T-cell-biased immune responses generated by a mucosally targeted adenovirus- σ 1 vaccine

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As most pathogens enter through the mucosa, it is important to develop vaccines that induce mucosal immunity. To this end, we generated a novel adenovirus (Ad) vaccine that displays the σ 1 protein from reovirus to target junctional adhesion molecule 1 and sialic acid. Replication-defective Ad5 vectors were modified by replacement of the Ad fiber protein with σ 1 (T3D σ 1) protein of reovirus T3D in previous work. Ad5 and Ad5- σ 1 were compared in mouse models for gene delivery and vaccination to monitor cytokine, antibody, and T-cell responses. The viruses were also tested for the ability to transduce and mature dendritic cells. Ad5- σ 1 was 40-fold less efficient at gene delivery *in vivo*, yet it was capable of inducing equal or greater cellular immune responses and systemic interferon- γ levels than Ad5 after intranasal administration. Despite weaker gross transduction, intranasal administration of Ad5- σ 1 produced more green fluorescent protein-positive (GFP+) major histocompatibility complex class II (MHC II) cells in the draining lymph nodes, less GFP+/MHC II+ cells in the lungs, and mediated modestly better maturation of dendritic cells *in vitro*. These data suggest that targeting gene-based vaccination via the σ 1 protein may enhance the T-cell immune response, perhaps by skewing immune responses to encoded antigens.

INTRODUCTION

Most pathogens enter the body at mucosal surfaces. It has been estimated that as much as 90% of HIV-1 infections occur by sexual transmission. In these cases, infection is thought to occur at vaginal, rectal, and urethral mucosal surfaces (reviewed in ref. 1). Given that the mucosal surface is the predominant entry route for pathogens, there has been increasing interest in the development of vaccines that can generate robust antibody and cellular responses at mucosal surfaces (reviewed in ref. 2).

When vaccine strategies have been applied for mucosal immunization, they have in most cases taken advantage of the potential to expose one mucosal site to antigens and evoke responses at other mucosal sites. This unique biology of the mucosal immune system allows one to deliver vaccines to less invasive sites (e.g., nasally, orally, etc.) in order to elicit responses at sites that are less accessible, but more relevant for protection (e.g., for HIV, antibody and cellular immune responses in the vagina or rectum³). Although many vectors could in theory be used for mucosal immunization, not all can deliver genes into the

nasal-associated lymphoid tissue and gut-associated lymphoid tissue as efficiently as they do when applied for systemic immunization.

Adenoviruses (Ads) are potent gene delivery vectors that elicit both systemic and mucosal responses as gene-based vaccines. Relevant to mucosal vaccination, the most commonly used Ad serotype, Ad5, is a respiratory virus that would be expected to elicit potent mucosal immune responses. Ad5 uses a well-characterized pathway for internalization *in vitro*. Ad5 binds to the Coxsackie and Ad receptor (CAR) at the knob domain of the fiber.⁴ After fiber-mediated attachment of the virus, further binding of the penton base to cellular α V integrins mediates internalization.⁵ Surprisingly, Ad5's primary receptor, CAR, is actually sequestered on the basolateral surface of airway epithelial cells,⁶ making Ad5 less efficient at infecting the nasal passages and the lungs.^{6,7} Therefore, Ad5 may not be optimal for gene delivery to mucosal epithelial cells to produce antigens for cross-priming of mucosal immune responses. An alternate strategy for mucosal vaccination is to deliver antigens directly into mucosal dendritic cells (DCs).

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Figure 1 Adenovirus (Ad) genomes expressing wild-type and chimeric viral protein structures. The portion of fiber, the tail, responsible for docking into the penton base of the Ad capsid is shown as a gray box. The Ad5 and Ad5- σ 1 genomes expressing either luciferase (Luc) or HIV-1 HXB2 p55 gag genes are shown. CMV, cytomegalovirus; ITR, inverted terminal repeat.

Unfortunately, DCs do not express CAR and are poorly transduced by Ad5 virus.⁸

Reoviruses are nonenveloped RNA viruses that infect mammals at respiratory or gastric mucosa.⁹ Initial binding and infection are mediated by the σ 1 protein of T3D reovirus that binds to α -linked sialic acid and junctional adhesion molecule 1 (JAM1).⁹ In contrast to the Ad5 receptor CAR, sialic acid is expressed ubiquitously on all cells. Moreover, JAM1 is expressed on mucosal surfaces including Peyer's patches and is also highly expressed on DCs.⁸

Both Ad5 and reovirus have evolved fiber and σ 1 to bind their receptors. Surprisingly, these distinctly different viruses have separately evolved their receptor binding proteins with strikingly similar structures with shafts containing β -spiral repeats.¹⁰ The two proteins are both trimers with shaft- and knob (or head)-type structures. Ad5 fiber proteins have varied shaft lengths owing to different numbers of β -spiral repeats fused to a knob domain that binds the receptor. It has been observed that σ 1 has a shorter β -spiral repeat domain fused to an α -helical coiled-coil domain on its N terminus and its head or knob domain on its C terminus. This head domain binds to JAM1, whereas the shaft binds to sialic acid.

Given σ 1's structural homology to fiber and its tropism for mucosa, we previously engineered a chimeric Ad that displays the σ 1 protein, beginning at amino acid 18 of reovirus fused to the N-terminal 44 amino acids of fiber (ref. 8 and **Figure 1**). This original Ad- σ 1 chimeric virus (Ad5-T3D σ 1) was shown *in vitro* to retarget to both sialic acid and JAM1 and to no longer target CAR.⁸ Notably, this virus was able to more efficiently transduce DCs than Ad5 given that DCs express JAM1 and sialic acid rather than CAR. In this work, we have characterized the *in vivo* transduction and immunization activity of Ad5- σ 1 in mice.

RESULTS

In vivo transduction by Ad- σ 1

Mice were injected with 1×10^{10} virus particles (v.p.) of Ad5 and Ad5-T3D σ 1 viruses expressing luciferase-IRES-hrGFP. Mice were injected intramuscularly (IM) to represent vaccination into the systemic compartment. Mice were inoculated intranasally (IN) to represent a mucosal vaccination route. Under standard *in vivo* imaging conditions for luciferase activity, Ad5 transduction was readily observed. In contrast, Ad5- σ 1 was not observed (**Figure 2a**). Quantitation of *in vivo* luminescence revealed that

Ad5- σ 1 expression was 10-fold lower by the IM route and 40-fold lower by the IN route ($P < 0.01$ and < 0.001 , respectively; **Figure 2b**).

Antibody responses generated by Ad5 and Ad5- σ 1

Groups of 10 female BALB/c mice were inoculated by the IM and IN routes with 1×10^{10} v.p. of Ad5 and Ad5- σ 1 expressing HIV-1 HXB2 p55 gag to evaluate cellular and humoral immune responses (**Figure 3**). These data largely mimicked differences observed by luciferase imaging. By both routes, Ad5 generated markedly stronger immunoglobulin (Ig)G and IgA levels in the serum than Ad5- σ 1. Of note for mucosal vaccination, only the IN route of Ad5 inoculation generated detectable vaginal IgA and IgG antibodies against HIV-1 gag (**Figure 3b** and **d**). No HIV gag pseudovirus-particles were detected in the adenovirus preparations (**Figure S1**).

Cellular immune responses generated by vectors expressing HIV-1 gag

The mice that were inoculated above were killed 2 weeks after immunization and their splenocytes and cervical lymph nodes (LNs) were analyzed for T-cell responses by enzyme-linked immune spot (ELISPOT) (**Figure 4**). A major histocompatibility complex class I (MHC I)-restricted gag peptide was used to evaluate CD8 T- (cytotoxic T lymphocyte (CTL)) cell responses. A three-peptide pool was used to evaluate MHC II-restricted T helper (Th) cell responses. Under these conditions, Ad5- σ 1 generated surprisingly robust CTL and Th responses in the spleens of the mice by both routes of inoculation. By the IM route, Ad5- σ 1 generated equal CTL and Th cell numbers as Ad5 in the spleen (**Figure 4a**), despite the fact that both luciferase and gag antibody responses were 10-fold lower than those by Ad5 (**Figures 2** and **3**). This effect was even stronger by the mucosal IN route, where Ad5- σ 1 actually generated stronger Th cell responses than Ad5 ($P \leq 0.0001$) (**Figure 4c**) under conditions of 40-fold weaker transduction (**Figure 2**). Ad5 induced stronger CTL and Th responses in the LNs as compared with Ad5- σ 1 when delivered IM (**Figure 4b**). However, Ad5 and Ad5- σ 1 induced equivalent CTL and Th cellular responses in the LNs of mice immunized IN (**Figure 4d**).

Immune responses are amplified when Ad5 and Ad5- σ 1 are combined

These data indicated that Ad5- σ 1 was 10- to 40-fold less efficient at transduction. This lower bulk gene delivery correlated well with reduced antibody responses generated by Ad5- σ 1 and was consistent with humoral responses being tied to raw antigen production. While Ad5- σ 1 appeared weak at transduction, it generated surprisingly stronger Th responses under conditions of drastically reduced gene delivery.

Given their differing activities with Ad5 generating antibody responses and Ad5- σ 1 generating stronger Th responses, we hypothesized that Ad5- σ 1 might be able to boost anti-gag antibody responses from Ad5 by using the increased Th responses induced by Ad5- σ 1. To test this, groups of 10 mice

Figure 2 *In vivo* transduction. (a) Mice were immunized with Ad5 or Ad5-σ1 expressing luciferase intramuscularly (IM) or intranasally (IN) and imaged 24 h later. (b) Quantitation of emitted luminescence showed statistically significant differences in overall transduction and protein expression between Ad5 and Ad5-σ1 IM (** $P < 0.01$) and IN (** $P < 0.001$) immunized mice. Groups of five mice were used and error bars indicate standard error. Ad, adenovirus.

were immunized mucosally by the IN route with Ad5 and Ad5-σ1 in different combinations. Mice were immunized either with Ad5, Ad5-σ1, or a combination of both. If two viruses were used in combination, then 2.5×10^9 v.p. of each were mixed and delivered IN to each mouse. The single vector control for this combination was delivery of 2.5×10^9 v.p. of the single virus supplemented with 2.5×10^9 v.p. with Ad5 control virus (Ad5 Cx) that did not express HIV-1 HXB2 p55 gag. To compare the combination, groups of mice were also inoculated with 5×10^9 v.p. of virus to equalize the maximum total virus delivered in the combination group (Figure 5).

As seen previously, the Ad5-σ1 virus was unable to induce anti-gag humoral immune responses as compared with the Ad5 virus (Figure 5a). When Ad5 Cx virus was used in combination with Ad5 or Ad5-σ1, there was no additive effect. However, when Ad5 and Ad5-σ1 expressing gag were mixed together, there was a synergistic effect on antibody responses, resulting in greater anti-gag humoral immunity than the sum of the viruses when used alone ($P = 0.001$). This synergistic effect was not observed for cellular immune responses (Figure 5b). However, again Ad5-σ1 generated stronger Th responses than Ad5 ($P = 0.05$). In fact, the combination of Ad5 and Ad5-σ1 virus had an antagonistic effect on Th

cellular responses when compared with Ad5 or Ad5-σ1 alone ($P = 0.001$) (Figure 5b).

Ad5-σ1 generates stronger systemic IFN-γ levels than Ad5

The vaccination data above suggested that although Ad5-σ1 was weaker at raw gene transfer, it generated stronger T-cell responses with weak antibody responses. To test if this effect might be related to changes in Th responses, groups of 10 BALB/c mice were immunized IN with 1×10^{10} v.p. of Ad5 or Ad5-T3Dσ1-gag virus. Dulbecco's phosphate-buffered saline (PBS) was used as a negative control. Sera were collected 7 days after immunization during T-cell expansion and were analyzed for interleukin-4 (IL-4) and interferon-γ (IFN-γ) levels by enzyme-linked immunosorbent assay (ELISA) (Figure 6a). Under these conditions, increased IL-4 and IFN-γ levels were observed in the Ad-immunized mice, but not in buffer-treated animals. IL-4 levels were not statistically different between the Ad5 and Ad5-σ1 groups ($P = 0.5$). In contrast, IFN-γ levels were significantly higher in the Ad5-σ1 group than in the Ad5 group ($P = 0.02$ by two-tailed *T*-test). These data indicated that Ad5-σ1 induced stronger systemic IFN-γ responses after IN administration than Ad5.

Ad5 and Ad5- σ 1 generate similar Th1, Th2, and Th17 responses

The data above suggested differences in Th cell responses after IN immunization with the two vectors. To test if differences in

Th1, Th2, or Th17 responses were being provoked by the vectors, 10 BALB/c mice were immunized IN with 1×10^{10} v.p. of Ad5 or Ad5-T3D σ 1-gag virus and splenocytes were analyzed for T-cell responses by ELISPOT 14 days later (**Figure 6b**). Two

Figure 3 Humoral immune responses. Mice were immunized intramuscularly (IM) and intranasally (IN) with Ad5 or Ad5- σ 1 expressing HIV-1 HXB2 p55 gag. At 2 weeks after immunization, sera and vaginal washes were obtained. Anti-gag humoral immune responses were determined by ELISA. (a) Plasma and (b) vaginal anti-gag whole immune responses were determined. Mucosal anti-gag IgA immune responses in (c) plasma and (d) vaginal washes were determined. Groups of 10 mice were used and error bars indicate standard error. Ad, adenovirus; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; OD, optical density; PBS, phosphate-buffered saline.

Figure 4 Cellular immune responses. Mice were immunized intramuscularly (IM) and intranasally (IN) with Ad5 or Ad5- σ 1 expressing HIV-1 HXB2 p55 gag. At 2 weeks after immunization, splenocytes and lymphocytes from the cervical lymph nodes were obtained. Cellular immune responses were detected in the (a) spleens and (b) lymph nodes of mice immunized IM. CTL responses were significantly lower in mice immunized with Ad5- σ 1 ($P < 0.01$). Cellular immune responses were detected in the (c) spleens and (d) lymph nodes of mice immunized IN. Mice immunized with Ad5- σ 1 had significantly higher Th responses ($P < 0.0001$). Groups of 10 mice were used and error bars indicate standard error. Ad, adenovirus; CTL, cytotoxic T-lymphocyte; PBS, phosphate-buffered saline; SFC, spot-forming cells; Th, T helper.

Figure 5 Combined effects of Ad5 and Ad5- σ 1 on immune responses. Mice were immunized intranasally with Ad5 and Ad5- σ 1 expressing HIV-1 HXB2 p55 gag individually or in combination. Values on the X axis indicate the concentration of virus used to immunize the mice (0 = none; 1 = 2.5×10^9 v.p.; and 2 = 5×10^9 v.p.). Ad5 Cx does not express HIV-1 HXB2 p55 gag and is a control virus. (a) Ad5 in combination with Ad5- σ 1 induces a synergistic humoral immune response in which greater than additive effects are produced. (b) Cellular immune responses were not shown to improve when Ad5 and Ad5- σ 1 were used in combination. Groups of five mice were used and error bars indicate standard error. Ad, adenovirus; CTL, cytotoxic T-lymphocyte; OD, optical density; SFC, spot-forming cells; Th, T helper; v.p., viral particles.

pools of conserved overlapping 15-mer peptides spanning gag were used to ensure that all T-cell responses might be detected. Under these conditions, both Ad5 and Ad5- σ 1 generated no IL-4 and similar IFN- γ responses after stimulation by the peptide pools (Figure 6b). This contrasted with stronger responses generated by Ad- σ 1 by the single peptides used at higher concentrations (Figure 4). Interestingly, Ad5 generated low IL-17 responses that were somewhat higher than was generated by Ad- σ 1 ($P = 0.08$ by one-tailed *T*-test).

Maturation of immature human DCs by Ad5 and Ad5- σ 1

The data above suggested that Ad5- σ 1 might interact with the immune system differently than Ad5. DCs express JAM1 but not CAR, translating into more efficient transduction of human DCs by Ad- σ 1 than by Ad5.⁸ Given this, we tested if Ad5- σ 1 affects the maturation of immature human DCs. CD14⁺ cells were isolated from human blood and these were incubated with Ad5 or Ad5- σ 1. After 72 h, treated and untreated cells were stained for the maturation marker CD83 and were analyzed by flow

Figure 6 Th1/Th2/Th17 responses. Groups of 10 mice were immunized intranasally with 1×10^{10} v.p. Ad5 or Ad5-T3Dσ1-gag viruses. (a) Sera were collected and assayed for cytokine expression 7 days post-immunization. Interleukin-4 (IL-4) and interferon- γ (IFN- γ) cytokine were measured by ELISA. (b) Groups of five mice were immunized with 1×10^{10} v.p. Ad5 or Ad5-T3Dσ1-gag viruses. At 2 weeks after immunization, splenocytes were obtained. The splenocytes were stimulated with the B consensus gag peptides (NIH AIDS Research and Reference Reagent Program Catalog Number: 8117) in two separate pools. IFN- γ , IL-4, and IL-17 cellular immune responses were detected by ELISPOT. Error bars show standard error. * $P \leq 0.05$ by two-tailed *T*-test. Ad, adenovirus; DPBS, Dulbecco's phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immune spot; SFC, spot-forming cells; Th, T helper; v.p., viral particle.

cytometry (Figure 7a). CD83 levels on untreated cells remained low (mean fluorescence intensity = 7.99). In contrast, positive control cells treated with tumor necrosis factor- α and immunoreactive prostaglandin E2 produced 20-fold increases in CD83 levels (mean fluorescence intensity = 188.0). Ad5 induced intermediate increases in CD83 (mean fluorescence intensity = 34.33). Ad5- σ 1 induced slightly higher CD83 levels with a mean fluorescence intensity of 38.80.

In vivo transduction of MHC II⁺ cells by Ad5 and Ad5- σ 1

Ad- σ 1 transduces human DCs more efficiently than Ad5⁸ and produces modest increases in DC maturation. To determine if virus interactions *in vivo* might also play a role, groups of three BALB/c mice were inoculated IN with Ad5 or Ad5- σ 1 expressing the luciferase-IRES-hrGFP cassette and cervical LNs and lung cells were harvested 24 h later. Single-cell suspensions were stained for MHC II and transduction of these cells was assessed by flow cytometry for hrGFP expression (Figure 7b). Owing to limited cell numbers, the LNs from the three mice were pooled from each group, whereas the lung

Figure 7 Interactions with antigen-presenting cells. **(a)** Dendritic cell maturation induced by the viruses was assayed. Fresh human dendritic cells (hDCs) were isolated using anti-CD14 magnetic beads and incubated for 72 h. The cells were then stimulated with Ad5 or Ad5-T3D σ 1-gag viruses. A maturation cocktail containing tumor necrosis factor- α (TNF- α) and immunoreactive prostaglandin E2 (IPGE2) was used as a positive control. The hDCs were analyzed by flow cytometry using anti-CD83-PE antibody. **(b)** *In vivo* transduction of lymph node and lung major histocompatibility complex class II+ (MHC II+) cells by Ad5 and Ad5- σ 1 (* P <0.05). Mice were immunized intranasally with 1×10^{10} v.p. Ad5 or Ad5-T3D σ 1 virus expressing luciferase-IRES-hrGFP. The lungs and cervical lymph nodes were analyzed for transduction (GFP) and MHCII using PE-labeled anti-mouse I-A/I-E antibody. Ad, adenovirus; GFP, humanized Renilla green fluorescent protein; NC negative control; PC, positive control; PE, phycoerythrin; v.p., viral particle.

samples from each were treated separately. In contrast to the much stronger bulk transduction observed by Ad5 (**Figure 2**), Ad5- σ 1 generated approximately two times as many green fluorescent protein-positive (GFP+)/MHC II+ cells in the draining LNs of the mice than Ad5. In the lungs, the two vectors generated 5–30 times more GFP+/MHC II+ cells than in the LNs. However in this case, Ad5 produced 2.5-fold higher lung GFP+/MHC II+ cells than Ad5- σ 1 (P <0.05). Therefore, in contrast to the 40-fold higher gross transduction of nasal and lung tissues by Ad5, transduction of MHC II+ cells by the two vectors were similar with Ad5- σ 1 mediating equal or better transduction of antigen-presenting cells in the draining LNs.

DISCUSSION

Mucosal immunity is of great importance for the induction of prophylactic immune responses against infectious diseases

by vaccination. This has been highlighted by the recent failure of the HIV-1 STEP (Study of the Therapeutic Effects of Intercessory Prayer) trial. The mucosal barrier is composed of highly specialized innate and adaptive immune complexes. It is at this barrier that infectious agents must be stopped. It is thought that stimulation of immune responses at the mucosal membrane will result in greater mucosal immunity. The most commonly used viral vector for vaccine studies, Ad5, generates robust systemic immune responses. However, Ad5 seems to be restricted to lung epithelial cells and not to antigen-presenting cells.

In this study, we re-engineered the Ad5 vector to express the reovirus σ 1 gene to increase transduction of mucosal cells and DCs. At first glance, using *in vivo* imaging, Ad5- σ 1 appeared to be nonfunctional or to have markedly reduced activity. The absence of luciferase activity indicated that relatively few bulk cells were transduced by the chimeric Ad5- σ 1 virus as compared with the wild-type Ad5. However, when we studied the ability of Ad5- σ 1 to induce systemic and mucosal immunity, we were surprised to find that it was more effective than Ad5 at inducing cellular immunity. Therefore, although it appeared that the retargeted virus had only low bulk tissue transduction, the small amount of transduction that did occur induced potent systemic and mucosal immune responses that were equivalent to or greater than that of wild-type Ad5 virus. The amount of antigen produced and the resulting immune responses did not seem to correlate in this case, suggesting that the chimeric Ad5- σ 1 and the wild-type Ad5 viruses were stimulating cellular immune responses via separate mechanisms.

Another striking difference between Ad5- σ 1 and Ad5 was observed in their abilities to drive humoral immune responses. Unlike the cellular immune responses, Ad5- σ 1 failed to induce anti-gag systemic or mucosal antibodies. One would expect that humoral immune responses would at least somewhat resemble that of the cellular immune responses as was seen for the Ad5 virus. Again, these results reiterate that Ad5- σ 1 is stimulating immune responses in a manner very different from that of wild-type Ad5 virus. If low levels of transduction are resulting in greater cellular immune responses than expected, it is possible that Ad5- σ 1 is targeting cells capable of inducing strong or altered immune responses as compared with Ad5. Indeed, the observations that Ad5- σ 1 drives increased systemic IFN- γ responses are consistent with this hypothesis.

It stands to reason that the different mechanisms of immunity induced by chimeric and wild-type viruses would not be mutually exclusive, and that by stimulating immunity via both mechanisms, there would at least be an additive effect. Therefore, we immunized animals with wild-type and chimeric viruses individually and in combination. Indeed, this was the case for humoral immunity. In fact, when used in combination, the chimeric and wild-type viruses induced a synergistic effect on the humoral immune response. To rule out any nonspecific additive effects of wild-type Ad5, we included the use of a control Ad5 expressing an irrelevant gene.

No nonspecific additive responses were seen for either wild-type or chimeric viruses. Ironically, there was no additive effect seen in the cellular immune responses. In fact, the combination of the viruses resulted in an antagonistic response and ablated the enhanced Th responses. However, when used alone Ad5- σ 1 again induced significantly stronger Th responses than Ad5 alone. The results indicate that the mechanism by which the chimeric and wild-type Ad5 induce cellular immunity may actually be mutually exclusive. Perhaps, this would explain the antagonistic response.

As mucosal immunity is paramount to vaccine development, it is essential that we explore vaccine vectors that stimulate strong mucosal immune responses. Here we show a retargeted chimeric vector that appears significantly impaired when studied using *in vivo* imaging. It was surprising to discover that this chimeric virus was capable of inducing cellular immune responses that were equivalent to or greater than that of wild-type Ad5. The mechanism by which Ad5- σ 1 could induce significantly greater Th responses in the absence of humoral responses is as yet unclear. It is well known that antibody responses after genetic immunization are proportional to the level of transduction by the vector. Therefore, it is not surprising that Ad- σ 1 that mediates less efficient bulk transduction also mediates weak antibody responses by itself.

In contrast, the better transduction and maturation of DCs and MHC II⁺ cells by Ad5- σ 1 may stimulate Th cells better than Ad5. This could explain the synergistic induction of humoral immunity when Ad5 and Ad5- σ 1 are used in combination as Ad5 was shown to induce more protein expression as measured by *in vivo* luciferase activity. The overall lack of Ad5- σ 1's ability to transduce bulk mucosal epithelial cells and lower overall protein expression may be the effect of structural constraint induced by the fusion of the two genes. Small changes in the chimeric fusion gene may alleviate these constraints and repair Ad5- σ 1's overall transduction levels. Alternately, the σ 1 protein may be acting as a protein adjuvant for immune responses. The relative roles of receptor targeting and adjuvant effects by σ 1 are under investigation.

Here we show the characterization of a chimeric Ad5 virus, Ad5- σ 1, to induce systemic and mucosal immune responses. While overall virus transduction and protein expression levels are significantly decreased, the cellular immune responses were equivalent to or greater than wild-type Ad5. Combining the two viruses reversed the lack of humoral immune responses and resulted in a synergistic effect. This effect indicates that the two viruses may be acting through different mechanisms to stimulate the immune system. It is possible that further manipulation of the chimeric fusion protein could potentially reverse the lack of overall transduction and would result in a superior viral vector for inducing both humoral and cellular immunity.

METHODS

Generation of chimeric fiber- σ 1 T3D proteins. The chimeric Fibtail- σ 1 protein from T3D reovirus was created as described previously.⁸ Briefly, the N-terminal 44 amino acids of Ad5 fiber (Fibtail) involved in docking into the penton base were fused to amino acid 18 of the T3D σ 1 protein (Figure 1). To detect the chimeric proteins, two c-Myc tags and

one hexahistidine tag were added to the C terminus of the chimera. The chimeric proteins were cloned into the plasmid pETPL bearing a cytomegalovirus promoter, the Ad tripartite repeat from pDV55,¹¹ and a bovine growth hormone polyadenylation sequence for maximal expression as well as the E4 domain, and an intervening zeocin resistance gene for homologous recombination in bacteria.¹²

Adenoviruses. First-generation replication-defective (E1/E3 deleted) Ad5 vectors were constructed using the Ad-Easy system in 293A cells as described in ref. 13. Ad5 viruses with wild-type fiber expressing a firefly luciferase-IRES-hrGFP cassette and the HIV-1 p55 gag codon-optimized gene of strain HXB2 cassette from the CMV promoter were used as controls. The pETPL plasmids bearing the fiber tail σ 1 chimera was linearized and recombined into these Ad plasmids using homologous recombination at fiber and E4 regions with zeocin selection as in ref. 8. Briefly, the linearized chimeric genomes were transfected into fiber expressing 633 cells¹¹ in the presence of 0.3 μ M dexamethasone and 4 μ g ml⁻¹ of polybrene. The chimeric viruses were amplified by serial passage in 633 cells until the last infection. Virus was purified from 20 100-mm dishes by CsCl gradient centrifugation to remove residual wild-type fiber and these were used to infect a cell factory of 293 cells to produce virions displaying only the virally encoded σ 1 chimeric protein. Recombinant Ads expressing wild-type fiber were purified twice by CsCl gradient centrifugation and quantitated by optical density 260.

Animals. Female BALB/c mice (6–8 weeks old) were purchased from Charles River Laboratories (Wilmington, MA) and housed in the Mayo Clinic Animal Facility under the Association for Assessment and Accreditation of Laboratory Animal Care (AALAC) guidelines with animal use protocols approved by the Mayo Clinic Animal Use and Care Committee. All animal experiments were carried out according to the provisions of the Animal Welfare Act, PHS Animal Welfare Policy, the Principles of the NIH Guide for the Care and Use of Laboratory Animals, and the policies and procedures of Mayo Clinic.

Mice were immunized IM or IN. Mice immunized by the IM route received 1×10^{10} v.p. per mouse in two 25- μ l injections into each mouse's quadriceps muscles. Mice immunized by the IN route received 1×10^{10} v.p. per mouse in 20 μ l total volume (10 μ l per nare). At 2 weeks after immunization, the mice were bled, euthanized, and spleens and LNs were collected.

***In vivo* luciferase imaging.** The luciferase-expressing viruses were administered as indicated in the text and the mice were imaged at varied times on a Lumazone Imaging System (Roper Scientific, Tucson, AZ) as in ref. 14. Mice were anesthetized with isoflurane, injected intraperitoneally (IP) with D-luciferin at a concentration of 20 mg ml⁻¹ in PBS in a volume of 200 μ l and the mice were immediately placed into the Lumazone Imager and images were captured. All images were taken with a 10-min exposure and 2 $\times$ 2 binning using no filters and no photo-multiplication. Data analysis was performed on each image using background subtracted mean intensities detected by the Lumazone Imaging Software (Tucson, AZ) at each time point and graphed using the Prism Graphing Software (La Jolla, CA).

Enzyme-linked immunosorbent assay. To measure humoral immune responses to transgenes, ELISAs were performed on mouse sera as described previously.¹⁵ Briefly, Immulon 4 HBX plates (Thermo, Milford, MA) were coated with 100 μ l of HIV-1 gag protein, SF162 gp120 (NIH AIDS Reagent and Repository) or FireFly luciferase (Roche, Switzerland) at 1 μ g ml⁻¹ in PBS for 2 h at room temperature (RT). The plates were blocked for 1 h with bovine serum albumin at 2 mg ml⁻¹ for 1 h. Sera were diluted 1:100 in PBS with bovine serum albumin (1 mg ml⁻¹) and added to the plate for 1 h at RT. The plates were washed five times with PBS and 100 μ l of goat anti-mouse horse radish peroxidase-conjugated antibody (Pierce, Rockford, IL) diluted 1:2,000 in PBS with bovine serum albumin (1 mg ml⁻¹) was added to the plate for 1 h at RT. The plates were washed five times with PBS and 100 μ l of 1 Step Ultra TMB-ELISA substrate

(Pierce) was added for 1 h at RT. The reaction was stopped with 50 μ l of 2 M sulfuric acid and analyzed at 450 nm using a Beckman Coulter DTX 880 Multimode Detector.

ELISPOT assay. To measure cellular responses to gag, splenocytes were incubated in the presence of peptides at a concentration of 5 μ g ml⁻¹. Gag CTL and Th responses were determined using the VGGHQAAMQMLKDTINEEAA peptide (containing the H-2Kd-restricted peptide AMQMLKDTI) and a peptide pool (ATLEEMM TACQGVGGPSHKA, TSNPPIPVGDYKRWILGL, and FKTLRAEQATQEVKNWMTDT), respectively. The spleens from individual mice were minced and then forced through a 40 μ m Nylon cell strainer (BD Labware, Franklin Lakes, NJ). Mesenteric and cervical LNs were dissected from individual mice and processed as described for splenocytes. Single-cell suspensions of splenocytes or lymphocytes were plated in 96-well polyvinylidene difluoride-backed plates (MultiScreen-IP; Millipore, Billerica, MA) coated with 50 μ l of anti-mouse IFN- γ monoclonal antibody AN18 (5 μ g ml⁻¹; Mabtech, Stockholm, Sweden) overnight at 4°C. The plates were blocked with HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)-buffered complete RPMI medium at 37°C for 2 h. Equal volumes (50 μ l) of each peptide pool and splenocytes (10⁷ cells per ml) were added to the wells in duplicate. Plates were incubated overnight (14–16 h) at 37°C with 5% CO₂. After the plates were washed six times with PBS, 50 μ l of 1:1,000-diluted biotinylated anti-mouse IFN- γ monoclonal antibody (Mabtech) was added to each well. Plates were incubated at RT for 2 h and then washed three times with PBS. A measure of 50 μ l of streptavidin-alkaline phosphatase conjugate (1:1,000 dilution; Mabtech) was added to each well. After incubation at RT for 1 h, the plates were washed five times with phosphate-buffered saline with Tween 20. Finally, 100 μ l of BCIP/NBT (Plus) alkaline phosphatase substrate (Moss, Pasadena, MD) were added to each well. The plates were incubated at RT for 10 min. After washing with water, plates were air-dried. Spots were counted using an automated ELISPOT plate reader (Immunospot counting system; CTL Analyzers, Cleveland, OH) and expressed as spot-forming cells per 10⁶ splenocytes.

Transduction of immature DCs. Peripheral blood mononuclear cells were collected under a Baylor College of Medicine institutional review board-approved protocol and informed consent was obtained from all donors. Peripheral blood mononuclear cells were used to generate human monocyte-derived immature DCs by the “adherence method” or by CD14 isolation as in refs 16,17. Adherent or CD14-selected monocytes were then cultured in Cell Genix/GlutaMAX-I media with 800 U ml⁻¹ granulocyte-macrophage colony-stimulating factor (Sargramostim Leukine; Immunex, Seattle, WA) and 1,000 U ml⁻¹ IL-4 (R&D Systems, Minneapolis, MN) for 5 days with IL-4 and granulocyte-macrophage colony-stimulating factor replenishment on days 2 and 4. On day 5, they were transduced with the indicated vectors.

Human DC maturation assay. Human peripheral blood mononuclear cells were collected from using a Trima cone. Lymphocytes were purified on a Histopaque 1077 density gradient (Sigma Aldrich, St Louis, MO). DCs were purified using anti-CD14 magnetic beads and cultured in Cell Genix DC media supplemented with 2,800 U ml⁻¹ of granulocyte-macrophage colony-stimulating factor and 1,000 U ml⁻¹ of IL-4. The cells were incubated for 72 h and then stimulated with Ad5 or Ad5-T3D σ 1-gag viruses. A positive control maturation cocktail containing tumor necrosis factor- α and immunoreactive prostaglandin E2 was used as a positive control. The DCs were then analyzed by flow cytometry on a FACSCalibur (BD Biosciences, Mountain View, CA) using anti-CD83-PE antibody (Immunotech IM2218, Marseille, France).

Cytokine ELISA. The Th1/Th2 cytokine assays were performed using the eBioscience (San Diego, CA) Th1/Th2 ELISA Ready-SET-Go kit. Groups of 10 mice were immunized IN with 1 $\times$ 10¹⁰ v.p. of Ad5 or Ad5-T3D σ 1-gag virus. Dulbecco's PBS was used as a negative control. Sera

were collected 7 days post-immunization, diluted 1:4 in Dulbecco's PBS and assayed for IL-4 and IFN- γ cytokine expression as described by the manufacturer (eBioscience).

In vivo transduction of antigen-presenting cells. Groups of three mice were immunized IN with 1 $\times$ 10¹⁰ v.p. of Ad5 or Ad5-T3D σ 1 virus expressing luciferase-IRES-hrGFP. Lungs and cervical LNs were harvested 24 h after immunization. The lungs from individual mice were washed twice with PBS and resuspended in 2 ml of PBS. The lungs were minced, vortexed, and then filtered through a 40 μ m Nylon cell strainer (BD Labware, Franklin Lakes, NJ). To increase the total cell numbers for analysis, all of the LNs were combined and processed as described previously. The LN and lung single-cell suspensions were then stained for MHC II using phycoerythrin-labeled anti-mouse I-A/I-E for 1 h at 4°C. The cells were washed with PBS and fixed in 1% formalin overnight. The cells were then analyzed by flow cytometry for GFP and phycoerythrin signals.

Statistical analyses. Data were evaluated using the GraphPad Prism 4 software (La Jolla, CA). Unpaired, two-tailed *T*-tests and one-way analysis of variance with Bonferroni post test were used to determine statistical significance. *P*-values $\leq$ 0.05 were considered statistically significant.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

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