RICE UNIVERSITY

Engineering Adenoviral Gene Delivery Vectors for Improved Gene-based Immunization

by

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Abstract

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Gene-based vaccines have been shown to elicit protective immune responses against a number of pathogens; however, conventional gene delivery methods fail to elicit potent systemic and mucosal responses required to prevent infection by certain pathogens. With the aim of inducing more potent responses, two complementary targeting strategies were employed for the delivery of antigen genes to immunologically-relevant cells, namely mucosal cells and antigen-presenting cells (APC). This thesis explores the identification and application of ligands that target Ad vectors to APC and mucosal sites using genetic engineering and biotin-avidin coupling methods with the aim of increasing immune responses to a model transgene. For APC-targeting, biotinylated Ad (Ad-BAP) was used as a novel ligand screening platform to identify the antigen uptake receptors as the most efficient targets for increased transduction. Specifically, Ad-BAP complexed to a mannosylated ligand demonstrated efficient transduction of mouse dendritic cells and macrophages. Vaccination experiments in mice indicate this vector elicits decreased antibody responses and similar cellular responses compared to unmodified Ad. More work is required to determine the benefit of targeting APC for vaccination, because not all types of APC in mice harbor mannose receptors in situ. For targeting mucosal sites, a chimeric Ad vector (Ad-σ1) was genetically engineered to
display the mucosal-targeting σ1 protein of reovirus. The striking structural homology between the Ad fiber protein and the reovirus σ1 protein was exploited for the development of a functional chimeric fiber-σ1 protein to allow virion encapsidation. Ad-σ1 binds and infects cells through the reovirus receptors, junctional adhesion molecule 1 (JAM1) and cell-surface sialic acid, and not through the Ad receptor, coxsackievirus and adenovirus receptor (CAR). However, Ad-σ1 transduction of mucosal cells in vitro shows markedly decreased efficiency as compared to unmodified Ad. Despite these defects, Ad-σ1 elicited similar immune responses compared to unmodified Ad after mucosal vaccination of mice. These results suggest that re-engineering of the fiber-σ1 chimera to better enable JAM1 interactions may offer promise for using Ad-σ1 for mucosal vaccination. Further work needs to establish the relative importance of the barriers to Ad-based vaccination, namely low pH, digestive enzymes, glycocalyx, and apical receptor expression.
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Chapter 1

Introduction and Specific Aims

The World Health Organization estimated that over 10 million people died from infectious and parasitic diseases in 2002 [1]. Currently, there is a lack of protective vaccines for human immunodeficiency virus (HIV), hepatitis C virus (HCV), human papillomavirus (HPV), herpes simplex virus (HSV), and malaria, to name a few. Vaccines are one of the most effective strategies at preventing infectious diseases. Treatment post-infection can include costly medicines that may have limited efficacy and significant host toxicity. One such example, HIV, alone accounted for 3.1 million deaths and 4.9 million new infections in 2005 [2]. Even with the advent of antiretroviral drug treatment, resistant viruses often can arise as a consequence of the rapid mutation rate of the HIV genome. Compounding the problem, access to medicines in developing countries is limited and the morbidity rate is high. The purpose of this thesis is to develop new vaccination strategies by combining advances in the fields of gene therapy, molecular biology, virology, and immunology.

Traditionally, vaccines are developed from inactivated pathogens or even purified component proteins, which generally elicit neutralizing antibodies and helper T cells, but fail to generate cytotoxic T lymphocytes (CTLs). Pathogen biology determines which types of immune responses best correlate with protection; there are acute viruses, which are normally cleared during natural infection (e.g. smallpox, polio, measles, mumps, rubella, and influenza), and chronic viruses, which do not elicit sufficient immunity to clear the infection (e.g. HIV, HCV, HPV, and HSV) [3]. Conventional vaccines generate
protection from acute viruses predominantly through generation of neutralizing antibodies. However, strong evidence indicates that controlling chronic infections is mediated through cellular immunity, namely CTLs [4]. In fact, extensive work in the study of HIV confirms the importance of CTLs in keeping individuals uninfected [5-7], and in controlling viral load in monkeys infected with the related simian immunodeficiency virus (SIV) [8, 9].

In addition to focusing on CTL generation, recent efforts have also been focused on generating immune responses at mucosal sites where pathogens like HIV usually first enter the body. The goal of these efforts is to produce antibody-secreting cells and CTL that localize to the mucosal immune compartment and remain in that compartment as memory cells. Most conventional vaccines are delivered intramuscularly (i.e. systemically) and generally fail at producing long-lived mucosal immunity [10]. However, current work has shown that delivery of vaccines at readily accessible sites (e.g. oral and intranasal routes) can evoke responses at sites more relevant for protection (e.g. vagina) [11-14].

A relatively new vaccine strategy called gene-based immunization allows for the tailoring of the immune response to include the generation of CTLs. This strategy more closely mimics natural infection, or even live-attenuated vaccines, by delivering pathogen genes directly to the host cells. By taking advantage of the host cell’s transcription machinery, the endogenous pathway of immune stimulation can occur with the generation of CTLs. Advantages of this strategy over traditional methods include rational design of the pathogen gene to specifically tailor the type of immune response and decreased health risks associated with live-attenuated vaccines.
Using viruses as gene delivery vectors are attractive because co-evolution has made them efficient at delivering their genes to all types of cells in order to take advantage of the cell's translational machinery. Replication-defective adenovirus type 5 (Ad5) is one of the most studied and efficient viral gene delivery vectors that holds great promise for use as a vaccine carrier. In fact, Merck has developed an Ad5-based HIV vaccine that shows promise in animal studies and is currently undergoing clinical trials [15]. Ad5 can be grown to high titers and can deliver approximately 35 kilobases of foreign genetic material to both dividing and non-dividing cells. The inherent immunogenicity of this vector limits its appeal for gene replacement therapy, but this same characteristic makes it more attractive as a vaccine vehicle. However, the broad tropism of this virus can limit its efficacy in vivo because gene delivery may not occur in the desired cells types.

Evidence indicates that the efficacy of gene-based vaccines depends on the direct in vivo gene delivery to antigen-presenting cells (APC) [16-19]. Adenovirus delivers its genes very inefficiently to cells that lack the coxsackievirus and adenovirus receptor (CAR), like APC [20]. This limited delivery to APC is thought to explain the ability of Ad vectors to provoke immune responses against the transgene products [21]. It is this ability that makes adenovirus an attractive vaccine vector, but less so as a gene therapy vector.

While much vaccine research is focused on eliciting specific immune responses to generate protective immunity, this work focuses on modification of the vaccine vector (Ad5) to direct delivery of genes to immunologically-relevant cells using a combination of genetic modifications and biotin-avidin coupling methods to introduce novel targeting
ligands. The hypothesis is that adenoviral vectors targeted to APC and mucosal sites can generate more potent mucosal and systemic immune responses than the corresponding non-targeted vectors. The following specific aims are addressed in this thesis:

1. Discover potential ligands for transduction of APC and mucosal cells using metabolically biotinylated adenoviral vectors \textit{in vitro}.

2. Develop a mannose receptor-targeted adenoviral vector based on the ligand screening method for use \textit{in vivo}.

3. Develop a chimeric adenoviral vector displaying the mucosal-targeting protein σ1 of reovirus using genetic engineering methods.

4. Characterize both targeted and non-targeted vectors \textit{in vivo}, including a comparison of the antibody and cellular immune responses to a model antigen.
Chapter 2

Background and Literature Review

2.1 Gene-based vaccines

Gene-based vaccination is a specific application of gene therapy in which immunity is elicited through the delivery of genes encoding pathogen proteins. The result is a novel type of vaccination that offers the opportunity to tailor the stimulation of a broad array of immune responses to produce antibodies, helper T lymphocytes, and even cytotoxic T lymphocytes (CTL).

The field of vaccinology has seen a number of advancements since the widespread use of vaccines by Jenner and Pasteur. First generation vaccines consisted of live attenuated pathogens. Safety concerns led to the development of the second generation consisting of inactivated whole pathogens. The third generation consists of purified or recombinant proteins, and more recently, the fourth generation consists of non-viral and viral gene-based vaccines [4]. Currently approved vaccines consisting of purified proteins or chemically-inactivated pathogens interact with B cells (Figure 2-1, #1), which stimulate antibody production and, to a lesser extent, antigen-presenting cells (APC) (Figure 2-1, #2) that stimulate helper T lymphocytes (Th) (Figure 2-1, #3). Live attenuated viruses and gene-based vaccines make use of host cells to produce proteins that additionally stimulate CTL (Figure 2-1B, #4).

Fourth generation, gene-based formulations have many advantages over the methods of previous generations. These novel vaccines more closely mimic the antigenicity of pathogens without the inherent dangers associated with live attenuated
viruses, like HIV [22]. Since host cells produce the encoded proteins in these vaccines, they do not suffer from the decreased antigenicity from manufacturing whole killed and live attenuated vaccines. Moreover, the use of genes allows for multiple epitopes to be included that can elicit a breadth of immune reactivity that has not been possible before, such as that seen with expression library immunization [23-25]. Lastly, gene-based vaccines have the advantage of accessing the endogenous pathway of immune presentation and are thus able to elicit CTL responses, unlike whole killed vaccines.

Figure 2-1. Schematic of immune stimulation by vaccines [adapted from 26]. A) Pure proteins or chemically inactivated pathogens predominantly stimulate antibody responses, and T helper (Th) responses to a lesser degree. B) Live, attenuated pathogens or gene-based vaccines additionally stimulate cytotoxic T lymphocyte responses (CTL).

Prophylactic vaccines for many pathogens that establish chronic infections, like HPV, HCV, and HIV, remain elusive. In most cases, chronic viruses fail to elicit sufficient immunity that is capable of clearing the infection. Therefore, vaccines that
mimic the natural infection like live attenuated viruses will most likely be inadequate for controlling the infection [3]. Traditional vaccines have been most effective towards the acute, self-limiting infections in which generation of neutralizing antibodies has been sufficient protective immunity. However, strong evidence indicates that protection from chronic infections is mediated through CTLs [4]. In particular, studies suggest that CTLs are important in controlling HIV infection or providing sterilizing immunity [5-7], and in controlling viral load in monkeys infected with the related simian immunodeficiency virus (SIV) [8, 9].

Gene-based vaccines offer a promise of establishing a new paradigm for vaccine development, one that applies advances in molecular biology, virology, and immunology. Building on these advances, researchers have harnessed the innate ability of viruses to efficiently deliver their genes to create what are known as viral vectors. These vectors are engineered to contain any antigen gene of interest and deleted of any undesirable genes. Usually this requires rendering the virus replication-deficient and unable to express endogenous genes to further increase the safety profile. Current HIV vaccine research is studying different prime-boost vaccine approaches to elicit immune responses that will protect and prevent disease. Table 2-1 outlines the different strategies used in ongoing HIV vaccine trials, and indicates that fourth generation vaccines consisting of viral-based gene delivery vectors are promising tools for developing HIV vaccines. Because viruses have evolved to bind specific cell types, they offer the opportunity for rational design of the outer capsid proteins to increase their efficacy in vivo. Specifically, adenoviral-based vaccines are promising as evidenced by the range of HIV clinical trials utilizing them (Table 2-1) and the extensive knowledge of their biology. This work
focuses on developing new approaches to the modification of adenoviral-based vaccines with the aim of increasing their efficacy.

<table>
<thead>
<tr>
<th>Generation of Vaccine</th>
<th>Product</th>
<th>Number of trials (completed, planned, and ongoing)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fourth, viral</td>
<td>Ad5</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>MVA</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Canarypox</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Fowlpox</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>VEE</td>
<td>3</td>
</tr>
<tr>
<td>Fourth, non-viral</td>
<td>Plasmid</td>
<td>8</td>
</tr>
<tr>
<td>Third</td>
<td>Protein</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Peptide</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 2-1. Current strategies for HIV vaccination. Ad5, Adenovirus type 5; MVA, Modified Vaccinia Ankara; VEE, Venezuela Equine Encephalitis virus [data taken from 27].

2.2 Role of antigen-presenting cells in vaccination

Professional APC include a broad array of leukocytes that are specialized in activating the adaptive immune responses to antigens. These cells, which include macrophages and many types of dendritic cells (DC), are able to initiate a primary immune response that allows for the establishment of immunological memory. DC are the most potent class of APC that, upon stimulation, can present antigen to T cells and provide costimulatory molecules required for activation of naïve T cells [reviewed in 28].
The four vaccine types differ in their ability to access antigen presentation pathways and thus differ in the resulting immune responses. Inactivated whole pathogens and protein vaccines are recognized by precursor B cells that differentiate to produce neutralizing antibodies. To a lesser degree, they can also be taken up into cells by phagocytosis, pinocytosis, or endocytosis and processed through the major histocompatibility complex (MHC) class II pathway. Presentation through this pathway results in CD4+ T helper responses. The inactivated pathogens are no longer capable of infection and therefore cannot access the endogenous MHC class I pathway for stimulation of CD8+ CTL. In contrast, live attenuated viruses and gene-based vaccines deliver their genes to cells in which endogenous protein expression allows entry into the MHC class I pathway (Figure 2-2).

Figure 2-2. Mechanism of activation of CTL [from 29]
There are three proposed mechanisms by which gene-based vaccines can elicit cell-mediated immunity: 1) direct priming by somatic cells (non-bone marrow-derived cells), 2) direct priming by APC, and 3) cross-priming to APC by somatic cells (Figure 2-3). No evidence exists for direct priming of T cells by somatic cells, but there is clear evidence that bone-marrow derived cells (including APC) are involved in T cell priming [30-32]. Alternatively, CTLs can be generated via cross-priming [33-35], a mechanism in which APC present exogenous antigen on MHC class I molecules. This phenomenon requires that the source of exogenous antigen be produced by infection of somatic cells and then transferred to APC [reviewed in 36], since vaccination with antigen or killed pathogens do not stimulate the production of CTLs. Other factors such as the levels of exogenous antigen introduced into APC or the amount of maturation of the APC may also influence how much reaches the MHC class I pathway [37].

Figure 2-3. Mechanisms of T cell priming [from 38].
The relative influence of direct priming versus cross-priming in the generation of CTLs is currently under investigation [37]. Some work supports the role of direct priming in which virus-infected APC activate naïve T cells in vivo [39]. With respect to gene-based vaccines, evidence indicates that the efficacy of such vaccines depends on the direct in vivo gene delivery to DC [16-19]. Moreover, transduction of somatic cells may lead to induction of immune tolerance by clonal deletion of specific T cells [40]. Thus, there is rationale for engineering gene-based vaccines to target APC while avoiding gene delivery to non-immune cells.

One aspect of this thesis explores targeting adenoviral gene-based vaccines to APC with the aim of increasing their potency. This work measured antibody levels as well as antigen-specific CD8+ T cell responses as an endpoint measure of effectiveness in a mouse model. Since there is some debate whether direct APC transduction or cross-priming by somatic cells is more important in generating CTLs [37], this work may offer further insight into which mechanism is more important in vivo. The success and future development of gene-based vaccines will depend on work that elucidates whether direct targeting of APC or somatic cells produces a more effective strategy.

2.3 Role of mucosa in gene-based vaccination

The immune response to any antigen encountered either in the airways or gastrointestinal (GI) tract depends upon processing through the follicle-associated epithelium (FAE), an important first step in the initiation of the mucosal immune response. Components of these special structures, Peyer’s patches are located in the GI tract and the airways and contain enterocyte-like cells and microvilli (M) cells. M cells
are specialized for antigen uptake and transcytosis, which is the delivery of macromolecules, particles, and microorganisms from the lumen to the immune cells located on the basolateral side of the epithelium. Deep invaginations of B and T lymphocytes, macrophages (MΦ), and dendritic cells (DC) form what is known as an intraepithelial pocket (Figure 2-4). Antigens delivered to this pocket are thought to be processed by APC for initiation of the mucosal immune response [reviewed in 41].

Figure 2-4. Diagram of an M cell in the follicle-associated epithelium. MΦ, macrophages [adapted from 41].

Mucosal surfaces are the first line of defense for most pathogens. The predominant, protective components of the mucosal immune response are secretory IgA and CTL [reviewed in 42]. The presence of sIgA can prevent infection through the epithelial cells at mucosal surfaces by binding and neutralizing pathogens. Mucosal
CTLs, on the other hand, specifically destroy infected cells and control replication of pathogens.

The mucosal immune system is often described as being separate and distinct from the systemic (or peripheral) immune system [43-45], a phenomenon known as compartmentalization. In practice, though, there is some measurable cross-talk between the two compartments. Regardless, the induction of IgA-secreting B cells at mucosal surfaces following antigen exposure results in trafficking in the common mucosal immune system (i.e. gut, airways, vagina) [44, 45]. Furthermore, antigen-specific mucosal T cells preferentially home to mucosal tissue and do so more readily when re-exposed to that antigen [46]. The molecular basis for the preferential homing of lymphocytes to the mucosa is associated with the expression of α4β7 [47] and also CCR9 [48-50]. Specifically, DC in gut-associated lymphoid tissue, namely mesenteric lymph nodes and Peyer’s patches, induce the expression of these homing receptors on T cells (Figure 2-5) [49, 50]. These imprinted lymphocytes are then able to bind to the mucosal addressin MAdCAM-1, selectively expressed on capillary endothelium in inductive and effector immune sites in the GI tract [51, 52].

There is a belief that mucosal immune responses and thus mucosal vaccination will be required for an effective HIV vaccine [53]. Evidence comes with the fact that most HIV infections occur through the mucosal route [54] and that the majority of CD4+ T cells are depleted from the GI tract, regardless of the stage of infection or when highly active antiretroviral therapy was initiated [55, 56]. Therefore, recent efforts have focused on generating effective immune responses at mucosal surfaces for preventing and controlling HIV infection [reviewed in 57]. The benefit of this approach stems from its
ability to elicit mucosal-homing B cells for IgA secretion and mucosal-homing CTLs for controlling mucosal infections. Studies have shown that mucosal delivery of an HIV vaccine produced both systemic and mucosal immune responses, but systemic immunization produced only systemic immune responses [58]. Moreover, it was found that only after mucosal immunization was long-term T cell memory found in the mucosal compartment [59]. It is imperative that mucosal CTLs be present in mucosal sites as systemic CTLs were not sufficient to protect against mucosal challenge [60]. Systemic vaccinations are also quite ineffective at producing sIgA [61, 62], another component of an effective mucosal response.

**Figure 2-5. Mucosal priming of naïve T cells** [adapted from 63].

Another benefit of mucosal vaccination is that the delivery of vaccines at readily accessible sites (e.g. oral or intranasal) can evoke responses at sites relevant for protection against pathogens (e.g. vagina) [13, 14]—a phenomenon known as cross-talk.
Thus, strategies to increase the effectiveness of oral or intranasal vaccination may lead to protective vaccines that are simpler and easier to administer. With the aim of increasing their potency, this thesis explores targeting adenoviral gene-based vaccines to mucosal sites.

2.4 Adenoviruses as cell-targeting vectors

Adenoviral vectors are one of the most studied gene therapy vectors to date. These vectors demonstrate the optimal characteristics of a gene delivery system: safe, efficient gene delivery to dividing and quiescent cells, and nononcogenic [64]. Replication-incompetent adenovirus type 5 (Ad5) is one of the most popular since it can be grown to high titers in complementing cells lines and is capable of carrying up to 36 kbp of therapeutic DNA. In addition, these vectors yield the most robust in vivo gene expression available [65].

Targeting adenoviral vectors to specific cells types or tissues entails the ablation of existing tropism and the addition of new ligands to modify the tropism to a certain cell type. Tropism can be limited by certain mutations in the viral capsid, and new ligands can be conjugated to the capsid using genetic, chemical, or non-covalent methods. The icosahedral capsid of adenovirus contains three major core proteins: hexon, penton base, and fiber, which protrudes from its 12 vertices (Figure 2-6). Fiber is a homotrimer that contains three domains: tail, shaft, and knob. Trimerization [66] and the tail domain [67] of fiber are required for non-covalent interaction with penton base to produce functional virus. The fiber knob domain gives adenovirus its wide tropism by high-affinity binding to the coxsackievirus and adenovirus receptor (CAR) [68]. In addition,
the arginine-glutamine-aspartamine (RGD) motif of penton base mediates internalization through $\alpha_\text{v}\beta_3$ and $\alpha_\text{v}\beta_5$ integrins [69-72]. Both the CAR and integrin binding sites can be ablated to reduce the natural tropism of the virus. Mutation of the FG loop of the fiber knob can reduce the transduction of CAR-positive cells over 10-fold [73] and deletion of the RGD motif in penton base can reduce transduction of $\alpha_\text{v}$ integrin-positive cells over 1-fold [74].

Figure 2-6. Diagram of an Adenovirus [adapted from 75].

After CAR-binding ablation, targeting ligands can be combined with adenovirus to redirect it to specific tissue sites. Three approaches for ligand addition include direct chemical cross-linking, genetic modification, and non-covalent coupling. Direct chemical cross-linking covalently links the ligand to the virus with a spacer moiety, such as polyethylene glycol (PEG) [76]. Genetic modification involves inserting the ligand at
the genetic level into a capsid protein so that it is displayed outward and available for binding after viral production. Some ligands available for genetic re-targeting include cell-binding peptides selected by phage display [77, 78] that are inserted into the HI-loop of the fiber protein [79, 80]. The HI loop of the fiber knob is the solvent-exposed set of amino acids that connects the β-strands H and I. Krasnykh et al. first demonstrated that an octapeptide sequence could be inserted into this loop without disruption of fiber trimerization and remains available for binding in the mature virions [79]. Non-covalent coupling utilizes bi-specific adapter molecules with one end targeted to the virus and the other targeted to a refractory cell type. Examples include bi-specific antibodies [81], CAR ectodomain-growth factor fusions [82], and CAR ectodomain-antibody fusions [83].

Another genetic re-targeting possibility is the creation of a chimeric adenovirus using the capsid proteins of different viruses. Fiber-swapping is a strategy in which the fiber domain of one serotype is swapped for another serotype by creating a chimeric fiber protein [84-88]. Other possibilities include incorporation of a structurally homologous protein from an evolutionarily diverse virus, such the bacteriophage T4 [89]. These types of genetic modification usually do not suffer from the inherent problems associated with ligand insertion since evolution has produced structurally homologous domains that function as cell-targeting ligands for diverse viruses. Unlike chemical cross-linking or non-covalent coupling, genetic modification does not require any additional steps other than viral production and purification. Part of this thesis describes the development of a novel mucosal-targeting ligand derived from the enteric pathogen reovirus and its application as a mucosal vaccine.
Not all ligands are amenable to genetic incorporation since they can disrupt viral assembly [90, 91] and lose their binding specificity when displayed on the virus. Non-covalent methods of ligand addition can overcome the problems inherent with some ligands since they are added after mature virions are formed and purified. Biotin-avidin coupling is one such method that involves non-covalently linking a biotinylated ligand to biotinylated adenovirus using a tetrameric avidin bridge [92]. Other non-covalent methods are considered too weak to work in vivo; however, the biotin-avidin interaction is one of the strongest non-covalent interactions in nature ($K_d = 10^{15}$ M) [93].

To prevent the problems associated with non-specific chemical biotinylation, Parrott et al. developed a method to metabolically biotinylate the fiber protein during viral production. A biotin acceptor peptide (BAP) was genetically engineered onto the carboxy-terminus of fiber (Figure 2-7) and was biotinylated by the host cell biotin ligase during fiber synthesis [94].

Figure 2-7. Structural Approximation of BAP on Fiber Knob. The BAP is represented by the multicolored domain and each fiber monomer is represented as blue, purple, and brown. Knob sits on the right of the illustration with the shaft extending off to the left [adapted from 95].
Re-targeting of biotinylated adenovirus (Ad-Fiber-BAP) to alternate cellular receptors using biotinylated antibodies, lectins, and glycoproteins increased transduction of refractory cells up to 1000-fold [95]. This strategy obviates the need to genetically engineer a new vector for each new ligand and also avoids context-specific disruption of ligand and viral function. Another aspect of this thesis develops the biotin-avidin targeting system as a high-throughput ligand screening platform for identification of ligands targeting APC and mucosal cells for the application of improved vaccination.

2.5 Adenoviruses as vaccine vectors

Adenoviruses are one of the most promising vectors in use for gene-based vaccination since they have proven efficacy in vivo. In a comparison study in rhesus monkeys of adenoviruses, poxviruses, alphaviruses, mycobacteria, and plasmid DNA, Merck found that Ad5 elicited the highest antigen-specific CTL responses [15, 96]. This characteristic is primarily responsible for the wide use of Ad vectors in clinical and preclinical trials for HIV vaccines (Table 2-1).

Unfortunately, pre-existing immunity to the Ad5 vector severely limits the efficacy in vivo. Neutralizing antibodies are generated upon wild-type infection or during prior vector administration and play a critical role in reducing vaccine efficacy in mice [97] and in humans [15]. Anti-Ad5 neutralizing antibodies are present in the majority of North Americans with up to one third having high titers [15]. Research is being conducted to overcome this impediment to vector use and re-administration; it has been shown that Ad vectors coated with PEG [98] and alginate [99] successfully mask the capsid from neutralizing antibodies. To allow vector re-administration, prime-boost
strategies have been developed using alternate serotypes, such as human Ad11 [100], human Ad35 [101], and chimpanzee serotypes [102, 103]. In addition, mucosal delivery can bypass pre-existing immunity to Ad5 [104]. See Figure 2-8 for a schematic of vector modifications for increasing the effectiveness of Ad-based vaccination.

The drawbacks of using Ad5 for gene therapy, however, present advantages for gene-based immunization. The Ad5 genome is maintained episomally so there is relatively short-term gene expression and no problems associated with the oncogenic potential of genomic integration. Overall, they are relatively safe as adenovirus has been used as a live oral vaccine in millions of military personnel with few complications [105] and as intramuscular vaccines in ongoing clinical trials. The questionable safety of adenovirus is related to high doses through the intravenous (i.v.) route. The recent human gene therapy death [106] was attributed to acute toxicity arising from the delivery of massive particle numbers needed for gene therapy applications.

Adenovirus delivers its genes efficiently to non-immune cells with delivery occurring to a lesser degree in DC, which is thought to explain the ability of this vector to provoke immune responses to the transgene products [21]. While this remains problematic for gene therapy, adenovirus remains an attractive gene-based vaccine vector [12, 105, 107-112].

While Ad vectors have proven their ability to elicit immune responses, they are relatively inefficient at transducing APC as evidenced by the requirement of high particle numbers [20, 113]. This lack of transduction is explained by the absence of CAR, which is the initial high affinity receptor for Ad5 binding and attachment [68, 114]. Any deficiency in initial attachment is overcome by the interaction of penton base with
integrins, which mediate internalization [70] and can serve as an attachment receptor where CAR is lacking [115]. Thus, there is rationale for engineering Ad vectors for greater transduction of APC since their role in vaccination has been widely established.

Figure 2-8. Adenoviral vector modifications for improved gene-based vaccination [modified from 116].

A number of strategies are in development for modifying Ad vectors for improved transduction of APC [reviewed in 116]. These involve developing re-targeted Ad vectors that utilize receptors which are either exclusively expressed or in more abundance on APC. Ad vectors based on other human serotypes, such as Ad35, infect dendritic cells more efficiently than Ad5 [117] and a chimeric Ad encoding the Ad35 fiber performs similarly well [118]. Since dendritic cells express high levels of $\alpha_v$ integrins, an arginine-glycine-aspartic acid (RGD) peptide was inserted into the HI loop of the knob domain to enhance transduction efficiency [119, 120]. The CD40 receptor on dendritic cells has been targeted using genetic manipulation [121] and bi-specific molecules [122, 123] with improved transduction efficiency of dendritic cells and activation of CTL.
This thesis explores two separate strategies for modifying adenoviral vectors for improving the potency for gene-based vaccination: re-targeting to immune effector cells of the systemic and mucosal immune sites. This work includes discovery and screening of potential ligands, physical and genetic incorporation of ligands, and in vivo characterization of gene delivery and vaccine effects.
Chapter 3

Ligand Discovery and Screening using Biotinylated Adenovirus

3.1 Introduction

Adenovirus type 5 (Ad5) vectors are one of the most robust gene delivery vectors and show promise for genetic correction, cancer therapy, and gene-based vaccination. However, their broad tropism limits the ability to deliver genes to relevant cell types. The loss of vector to irrelevant cells decreases the efficacy and requires the use of higher doses to achieve a therapeutic effect. Increasing doses have been associated with increasing side effects, like acute toxicity and immune activation [124, 125]. Engineering Ad5 with altered tropism that narrows the specificity to desired cell types should increase the efficacy for a smaller dose, while decreasing the toxicity.

Expression of the coxsackievirus and adenovirus receptor (CAR) determines the tropism of Ad 5 [68]. A number of strategies have been employed to add new ligands to the virus to re-direct cellular tropism. Ligands can be incorporated by chemical biotinylation [92], chemical cross-linking [126], or genetic engineering [121, 127, 128]. However, chemical conjugation can inactivate capsid proteins and genetic insertion is problematic because ligands may disrupt virion assembly, and the ligand may no longer function in the context of the capsid [91].

Parrott et al. developed a method of metabolically biotinylating Ad vectors [95] using a biotin-acceptor peptide (Ad-Fiber-BAP). Biotinylated ligands can successfully re-target Ad-Fiber-BAP to various cell types using an avidin bridge. This method obviates the need for engineering a new vector each time a new receptor target needs to
be tested. In addition, the problems associated with inactivation by non-specific chemical biotinylation are eliminated [129].

To develop successful re-targeted Ad vectors, a comprehensive knowledge of the receptors for a given cell type and their tissue distribution is required. Not all of these receptors will be amenable to re-targeting. Many other factors are involved in gene expression after initial Ad5 binding: internalization, endosomal escape, intracellular trafficking and nuclear localization [130]. Transduction is defined as the summation of all these events and subsequent quantifiable gene expression. Even though a ligand may re-target adenoviral vectors, some receptors may be “dead-end” in the sense that they are incompatible with Ad5 biology. The binding of some ligands to these receptors may induce altered trafficking and inefficient transduction [131-133].

To bypass these concerns, we developed a ligand screening method whereby Ad-Fiber-BAP transduction efficiency was used as the measure of success. If a receptor-ligand interaction causes aberrant Ad intracellular trafficking and transduction, the ligand would compare poorly to other candidate ligands and subsequently eliminated from further consideration. In this chapter, various ligands for antigen-presenting cells (APC) and mucosal cells were identified in the literature and tested for binding by flow cytometry. A diverse set of ligands including antibodies, glycoproteins, oligonucleotides, and lectins were used successfully with varying degrees of re-targeting efficiency. This screening method was used to identify ligands and receptors that were used in subsequent studies to develop re-targeted vectors to APC and mucosal sites. The screened ligands that provide the most efficient re-targeting capability can potentially be attached by
genetic insertion, non-covalent coupling, or chemically cross-linking to create a re-targeted Ad for use in vivo.

Furthermore, the ligands identified by this screening method that increase transduction can lead to the discovery of other targeting ligands that cannot easily be tested but may work better in the context of the Ad capsid. For example, Belousova et al. genetically inserted CD40 ligand into the fiber protein to create a CD40-targeted adenoviral vector [121]. Moreover, biotinylated anti-CD40 antibody is also a target for CD40 and can easily be screened with Ad-Fiber-BAP. This work shows that Ad-Fiber-BAP can be used as a versatile ligand screening platform to identify receptor targets in vitro that may lead to the development of genetically re-targeted Ad vectors for use in vivo.

3.2 Materials and Methods

Cells and viruses

Bone marrow-derived dendritic cells (BMDC) were maintained in RPMI-1640 with 10% fetal bovine serum (FBS) (Hyclone, Salt Lake City, UT), antibiotic/antimycotic (Gibco BRL, Gaithersburg, MD), and 50 μM β-mercaptoethanol (Sigma, St. Louis, MO). Caco-2 cells (ATCC, Manassas, VA) were maintained in RPMI-1640 with 10% FBS, antibiotic/antimycotic, sodium pyruvate, and HEPES (Gibco BRL, Gaithersburg, MD).

Ad vectors used in this study are based on the Ad-Easy system (Quantum Biotechnologies, Montreal, QC, Canada) and carry the full E1- and E3-deleted Ad5 genome. Viral propagation, purification, and plaque formation were performed as
described [134]. Ad-Fiber-BAP and the CAR binding-ablated vector, Ad-Fiber-TR-BAP, expressing enhanced green fluorescent protein (EGFP) were constructed as previously reported [95]. Ad-Fiber-BAP expressing the firefly luciferase gene, an internal ribosome entry site, and the humanized Renilla green fluorescent protein were constructed with the Ad-Easy system. Viral particle number was calculated using spectrophotometry by measuring the absorbance of purified viral particles at 260 nm [134].

**Generation of bone marrow-derived dendritic cells**

Femurs and tibia of 4- to 6-week-old C57BL/6 mice were harvested and soaked in 70% ethanol for 1 min. One end was cut from each bone and the marrow was flushed with medium using a 0.45-mm needle. Cells were pipetted vigorously, strained to remove debris, and plated on 150-mm dishes at $10^6$ per milliliter. After overnight incubation, non-adherent cells were removed and 20 ml of medium with 20 ng/ml recombinant mouse granulocyte macrophage colony stimulating factor (rmGM-CSF) (PeproTech, Rocky Hill, NJ) was added to the plates on day 1. At days 4, 6, 8, and 10, half of the medium was removed, and non-adherent cells were pelleted and returned to the plates with fresh medium containing rmGM-CSF. Dendritic cells were harvested for experiments on days 11 to 13. Purity was confirmed by CD11c expression and blocked with 0.25 μg Fe Block (Pharmingen, San Diego, CA) per 1 million cells before staining and transduction assays.
**Ligand binding on bone marrow-derived dendritic cells**

BMDC were generated as indicated. On Day 12, BMDC were incubated with 5 μg/ml biotinylated anti-CD40, anti-CD86, anti-CD11b, anti-CD11c, anti-IA/IE (MHC II), and isotype (Rat IgG2a) monoclonal antibodies (mAb) (PharMingen, San Diego, CA) in staining buffer (1X phosphate-buffered saline (0.1 M sodium phosphate, 0.15 M sodium chloride, pH 7.2) containing 2% FBS) at 4°C. On Day 13, BMDC were incubated with biotinylated bovine serum albumin (BSA), asialofetuin (Sigma, St. Louis, MO), and heat shock cognate protein (Hsc70) (StressGen Biotechnologies, San Diego, CA) at the indicated concentration. Cells were detected with 5 μg/ml streptavidin-Alexa Fluor 488 conjugate (Molecular Probes, Eugene, OR). Fluorescence was analyzed by FACScan and quantified as mean fluorescence intensity (MFI) using CellQuest software (Becton-Dickinson, Franklin Lakes, NJ). Asialofetuin was biotinylated as described [95].

**Ligand screening using Ad-Fiber-BAP on bone marrow-derived dendritic cells**

BMDC were generated as indicated. On day 12, the cells were resuspended to 250 thousand per 50 μl in transduction buffer (Hank’s Balanced Salt Solution (Gibco BRL, Gaithersburg, MD) with 1% BSA). Biotinylated antibodies (50 μl) at a final concentration of 5 μg/ml, Hsc70, asialofetuin, macroaggregated albumin [135], mannosylated BSA (Sigma, St. Louis, MO), or buffer was added to the cell suspension and incubated for 30 min. at 4°C. Other ligands included biotinylated phosphorothioate oligonucleotides containing unmethylated CpG and GpC motifs (Operon Technologies, Alameda, CA). The negative control ligand was biotinylated rat IgG2a isotype mAb. Following the incubation, the cells were washed and pelleted two times before the
addition of 50 µl of buffer containing neutravidin (0.01 ng per cell) (Pierce Chemical, Rockford, IL). The incubation and washes were then repeated as detailed above. Ad-Fiber-BAP and Ad-Fiber-TR-BAP expressing EGFP was added at 3000 particles per cell in a volume of 50 µl of buffer. The cells were then incubated and washed as above. Finally, the cells were resuspended in 0.5 ml of complete RPMI with 20 ng/ml of rmGM-CSF. After 24 hr., EGFP expression was analyzed by FACScan and quantified as percent positive using CellQuest software. Ten thousand events per sample were collected, and gates between transduced and non-transduced cells were determined on a one-dimensional histogram by excluding 98–99% of mock-infected cells from the threshold value for determining positively transduced cells. The trends are representative of two independent experiments.

**Lectin binding on Caco-2 cells**

Caco-2 cells were incubated at 250 thousand per condition with biotinylated bovine serum albumin (BSA), wheat germ agglutinin (WGA), Ulex Europaeus agglutinin (UEA), or Griffonia Simplicifolia Lectin isolectin B4 (GSL-I-B4) (Vector Laboratories, Burlingame, CA) at 10 and 100 µg/ml at 4°C overnight. As a positive control, anti-CD59 mAb (Pharmingen, San Diego, CA) was used at 10 µg/ml. The cells were washed three times with staining buffer. Next, labeling was performed with streptavidin-phycoerythrin conjugate (Molecular Probes, Eugene, OR), at 20 µg/ml for 20 minutes at 4°C. After washing two times with 1 ml of wash buffer, cells were analyzed by FACScan and quantified as mean fluorescence intensity (MFI) using CellQuest software.
**Ligand screening using Ad-Fiber-BAP on mucosal cells**

Three 24-well plates were seeded with Caco-2 cells. One day after confluency, cells were washed with transduction buffer and incubated with 200 µl of varying concentrations of biotinylated WGA for 30 min. at 4°C. Following the incubation, the cells were washed two times before the addition of 200 µl of buffer containing neutravidin (0.01 ng per cell). The incubation and washes were then repeated as detailed above. Ad-Fiber-BAP expressing luciferase was added at 10,000 particles per cell in a volume of 200 µl of buffer. The cells were then incubated and washed as above. Finally, the cells were incubated with 0.5 ml of complete RPMI. After 24 hr., cells were lysed and analyzed by luciferase assay as indicated [95]. Results are reported as the mean and standard deviation of triplicates of total lumens. The trends are representative of three independent experiments. For cholera toxin B subunit (CTB) targeting, a slight modification of the protocol was performed. Caco-2 cells were seeded onto 24-well plates in half concentrations so that one side would become confluent before the other. Upon confluence on one side, all wells were infected in triplicate by layering approach as above with 2.5 µg/ml final concentration of biotinylated CTB (Sigma, St. Louis, MO). Cells were processed for luciferase assay as above.

**3.3 Results and Discussion**

**A diverse set of ligands specifically bind APC**

The first part of ligand discovery for adenovirus involved scanning the literature for various receptor targets on APC and mucosal cells and testing the potential ligands for
binding by flow cytometry. The obvious targets are those that identify APC by routine immunochemistry: MHC class II, CD40, CD11c, CD11b, and CD86. The MHC class II receptor is expressed solely by professional APC and used to present peptide antigens to CD4+ T cells. The CD11c receptor is an integrin expressed on dendritic cells, but not expressed on macrophages, B cells, or T cells [136]. The CD11b receptor is a marker for monocyte lineage, expressed predominantly on macrophages and dendritic cells. The CD40 and CD86 receptors belong to the family of costimulatory molecules that are involved in APC-T cell stimulation. Using antibodies, the expression level of these receptors were analyzed on mouse bone marrow-derived dendritic cells (BMDC) (Figure 3-1A). MHC class II and CD11b were the highest as measured by mean fluorescence intensity (MFI) and considered promising for Ad-Fiber-BAP targeting.

It was hypothesized that other receptors might be better equipped to serve as receptors for increased Ad uptake and subsequent transduction. In addition to macropinocytosis and phagocytosis, APC use various C-type lectin receptors [137] and scavenger receptors [138] to internalize antigen for processing and presentation to T cells. Asialofetuin targeting the dendritic cell asialoglycoprotein receptor (DC-ASGPR) [139] and heat shock cognate protein 70 (Hsc70) targeting CD91 [140] were chosen to characterize the expression of two antigen uptake receptors (Figure 3-1B). DC-ASGPR expression was more than 40 times lower than MHC class II (y axes not on same scale). Antigen uptake receptors presumably need not be expressed at high levels for their function.
Figure 3-1. Receptor expression on bone marrow-derived dendritic cells. A) On Day 12, BMDC were stained with various biotinylated monoclonal antibodies at 5 μg/ml. Biotinylated rat IgG2a isotype antibody was used as a negative control ligand. B) On Day 13, BMDC were stained with indicated biotinylated ligands. BSA was used as a negative control ligand. Streptavidin-Alexa Fluor 488 conjugate was used for detection. Cells were analyzed by FACScan and quantified as mean fluorescence intensity (MFI) using CellQuest software by subtracting the untreated condition.
**Ad-Fiber-BAP works as a versatile ligand screening platform on APC**

After determining the expression level of varying receptors, Ad-Fiber-BAP was used as a ligand screening platform for the enhanced transduction of mouse BMDC. The ligands were tested by the layering approach as described previously [95]. Briefly, the ligands and Ad-Fiber-BAP are bound to the cells using an avidin bridge. This allows the virus to be targeted to any receptor for which there exists a biotinylated ligand. Biotinylated antibodies were used to target MHC class II, CD11b, CD11c, CD40, and CD86. Other biotinylated ligands included those that are directed to the uptake receptors of APC: Hsc70 targeting CD91 [140] and possibly TLR-2/4 [141, 142], macroaggregated albumin targeting scavenger receptors [135, 138], mannose-BSA targeting the mannose receptor [143, 144], and asialofetuin targeting the DC-ASGPR [139]. In addition, we used biotinylated phosphorothioate oligonucleotides containing unmethylated CpG and GpC motifs for targeting CD11b [145] and possibly TLR-9 [146, 147]. Transduction was measured by flow cytometry 24 hr later and reported as percent GFP-positive cells (Figure 3-2).

We observed varied levels of transduction based on percentage positive BMDC with the highest levels seen using the ligands targeting the uptake receptors. There are two interesting comparisons made between the binding in Figure 3-1 and the re-targeting in Figure 3-2. First, the anti-MHCII and -CD11b mAb conferred over 40-fold increased binding as compared to asialofetuin and Hsc70; however the re-targeting efficiency with mAbs conferred an approximate 4-fold decrease in transduction efficiency. These trends may be related to how well each receptor binds and internalizes its ligand, providing evidence that the uptake receptors are honed to process and present exogenous antigen
more efficiently. Binding assays were not performed for mannosylated BSA, macroaggregated albumin, or the GpC and CpG oligos. It remains to be seen if these ligands have lower affinity than the antibodies but perform better for re-targeting Ad-Fiber-BAP. Second, the anti-CD11b mAb mediated weak transduction, whereas the oligonucleotides that presumably bind CD11b mediated substantially better transduction. Assuming that the mAb would have equal to or better affinity for CD11b, the difference could be explained by other factors, such as steric hindrance and additional nucleic acid-binding receptors. Overall, these results seem to contradict the conventional wisdom that high-affinity antibodies are ideal for targeting and suggest that native ligands might be superior in trying to re-target adenovirus for internalization into dendritic cells.

![Figure 3-2. Ligand screening on bone marrow-derived dendritic cells using Ad-Fiber-TR-BAP. BMDC were first incubated with the indicated biotinylated ligands, then with neutravidin, and finally with 3000 particles per cell of Ad-Fiber-TR-BAP expressing EGFP. At 24 hr, fluorescence was analyzed by flow cytometry and reported as percent positive relative to mock-infected control. Biotinylated antibodies were used at 5 μg/ml. The negative control ligand was biotinylated rat IgG2a isotype antibody. The trends are representative of two independent experiments.](image-url)
Ligand binding on mucosal cells

A review of the literature indicated several potential ligands for binding mucosal epithelial and M cells of the Peyer’s patches. Of these, cholera toxin and various lectins were considered the most promising. The non-toxic, pentameric binding (B) subunit of cholera toxin (CTB) binds the ubiquitous ganglioside GM1 receptor [148] found on the apical membrane of intestinal epithelial cells [149]. Lectins are proteins and glycoproteins that bind to specific carbohydrate residues, sometimes requiring a certain orientation on the cell surface receptor [reviewed in 150]. The lectins tested include WGA, UEA, and GSL-I-B4. WGA targets mucosal epithelial cells, like Caco-2 [151, 152] and M cells [153] by binding N-acetylglucosamine or cell-surface sialic acid. GSL-I-B4 [153] and UEA [154] selectively target M cells by binding α-galactose and α-fucose, respectively. However, some of these interactions are highly species-dependent.

Biotinylated lectins were first tested for binding on a model mucosal epithelial cell line, Caco-2. The cells were incubated with GSL-I-B4, UEA, and WGA. Biotinylated BSA was used as a negative control and anti-CD59 mAb was used as a positive control. WGA was found to bind to a greater degree than the other lectins and even the positive control (Figure 3-3). These results suggest that the density of sialic acid is much greater than other carbohydrates and even the complement-protecting CD59 receptor. However, the number of biotin molecules per ligand also plays a role in determining the total fluorescence as a tetravalent streptavidin conjugate was used for detection. WGA binding was verified in other experiments in a dose-dependent manner and repeatedly demonstrated its superiority over other lectins (data not shown).
Figure 3-3. Binding of lectins on Caco-2 cells. Cells were incubated with various biotinylated ligands and detected with a streptavidin-phycoerythrin conjugate. Cells were analyzed by FACScan and quantified as mean fluorescence intensity (MFI) using CellQuest software by subtracting the untreated condition.

Ad-Fiber-BAP as a ligand screening platform on mucosal cells

Since WGA binds to a greater degree than all the lectins tested, it was used for screening increased transduction of Ad-Fiber-BAP. WGA was used to target Caco-2 cells by layering approach. Expression was enhanced 20-fold as measured by raw lumens (Figure 3-4). At concentrations above 1 μg/ml, WGA decreases transduction efficiency. This effect may be attributed to receptor cross-linking and inhibition of internalization of the viral targeting complex.

Since other work has shown that CTB conjugates bind Peyer’s patch epithelium [155], CTB was used to screen for enhanced transduction of Ad-Fiber-BAP. Biotinylated CTB was used to target confluent and subconfluent monolayers of Caco-2 cells by layering approach. Expression was enhanced approximately 10-fold regardless of whether the cells were confluent or subconfluent (Figure 3-5).
Figure 3-4. WGA-targeting Caco-2 cells using Ad-Fiber-BAP. Caco-2 cells were incubated with the indicated amount of ligand. The cells were transduced by layering approach with Ad-Fiber-BAP using an avidin bridge. Transduction efficiency was measured by luciferase assay, reported as lumens [129], and presented as the mean and standard deviation of three independent measurements.

Figure 3-5. CTB-targeting of Caco-2 cells using Ad-Fiber-BAP. Subconfluent and confluent Caco-2 cells were incubated with and without 2.5 μg/ml of biotinylated CTB. The cells were transduced by layering approach with Ad-Fiber-BAP using an avidin bridge. Transduction efficiency was measured by luciferase assay, reported as lumens, and presented as the means and standard deviations of three independent measurements. A paired student's t-test was performed to compare non-targeted to targeted Ad-Fiber-BAP (* P < 0.01).
The lack of a significant difference in CTB-targeting as related to confluency is encouraging since ganglioside GM1 has been shown on the apical side of polarized epithelium [149], and CAR has been shown sequestered in tight junctions [156]. Moreover, CAR was further shown to be inaccessible on the basolateral side of polarized epithelium [157]. These results suggest that CTB may be used to target Ad-Fiber-BAP in vivo where CAR may be inaccessible.

3.4 Conclusions

The ligand screening assays using Ad-Fiber-BAP for APC and mucosal targeting were performed as proof of principle. These data demonstrate the utility of metabolically biotinylated vectors as a screening platform for a diverse set of ligands, most of which could never be genetically engineered into the capsid proteins of a virus.

The results from Ad-Fiber-BAP ligand screening experiments offer an alternative to the conventional targeting dogma. First, targeting Ad-Fiber-BAP using high-affinity antibodies does not always guarantee higher transduction efficiency. Some ligands may be better suited for Ad-Fiber-BAP re-targeting as they have co-evolved with the antigen-sampling cells of the immune system. Second, not all receptors are amenable to Ad-Fiber-BAP re-targeting because some may internalize more efficiently and have varying compatibility with Ad intracellular trafficking.

This work has demonstrated that the versatile Ad-Fiber-BAP ligand screening platform may indicate which receptors to re-target in developing an Ad vector for in vivo applications. Potentially, a ligand could be screened first with Ad-Fiber-BAP and then chemically or genetically linked to Ad to create a therapeutic vector for in vivo
applications. The results in this chapter were used to develop re-targeted Ad vectors to APC and mucosal sites \textit{in vivo}. Ad-Fiber-BAP ligand screening indicated that conjugating mannose to the vector may be optimal for enhancing transduction of APC and incorporating a lectin-like ligand onto the vector for targeting cell-surface sialoglycoconjugates may be optimal for transduction of mucosal sites.
Chapter 4

Mannose Receptor-Targeted Transduction of Antigen-presenting Cells

4.1 Introduction

Metabolically biotinylated adenoviral vectors (Ad-Fiber-BAP) are a successful ligand-screening platform for increased transduction of antigen-presenting cells (APC). A diverse set of biotinylated ligands including antibodies, proteins, glycoproteins, and oligonucleotides have been used with varying degrees of success [95]. In particular, the uptake receptors on APC seem to be quite adept in enhancing Ad5 transduction, possibly owing to the fact that they have evolved to sample extracellular antigen for presentation to the immune system.

The candidate ligand for targeting APC identified by the Ad-Fiber-BAP screening platform was mannosylated bovine serum albumin (ManBSA) [95]. ManBSA is a synthetic glycoprotein prepared by reacting α-D-mannopyranosylphenylisothiocyanate with the free amines in BSA [158]. This neoglycoprotein binds specifically to cell-surface lectins in the mannose receptor family.

The mannose receptor family is a subgroup of the calcium-dependent (C-type) lectin superfamily that consists of four members: the mannose receptor (MR), the phospholipase A2 receptor (PLA2R), DEC-205, and Endo180 [reviewed in 159]. Of particular interest in innate and adaptive immunity, the MR is an antigen uptake receptor expressed on macrophages [160] and dendritic cells [161, 162]. It binds glycoproteins and glycoconjugates containing terminal mannose, fucose, and N-acetylglucosamine [163]. It contains multiple carbohydrate-recognition domains (CRDs) [164] that
individually bind monosaccharides with low affinity, while the cumulative contribution of all CRDs contribute to the high avidity towards oligosaccharides.

While Ad-Fiber-BAP transduction of cells by the layering approach is an effective method for ligand screening, it is not useful for in vivo applications. However, biotinylated ligands can be non-covalently linked to Ad-Fiber-BAP using the tight interaction of an avidin bridge during the purification process to form a three-component targeting complex. Since fewer steps or components are generally easier during production, a number of additional strategies were explored for creating a mannose-containing Ad vector to target the MR on APC. They included direct mannosylation of the adenoviral capsid and two-component complex formation using mannose-conjugated avidin. These studies were explored with the aim of creating an easily-modified vector for eventual use in vivo.

4.2 Materials and Methods

Cells and viruses

D2SC/1 cells [165] were maintained in RPMI-1640 with 10% fetal bovine serum (FBS) (Hyclone, Salt Lake City, UT), antibiotic/antimycotic (Gibco BRL, Gaithersburg, MD), sodium pyruvate, and HEPES (Gibco BRL, Gaithersburg, MD). XS52 and XS106 cells [166] were maintained as above with the addition of 50 μM β-mercaptoethanol (Sigma, St. Louis, MO) and 2 ng/ml recombinant mouse GM-CSF (PeproTech, Rocky Hill, NJ). Ad vectors used in this study are based on the Ad-Easy system (Quantum Biotechnologies, Montreal, QC, Canada) and carry the full E1- and E3-deleted Ad5
genome. Viral propagation, purification, and plaque formation were performed as described [134]. Ad-Fiber-BAP was constructed as previously reported [95]. Ad-Fiber-BAP encoding jellyfish green fluorescent protein (GFP) gene and firefly luciferase gene, an internal ribosome entry site, and the humanized Renilla green fluorescent protein were constructed with the Ad-Easy system. Viral particle number was calculated using spectrophotometry by measuring the absorbance of purified viral particles at 260 nm [134].

*Mannosylation of Ad capsid and in vitro transduction*

A luciferase-expressing Ad in 10 mM phosphate buffer (pH 8.0) with 1 M sucrose was reacted with $10^5$, $10^6$, and $10^7$ molecules of α-D-mannopyranosylphenylisothiocyanate (Man-ITC) (Sigma, St. Louis, MO) per viral particle at 4°C for ~18 hr. Un-reacted mannose reagent was removed by desalting with an Econo-Pac 10DG column (Bio-Rad, Hercules, CA). Biotin N-hydroxysuccinimide (Vector Labs, Burlingame, CA) was used at $10^4$ molecules per viral particle as a negative control ligand. For transduction, 5000 particles/cell of conjugated virus was added to D2SC/1 cells in transduction buffer (Hank’s Balanced Salt Solution (HBSS) with 1% BSA) for 30 min. at 4°C. The cells were washed with 1 ml of buffer and resuspended in complete media. After 24 hr., cells were lysed and analyzed by luciferase assay as indicated [95]. The results are reported as the mean and standard deviation of total lumens. The trends are representative of two independent experiments.
**Streptavidin mannosylation**

Streptavidin (Pierce Chemical, Rockford, IL) dissolved to 1 mg/ml in mannosylation buffer (0.3 M NaCl, 0.1 M sodium carbonate, pH = 9.0) was reacted at 4°C with 154 µg of Man-ITC for 20 hr. After dialysis against PBS, the conjugation was resolved by SDS-PAGE and stained with Coomassie blue. The conjugate migrated against free streptavidin as reported previously [167].

**Mannosylation of lysine-avidin conjugates**

First, 10 µl of a 100 mg/ml solution of α-D-mannopyranosylphenylisothiocyanate in DMSO was added to 1 ml of mannosylation buffer. Slowly, this solution was added drop-wise to 1 ml of a 2 mg/ml solution of a 20-mer lysine peptide with a cysteine at the N-terminus (C-20K) (synthesized by ResGen, Huntsville, AL). The resulting solution was allowed to react for 20 hr. at 4°C and then dialyzed against PBS with a 10K molecular weight cut-off dialysis cassette (Pierce Chemical, Rockford, IL). Second, 500 µl of the mannosylated C-20K solution was reacted with 500 µl of a 1 mg/ml solution of EZ-Link Maleimide Activated NeutrAvidin (Mal-NA) (Pierce Chemical, Rockford, IL) for 2 days at 4°C. After dialysis as above, the protein concentration of the conjugate (Man-lys-NA) was determined by BCA assay (Pierce Chemical, Rockford, IL).

**Fluorescamine assay for determining mannosylation and free amines**

Fluorescamine assay was used to measure free amines for estimating the extent of mannosylation by modification of a previously described method [168]. Briefly, serial dilutions of Mal-NA and Man-lys-NA (150 µl) were allowed to react with 50 µl of 0.3
mg/ml fluorescamine (Sigma, St. Louis, MO) in acetone. An avidin that was reacted with C-20K without mannosylation (Lys-NA) was used as a non-mannosylated positive control. The reactions were carried out in the dark at room temperature for 15 min. Fluorescence measurements were then performed using an HTS 7000 Plus plate reader (Perkin–Elmer, Inc., Boston, MA) on the samples, with an excitation wavelength of 360 nm and emission wavelength of 465 nm. Fluorescence measurements were then plotted against protein concentration. The amount of conjugation was determined by comparing the slope calculated by linear regression of mannosylated and unmodified controls. The percentage of additional free amines was expressed as 100 x (slopeman-lys-NA/slopeman-NA - 1) and the extent of mannosylation as 100 x (1 - slopeMan-lys-NA/slopeLys-NA).

**Streptavidin-mannan fusion construct**

Mannan from *Saccharomyces cerevisiae* (Sigma, St. Louis, MO) was resuspended to 14 mg/ml in PBS. 500 µl of the mannan solution was oxidized by incubating 500 µl of 20 mM sodium meta-periodate (Sigma, St. Louis, MO) for 60 min. at 4°C in the dark. To stop the reaction, 5 µl of glycerol was incubated for 5 min. The reaction was desalted with an Econo-Pac 10DG column. Conjugation (ligand termed SA-mannan) was verified by SDS-PAGE analysis and immunoblotting.

**Binding and competition assays for mannosylated ligands**

D2SC/1 cells were incubated with indicated ligands in HBSS with 1% BSA for 30 min. at 4°C. After washing, streptavidin-based ligands were detected with 20 µg/ml of biotinylated phycoerythrin (Molecular Probes, Eugene, OR), and biotinylated ligands
were detected with 20 μg/ml of NeutrAvidin-phycoerythrin conjugate (Molecular Probes). Where ligand competition is indicated, mannan from \textit{Saccharomyces cerevisiae} was co-incubated with ligands at the indicated concentrations. Cells were analyzed by FACScan and quantified as mean fluorescent intensity or percent positive using CellQuest software by setting the background condition without ligand to zero.

\textit{Ad-Fiber-BAP complexing}

Ad-fiber-BAP was propagated on 293A cells and the crude lysate was loaded onto a large CsCl gradient as previously described [134]. After the first CsCl gradient, the vector was diluted to 4 ml in 50 mM Tris-HCl, pH 8 with saturating amounts of tetrameric NeutrAvidin (corresponding to a 100:1 molar ratio of avidin to capsid biotins, assuming 36 biotins/virion). The sample was incubated for 30 min. at 4°C with occasional mixing, then loaded onto a second CsCl gradient and spun at 20,000 rpm for three hours. The band was collected and diluted to 4 ml in buffer with excess biotinylated ManBSA (corresponding to a 100:1 molar ratio of ManBSA to capsid biotins, assuming 36 biotins per virion). The sample was incubated for 30 min. at 4°C to allow complex formation and then banded in a third CsCl gradient overnight. Alternatively, to remove free ManBSA, the complexes were dialyzed using a 1 MDa-cutoff PVDF membrane (Spectrum Laboratories, Rancho Dominguez, CA). Bands were collected and desalted in an Econo-Pac 10DG column. Alternatively, SA-mannan was added in place of NeutrAvidin as above and a final CsCl gradient purification step was performed.
Transduction of dendritic cells with Ad-Fiber-BAP complexes

Each cell type was transduced as indicated with 5000 part./cell of vector complex. Transduction was analyzed by luciferase assay as indicated previously [95]. The results were reported as the mean and standard deviation of total lumens. Alternatively, transduction was measured by fluorescence microscopy and FACScan analysis. Data were reported as the mean and standard deviation of percent GFP-positive using CellQuest software by setting mock-transduced cells to zero.

4.3 Results and Discussion

Direct mannosylation inactivates adenoviral transduction

Since carbohydrate moieties cannot be genetically engineered into the Ad capsid, the most obvious and direct approach is to chemically conjugate mannose to lysine residues exposed on the surface of the viral capsid proteins. To test direct conjugation to Ad, an amine-reactive mannose was reacted at a low ($10^5$ molecules), medium ($10^6$ molecules), and high ($10^7$ molecules) amounts per particle. The doses were chosen based on previous experiments with the PEGylation of Ad [169]. After purification, a splenic DC line (D2SC/1) was transduced with each vector (Figure 4-1). These cell lines were shown previously to be re-targeted by layering approach with Ad-Fiber-BAP and biotinylated ManBSA (data not shown). No increase in transduction was seen for the mannosylated vectors, even when tested on a Langerhans cell line, a type of epidermal DC (data not shown). In fact, conjugation of high amounts of mannose reagent to Ad completely ablated vector transduction. These results are consistent with previous work
with the capsid inactivation of Ad using high amounts of PEG [169]. Results with Ad-
Fiber-BAP transduction using ManBSA suggest that mannose confers increased targeting
when the ligand is displayed from the fiber protein. Since the mannose reagent is amine-
reactive, much of the ligand may react with hexon, disrupting virion function.

![Graph showing transduction levels with different conjugates](image)

**Figure 4-1. Transduction of dendritic cells with mannosylated adenovirus.**
Ad vector mannosylated with $10^5$ (low), $10^6$ (med.), and $10^7$ (high) molecules
was used to transduce D2SC/1 cell lines at 5000 part./cell. After 24 hr.
incubation, cells were harvested for luciferase assay and reported as the mean
and standard deviation of total lumens. NHS-biotin was used as a negative
control conjugate. The trends are representative of two independent
experiments.

**Direct mannosylation of avidin is insufficient for targeting**

Since direct mannosylation of capsid proteins results in disruption of virion
function, a number of strategies for displaying mannose ligands using the Ad-Fiber-BAP
system were explored. Biotinylated ManBSA can successfully re-target Ad-Fiber-BAP
using an avidin bridge. The hypothesis was that conjugating mannose to avidin could accomplish the same goal while turning the avidin bridge into the ligand and making the system a two-component targeting system. A biotinylated ligand would no longer be needed since the targeting moieties would be directly linked to avidin.

The first strategy was to directly conjugate mannose to the free lysines on streptavidin using the amine-reactive mannose reagent used for viral chemical conjugation. This method was found to substitute streptavidin with 12 mannose residues and did not impair the accessibility of the biotin binding sites [167]. The binding of mannosylated streptavidin was compared to ManBSA on dendritic cells (Figure 4-2).

**Figure 4-2.** Binding comparison of mannosylated streptavidin and biotinylated mannosylated BSA. D2SC/1 cells were bound with 50 μg/ml of indicated ligands. Streptavidin-based ligands were detected with 20 μg/ml of biotinylated phycoerythrin, and biotinylated ligands were detected with 20 μg/ml of NeutrAvidin-phycoerythrin conjugate. Cells were analyzed by FACScan and quantified as percent positive using CellQuest software by setting the background condition without ligand to zero.
ManBSA binds 40 times better as measured by percent positive. Unconjugated streptavidin and biotinylated BSA were included as negative controls. The lower affinity of mannosylated streptavidin may be due to the lower substitution of mannose residues. Mannosylated BSA contains 20 to 25 mannose residues and this higher extent of mannosylation was found to be required for high affinity binding to the mannose receptor [170]. Streptavidin can only theoretically contain 16 mannose residues based on the presence of lysines, though only 12 may be solvent-exposed for chemical modification [167]. Therefore, mannosylation of streptavidin may be an inadequate strategy for generating a dendritic cell-targeting ligand for replacement of mannosylated BSA.

Mannosylated lysine-conjugated avidin is a promiscuous targeting ligand

The second strategy involved increasing the number of mannose residues that could be displayed on an avidin molecule. It was noticed that mannosylated polylysine [171, 172] could target the mannose receptor on macrophages. Furthermore, mannosylated di- and tri-lysine peptides [173] and a mannosylated hexa-lysine peptide [174] could bind the mannose receptor with high affinity. It was hypothesized that an oligolysine peptide cross-linked to avidin could be sufficiently mannosylated to target the mannose receptor with high affinity.

To accomplish this feat, a two-step method in protein chemistry was devised taking advantage of amine- and sulfhydryl-reactive molecules to increase the number of mannoses that could be displayed on an avidin molecule. First, the free amines of a 20-mer lysine peptide with a cysteine at the N-terminus (C-20K) were reacted with a mannosylating reagent. The mannosylated C-20K was then cross-linked to
commercially-available maleimide-activated avidin, and the conjugate was termed Man-lys-NA. Maleimides react specifically and irreversibly with sulfhydryls on proteins. Since there are approximately four maleimide molecules per molecule of avidin, there are theoretically 80 possible mannose residues. Due to steric hindrance, it was anticipated that the actual degree of conjugation would be lower than 80.

To assess the extent of mannosylation of the lysine residues, a fluorescamine assay was employed to quantitatively measure the remaining free amines (Figure 4-3).

![Figure 4-3. Fluorescamine assay for extent of mannosylation. Serial dilutions of each molecule were reacted with fluorescamine and the fluorescence was measured in arbitrary units. The slopes of the lines were calculated by linear regression and used to represent the percent of mannosylation as 100 x (1 - slope_{Man-lys-NA}/slope_{Lys-NA}) and the percent of additional free amines as 100 x (slope_{Man-lys-NA}/slope_{Mal-NA} - 1).

Fluorescamine is a non-fluorescent reagent that reacts readily with primary amines to form a highly fluorescent conjugate. The increase in fluorescence is proportional to how
many free amines are available on the protein. Comparison to an unmodified control reveals the percentage of amines that were not modified during mannosylation. Avidin conjugated to the C-20K peptide without mannosylation (Lys-NA) served as the control for measuring extent of mannosylation. Maleimide-activated avidin (Mal-NA) served as a control for measuring free amines as this was the base molecule on which the lysines and mannoses were added. Based on the slopes of each trend line in Figure 4-3, it was estimated that 79% of the Man-lys-NA was mannosylated with 38% remaining free amines. With the theoretical limit of 80 residues of mannose per avidin, the actual number was approximately 64.

Next, the binding of Man-lys-NA was assessed on dendritic cells and compared to the model mannose receptor ligand, ManBSA (Figure 4-4). Man-lys-NA labels cells as efficiently as ManBSA with up to 100% of the total cells stained. To determine specificity for the mannose receptor, a competition binding assay was performed with mannan, a high molecular weight oligomannose structure isolated from yeast (Figure 4-5). This carbohydrate is the standard competitor for assays involving binding studies with the mannose receptor. Even at 1 mg/ml concentration of mannan there is an insignificant reduction in the amount of cells stained positive, though concentrations of 0.3 mg/ml of mannan are sufficient for maximum blockade of the mannose receptor [144]. Man-lys-NA seems to be interacting with cell surface receptors independent of the mannose receptor. In fact, binding assays on intestinal epithelial cells, fibroblasts, and dendritic cells indicate that all stain equally well with Man-lys-NA (data not shown).
Figure 4-4. Binding comparison of mannosylated lysine-avidin conjugate and mannosylated BSA. D2SC/1 cells were bound with indicated concentrations of ligands. Streptavidin-based ligands were detected with 20 μg/ml of biotinylated phycoerythrin, and biotinylated ligands were detected with 20 μg/ml of NeutrAvidin-phycoerythrin conjugate. Cells were analyzed by FACSscan and quantified as percent positive using CellQuest software by setting the background condition without ligand to zero.

Research has shown that higher substitution of neoglycoproteins with mannose residues can cause non-specific binding and lower substitution can cause inefficient binding [170]. Monsigny et al. have determined that neoglycoproteins containing approximately 25 mannose residues specifically recognize lectin receptors [158]. With approximately 60 mannose residues per molecule, Man-lys-NA potentially interacts non-specifically (e.g. via charge interactions) with the cell surface as the inability of mannan to effectively inhibit suggests. This ligand will ultimately have to be modified to decrease the extent of lysines for amine-reactive mannosylation.
Figure 4-5. Mannan competition of mannosylated lysine-avidin conjugate. D2SC/1 cells were bound with indicated concentrations of mannan and 25 μg/ml of Man-lys-NA. Cells were detected with 20 μg/ml of biotinylated phycoerythrin, analyzed by FACScan, and quantified as percent positive using CellQuest software by setting the background condition without ligand to zero.

Mannan-conjugated avidin is incapable of re-targeting adenovirus

Since increasing the mannose residues on avidin using an oligolysine peptide failed to produce specific targeting, a mannan-avidin conjugation strategy was employed. Mannan has been used extensively to block the mannose receptor to show the specificity of mannose-containing ligands. Additionally, mannan conjugates have been used previously to target antigen to the mannose receptor [175-177]. Reactive aldehydes were generated on mannan using periodate oxidation. The oxidized mannan was conjugated to hydrazide-modified streptavidin, termed SA-mannan. Hydrazide groups react with aldehydes on carbohydrates to form a covalent hydrazone linkage. Conjugation was confirmed by SDS-PAGE analysis and the molecular weight migrated at approximately 200,000 Daltons by comparison to standards (data not shown). Next,
the targeting characteristics of SA-mannan was assessed on dendritic cells and compared to unmodified streptavidin (SA) (Figure 4-6). SA-mannan efficiently binds D2SC/1 cells in a dose-dependent manner with greater than 80% positive for all concentrations tested (data not shown). To test targeting, a layering approach has been employed in previous experiments. This method is useful for screening in vitro but would be insufficient for assessing any in vivo gene delivery. Therefore, a method for creating a two-component targeting complex was developed during vector production. To do this, SA-mannan was added to Ad-Fiber-BAP in excess after the first CsCl gradient purification step.

![Graph showing binding of streptavidin-mannan conjugate](image.png)

**Figure 4-6. Binding of streptavidin-mannan conjugate.** D2SC/1 cells were bound with indicated concentrations of ligands and detected with 20 μg/ml of biotinylated phycoerythrin. Cells were analyzed by FACScan and quantified as mean fluorescence intensity (MFI) using CellQuest.
Free ligand was removed by the final CsCl gradient purification step. Purified complexes were subjected to SDS-PAGE and immunoblotting to show that the ligands are physically bound to the Ad particles (data not shown). The Ad-Fiber-BAP/SA-mannan targeting complex was tested for the ability to increase transduction of D2SC/1 cells (Figure 4-7). SA-mannan confers no increase in transduction efficiency as compared to unmodified or SA-complexed Ad. D2SC/1 cells were also transduced by layering approach with similar results (data not shown). It was hypothesized that the high molecular weight of SA-mannan might inhibit the internalization of the Ad particles, as do high concentrations of lectins (Figure 3-4). This argument is supported by the fact that SA-mannan binds dendritic cells (Figure 4-6) but cannot confer increased transduction.

![Graph showing transduction levels](image)

**Figure 4-7. Transduction of dendritic cells with Ad-Fiber-BAP/SA-mannan complexes.** D2SC/1 cells were transduced with 5000 part./cell of indicated vector. After 24 hr. incubation, cells were harvested for luciferase assay and transduction is indicated as total lumens. The results are presented as the means for three independent experiments and error bars indicate standard deviations. A paired student's t-test was performed to compare transduction of Ad-BAP vs. Ad-BAP/SA-mannan (ns, not significant).
Mannosylated BSA is a functional targeting ligand for adenovirus

Based on the fact that 25 mannose residues are optimal for specific targeting of the mannose receptor [158], biotinylated ManBSA was tested as proof of principle for re-targeting Ad. Since ManBSA had been used successfully in the ligand screening method by layering, two separate complex formation strategies were developed. Both strategies relied on the successful complexing of avidin to Ad-Fiber-BAP described previously during vector production and purification. The first strategy included a dialysis step to remove excess biotinylated ManBSA, whereas the second strategy included a third CsCl gradient purification step. For dialysis purification, Ad-Fiber-BAP expressing luciferase complexed to ManBSA was used to transduce D2SC/1 cells and a Langerhans cell line (XS52) (Figure 4-8).

![Figure 4-8. Transduction of dendritic cells with Ad-Fiber-BAP/ManBSA complexes expressing luciferase. XS52 and D2SC/1 cells were transduced with 5000 part./cell of indicated vector. After 24 hr. incubation, cells were harvested for luciferase assay and reported as the mean and standard deviation of total lumens.](image-url)
To mimic in vivo conditions, all transductions were done without washing off the complexes. ManBSA increased transduction more than a log over unmodified Ad. For CsCl gradient purification, Ad-Fiber-BAP expressing GFP complexed with ManBSA was used to transduce D2SC1 cells (Figure 4-9).

Figure 4-9. Transduction of dendritic cells with Ad-Fiber-BAP/ManBSA complexes expressing GFP. D2SC1 cells were transduced with 5000 part./cell of indicated vector. Cells were monitored by fluorescence microscopy (A) and at 48 hr. harvested for FACScan analysis (B). Transduction was quantified as percent GFP-positive using CellQuest software by setting mock-transduced cells to zero.
Fluorescence microscopy demonstrated that expression of GFP increased steadily over 48 hr. with the highest seen with the Ad-Fiber-BAP/ManBSA complexes (Figure 4-9A). After 48 hr., the cells were harvested and the percent GFP-positive was enumerated by flow cytometry (Figure 4-9B), indicating that the ManBSA complexes were almost five times more efficient than unmodified Ad-Fiber-BAP. Moreover, these trends hold true for XS52 cells, XS106 cells and primary mouse bone marrow-derived DCs (data not shown).

4.4 Conclusions

The goal of the work just described was to develop an effective re-targeted adenoviral vector for in vivo applications. The mannose receptor was discovered through the ligand screening method as an effective target for APC. Various strategies were explored for the modification of Ad with mannose residues. Direct chemical conjugation was shown to destroy the transduction capability of Ad; therefore, methods of adding mannose through an avidin bridge were studied. A two-component strategy was the preferred method as it would be the easiest to produce and purify. This strategy would have entailed conjugating mannose directly to avidin and then complexing this modified avidin to Ad-Fiber-BAP. However, the various strategies for conjugating mannose residues to avidin failed at specific binding to the mannose receptor and/or conferring increased transduction to Ad. The model ligand, ManBSA, was thus used to create a three-component complex using an avidin bridge. The Ad-Fiber-BAP/ManBSA targeting complex showed enhanced transduction of dendritic cells in vitro. Since others have
successfully used ManBSA to target APC in vivo [178, 179], this vector was selected as a model targeting vector for in vivo characterization in Chapter 6.
Chapter 5

Ad-σ1 Chimera for Targeting Mucosal Sites

5.1 Introduction

Adenovirus (Ad) vectors are potent gene delivery vehicles capable of eliciting both mucosal and systemic immune responses [15]. Human Ad serotypes 2 and 5 (Ad2 and Ad5) bind and enter cells using the combined interactions of the fiber and penton base proteins with cellular receptors. The fiber protein is an elongated trimer with an N-terminal fibrous tail domain (shaft) and a C-terminal globular head domain (knob). Ad2 and Ad5 bind to the coxsackievirus and adenovirus receptor (CAR) [68, 180] via a binding site located in the knob [114]. CAR is a member of the immunoglobulin superfamily [68, 180] expressed at regions of cell-cell contact [156]. Following fiber-mediated attachment, the penton base binds to cell surface αv integrins to induce internalization [70].

While Ad5 vectors transduce many types of cells, the efficiency of these vectors is limited if cells lack one or more of its receptors [181]. For example, dendritic cells (DCs) do not express CAR and are poorly transduced by Ad5 [20]. This relatively poor transduction of DCs can be enhanced by re-engineering the vector to target alternative receptors [95, 121]. Ad serotypes that bind to other receptors (e.g., CD46 [182]) mediate increased transduction of immunologically-relevant cells [117], but these vectors are more promiscuous than Ad5 and deliver genes into cells that may not contribute to vaccination and thus may increase toxicity. Therefore, while potent, current Ad vectors lack sufficient specificity to function in some applications.
Mammalian reoviruses are nonenveloped, double-stranded RNA viruses with a broad host range [183]. Reovirus infections are common but most are asymptomatic. Reovirus enters the host by either the respiratory or enteric routes and infects epithelium and associated lymphoid tissue [184]. The reovirus attachment protein, σ1, plays a key role in targeting the virus to distinct cell types, including those at mucosal surfaces [185-188]. Like the Ad fiber, reovirus σ1 is an elongated trimer with head-and-tail morphology [189-191]. A domain in the fibrous tail of serotype 3 Dearing (T3D) σ1 binds to α-linked sialic acid [192-195], whereas the head binds to junctional adhesion molecule 1 (JAM1) [195]. JAM1 is an immunoglobulin-superfamily member expressed in a variety of tissues including DCs [196] and epithelial and endothelial barriers [197-199].

The structures of the Ad fiber [200] and reovirus σ1 [201] proteins are strikingly similar (Figure 5-1). The two proteins are to date the only structures known to form trimers using triple β-spiral motifs. The fiber shaft is most likely composed entirely of β-spiral repeats [200], whereas the σ1 tail is predicted to also contain an α-helical coiled coil N-terminal to the β-spiral region [201]. The head domains of both proteins are formed by eight antiparallel β-strands with identical interstrand connectivity. Therefore, while Ad and reovirus belong to different virus families and have few overall properties in common, the observed similarities between the attachment proteins and receptors of these viruses suggest a conserved mechanism of binding.

Based on the structural similarities between Ad fiber and reovirus σ1, we engineered chimeric fiber-σ1 attachment proteins to utilize the JAM1- and sialic acid-binding properties of σ1. Of those tested, only a near full-length version of σ1 grafted
onto the virion-insertion domain of Ad fiber (Fibtail-T3Dσ1) formed trimers and assembled onto Ad particles. We show here that when the fiber gene in the Ad5 genome is replaced with Fibtail-T3Dσ1, the resulting virus, Ad5-T3Dσ1, is capable of infecting intestinal epithelial cells expressing JAM1 and sialic acid and primary human DCs expressing JAM1. These data provide proof of principle for the development of chimeric Ad vectors encoding reovirus σ1 for gene delivery to mucosal surfaces. This work also establishes a foundation for the use of Ad-σ1 chimeric viruses as a genetic platform to enable facile reverse genetic manipulation of the reovirus attachment protein for studies of virus-cell and virus-host interactions.

5.2 Materials and Methods

Cells, antibodies, and viruses

293A (Q-Biogene) and Chinese hamster ovary (CHO) cells (American Type Culture Collection (ATCC), Manassas, VA) were maintained as described [95]. CHO cells stably expressing hJAM1 (CHO-JAM1) were created and maintained as described [202]. 633 cells, a derivative of A549 cells expressing E1, E2A, and Ad5 fiber, were provided by Dan Von Seggern (The Scripps Research Institute, La Jolla, CA) and maintained as described [203]. Caco-2 cells (ATCC) were maintained in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD) with 20% fetal bovine serum. Primary human DCs (NHDC, Cambrex, East Rutherford, NJ) were maintained per vendor’s protocol.

The human (h) CAR-specific monoclonal antibody (mAb) RmcB was purified from CRL-2379 hybridoma cells (ATCC). The hJAM1-specific mAb J10.4 was provided
by Chuck Parkos (Emory University School of Medicine, Atlanta, GA). Rabbit polyclonal serum 1561 was raised against the N-terminal region of Ad5 fiber (peptide ARPSEDTFNPVY). The c-myc-specific mAb was purchased from Pharmingen (San Diego, CA).

Ad vectors used in this study are based on the Ad-Easy system (Quantum Biotechnologies, Montreal, QC, Canada) and carry the full E1- and E3-deleted Ad5 genome with the firefly luciferase gene, an internal ribosome entry site, and the humanized Renilla green fluorescent protein (hrGFP) expressed from a CMV immediate-early promoter in the E1 region.

**Generation of chimeric fiber-σ1 attachment proteins**

Fiber-σ1 fusion constructs were generated using the λ phage Red recombinase system [204] expressed in the BW25113/pKD46 strain [205] obtained from the E. coli Genetic Stock Center (http://cgsc.biology.yale.edu/) as follows: Fibshaft-T3Dσ1, consisting of the N-terminal 396 amino acids of Ad5 fiber fused to amino acid 292 of T3D σ1; Fib8-T3Dσ1, consisting of the N-terminal 170 amino acids of Ad5 fiber fused to amino acid 167 of T3D σ1; and Fibtail-T3Dσ1, consisting of the N-terminal 44 amino acids of Ad5 fiber fused to amino acid 18 of T3D σ1. Sequences encoding the reovirus T3D σ1 protein flanked by a bovine growth hormone polyadenylation signal and a zeocin-resistance gene were amplified using Pfu polymerase (Stratagene, La Jolla, CA) and primers containing 39-nucleotide overhangs homologous to the pCMVfiber plasmid. The pCMVfiber plasmid, containing the Ad5 fiber gene expressed from a CMV immediate-early promoter, was cotransformed with the PCR product into the λ phage
Red strain BW25113/pKD46. Recombinants were selected using zeocin-containing agar plates.

FibTail-T3Dσ1 was subcloned into a plasmid containing sequences homologous to E4 and then recombined into the Ad5 genome to replace the fiber gene using Red recombinase. To aid in detection of the chimeric protein, two c-myc tags (C2) and one hexahistidine tag (H6) were added to the C-terminus of the chimera (FibTail-T3Dσ1C2H6) prior to recombination. The recombinants were screened for loss of the fiber gene by restriction endonuclease mapping and sequencing.

To remove the JAM1-interacting head domain, primers were designed to amplify FibTail-T3Dσ1 created previously that would remove all amino acids past 292 of the T3D σ1 protein. The PCR product was cloned into pCMVfiber by restriction endonuclease digestion to create a chimeric protein called FibTail-T3Dσ1-ΔH. This construct was cloned into the Ad5 genome as above.

**Protein expression and characterization**

CHO cells were transfected with plasmids encoding fiber-σ1 chimeras using LipofectAmine-PLUS (Invitrogen, Carlsbad, CA), and cell extracts were harvested for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Immunoblots were performed as described [95].

**Generation of a chimeric Ad vector**

Linearized Ad genome encoding the FibTail-T3Dσ1C2H6 chimera was transfected into 633 cells and maintained in the presence of 0.3 μM dexamethasone and 5 μg/ml of
Polybrene. Virus was propagated, purified by CsCl-gradient centrifugation, and quantified as described [134]. The resultant recombinant virus, Ad5-T3Dσ1, was amplified for a final round using 293A cells to remove any residual fiber from newly assembled virions. Ad genome encoding Fibtail-T3Dσ1-ΔH was used in a similar manner to create the virus, Ad5-T3Dσ1-ΔH.

CsCl-banded Ad5, Ad5-BAP-TR (CAR-ablated biotinylated Ad [95]), and Ad5-T3Dσ1 were precipitated with trichloroacetic acid (TCA). Pellets were resuspended in loading buffer, and 4x10^{10} particles/lane were resolved by SDS-PAGE and immunoblotting. For total protein analysis, precipitated virus (1.5x10^{11} particles/lane) was resolved by SDS-PAGE, and gels were stained with Coomassie blue.

**Transduction of CHO cells transfected with receptor constructs**

CHO cells were transfected with plasmids expressing hCAR, hJAM1, or murine (m) JAM1 [206, 207]. After 48 h, the cells were washed once with Hank’s Balanced Salt Solution (Gibco) with 1% bovine serum albumin (HBSS-BSA) and adsorbed with 5000 particles/cell of Ad5-T3Dσ1 at 4°C for 30 min. Cells were washed twice with HBSS-BSA, and fresh medium was added. After incubation at 37°C for 24 h, cells were harvested, lysed and luciferase activity (in lumens) was measured as in [95]. For comparison of Ad5 and Ad5-T3Dσ1, cells were transfected and transduced as above without using mJAM1. Transduction efficiency for each vector as measured in lumens was compared to mock-transfected to generate fold-increase values. Receptor expression was confirmed by flow cytometry.
Transduction of Caco-2 cells and primary DCs following receptor blockade

Cells were harvested, washed with HBSS-BSA, and incubated in suspension with 10 μg/ml of either hCAR-specific mAb RmcB or hJAM1-specific mAb J10.4 at 4°C for 30 min. Alternatively, Caco-2 cells were treated with 333 mU/ml of Clostridium perfringens neuraminidase type X (Sigma, St. Louis, MO) at 37°C for 30 min to remove cell-surface sialic acid, followed by two washes with HBSS-BSA. Cells were then adsorbed with 5000 particles/cell of Ad5-T3Dσ1 at 4°C for an additional 30 min, washed twice, and seeded onto 24-well plates in fresh medium. After incubation at 37°C for 24 h, cells were harvested for determination of luciferase activity. For comparison of Ad5 and Ad5-T3Dσ1, and Ad5-T3Dσ1 and Ad5-T3Dσ1ΔH, Caco-2 cells were transduced as above without neuraminidase treatment.

Binding assay of Ad vectors using real-time PCR

Caco-2 cells were seeded at a density of 100 thousand cells per well in a 24-well plate. The following day, the cells were then adsorbed with 1000 particles/cell of Ad5 and Ad5-T3Dσ1 at 4°C for 30 min, washed three times, and harvested with reporter lysis buffer (Promega, Madison, WI). Genomes were amplified using hexon-specific primers and the QuantiTect SYBR green PCR kit (Qiagen, Valencia, CA). Five concentrations of a hexon-containing plasmid with log intervals were used as standards. The real-time PCR reaction was carried out in an ABI Prism 7900HT (Applied Biosystems, Foster City, CA) and analyzed using the SDS 2.2P1 software.
Fluorescence deconvolution microscopy of Ad-T3Dσ1 trafficking

Ad5-T3Dσ1 (2x10^{11} particles) was labeled with 100 μg Alexa Fluor 488 succinimidyl ester (Molecular Probes, Eugene, OR) for 1 hr. in the dark at room temp. Free dye was removed by desalting with an Econo-Pac 10DG column (BioRad, Hercules, CA). CHO-JAM1 cells were plated in permanox 8-well chamber slides at 15,000 cells per well. The next day, cells were washed with buffer (HBSS with 1% BSA) and incubated with 15,000 particles per cell at 4°C for 30 min. After thorough washing, cells were incubated at 37°C with complete media until fixation. Cells were fixed with 10% buffered formalin after 20 min., 2 hr., and 4 hr. to allow differing levels of trafficking. Nuclei were stained with DAPI (Molecular Probes) and mounted for imaging on a DeltaVision Restoration (Deconvolution) microscope (Applied Precision, Issaquah, WA).

5.3 Results and Discussion

Generation of Ad5 fiber model

The Ad5 fiber shaft is predicted to consist of 21 β-spiral repeats [200]. The Ad5 fiber model (Fiber 5-1, top) was generated by adding 17 β-spiral repeats to the four present in the crystal structure of an Ad2 fragment, which also has 21 β-spiral repeats [200]. Sequence predictions suggest that σ1 contains an N-terminal ~135-residue α-helical coiled coil followed by eight β-spiral repeats and the globular head domain [201, 208]. The σ1 model (Figure 5-1, bottom) was generated by first adding five β-spiral repeats to the N-terminus of the crystallized fragment [201]. This model was then joined with a 135-residue trimeric coiled coil formed by elongating an existing coiled-coil structure.
The N-terminal 45 and 39 residues of fiber and σ1, respectively, are not included in the model as they form a virion-anchoring structure (indicated by grey lines). The overall lengths of the fiber and σ1 models are about 325 Å and 385 Å, respectively, consistent with data from electron microscopy studies.

Figure 5-1. Full-length models of Ad5 fiber (top) and reovirus σ1 (bottom). The three monomers within each trimer are shown in red, orange, and blue. Both proteins have head-and-tail morphology, with an eight-stranded β-barrel domain forming the head. This figure was prepared using RIBBONS [210].

Design and characterization of a functional fiber-σ1 chimera

Based on the structural similarities between Ad5 fiber and reovirus σ1 (Figure 5-1), we engineered three Ad fiber-reovirus σ1 chimeras with increasingly larger portions of σ1 protein replacing structurally homologous regions of fiber (Figure 5-2A). Fibshaft-T3Dσ1 contains the N-terminal 21 β-spiral repeats of fiber fused to the head domain of T3D σ1. Fib8-T3Dσ1 contains the N-terminal eight β-spiral repeats of fiber fused to the T3D σ1 β-spiral and head domains. Fibtail-T3Dσ1 contains the N-terminal 44 amino acid virion-anchoring domain [211] fused to T3D σ1 lacking only the N-terminal 17 amino acids. Following transfection of CHO cells, each of the chimeric attachment
proteins was expressed, but only Fibtail-T3Dσ1 formed trimers (Figure 5-2B and data not shown), suggesting that only this chimera maintains native folding.

Figure 5-2. Design and expression of chimeric fiber-σ1 attachment proteins. (A) Schematic diagram of the chimeric fiber σ1 proteins described in the text. Regions corresponding to fiber and σ1 in the diagrams are shaded black and grey, respectively (not drawn to scale). Fiber tail, which mediates virion anchoring, is represented as a cylinder, the α-helical coiled-coils as ovals, the β-spiral repeats as cylinders, and the head domain as three large ovals. (B) Western blots of boiled (denatured) and unboiled (native) lysates of CHO cells transfected with plasmid expressing Fibtail-T3Dσ1 probed with 1561 serum, which recognizes the N-terminal region of Ad5 fiber.
The lack of trimerization of Fib8-T3Dσ1 and Fibshaft-T3Dσ1 was surprising since both the head and tail regions of σ1 contain trimerization domains [212], whereas the fiber knob domain initiates and maintains trimerization [213]. Since only Fibtail-T3Dσ1 formed trimers, it is likely that the C-terminal trimerization domain of σ1 is insufficient for trimerization of the fiber shaft. Alternatively, it is possible that the chimeric Fib8-T3Dσ1 and Fibshaft-T3Dσ1 proteins do not form trimers because the fused β-spiral junctions are imperfectly matched.

Creation of an Ad vector expressing a chimeric fiber-σ1 attachment protein

The Fibtail-T3Dσ1 gene was recombined into an Ad5 genome lacking E1 and E3 to replace the fiber gene using λ phage Red recombinase [204]. During the cloning process, two c-myc tags (C2) and one hexahistidine tag (H6) were added to the C-terminus of Fibtail-T3Dσ1 (Fibtail-T3Dσ1C2H6) to facilitate protein detection. The resulting virus, Ad5-T3Dσ1, was rescued by transfection and production in 633 fiber-expressing cells [203]. After amplification in 633 cells, the virus was passaged in 293A cells to eliminate fiber from the virions and allow only Fibtail-T3Dσ1C2H6 to be encapsidated.

To determine whether Fibtail-T3Dσ1C2H6 was encapsidated onto Ad5 virions, CsCl-purified Ad5, Ad5-BAP-TR, which displays biotinylated fibers [95], and Ad5-T3Dσ1 were analyzed by immunoblotting using antibodies specific for either the fiber N-terminus (1561) or the c-myc epitope tag (Figure 5-3A). Comparison of the immunoblots demonstrated that Fibtail-T3Dσ1C2H6 was encapsidated onto Ad5 virions at levels similar to those of fiber on Ad5 and Ad5-BAP-TR. As anticipated, the anti-c-myc
antibody recognized both Ad5-BAP-TR and Ad5-T3Dσ1C2H6, which contain c-myc tags, but not wild-type fiber. Coomassie blue staining demonstrated that relative amounts of the capsid proteins of wild-type Ad5 and Ad5-T3Dσ1 were indistinguishable (Figure 5-3B). Thus, Fibtail-T3Dσ1C2H6 is encapsidated onto Ad virions and enables normal virion maturation.

**Figure 5-3. Characterization of Ad5-T3Dσ1.** Ad5 virions expressing wild-type fiber (Fiberwt), CAR-ablated biotinylated fiber (Fiber-BAP-TR) [95], and Fibtail-T3Dσ1C2H6 were precipitated with TCA. (A) Precipitated particles (4 x10^10/lane) were resolved by SDS-PAGE and immunoblotted with anti-c-myc mAb 9E10 or antiserum 1561, which recognizes the N-terminal region of Ad5 fiber. (B) Precipitated particles (1.5 x 10^11/lane) were resolved by SDS-PAGE and stained with Coomassie blue.

**Transient transfection of CHO cells with JAM1 rescues infection by Ad5-T3Dσ1**

To determine whether the chimeric Fibtail-T3Dσ1 attachment protein could bind to JAM1, CHO cells were transfected with plasmids expressing hCAR, hJAM1, and mJAM1 and tested for infection by luciferase-expressing Ad5-T3Dσ1. CHO cells were chosen for these studies since they lack both CAR and JAM1 and are poorly infected by
both Ad and reovirus [207]. Transduction of CHO cells by Ad5-T3Dσ1 was increased substantially by expression of either hJAM1 or mJAM1 but not by expression of hCar (Figure 5-4A), the cognate receptor for Ad5 [114]. These data indicate that the JAM1-binding domain of Ad5-T3Dσ1 is functional and can target JAM1-expressing cells in a species-independent fashion.

Inhibition of binding to JAM1 and sialic acid blocks Ad5-T3Dσ1 infection

We next tested the capacity of hJAM1-specific mAb J10.4 and C. perfringens neuraminidase to inhibit transduction by Ad5-T3Dσ1. Caco-2 intestinal epithelial cells, a model of mucosal surfaces [214, 215], were used for these experiments since these cells express JAM1, CAR, and sialic acid [195, 216]. Transduction by Ad5-T3Dσ1 was inhibited 50% by JAM1-specific mAb J10.4 and 80% by neuraminidase (Figure 5-4B). Combined treatment with both mAb J10.4 and neuraminidase reduced transduction nearly 95%. In contrast, isotype-matched hCAR-specific mAb Rmcb, used as a negative control, did not diminish luciferase transduction (Figure 5-4B).

To ensure that JAM1-dependent transduction by Ad5-T3Dσ1 is dependent on σ1 and not another Ad protein, we tested the capacity of the T3D σ1-specific mAb 9BG5 [194] to block infection. In contrast to T1L σ1-specific mAb 5C6 [194], mAb 9BG5 inhibited transduction in a dose-dependent fashion (data not shown). We noted a similar decrease in transduction efficiency following incubation of Ad5-T3Dσ1 with sialoglycoporphin, which is known to interact with reovirus T3D σ1 [192], prior to infection of Caco-2 cells (data not shown). These results demonstrate that transduction by Ad5-T3Dσ1 requires σ1 and its receptors, JAM1 and sialic acid.
Figure 5.4. Ad5-T3Dσ1 transduction is mediated by JAM1 and sialic acid. (A) CHO cells were transiently transfected with plasmids encoding hCAR, hJAM1, or mJAM1 and then transduced with 5000 particles/cell of Ad5-T3Dσ1. Transduction was measured in lumens. (B) Caco-2 cells were either untreated or treated with 10 μg/ml of hCAR-specific mAb RmcB (CAR mAb), 10 μg/ml of hJAM1-specific mAb J10.4 (JAM1 mAb), 333 mU/ml of C. perfringens neuraminidase, or both JAM1 mAb and neuraminidase. Cells were transduced and assessed as before. The results are presented as the means for three independent experiments. Error bars indicate standard deviations. A paired student’s t-test was performed to compare transduction of transfected or treated cells versus mock or untreated cells (* P < 0.01; ** P < 0.05; ns, not significant).

Ad5-T3Dσ1 transduces primary human DCs

DCs play important roles in induction of adaptive immune responses [28]. To determine whether Ad5-T3Dσ1 is capable of transducing DCs, we infected primary cultures of human DCs with Ad5 and Ad5-T3Dσ1. DCs express JAM1 but not CAR (Figure 5.5A), consistent with previous observations [196]. Transduction of DCs by Ad5-T3Dσ1 was substantially more efficient than by Ad5 (Figure 5.5B). Moreover, this difference was almost completely eliminated by treatment with hJAM1-specific mAb J10.4 (Figure 5.5B). These findings suggest that Ad5-T3Dσ1 may have utility for transducing CAR-negative DCs at mucosal and other sites.
Figure 5-5. Ad5 and Ad5-T3Dσ1 transduction of primary human DCs. (A) DCs were assessed for surface expression of CAR and JAM1 by flow cytometry using hCAR-specific mAb RmcB and hJAM1-specific mAb J10.4, respectively [207]. The results are presented as the mean fluorescence intensity. (B) DCs were either untreated or treated with 10 μg/ml of hCAR-specific mAb RmcB (CAR mAb) or hJAM1-specific mAb J10.4 (JAM1 mAb) prior to adsorption with 5000 particles/cell of either Ad5 or Ad5-T3Dσ1. Transduction was measured in lumens. The results are presented as the means for three independent experiments. Error bars indicate standard deviations. A paired student's t-test was performed to compare transduction by Ad5 versus Ad5-T3Dσ1 (* P < 0.01; ** P < 0.05).

Ad5-T3Dσ1 transduces Caco-2 cells less efficiently than Ad5

We next wanted to compare the transduction of Caco-2 cells by Ad5 and Ad5-T3Dσ1. Transduction efficiency by Ad5 is over a log more efficient than Ad5-T3Dσ1 (Figure 5-6A), indicating that a binding, internalization, or intracellular trafficking is defective. It was hypothesized that the fiber-σ1 chimera may create a vector that binds to JAM1 less efficiently than Ad5 binds to CAR. Both vectors were adsorbed onto Caco-2 cells, washed, and the genomes were detected using real-time PCR of the hexon gene (Figure 5-6B). In contrast, the data suggest that the chimeric vector actually binds a log more efficiently to Caco-2 cells. Further work using neuraminidase-treated cells and varying concentrations of vector will be required to confirm the binding effectiveness.
Figure 5-6. Comparison of Ad5 and Ad5-T3Dσ1 on Caco-2 cells. (A) Caco-2 cells were either untreated or treated with 10 μg/ml of hCAR-specific mAb RmcB (CAR mAb) or 10 μg/ml of hJAM1-specific mAb J10.4 (JAM1 mAb), and then transduced with 5000 particles/cell of Ad5 or Ad5-T3Dσ1. Transduction was measured in lumens. (B) Caco-2 cells were adsorbed with 1000 particles/cell of Ad5 or Ad5-T3Dσ1 and washed thoroughly. Genomes were amplified with hexon primers and quantified with SDS 2.2P1 software by real-time PCR using SYBR green and an ABI Prism 7900HT. The results are presented as the means for three independent experiments and error bars indicate standard deviations. A paired student’s t-test was performed to compare Ad5 versus Ad5-T3Dσ1 (* P < 0.01).

Ad5-T3Dσ1 remains in intracellular vesicles at long time points

We next hypothesized that the Ad-T3Dσ1 interaction with JAM1 may disrupt normal Ad trafficking. In fact, researchers have discovered that interaction with the primary receptor determines the intracellular trafficking of adenoviruses [131-133]. Retargeting Ad5 to certain receptors may lead to ineffective intracellular trafficking and thus lower transduction efficiency. In a preliminary study, we chose to image Ad5-T3Dσ1 trafficking by fluorescence deconvolution microscopy. Amine-reactive Alexa Fluor 488-was used to label the virus to allow tracking of green fluorescence. Previous experiments in our lab and others have determined this labeling technique to have
negligible effects on Ad infectivity (unpublished observations). CHO cells stably-expressing hJAM1 were infected with labeled virus for 2 and 4 hr before fixation and imaging (Figure 5-7).

![Ad5-T3Dσ1 (2 hr)](image1)
![Ad5-T3Dσ1 (4 hr)](image2)

**Figure 5-7. Fluorescence deconvolution microscopy of Ad5-T3Dσ1 trafficking.** Alexa Fluor 488-labeled Ad5-T3Dσ1 (green) was bound at 15,000 particles per cell on CHO-JAM1 stable transfectants. Virus was allowed to infect cells 2 hr and 4 hr after binding and the cells were fixed for imaging. Cell nuclei (blue) were stained with DAPI.

Mock-infected cells displayed no green fluorescence and binding was confirmed from images taken at time zero (data not shown). At 2 hr and 4 hr after infection, the majority of Ad5-T3Dσ1 remains at the cellular periphery. It is unclear if the virus would reach the nucleus at further time points; however, Ad5 normally reaches the nucleus within 2 hr [131]. As Ad5 was not similarly imaged, it cannot be concluded without a doubt that Ad5-T3Dσ1 has altered trafficking.

The mechanism of influence of fiber and the primary receptor interaction on trafficking is currently unknown. If fiber remains attached to the virion after entry, one
could argue that the Ad particle might follow the normal trafficking of the primary receptor. Ad5 and the fiber-CAR interaction have evolved to provide an efficient way for delivery of its genome to the nucleus. On the other hand, studies have shown that fiber is shed from the capsid immediately upon internalization [130] so it is unlikely to be related to CAR trafficking. There is some evidence that the fiber domain provides signals to allow endosomal escape and trafficking to the nucleus [132]. Moreover, the sequence may be located within the knob domain [133]. Since Fibtail-T3Dσ1 contains only a small N-terminal region of fiber, perhaps the inefficiency of Ad5-T3Dσ1 transduction is related to alterations in trafficking.

**Fibtail-T3Dσ1 and JAM1 interaction is not efficient for Ad transduction**

To elaborate on the altered intracellular trafficking data, we wanted to quantify the effectiveness of targeting the primary receptor for Ad5 and Ad5-T3Dσ1. Thus, we transfected non-permissive CHO cells with hCAR and hJAM1-expressing plasmids and then subjected them to Ad5 and Ad5-T3Dσ1 infection. CAR expression confers a 400-fold increase in transduction for Ad5; however, JAM1 expression only confers a 4-fold increase for Ad5-T3Dσ1 (Figure 5-8A). Next, to determine the relative importance of the JAM1 interaction, we removed the head domain [195] from the Fibtail-T3Dσ1 chimera and produced a chimeric virus, termed Ad5-T3Dσ1-ΔH. Fibtail-T3Dσ1-ΔH protein expression and viral encapsidation was confirmed by SDS-PAGE and immunoblotting (data not shown). Transduction of Caco-2 cells by Ad5-T3Dσ1-ΔH increased 60% over Ad5-T3Dσ1 and proceeded in a JAM1-independent manner as evidenced by JAM1-specific mAb J10.4 blockade (Figure 5-8B). These data indicate that
the chimeric Ad does not utilize JAM1 as efficiently as Ad5 uses CAR for transduction. Since there is evidence that internalization is as efficient as Ad5 (unpublished observations), the data also suggest that JAM1 interaction is detrimental for viral trafficking.

![Graph A](image1)

![Graph B](image2)

**Figure 5-8. JAM1 interaction is not efficient for Ad chimera transduction.** (A) CHO cells were transiently transfected with plasmids encoding hCAR or hJAM1, and then transduced with 5000 particles/cell of Ad5 or Ad5-T3Dσ1. Fold-increase in transduction was calculated by dividing by the values of the mock-transfected condition and the trends are representative of three independent experiments. (B) Caco-2 cells were either untreated or treated with 10 μg/ml of hCAR-specific mAb RmcB (CAR mAb) or 10 μg/ml of hJAM1-specific mAb J10.4 (JAM1 mAb), and then transduced with 5000 particles/cell of Ad5-T3Dσ1 or Ad5-T3Dσ1-ΔH. Transduction data measured in lumens is presented as means and representative of three independent experiments. Error bars indicate standard deviations.

**5.4 Conclusion**

In this study, we fused two structurally homologous viral attachment proteins, Ad fiber and reovirus σ1, to produce a functional chimeric virus, Ad5-T3Dσ1. Of the three fiber-σ1 chimeras tested, only Fibtail-T3Dσ1 bearing the Ad5 fiber virion-insertion domain fused to an almost full-length version of T3D σ1 protein formed trimers and assembled onto Ad virions.
In Ad5-T3Dσ1 virions, Fibtail-T3Dσ1 was encapsidated at levels comparable to wild-type fiber. Furthermore, the capsid protein profile of Ad5-T3Dσ1 is identical to that of wild-type Ad5. Experiments using receptor-transfected cells and antibody and sialic acid-blocking reagents provide compelling evidence that Ad5-T3Dσ1 displaying Fibtail-T3Dσ1 retains both the JAM1- and sialic acid-binding functions of the T3D σ1 protein.

In spite of the re-targeting capability, Ad5-T3Dσ1 was strikingly less efficient than Ad5 at transducing intestinal epithelial cells \textit{in vitro}. We provided evidence that this deficiency is related to JAM1 interaction and altered trafficking, but not altered binding to JAM1. We propose that the Fibtail-T3Dσ1 chimera may be suboptimal for transduction through JAM1. There is some evidence that the Fibtail-T3Dσ1 chimera is not encapsidated as efficiently as fiber (unpublished observations), which may affect the stability of virus during infection. Thus, it remains to be seen if re-engineering of the fiber-σ1 fusion may produce a chimera that does not cause defects in Ad5 biology.

We envision at least four applications for chimeric Ad vectors in which the CAR-binding functions of fiber have been replaced with the JAM1- and sialic acid-binding functions of σ1. First, Ad vectors based on fiber-σ1 chimeras may serve to efficiently target mucosal sites for enhanced induction of immune responses at mucosal surfaces. Second, since JAM1 and sialic acid are expressed on a variety of cells, Ad5-T3Dσ1 and its derivatives may have utility for transducing cells deficient in CAR (e.g., DCs and certain types of cancer cells). Third, since σ1 incorporates its own trimerization motifs, fiber-σ1 fusions may provide a trimeric scaffold for the display of other cell targeting ligands in a manner analogous to fiber-fibrin chimeras [89]. In support of this, we have recently displayed single-chain antibodies on truncated forms of FigTail-σ1 (unpublished
data). Fourth, Ad vectors based on Ad5-T3Dσ1 can be used as a simple genetic platform for directed mutagenesis of σ1 for studies of reovirus tropism and receptor-linked signaling.

The opportunity to use Ad vectors encoding fiber-σ1 chimeras for mucosal targeting is especially appealing. Increased delivery of antigens to intestinal epithelial cells and Peyer’s patch lymphocytes by such vectors might result in more potent and less toxic gene-based vaccines. Reovirus binds to murine microfold (M) cells [185, 186, 188], and the σ1 protein plays an important role in conferring this tropism [188, 217]. Interactions of Ad5-σ1 vectors with M cells may facilitate efficient delivery to underlying Peyer’s patches for induction of immune responses in the gut. Alternatively, σ1-bearing Ad vectors may directly infect DCs at the luminal surface, which are known to shuttle bacteria across epithelial monolayers by opening tight junctions and sampling the intestinal lumen [218]. DCs express tight junction proteins, including JAM1 [196], which are hypothesized to facilitate epithelial barrier penetration. Our finding that Ad5-T3Dσ1 transduces primary DCs more efficiently than wild-type Ad5 suggests that Ad5-σ1 vectors may be useful for antigen gene delivery to DCs in the intestine and other sites.

By virtue of the capacity to infect both intestinal epithelial cells and DCs, Ad5-σ1 vectors may have utility in the induction of immune responses at mucosal surfaces and thus prevent infection at the site of pathogen entry. These vectors also will allow a precise determination of the contribution of the JAM1- and sialic acid-binding properties of σ1 to interactions of σ1 with cells in vivo. This novel approach should lead to improved Ad vectors for gene delivery and enhance an understanding of σ1 biology.
Chapter 6

*In Vivo* Characterization of Targeted and Non-targeted Adenovirus

6.1 Introduction

Adenoviruses are one of the most promising vectors in use for gene-based vaccination since they have proven efficacy *in vivo*. In a comparison study in rhesus monkeys of adenoviruses, poxviruses, alphaviruses, mycobacteria, and plasmid DNA, Merck found that adenovirus type 5 (Ad5) elicited the highest antigen-specific cytotoxic T lymphocyte (CTL) responses [15, 96].

Ad5 delivers its genes efficiently to non-immune cells with delivery occurring to a lesser degree in antigen-presenting cells (APC), which is thought to explain the ability of this vector to provoke immune responses to the transgene products [21]. While Ad vectors have proven their ability to elicit immune responses, they are relatively inefficient at transducing APC as evidenced by the requirement of high particle numbers [20, 113]. For example, dendritic cells (DCs) do not express CAR and are poorly transduced by Ad5 [20]. This relatively poor transduction of DCs can be enhanced by re-engineering the vector to target alternative receptors [95, 121]. Thus, there is rationale for engineering Ad vectors for greater transduction of APC since their role in vaccination has been widely established.

In prior work [95], ligand screening demonstrated that biotinylated Ad5 vectors targeted to the mannose receptor (MR) on dendritic cells (DCs) provided for the highest amount of transduction. We chose to use complexes of biotinylated Ad (Ad-Fiber-BAP) and biotinylated mannosylated bovine serum albumin (ManBSA) as proof of principle for
targeting mouse antigen-presenting cells (APC) \textit{in vivo} for enhancement of gene-based vaccination. Gene delivery of non-targeted and targeted vectors was assessed after systemic delivery and in an \textit{ex vivo} mouse model of macrophage transduction. How the gene delivery \textit{in situ} relates to the subsequent immune responses was compared and discussed. Though inconclusive, the data suggest that targeting APC may not be adequate for enhancing immune responses.

As it relates to mucosal delivery, Ad5 is a respiratory pathogen and thus not adapted to infection of the gut [219]. Studies have shown that oral vaccination produces much lower CD8 responses than systemic [220] and requires much higher particle numbers for eliciting strong responses [221, 222]. Moreover, oral vaccination studies with Ad have shown that some animals do not mount an immune response, indicating that intestinal mucosal surfaces cannot be reliably infected [221, 223]. The difficulty of mucosal gene delivery is not surprising considering that mucosal surfaces have evolved to escape infection by pathogens. Since the importance of mucosal immune responses in protection has been widely established, there is rationale for targeting mucosal surfaces for delivery of adenoviral gene-based vaccines.

Previous work demonstrated the development of a chimeric Ad vector displaying the reovirus $\sigma1$ protein [224] that targets sialic acid and junctional adhesion molecule 1 (JAM1) on mucosal epithelial cells \textit{in vitro}. Reovirus binds to murine microfold (M) cells [185, 186, 188], and the $\sigma1$ protein plays an important role in conferring this tropism [188, 217]. The opportunity to use Ad vectors encoding fiber-$\sigma1$ chimeras for mucosal targeting is especially appealing. Interactions of Ad5-$\sigma1$ vectors with M cells may facilitate efficient delivery to underlying Peyer's patches for induction of immune
responses in the gut. Alternatively, σ1-bearing Ad vectors may directly infect DCs at the luminal surface, which are known to shuttle bacteria across epithelial monolayers by opening tight junctions and sampling the intestinal lumen [218]. DCs express tight junction proteins, including JAM1 [196], which are hypothesized to facilitate epithelial barrier penetration. Since DCs and M cells are implicated in mucosal immune responses [41], Ad5-σ1 vectors might result in more potent and less toxic gene-based vaccines.

Ad5-σ1 vectors were used as proof of principle for targeting mouse mucosal sites \textit{in vivo} for testing the enhancement of gene-based vaccination. Gene delivery of Ad5 and Ad5-σ1 vectors were compared after systemic and mucosal delivery. How the gene delivery \textit{in situ} relates to the subsequent immune responses was discussed. Though inconclusive, the data suggest that Ad5-σ1 vectors may have utility for mucosal targeting and enhancement of immune responses.

\subsection*{6.2 Materials and Methods}

\textit{Animals, cell lines, and viruses}

RAW264.7 cells (ATCC, Manassas, VA) were maintained in cRPMI (RPMI-1640 with 10\% fetal bovine serum (FBS) (Hyclone, Salt Lake City, UT), and antibiotic/antimycotic, sodium pyruvate, and HEPES (Gibco BRL, Gaithersburg, MD)).

Ad vectors used in this study are based on the Ad-Easy system (Quantum Biotechnologies, Montreal, QC, Canada) and carry the full E1- and E3-deleted Ad5 genome with all transgenes expressed from a cytomegalovirus (CMV) immediate-early promoter in the E1 region. Viral propagation, purification, and determination of particle
number were performed as described [134]. Where indicated, genomic titers were determined by real-time PCR amplification of the hexon gene as described below. Ad-Fiber-BAP expressing enhanced green fluorescent protein (EGFP) were constructed as previously reported [95]. Ad-Fiber-BAP expressing the firefly luciferase gene, an internal ribosome entry site, and the humanized Renilla green fluorescent protein were constructed with the Ad-Easy system. Ad5-T3Dσ1 vectors expressing luciferase were constructed as previously described [224]. To create Ad5-T3Dσ1 vectors expressing HIV-1 gag, Fibtail-T3Dσ1C2H6 was recombined using fiber homology as before [224] into a shuttle plasmid containing a blue fluorescent protein (BFP) gene in the Ad5 E3 region. The resulting plasmid was then recombined into an Ad genome based on the AdEasy system with the HIV-1 gag gene. BFP expressed from the E3 region allows visual confirmation of viral propagation. Ad-Fiber-BAP/ManBSA complexes expressing EGFP were created by CsCl gradient purification as described in Chapter 4.

Female BALB/c mice (4-6 weeks old) and female ICR mice (4-6 weeks old) were purchased from Harlan Sprague-Dawley (Indianapolis, IN, USA) and kept in the Center for Comparative Medicine at Baylor College of Medicine (Houston, TX). Mice transgenic for HLA-A*0201/Kb (line 6) were provided by Dr. L. A. Sherman (The Scripps Research Institute, La Jolla, CA) [225]. These mice express α1 and α2 domains of HLA-A*0201 fused to the α3 domain of H-2Kb, allowing the mice to generate HLA-A*0201-restricted T cell responses. The line 6 mice are backcrossed onto C57BL/6 mice such that HLA-A*0201 is expressed in an MHC background of H-2b. These mice were further bred with C3H mice to generate the F1 crosses (HLA-A2.1 mice), used in the bulk of the experiments. All animal procedures were done according to approved
protocols and in accordance with the American Association for the Accreditation of Laboratory Animal Care (AAALAC).

**Footpad injection of Ad-Fiber-BAP/ManBSA**

Both hind footpads of female HLA-A2.1 mice (4-8 weeks old, two per group, n=2) were injected with 50 μl of $10^{10}$ particles of Ad and Ad-Fiber-BAP/ManBSA. After 24 and 48 hr, popliteal lymph nodes were harvested and pooled. Cells were separated by crushing through a 75-um strainer, washed, and resuspended for fluorescence analysis using a FACScan (BD Biosciences, Franklin Lakes, NJ) flow cytometer. Cells were counted as percent positive using CellQuest software by setting the mock-transduced cells to zero. The trends are representative of two separate experiments.

**Peritoneal macrophage isolation and macrophage transduction**

BALB/c mice (three per group, n=3) were sacrificed humanely and dipped in ethanol for sterilization. Five ml of cRPMI were injected into the peritoneum and the stomach was massaged repeatedly. The peritoneal cavity was then cut open carefully and the fluid was collected and kept on ice. After washing, the cells were subjected to magnetic-activated cell sorting (MACS) using a rat monoclonal antibody towards mouse F4/80 (Abcam, Cambridge, MA), goat anti-rat IgG MicroBeads, and MS columns (Miltenyi Biotec, Auburn, CA). Peritoneal macrophages and RAW264.7 (100,000 per condition) were infected with 5000 genomes/cell of Ad-Fiber-BAP/ManBSA expressing GFP. Fluorescence was assessed by microscopy 24 hr later and RAW264.7 cells were subsequently harvested for real-time PCR detection of viral genomes as below.
**Imaging gene expression upon systemic and mucosal delivery of Ad5**

Ad5 expressing luciferase (1.5x10^{10} particles) was given to two sets of ICR mice (at least three per group, n=3) by tail-vein injection, intraperitoneal injection, mouth pipetting, or oral gavage. Cimetidine (Sigma, St. Louis, MO) was included at 0.4 mg/ml in the water of one set of mice for 24 hr prior to administration of Ad. Twenty four hr later, mice were anesthetized with avertin (1 g/ml 2,2,2-tribromoethanol in tert-amyl alcohol) and given 100 μl of 10 mg/ml D-luciferin (Biosynth International, Naperville, IL) by intraperitoneal injection. Images were taken 1, 8, and 15 min after D-luciferin injection on the NightOWL imaging system (Berthold Technologies USA, Oak Ridge, TN) using WinLight software with 2 min exposure and low resolution. Mice given Ad by oral gavage were sacrificed and the intestines were removed and imaged separately.

**Assessing gene expression and delivery after oral gavage of Ad5 and Ad5-T3Dσ1**

For determining gene expression in the intestinal tract, HLA-A2.1 mice (three per group, n=3) were given 10^{11} particles of Ad5 and Ad5-T3Dσ1 expressing luciferase by oral gavage. At 24 hr, the stomach and small intestine were removed and thoroughly cleaned of all debris. Each organ was processed in Reporter Lysis Buffer (RLB) (Promega, Madison, WI) according to the manufacturer’s instructions. Luciferase activities were assayed with luciferase assay reagent (Promega) in a TD-20/20 single-tube luminometer (Turner Design, Sunnyvale, CA).

For determining the presence of Ad DNA in Peyer’s patches, HLA-A2.1 mice (seven per group, n=7) were given 5x10^{10} particles of Ad5 and Ad5-T3Dσ1. After 24 hr, total DNA was isolated from pooled Peyer’s patches using a DNeasy Tissue Kit (Qiagen,
Valencia, CA) according to the manufacturer’s protocol. Real-time PCR was performed on the total DNA in an Applied Biosystems Prism 7900HT sequence detection system with SDS 2.1 software. The parameters used were 1 cycle of 95°C for 15 min, and 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. Amplifications were carried out in a total volume of 50 μl with Qiagen’s QuantiTect SYBR Green PCR Kit using extracted DNA, forward hexon (5'-GAACAAGCGAGTGGGCTC-3') primers, and reverse hexon (5'-GCATTGCGGTGGTGTTAA-3') primers.

For determining the presence of luciferase RNA in Peyer’s patches, BALB/c mice (five per group, n=5) were given 5x10^10 particles of Ad5 and Ad5-T3Dσ1. After 24 hr, total RNA was isolated from pooled Peyer’s patches using an RNaseasy Protect Mini Kit (Qiagen) according to the manufacturer’s protocol. Real-time PCR was performed as before except for an additional cycle of 30 min at 50°C in the beginning to convert the RNA to cDNA using Qiagen’s QuantiTect SYBR Green RT-PCR Kit. Luciferase was detected by amplifying with forward luciferase (5'-TTTGTGCCAGAGTCCTTGA-3') and reverse luciferase (5'-CACGCAGGCTTATGAGG-3') primers.

The results for gene expression and presence of DNA or RNA in the intestinal tract are reported as the number of responding mice per total mice in each experiment. Responding mice were determined by a result that was at least five-fold higher than the mean of the non-treated mice.
**Immunizations**

For APC-targeting, BALB/c mice (five per group, n=5) were immunized with $10^9$ genomes of Ad5 and Ad5-Fiber-BAP/ManBSA expressing GFP. After 4 weeks, mice were sacrificed humanely and spleens and blood were harvested for immune assays.

For route study, BALB/c mice (five per group, n=5) were immunized intranasally (5 µl per nostril), intramuscularly (50 µl in quadriceps), and orally (200 µl) with $10^{10}$ particles of Ad5 expressing HIV-1 gag. After 4 weeks, mice were sacrificed humanely and spleens were harvested for immune assays.

For oral mucosal-targeting, BALB/c mice (five per group, n=5) were immunized by oral gavage with $5\times10^{10}$ particles of Ad5 and Ad5-T3Dσ1 expressing HIV-1 gag. After 8 weeks, mice were boosted by same route with same dose of Ad5. Three weeks later, mice were sacrificed humanely and spleens and blood were harvested for immune assays.

For intranasal mucosal-targeting, BALB/c mice (five per group, n=5) were immunized intranasally (5 µl per nostril) and intramuscularly (50 µl in quadriceps) with $5\times10^9$ particles of Ad5 and Ad5-T3Dσ1 expressing HIV-1 gag. After 4 weeks, mice were boosted by same route with same dose. After another 4 weeks, mice were sacrificed humanely and spleens and blood were harvested for immune assays.

**ELISA for detection of antibody responses**

Enzyme-linked immunosorbent assay (ELISA) was used for determination of GFP-specific and gag-specific antibody responses in mouse sera. NUNC MaxiSorp™ ELISA plates (Rochester, N.Y.) were coated with 100 ng/well of recombinant EGFP
protein (Clontech, Mountain View, CA) or HIV-1 p55 gag (LAV/SF2, NIH Research and Reference Reagent Program) in Plate Coating Reagent (Hybridoma Subisotyping Kit, Mouse, Calbiochem, San Diego, CA). After overnight incubation at 4°C, plates were washed with PBS with 1% Tween (PBST) and then blocked with 5% milk in PBST at room temp. for 2 hr. Plates were then washed with PBST and serum diluted 1 to 100 in PBS were applied in duplicate. After washing, the samples were detected with horseradish peroxidase-conjugated anti-mouse IgA, IgG, and IgM antibody (Abcam, Cambridge, MA). Plates were developed with tetramethylbenzadine (TMB) (Calbiochem) and the reaction was stopped by addition of 1N sulfuric acid. Absorbance was read directly with an HTS 7000 series BioAssay Reader (Perkin-Elmer, Norwalk, CT). Antibody responses were reported as the means and standard deviations of absorbance at a wavelength of 450 nm.

**ELISPOT for detection of CD8+ T cell responses**

Interferon-γ enzyme-linked immunosorbent spot (ELISPOT) assays (MABTECH, Mariemont, OH) were used for determination of GFP-specific and gag-specific CD8+ T cell responses in mouse splenocytes. Briefly, coating antibody (AN-18) diluted to 5.0 μg/ml in PBS was added (50 μl per well) to each well of 96-well PVDF (polyvinylidene difluoride) plates (Millipore Corp., Bedford, MA) and incubated overnight at 4°C. After washing, cRPMI was used to block the plate for 4 hr at 37°C. For isolation of mononuclear cells, spleens were crushed through 70-μm mesh cell strainers. Red blood cells were lysed with ACK buffer (8.29 g/l NH₄Cl, 1.00 g/l KHCO₃, 0.037 g/l EDTA, pH 7.3) and mononuclear cells were resuspended to 10,000 cells per ml in cRPMI. Blocking
medium was removed from plates, 50 μl of stimulating agents, and 50 μl of cells were added to each well in duplicate. Depending on the antigen, either the immunodominant H2-K<sup>d</sup>-restricted GFP peptides (HYLSTQSAL) or the immunodominant H2-K<sup>d</sup>-restricted HIV-1 gag [226] peptides (both synthesized by ResGen, Huntsville, AL) were used at 10 μg/ml. The immunodominant H2-K<sup>d</sup>-restricted influenza nucleoprotein (Flu NP) [227] peptides were used as negative controls, and concanavalin A (Sigma, St. Louis, MO) was used as a positive control. Cells were incubated at 37°C overnight (~18 hr) and then removed by washing. The positive cells were detected with biotinylated mAb (R4-6A2, 1 μg/ml) in PBS with 1.0% FBS for 2 hr. The wells were washed and streptavidin-alkaline phosphatase (diluted 1 to 1000 in PBS with 1.0% FBS) was added for 1 hr. After washing, spots were developed with 100 μl of BCIP/NBT substrate (BioRad, Hercules, CA). Color development was stopped by washing extensively in tap water. Spots were read by ZellNet Consulting (Fort Lee, NJ) and reported as the means and standards deviations of spot-forming colonies (SFC) per 10<sup>6</sup> splenocytes.

6.3 Results and Discussion

Footpad injection of Ad-Fiber-BAP/ManBSA fails to target DC in vivo

Assessing gene delivery of targeted vectors in situ is important for understanding the impact of re-targeting to alternate receptors. We hypothesized that enhancing transduction of dendritic cells after systemic delivery (e.g. footpad injection) of Ad-Fiber-BAP/ManBSA would also enhance the immune responses to the encoded transgene. Therefore, we first tested the ability of Ad-Fiber-BAP/ManBSA to transduce
DCs after intradermal injection. Footpad injection is a type of intradermal delivery that can be used to study transduction of DCs after migration to the draining popliteal lymph nodes in mice [228].

Mice were injected with $10^{10}$ particles of Ad and Ad-Fiber-BAP/ManBSA expressing dsRed in their hind footpad. Popliteal lymph nodes were harvested 24 and 48 hr later for evaluation of fluorescence by flow cytometry. No increase in gene expression was detected from injection of the re-targeted vector at either time point (Figure 6-1).

![Graph showing transduction percentage](image)

**Figure 6-1. Percent positive transduction after footpad injection of Ad and Ad-Fiber-BAP/ManBSA.** Hind footpads of transgenic HLA-A2.1 mice [225] (n=2) were injected with $10^{10}$ particles of each vector. Transduction was quantified at the indicated time points in the draining popliteal lymph nodes as percent positive using CellQuest software by setting the mock-transduced cells to zero. The results are reported as the means and standard deviations. The trends are representative of two separate experiments.

It is important to note that the majority of gene expression for both vectors is limited to CD11c$^+$ DCs, as measured by injection of the respective vectors expressing luciferase (data not shown). Clearly, Ad-Fiber-BAP/ManBSA shows a 5- to 10-fold increase in
transduction efficiency of DCs in vitro (Figure 4-9 and 4-10). The fact that there was no significant targeting effect in vivo may be attributable to the lack of the MR on DCs in situ. Recent work suggests that the MR is almost entirely absent on lymph node and dermal DCs in vivo [229]. This contrasts sharply with all the other literature indicating that DCs strongly express the MR; however, this is most likely attributed to phenotypic changes upon culturing DCs in vitro [230]. The lack of the MR on dermal DCs may explain why Ad-Fiber-BAP/ManBSA shows no enhancement after dermal footpad injection in mice.

**Ad-Fiber-BAP/ManBSA targets macrophages in vitro and ex vivo**

Since the MR is widely expressed on macrophages in vivo [230], another type of professional APC, we decided to further test the performance of Ad-Fiber-BAP/ManBSA on these cells.

A mouse macrophage cell line, RAW264.7, was used for in vitro transduction and primary macrophages were used for ex vivo transduction. Primary macrophages were isolated using magnetic activated cell sorting (MACS) of F4/80⁺ cells from mouse peritoneal washings. Ad, Ad-Fiber-BAP, and Ad-Fiber-BAP/ManBSA expressing GFP were used to infect the two cell types and analyzed by fluorescence microscopy at 24 hr (Figure 6-2). MR-targeting clearly shows the highest enhancement of transduction as evidenced by increased green fluorescence. Moreover, real-time PCR indicates a 30-fold increase in the number of genomes delivered to RAW264.7 cells (data not shown), which is likely the same or more for the primary macrophages.
Figure 6-2. *In vitro* and *ex vivo* transduction of macrophages. RAW264.7 and peritoneal macrophages were transduced with 5000 part./cell of Ad, Ad-Fiber-BAP, and Ad-Fiber-BAP/ManBSA expressing GFP. Transduction was assessed 24 hr later using fluorescence microscopy. Images shown are representative of three independent experiments.
Ad-Fiber-BAP/ManBSA fails to elicit increased immune responses

With the efficient transduction of macrophages in vitro and ex vivo, we set out to determine if Ad-Fiber-BAP/ManBSA could also enhance anti-transgene immune responses. Apostolopoulos et al. show that targeting antigen to peritoneal macrophages ex vivo generates increased antigen-specific cytotoxic T lymphocytes (CTLs) [175]. To test this, BALB/c mice were immunized intraperitoneally with Ad, Ad-Fiber-BAP, and Ad-Fiber-BAP/ManBSA expressing GFP so as to target the macrophages that lie within the peritoneum. Serum GFP-specific antibody responses measured by ELISA indicate that MR-targeting significantly reduced antibody levels (Figure 6-3A). CD8+ T cell responses measured by ELISPOT assay indicate that there is no significant change in interferon-γ-producing cells upon MR-targeting (Figure 6-3B). There is no enhancement of the antibody and cellular immune responses when an MR-targeted Ad vector is delivered into the peritoneum of mice. The first explanation is that this particular immunization model is not sufficient. There is evidence that DCs are the most potent APC for eliciting immune responses [231], and thus macrophages may not produce higher CD8+ T cell responses. Furthermore, we did not measure in situ gene delivery and determine if in fact macrophages are transduced more efficiently than somatic cells. Other effects like cross-presentation could play a more dominant role and limit the ability to elicit higher immune responses by sequestering antigen in macrophages.
Figure 6-3. GFP-specific immune responses after Ad-Fiber-BAP/ManBSA immunization. BALB/c mice (n=5) were immunized with 10⁷ particles of Ad, Ad-Fiber-BAP, and Ad-Fiber-BAP/ManBSA expressing GFP. Serum and splenocytes were harvested four weeks later. A) GFP-specific IgG, IgM, and IgA antibodies were measured by ELISA. B) GFP-specific CD8⁺ T cell responses were measured by ELISPOT and reported as interferon-γ spot-forming colonies (SFC). An irrelevant influenza peptide (Flu NP) was used as a negative control. The results are reported as the means and standard deviations. A paired student's t-test was performed to compare Ad vs. Ad-Fiber-BAP/ManBSA (* P < 0.01; ns, not significant).
Mucosal administration of Ad5 encounters significant barriers to gene delivery

In order to demonstrate that mucosal delivery of Ad5 vectors is indeed less efficient than systemic delivery, we designed an experiment that would image gene expression after administration by varying routes. Particularly, we wanted to assess oral delivery since this route is considered the easiest for adherence in a clinical setting as it obviates the need for needles and trained personnel. We wanted to avoid another easily-administered mucosal route, intranasal delivery, as there are potential safety issues concerning reported gene delivery to the central nervous system [11].

Ad5 expressing luciferase was given to female ICR mice (1.5x10¹⁰ particles each) by tail-vein injection (intravenous, i.v.), intraperitoneal (i.p.) injection, mouth delivery, or by oral gavage into the stomach. Twenty four hours prior to delivery, cimetidine was included in the water of mice given Ad5 by mouth or oral gavage with the hope of preventing pH-dependent destruction of Ad. Cimetidine is a histamine H₂-receptor antagonist that works by decreasing acid production in the stomach. Gene expression was assessed 24 hr after delivery on a NightOWL system by imaging luminescence upon i.p. injection of D-luciferin (Figure 6-4A). Systemic delivery (i.v. and i.p.) produced the strongest gene expression localized in the liver and peritoneal cavity, respectively. Other reports confirm Ad5 gene delivery localizes predominantly to the liver upon tail-vein injection [232]. Mouth application represented the best gene delivery by a mucosal route; however, oral gavage demonstrated no detectable gene expression from whole-animal imaging. There is a minimum amount of luciferase gene expression that must occur in any one site for sufficient light production to penetrate the entire animal and reach the
detector. Thus, to verify any low-level gene expression, the intestines of mice treated by oral gavage were removed for luminescence imaging (Figure 6-4B).

![Image of in situ luciferase imaging of Ad5 transduction by varying routes.](image)

**Figure 6-4. In situ luciferase imaging of Ad5 transduction by varying routes.** A) Female ICR mice were given 1.5x10^10 particles of Ad5 expressing luciferase by tail-vein injection (intravenous, i.v.), intraperitoneal (i.p.) injection, mouth delivery, or oral gavage. Cimetidine was included to block potential inactivation by stomach acids. Luminescence images were taken using the NightOWL system upon i.p. injection with D-luciferin. B) The intestines of mice treated with and without cimetidine by oral gavage were removed for imaging. Each image is representative of at least 3 mice per route.

No luciferase expression was detected in the intestines (or the liver, as a control) of mice treated with or without cimetidine, suggesting that stomach acids do not represent a significant barrier to Ad5 gene delivery by the oral route. Furthermore, the use of bicarbonate buffer to neutralize stomach acids failed to allow for Ad genome detection in the intestines using real-time PCR (data not shown). Flanagan *et al.* offers further evidence for a minor role of stomach acids since bicarbonate had no effect on increasing the effectiveness of an Ad5-based oral vaccine nor did altering the feeding regimen before and after oral delivery [223].

Since no gene expression could be detected by the methods used, we decided to take advantage of the natural signal amplification of the immune system. The
mammalian immune system has evolved to detect traces of foreign antigen and amplify that signal by producing antibodies and cellular responses. Therefore, we immunized mice with an Ad5 vector expressing HIV-1 gag intramuscularly (i.m.), intranasally (i.n.) and orally (by gavage). Two weeks later, interferon-γ-producing gag-specific CD8+ T cell responses in splenocytes were measured by ELISPOT assay (Figure 6-5).

![Graph](graph.png)

**Figure 6-5. Efficiency of Ad5 vaccination by varying routes.** BALB/c mice (n=5) were immunized with 10^10 particles of Ad5 expressing HIV-1 gag. Splenocytes were harvested two weeks later. Gag-specific CD8+ T cell responses were measured by ELISPOT and reported as interferon-γ+ spot-forming colonies (SFC). An irrelevant influenza peptide (Flu NP) was used as a negative control. The results are reported as the means and standard deviations. A paired student’s t-test was performed to compare i.m vs. mucosal (i.n. and oral gavage) and i.n. vs. oral gavage (* P < 0.01).

As expected, systemic delivery (i.m.) produced a significantly higher systemic T cell response. Mucosal delivery was lower with i.n. immune responses being significantly better than oral gavage. The data suggest that the oral route of delivery of Ad5 vectors may encounter more impediments to gene delivery than even the i.n. route.
Alternatively, the nasal-associated lymphoid tissue may be more adept at responding to antigens expressed in the context of Ad5 vectors.

These data suggest that there are many physical and biochemical barriers to in vivo Ad5 gene delivery to the intestinal epithelium. The striking differences between gene expression after mouth and oral delivery and the differences between the immune responses after i.n. and oral delivery could be attributed to the difference in the presence of digestive enzymes. Even though our data do not suggest a role for stomach acids, the low pH and digestive enzymes of gastric fluid have been attributed to inactivation of Ad vectors in vitro [216]. Moreover, the protective glycocalyx covering the mucosal epithelium may sequester Ad5 through charge effects and impede interaction with cellular receptors [233].

Not only are there physical barriers just described, there are significant biochemical barriers related to receptor expression on apical surfaces of the intestinal epithelium. The primary attachment receptor, CAR, is implicated in tight junction (TJ) formation between polarized epithelial cells and possibly limits viral infection of mucosal surfaces [156]. Additionally, CAR has been localized to the basolateral side of mucosal airway epithelia making it inaccessible for virion infection [157]. Also, the sequestering of the internalization receptors into regions of cell-cell contact and basolateral substrate attachment significantly affects adenoviral transduction in polarized Caco-2 monolayers [234].
Comparison of Ad5 and Ad5-T3Dσ1 vectors after mucosal and systemic delivery

Our previous work indicates that Ad5 vectors displaying the reovirus σ1 protein (Ad5-T3Dσ1) may have promise for mucosal gene delivery by targeting JAM1 and sialic acid [224]. We wanted to compare the relative efficiency of in vivo gene delivery to the intestines and the Peyer’s patches, the principal immune induction site for the mucosa and the target of reovirus infection. Previous results in vitro (Figure 5-6A) indicated that we may see a severe impairment of transduction efficiency of this vector in vivo.

A set of experiments were performed that would measure the gene expression, delivery, and transcription after oral delivery in mice. Ad5 and Ad5-T3Dσ1 vectors expressing luciferase were given orally to mice in all experiments. For gene expression, the stomachs and small intestines were removed and homogenized for luciferase assay. No luciferase activity was detected in any of the three mice tested (Table 6-1). Next, we considered the Peyer’s patches to be more relevant for studying vectors useful for vaccination and thought they might harbor more vector and thus increased sensitivity. Therefore, we repeated the experiment and employed a more sensitive real-time PCR assay for detecting Ad genomes. Only one mouse out of seven tested positive for hexon DNA in the total DNA extracted from the Peyer’s patches. Moreover, RNA levels were just as inconsistently positive when detecting transcription of RNA from the luciferase transgene (Table 6-1). In light of the luciferase imaging data (Figure 6-4) and vaccination data (Figure 6-5), the lack of consistent detection of gene delivery offers further proof that the intestinal tract is quite refractory to Ad5 vectors and gene delivery in general. Even so, the results are surprising considering the increased sensitivity of
real-time PCR, especially that assumed for the level of transcriptional RNA in relation to the corresponding gene.

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<th>Vector</th>
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<th>Hexon DNA</th>
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<td>Ad5</td>
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<td>Ad5-T3Dσ1</td>
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Table 6-1. Ad5 and Ad5-T3Dσ1 comparison after oral delivery in mice. Mice were given Ad5 and Ad5-T3Dσ1 vectors expressing luciferase. The stomachs and small intestines were harvested for luciferase activity. In separate experiments, Peyer’s patches (PP) were harvested for determination of hexon DNA and luciferase RNA by real-time PCR using SYBR green and an ABI Prism 7900HT. The results are presented as the number of mice responding in parentheses out of the total number tested outside of parentheses.

Vaccination with Ad5 and Ad5-T3Dσ1 produces similar immune responses

In addition to the gene delivery to the mucosa, we wanted to compare the immune responses after oral mucosal vaccination with Ad5 and Ad5-T3Dσ1 vectors. The σ1 protein was engineered onto the Ad capsid with the aim of increasing mucosal gene delivery to the Peyer’s patches of the gut and thus mucosal vaccination. Also, vaccination studies can serve as an indirect measure of gene delivery where other assays have failed. Measuring the immune responses to the transgene can be a way to increase the sensitivity of detection

BALB/c mice were immunized orally with 5x10^10 particles of either Ad5 or Ad5-T3Dσ1 expressing HIV-1 gag. Due to the difficulty in measuring gene expression (Table 6-1) and even immune responses by this route (Figure 6-5), the mice were boosted orally
after 8 weeks with the same dose of Ad5. Three weeks post-boost, serum gag-specific antibody responses measured by ELISA indicate that vaccination with Ad5-T3Dσ1 was not significantly different than Ad5, nor different from background levels on naïve animals (Figure 6-6A). CD8⁺ T cell responses measured by ELISPOT assay indicate that both vectors are comparable and thus not significantly different (Figure 6-6B). Antigen-specific CD8⁺ T cell frequencies of 0.01% are relatively low when compared to systemic vaccination, and the spread of the data in both cases indicate that gene delivery was highly variable. The lack of measurable antibody responses combined with lower cellular responses is supported by similar studies of oral immunization with Ad5 expressing simian immunodeficiency virus gag [223].

Since the oral mucosal route has additional barriers related to the digestive system, we also chose the intranasal mucosal route to study the effects of JAM1 and sialic acid targeting. The intramuscular route (systemic) was included since this it the most common route of vaccination. BALB/c mice were immunized either intramuscularly or intranasally with 5x10⁹ particles of Ad5 and Ad5-T3Dσ1 expressing HIV-1 gag. After four weeks, the mice were boosted by the same route with the same dose. Four weeks post-boost, serum gag-specific antibody responses measured by ELISA indicate that vaccination with Ad5-T3Dσ1 significantly reduced antibody levels in both routes (Figure 6-7A). CD8⁺ T cell responses measured by ELISPOT assay indicate that both vectors are comparable intramuscularly but that Ad5-T3Dσ1 produces a 50% lower interferon-γ response intranasally (Figure 6-7B). The lower antibody responses elicited by the Ad5-T3Dσ1 vector is not surprising considering its lack of transduction efficiency compared to epithelial cells in vitro (Figure 5-6).
Figure 6-6. Oral vaccination with Ad5 and Ad5-T3Dσ1. BALB/c mice (n=5) were immunized with 5x10^{10} particles of Ad5 and Ad5-T3Dσ1 expressing HIV-1 gag and both were boosted with same dose of Ad5 8 weeks later. Serum and splenocytes were harvested 3 weeks post-boost. A) Gag-specific IgG, IgM, and IgA antibodies were measured by ELISA. B) Gag-specific CD8^+ T cell responses were measured by ELISPOT and reported as interferon-γ^+ spot-forming colonies (SFC). An irrelevant influenza peptide (Flu NP) was used as a negative control. The results are reported as the means and standard deviations. A paired student's t-test was performed to compare Ad5 vs. Ad5-T3Dσ1 (ns, not significant).
Figure 6-7. Systemic and mucosal vaccination with Ad5 and Ad5-T3Dσ1. BALB/c mice (n=5) were immunized with 5x10^9 particles of Ad5 and Ad5-T3Dσ1 expressing HIV-1 gag and boosted with same dose four weeks later. Serum and splenocytes were harvested four weeks post-boost. A) Gag-specific IgG, IgM, and IgA antibodies were measured by ELISA. B) Gag-specific CD8+ T cell responses were measured by ELISPOT and reported as interferon-γ+ spot-forming colonies (SFC). An irrelevant influenza peptide (Flu NP) was used as a negative control. The results are reported as the means and standard deviations. A paired student’s t-test was performed to compare Ad5 vs. Ad5-T3Dσ1 by each route (* P < 0.01; ns, not significant).
However, the fact that the CD8+ T cell responses after intramuscular and intranasal delivery are so comparable was not expected. Overall, the immune responses are not indicative of a 20-fold decrease in transduction efficiency of the Ad5-T3Dσ1 vector on Caco-2 cells (Figure 5-6). One explanation for such similar performance after mucosal delivery is the accessibility of the major attachment receptors for each vector, CAR and JAM1. As stated previously, CAR is implicated in TJ formation and most likely limits Ad5 interaction with mucosal surfaces [156]. JAM1 is also implicated in TJ formation [197, 199] and possibly limits Ad5-T3Dσ1 interaction with mucosal surfaces. The sequestering of CAR and JAM1 would make both vectors dependent on other mechanisms for interacting with mucosal surfaces. Both vectors interact with α, integrins; however, Ad5-T3Dσ1 additionally interacts with sialic acid as a method of entry. This additional interaction could provide the Ad5 chimera with the advantage it needs to overcome its defects in transduction related to altered intracellular trafficking (Figure 5-7) and JAM1 interaction (Figure 5-8).

The similarities in the vaccination response after intramuscular vaccination may be more related than at first thought. CAR is virtually absent on adult skeletal muscle [235] and it is not reported whether JAM1 is even present on muscle. The crippling of Ad5 by the lack of CAR on muscle may give Ad5-T3Dσ1 the advantage it needs to perform comparably after intramuscular vaccination.

The results also suggest that targeting sialic acid may be beneficial for targeting epithelial mucosa. A recent report indicates sialic acid targeting to be important for transduction of intestinal epithelia by various Ad serotypes [236]. Cell-surface sialoglycoconjugates are presumably not sequestered in TJ or localized to the basolateral
membrane as evidenced by the transduction of polarized Caco-2 cells with various Ad-σ1 chimeras. In fact, Ad5 and Ad5-T3Dσ1 transduction efficiency differ by only five-fold when compared on polarized Caco-2 cells (data not shown). Furthermore, removal of the JAM1-binding domain to create a sialic-acid binding chimera, Ad5-T3Dσ1-ΔH, decreases the difference in transduction to only two-fold (data not shown).

6.4 Conclusion

In this work, we tested two re-targeted Ad5 vectors for increasing the immune responses to an encoded transgene. Both vectors were designed with the hypothesis that increasing gene delivery to immunologically-relevant sites would increase immune responses to the gene products. These studies aimed to characterize the gene delivery in vivo and determine whether the immune responses were enhanced. Increased expression of the transgene in the intended target cells in vivo would have provided evidence for verifying the hypothesis. Since gene expression was not fully assessed in vivo, it cannot be concluded that APC-targeting or mucosal-targeting is beneficial for enhancing vaccination.

The mannose receptor-targeted vector was originally designed to increase transduction of antigen-presenting cells, specifically DCs. Increased DC transduction was clearly demonstrated in vitro most likely due to extensive mannose receptor expression after culturing techniques (Chapter 4). After failed transduction of DCs after intradermal injection in mice, it was discovered that mannose receptor expression is quite limited in mouse DCs. Since this is not the case for macrophages, an ex vivo model of transduction was developed. It is unclear how potent macrophages are in vaccination as
most studies have employed DCs in eliciting immune responses. It might be more relevant to use another animal model in which mannose receptors are widely expressed on all professional APC.

Nevertheless, Ad-Fiber-BAP/ManBSA transduced mouse peritoneal macrophages \textit{ex vivo} significantly more efficiently than a non-targeted Ad. Transgene-specific immune responses were not subsequently enhanced after intraperitoneal vaccination with this vector. One caveat is that macrophage transduction was not assessed after direct injection into the peritoneum. Thus, it cannot be concluded that targeting APC, specifically macrophages, is detrimental for Ad-based vaccination. This conclusion would require that increased transduction \textit{in vivo} correlates with decreased immune responses.

If in fact macrophage transduction \textit{ex vivo} does mimic transduction \textit{in vivo}, then it raises the question of the relative importance of direct priming and cross-presentation in gene-based vaccination. There is no question that \textit{ex vivo} targeting of millions of DCs and subsequent administration into humans make for a potent vaccination strategy. The possible limitations of injecting APC-targeted gene delivery vectors as it relates to potency will be discussed in Chapter 7.

The chimeric adenoviral vector encoding the reovirus \(\sigma_1\) protein was developed for targeting JAM1 and sialic acid on mucosal sites. Reovirus \(\sigma_1\) protein plays an important role in conferring tropism to murine M cells [188, 217]. It was rationalized that Ad5-\(\sigma_1\) vectors may interact more efficiently than Ad5 with M cells in Peyer's patches for induction of increased systemic and mucosal immune responses in the gut. Both targeted and non-targeted vectors elicited systemic immune responses that do not
differ substantially; however, mucosal anti-gag IgG and IgA responses in vaginal washes were inconsistent and usually not significant above background (data not shown). Ad5-T3Dσ1 may still offer hope in eliciting better mucosal cellular immunity, including αβ+ T cell responses, which will need to be determined in future work. Nonetheless, the similarities in measured immune responses argue that, despite the defects in Ad5-T3Dσ1 biology (Chapter 5), this vector has promise for use as gene-based vaccine. Studies suggest that re-targeting Ad5 to apical receptors in epithelial barriers is sufficient to overcome lack of accessible CAR [237]. Since JAM1 is presumably inaccessible due to its presence in TJ [197, 199], interaction of Ad-T3Dσ1 with apical sialoglycoconjugates on mucosal surfaces may contribute to its comparable results as a vaccine vector.

Since various methods for assessing gene delivery and expression of the targeted and non-targeted vectors failed in vivo, it is difficult to make conclusions regarding the hypothesis that targeting mucosal sites, particularly Peyer’s patches, increase immune responses. Further work needs to establish the relative importance of the barriers to Ad gene delivery in vivo, namely low pH, digestive enzymes, glycocalyx, and apical receptor expression. These barriers and potential future schemes for overcoming these barriers are outlined in Chapter 7.
Chapter 7

Conclusions and Future Directions

This thesis set out to develop Ad5-based gene delivery vectors with enhanced tropism for immunologically-relevant cells with the goal of enhancing the antibody and cellular immune responses to the vector transgene. To accomplish this goal, two strategies were undertaken to create Ad5 vectors targeted to antigen-presenting cells (APC) and mucosal sites. Professional APC are the sentinels of the immune system that are unique in their ability to activate naïve T cells. Mucosal surfaces are the portal of entry for most pathogens and immune responses at these surfaces provide the first line of defense against most natural infections. Targeting any one of these sites with genes encoding pathogen proteins was hypothesized to enhance immune responses.

7.1 Antigen-presenting cell targeting

Metabolically biotinylated adenoviral vectors (Ad-Fiber-BAP) were used to screen a repertoire of diverse ligands, including biotinylated antibodies, glycoproteins, and oligonucleotides for increased transduction of APC. This method is a facile way of screening many receptor-ligand pairs without having to genetically engineer new Ad vectors and avoid context-specific disruption of capsid proteins. Since the mannose receptor was found to be the most efficient target for Ad transduction, ligands containing mannose were considered for engineering into the Ad capsid. Ultimately, Ad-Fiber-BAP complexed with avidin, and biotinylated mannosylated bovine serum albumin (ManBSA), termed Ad-Fiber-BAP/ManBSA, was created for APC-targeting. This vector
demonstrated improved transduction of mouse dendritic cells (DC) and macrophages in vitro.

Originally, this vector was to be used for targeting DC in mice, since they are the most potent type of APC. After intradermal injection into mouse footpads, the mannose receptor-targeted vector showed no improvement in gene delivery to DC. The skin contains numerous Langerhans cells and dermal DC; however, as found in a very recent study [229], mannose receptor expression is almost absent on mouse DC in situ. This fact could explain why Ad-Fiber-BAP/ManBSA works so well in vitro but performs poorly when injected in vivo. This limited expression contrasts sharply with all the other literature indicating that DC strongly express the mannose receptor; however, this is most likely attributed to phenotypic changes upon culturing DCs in vitro [230]. This work highlights important lessons when trying to develop technologies for biomedical purposes. Certain biochemical factors that are important in one animal model may not translate directly to humans.

Regardless of the poor intradermal transduction of DC, this vector efficiently transduced another type of professional APC ex vivo, mouse peritoneal macrophages. Although the potency of macrophages in vaccination is not well established, Ad-Fiber-BAP/ManBSA failed to produce stronger immune responses than the corresponding non-targeted Ad.

It is unclear whether targeting APC for gene delivery in vivo will actually generate increased immune responses, especially CD8+ cytotoxic lymphocytes. Recent work suggests that increased in vitro Ad transduction of DC does not correlate with higher elicited immune responses in vivo [238, 239]. Before continuing with developing
Ad vectors targeted to APC, more work needs to be done determining the relative influence of direct priming and cross-presentation on eliciting CD8$^+$ T cell responses \textit{in vivo}.

If direct priming plays a more dominant role in Ad-based vaccination, then targeting APC might be beneficial. However, if cross-presentation is more dominant, then targeting somatic cells might be beneficial. In fact, Ad infection of professional APC is not required for generating CD8$^+$ T cell responses [240], indicating that cross-presentation can play a dominant role. Moreover, APC use cross-presentation as the obligatory mechanism of CD8$^+$ T cell priming for viruses that infect only somatic cells [241].

These results suggest that Ad-based vaccination could be improved by targeting as many cells as possible upon injection. For example, upon intradermal injection, an Ad vector would encounter only a small number of APC relative to a large number of somatic cells, like keratinocytes. Targeting the keratinocytes would increase delivered genes and thus, the antigen load on the animal. If cross-presentation is as efficient as proposed in the literature, then this method could generate superior CD8$^+$ T cell responses.

The most important way to elucidate these mechanisms is to assess the specific tissue types that are transduced after \textit{in vivo} delivery of a targeted vector. To conclude that targeting DC will produce a more effective vaccine, one has to determine that DC are in fact expressing the transgene from the targeted vector. Previous studies, including this one, using \textit{in vitro} targeted Ad vectors have failed in determining whether DC are actually transduced \textit{in vivo}. Future work should include assessing gene delivery after
delivery of a targeted and non-targeted vector and subsequent measurement of the immune response.

The ultimate goal for engineering Ad vectors for improved immune responses is to develop a vaccine that can be targeted in situ. This would avoid the ex vivo manipulation of a patient’s cells in so-called DC vaccines. These require labor-intensive isolation and expansion of each patient’s DC, treatment with vaccine, and re-administration. This process is time-consuming and expensive and not feasible for effective worldwide vaccines for infectious diseases, especially HIV.

7.2 Mucosal targeting

Surface carbohydrate expression on intestinal epithelial is currently a target in many lectin-mediated drug delivery and vaccine applications [reviewed in 242]. Lectins and many other mucosal microbial bioadhesins can serve as ligands for targeting vaccines. One such ligand with lectin-like activity, the reovirus σ1 protein, plays an important role in conferring tropism to murine M cells [188, 217]. Moreover, this protein shares strong structural homology with the Ad5 fiber protein. Thus, an Ad5 vector displaying a fiber-σ1 chimera, termed Ad5-T3Dσ1, was created to take advantage of the mucosal-targeting ability of reovirus. This vector transduced mucosal epithelial cells in vitro through the reovirus receptors, junctional adhesion molecule 1 (JAM1) and cell-surface sialic acid.

However, Ad5-T3Dσ1 lacks considerable transduction efficiency when compared to Ad5 on cells permissive to both Ad and reovirus. These defects in transduction seem to be related to altered intracellular trafficking and inefficient use of JAM1. A number of
reasons are proposed for the disruptions in Ad5 biology and possible solutions. First, targeting JAM1 may not be amenable to Ad5 trafficking as fiber-CAR interactions are intertwined with Ad5 infection through evolution. Further studies need to address the affinity of the fiber-σ1 chimera for JAM1. Unpublished observations suggest that removing the c-myc and hexahistidine tags from the C-terminus cause further lowering of transduction efficiency. Perhaps the removal of the tags causes stronger binding and more ability of JAM1 to disrupt normal trafficking. Targeting JAM1 cannot be only to blame as removing the head domain while keeping the sialic-acid binding intact only partially restores transduction compared to Ad5. Second, domains in the fiber protein may contain signals that allow endosome escape, endosome disruption, and proper Ad5 trafficking. If this is a dominant effect, then it is unlikely to be corrected because adding more fiber domain into the fiber-σ1 chimeras causes disruptions in trimerization (data not shown). Third, some evidence suggests that the KKTK motif in fiber serves as flexibility domain (personal communication) and not for binding heparan sulfate glycosaminoglycans [243, 244]. Defects in the Fibtail-T3Dσ1 fusion domain could be causing virus instability or an inability to interact efficiently with JAM1. Moreover, unpublished observations point to an extreme variability in the efficiency of encapsidation of Fibtail-T3Dσ1. Thus, a new fiber-σ1 chimera was created by fusing the T3D σ1 shaft and head domain to the KKTK motif on fiber to create a new chimera termed FibKKTK-T3Dσ1. This chimera trimerizes by semi-native SDS-PAGE and immunoblotting. These results are encouraging because this is the second one to trimerize out of a total of nine tested so far. A chimeric Ad5 vector encoding FibKKTK-T3Dσ1 is currently under production.
Despite the inefficient transduction compared to Ad5, both vectors were compared after oral delivery into mice. Whole-animal luciferase imaging, tissue luciferase activity, and real-time PCR failed to provide a reliable measure of gene delivery. These failures point to many barriers (Figure 7-1) to intestinal Ad5 and Ad5-T3Dσ1 gene delivery that include but are not limited to digestive enzymes [216], stomach acids [216], glycocalyx [233], and inaccessibility of receptors [156, 197, 199].

![Figure 7-1. Oral barriers to intestinal gene delivery by Ad vectors. Digestive enzymes, stomach acids, and glycocalyx are represented as indicated. Epithelial (E) cells form an impermeable barrier to macromolecules through receptor tight junctions indicated as receptor doublets. E cells also contain a regular brush border that is absent on microfold (M) cells.](image)

This work used the reovirus σ1 protein as a novel targeting ligand in an attempt to solve the receptor barriers to Ad5 mucosal gene delivery. However, the contribution of the other barriers, namely low pH, digestive enzymes, and glycocalyx, is currently unknown. These barriers may be insurmountable using Ad-based vectors or they may
require novel strategies to overcome. Future work towards enhancing mucosal gene delivery should focus on assessing the relative influence of the in vivo barriers to mucosal gene delivery and possible solutions (Table 7-1).

<table>
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<tr>
<th>Barrier</th>
<th>Possible solution</th>
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<td>Stomach acids</td>
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<td>Digestive enzymes</td>
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<td>Receptor expression</td>
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Table 7-1. Overcoming and assessing contribution of oral gene delivery barriers.

Studies have shown that adenoviral vectors are extremely sensitive to low pH and digestive enzymes [216]. A change in pH from 8 to 5 can decrease viral titer by $10^7$-fold [245]. Stomach acids can reach a pH as low as 1.2. Enteric coatings are composed of polymers that are designed to dissolve in aqueous solutions between a pH of 6 and 8. Recent work in our lab has determined that lyophilized Ad5 preparations in enteric-coated hydroxypropyl-methylcellulose (HPMC) capsules preserves transduction efficiency. More work using sufficiently large animal models such as hamsters or rats needs to be done to compare the effectiveness of the enteric coatings after oral vaccination. Many enzymatic barriers are described for oral delivery of proteins [reviewed in 246] and overcoming these barriers may be achieved through the co-
administration of enzyme inhibitors [247]. Rectal delivery may be an additional way to assess the pH- and enzyme-dependent loss of Ad after mucosal vaccination.

The protective glycocalyx covering the intestinal epithelium is mostly absent on M cells [155], which are presumably more accessible than their neighboring enterocytes. Nevertheless, some M cells do harbor thin glycocalyx that has an upper pore size of 29 nm [155]. Adeno-associated virus (AAV) is approximately 25 nm in diameter and more resistant to low pH, so this would potentially serve as a more potent oral vaccine. In fact, AAV has been tested in vivo as an oral vaccine for HIV [248] and stroke [249]. It would be interesting to see if AAV would be more efficient than Ad5 after oral delivery.

Despite the physical barriers, apical receptor expression remains a major barrier to Ad gene delivery in differentiated intestinal epithelia [236]. Studies suggest that re-targeting Ad5 to apical receptors in epithelial barriers [237] is sufficient to overcome lack of accessible CAR. Many cell-surface glycoconjugates identified through lectin binding [242] and sialoglycoconjugate receptors identified through diverse Ad serotypes [236] are important for binding intestinal epithelia from the apical pole. Accordingly, Ad5-T3Dσ1 mediates transduction through sialic acid-linked receptors and deletion of the head domain to create Ad5-T3Dσ1-ΔH actually improves transduction efficiency. Other Ad-σ1 possibilities include using the Type 1 Lang (T1L) serotype [194], which has been shown to bind murine M cells through alternative sialic acid linkages [217]. Still other intestinal Ad serotypes like 40 and 41 [250] hold promise for vaccine applications because they are adapted to the harsh conditions of the gut [251].

Since gene delivery was not assessed in vivo, the conclusion cannot be made that targeting mucosal sites, specifically M cells of the Peyer’s patches, increases the immune
responses to the vector transgene. As a mucosal vaccine, both Ad5 and Ad5-T3D\sigma\text{I} vectors elicited immune responses that do not differ substantially. This fact implies that chimeric Ad-\sigma\text{I} vectors hold great promise if the defects in their biology can be corrected through re-engineering of the fiber-\sigma\text{I} chimera.
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