cell of Ad5, Ad35, or Ad26. Chemical inhibitors Chloroquine, Ammonium chloride, Ca074-Me, CAA0225, or Z-FA were added at the indicated times following infection. Levels of elicited cytokines and chemokines were measured 24 h following infection by Luminex assays and compared to an infected, mock-inhibited control (Media).

Figure 6. Chemical inhibition of Ad5, Ad35, or Ad26 innate immune signaling does not block Ad transduction. Fresh human PBMC (n=4/group) were synchronously inhibited with chloroquine, ammonium chloride, Ca074-Me, CAA0225, Z-FA, or DMSO vehicle control at same
concentrations as above, and stimulated with 1,000 vp/cell of Ad35 or Ad26 expressing eGFP. 24 h following infection percent eGFP cellular transduction was measured by flow cytometry.

Figure 7. Both fiber and capsid components influence Ad vector intracellular trafficking patterns. A549 cells were incubated with 50,000 vp/cell of Atto-633 labeled Ad35k5 or Ad5f35 and fixed at indicated times post infection by immersion in 4% paraformaldehyde. Cells were stained for the indicated markers by immunohistochemistry and virion colocalization assessed as described above. Graphs indicate virions/cell colocalized with the indicated compartment at indicated timepoints with Ad5 and Ad35 data from Figure 1 shown as dashed lines for reference. Symbols indicate timepoints where both Ad35k5 and Ad5f35 colocalization values were both significantly different than Ad5, with the less significant value indicated (*p<0.01, "p<0.001; Student’s t tests).

Figure 8. Proposed model of differential innate immune stimulation elicited by Ad5, Ad35, and Ad26. Ad5, Ad35, and Ad26 virions enter the cell through an EEA-positive early endosomal compartment. Upon entry to this location, the majority of Ad5 rapidly exits the endocytic pathway into the cytosol where it subsequently traffics to the nucleus by 60 min post-infection. Ad35 and Ad26, as well as a small subset of Ad5 virions, accumulate in the late endosomal compartment 2-6 h following infection. At approximately 2-6 h post-infection, late endosomal Ad virions exit the late endosome and traffic to the nucleus. Accumulation of Ad virions in late endosomes and their release into the cytosol stimulates both an endosomal acidification-dependent as well as a cathepsin-dependent sensor.
Figure 1A

Virions Colocalized / Cell (%) vs Minutes Post Infection

- Nuclear
- EEA1
- LAMP1
- M6P

Ad5, Ad35, Ad26
Figure 1B

B

<table>
<thead>
<tr>
<th></th>
<th>Nucleus</th>
<th>LAMP1</th>
<th>M6P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad35</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad26</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4

![Graphs showing cytokine expression levels](image-url)
Figure 5

The graph shows the levels of various cytokines (IFNα2, IFNγ, IL-1RA, TNFα) over time post infection for Ad5, Ad35, and Ad26. The x-axis represents time post infection (h), while the y-axis represents the concentration in pg/mL. Different lines represent different treatments or conditions, with distinct markers for each.

Key:
- Red: Chioropamine
- Blue: NMDA
- Green: CaMKII-Mc
- Orange: CAAX235
- Purple: ZPA