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# Recombinant Adenovirus Infection of Human Dendritic Cells

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## 1. Introduction

Recombinant Adenoviruses (rAd) are widely used as gene delivery vectors in gene therapy and vaccination (Hall et al., 2010, Liu, 2010). These replication incompetent vectors have established safety in humans and possess a number of advantages, such as that high viral titres can be produced efficiently. Several different human rAd types are being intensively investigated in clinical trials for their usefulness. In addition, there is an ever-expanding body of literature covering basic virology of Ad and interactions with host immune and other cells. It is important to understand how rAd vectors interact with specific cells of the immune system in order to improve their clinical efficacy. In particular, studies on the interaction of rAd and professional antigen presenting cells (pAPC), which specialize in recognizing pathogens and initiating a cascade of events that lead to specific immunity, are highly relevant. The most potent type of pAPC are dendritic cells (DC) that possess a unique ability to prime adaptive immune responses (Palucka & Banchereau, 1999, Palucka et al., 2010). rAd vectors likely contact DC early following inoculation and thus these cells may play a major role in regulating immunity towards the vector itself and encoded transgenes.

This chapter will include a review of the current literature on the interactions between DC and rAd vectors reported by ourselves and others, in addition to a presentation of novel data. We will first summarize the phenotype and function of specific human DC subsets and methods to isolate or differentiate DC, which are crucial tools to study the interplay of DC and rAd vectors in physiologically relevant systems. Further, we will discuss basic virological aspects of rAd including vector generation and cellular receptor usage among different rAd species. While a multitude of receptors have been described for rAd, we will focus on those relevant for DC. In addition to how rAd vectors bind and infect DC, the extent by which different rAd types infect different DC subsets will be examined. Finally we will give an overview of the functional response of DC to rAd vectors, including maturation, cytokine production, and antigen presentation. Understanding how human DC sense and respond to rAd vectors will assist in guiding the use of these gene delivery vehicles in their many different clinical applications.

## 2. Human dendritic cells

### 2.1 Function in innate and adaptive immunity

DC participate centrally in the initiation of immune responses towards foreign antigen and in this way link the innate and adaptive arms of the immune system (Palucka & Banchereau,

1999). During the steady state, DC have an immature phenotype, possess high endocytic capacity, and express a diverse array of pathogen recognition receptors (PRR) to sense extracellular and intracellular foreign antigen. Recognition of specific viral nucleic acid signatures by cytosolic and endosomal PRR enables DC to initiate downstream signalling cascades that lead to phenotypic maturation and production of cytokines such as type-1 interferons (IFN) (Pichlmair & Reis e Sousa, 2007). DC activation is also characterized by upregulation of chemokine receptors that facilitate their migration from the periphery to the spleen or lymph nodes, the primary sites for presentation of antigens, to activate antigen-naïve T lymphocytes. The morphological and phenotypic changes that occur upon maturation endow DC with a notable capacity to activate lymphocytes in an antigen specific manner (Steinman & Witmer, 1978). Specifically, DC have a unique capacity to present foreign peptides on MHC (major histocompatibility complex) I and II to activate both cytotoxic CD8+ T cells and helper CD4+ T cells, respectively. In addition to efficient induction of antigen-specific T cell responses, DC are also becoming increasingly appreciated for their role in shaping the function of innate immune cells such as NK cells (Medzhitov, 2007). Since DC have a multifaceted role in both innate and adaptive immunity, they likely respond to and influence the efficacy of rAd vector administration. While DC may facilitate the induction of systemic immunity towards vector transgenes, local immune responses may in contrast blunt the desired effect of the delivered gene. Part of the diversity in DC function is attributable to the presence of distinct DC subsets present in blood and other tissues.

### 2.2 Overview of DC subsets, phenotype, and function

Human DC are classified into subsets based on characteristics such as surface phenotype, anatomical location, cytokine and maturation profiles, and the capacity to present antigen to activate antigen specific lymphocytes. In this section, we will describe the phenotypes and functions of subsets of DC derived from human blood and skin.

### 2.2.1 Blood DC subsets

Human DC from blood can be broadly separated into three distinct subsets: plasmacytoid DC (pDC) and two types of myeloid DC (mDC) (Ueno et al., 2011, Ziegler-Heitbrock et al., 2010) (Table I). These subsets are distinguished by their unique expression of different blood DC antigens (BDCA) (Dzionek et al., 2000, Palucka et al., 2010). mDC are CD1c+ (BDCA-1+), while pDC co-express CD303 (BDCA-2) and CD304 (BDCA-4) (Table I). Another recently identified mDC subset expressing CD141+ (BDCA-3) is notably adept at presenting exogenous foreign peptides on MHC I molecules, in a process termed cross-presentation (Bachem et al., 2010, Crozat et al., 2010, Jongbloed et al., 2010, Poulin et al., 2010). Because there is very limited data on the interaction of rAd vectors and the CD141+ mDC subsets this chapter will focus on pDC and CD1c+ mDC. While mDC and pDC are similar in that they share several classical DC functions, such as mechanisms for efficient uptake of antigen, expression of PRR, ability to phenotypically mature, migrate and activate naïve T cells, they also differ in a number of critical aspects. For example, their expression repertoire of PRR differs. mDC express toll like receptors (TLR) 1 through 8, and 10 whereas pDC express TLR 7 and 9. mDC are generally considered more potent antigen presenting cells, while pDC specialize in the production of rapid and copious type-1 IFN (IFN$\alpha/\beta$) and may thus have a particularly important role in viral immunity (Liu, 2005). Both pDC and mDC are also defined as being mostly CD14- (Fig. 1). A surrogate for DC of myeloid lineage may

also be differentiated *in vitro* from CD14+ monocytes (termed monocytes derived DC or MDDC). These cells lose CD14 expression, but concurrently gain expression of CD1a and DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) (Table I and Fig. 2). It is currently unclear whether MDDC represent any single primary DC subset, but recent a report indicates that they in part mimic skin resident interstitial dermal DC in that they produce similar cytokines and express DC-SIGN (Klechevsky et al., 2008). In **section 2.3** we will briefly discuss the methods for differentiating MDDC *in vitro* and isolating mDC and pDC from blood.

| | DC Subset | Phenotype | Cytokines Produced | Selection method | Culture media |
|---|---|---|---|---|---|
| **Blood Subsets** | **CD1c+ Myeloid DC (mDC)** | **CD1c+ (BDCA-1)** CD11c+ CD14+/- HLA-DR+ | IL-12p70 TNF IL-6 | anti-CD1c magnetic microbeads with positive selection on Automacs (Miltenyi) | RPMI media 10 % fetal calf sera GM-CSF |
| | **Plasmacytoid DC (pDC)** | **CD303+ (BDCA-2)** **CD304+ (BDCA-4)** CD123+ (IL-3Rα) CD14- HLA-DR+ | IFNα/β IL-6 | anti-CD304 magnetic microbeads with positive selection on Automacs (Miltenyi) | RPMI media 10 % fetal calf sera IL-3 |
| *in vitro* derived | **Monocyte derived DC (MDDC)** | **CD1a+** **CD209+ (DC-SIGN)** HLA-DR+ CD14- | IL-12p70 TNF IL-6 | Monocyte isolation followed by 6 day culture with IL-4 and GM-CSF | RPMI media 10 % fetal calf sera GM-CSF + IL-4 |
| **Cutaneous Subsets** | **Dermal Interstitial DC (dDC)** | **CD209+/- (DC-SIGN)** **CD14+/-** HLA-DR+ CD1a +/- | TNF IL-1 IL-6 IL-12p40 | Collagenase digestion of skin or GM-CSF induced migration from dermal skin layer | RPMI media 10 % fetal calf sera |
| | **Epidermal Langerhans Cells (LC)** | **CD207+ (Langerin)** CD1a+ HLA-DR+ | TNF IL-1 IL-15 IL-8 | Collagenase digestion of skin or GM-CSF induced migration from dermal skin layer | RPMI media 10 % fetal calf sera |

Table 1. Overview of DC, phenotype, cytokines, methods for selection, and culturing.

### 2.2.2 Cutaneous DC subsets

Cutaneous DC are commonly divided into two main subsets based on the tissue in which they reside in steady state conditions: dermal interstitial DC (dDC) resident in the dermal layer, and Langerhans cells (LC) resident in the epidermal layer. Both subsets express HLA-DR and are of myeloid origin. LC are distinguished by expression of Langerin and CD1a, while dDC consist of a more diverse population based on differential expression of DC-SIGN, CD1a and CD14 (Bond et al., 2009, Klechevsky et al., 2008). A more complete phenotypic characterization of these cells is provided in Table I. The unique roles that each of these skin DC play in detecting viral infection and initiating immune responses likely depends on both the route of inoculation and the nature of the particular virus (Palucka et al., 2010). It has been shown that dDC, in particular the CD14+ subset, stimulate humoral immunity (i.e. antibody producing B cells) while LC specialize at inducing cellular immunity (i.e. cytotoxic CD8+ T cells) (Klechevsky et al., 2008). The methods for isolating cutaneous DC subsets from healthy skin tissue will be briefly discussed in **section 2.3.3**.

**2.3 Differentiation and isolation of DC from blood and skin**
**2.3.1 Isolation of primary blood DC subsets**
We have developed methods that yield significant numbers of highly pure and immature CD303+ pDC and CD1c+ mDC (Adams et al., 2009, Douagi et al., 2009, Lore, 2004, Lore et al., 2003). These cells allow for studies of more physiologically relevant primary human DC than *in vitro* surrogate DC (i.e. MDDC). As discussed earlier, isolation of pDC and mDC is facilitated by differential BDCA expression. A series of sequential separations is necessary to yield sorted cells of high purity. We have developed two means of first enriching DC and monocytes from the total peripheral blood mononuclear cell (PBMC) population: (i) aphaeresis of donor leukocytes followed by counterflow centrifugation elutriation to separate monocytes and lymphocytes based on cell size and sedimentation density (Lore et al., 2003, Lore et al., 2005), or (ii) treatment of PBMC with RosetteSep CD14+ enrichment kit (Lambert et al., 2009). Both these methods result in a fraction of cells highly enriched of monocytes and DC, and depleted of lymphocytes. Subsequently, the pDC are positively selected by staining with anti-CD304 monoclonal antibodies (mAb) directly conjugated to magnetic microbeads (Miltenyi). Since a subset of B cells expresses CD1c, these cells are depleted by staining with anti-CD19 mAb directly conjugated to magnetic microbeads (Miltenyi). mDC may thereafter be positively selected with mAb against CD1c. Cell separation based on magnetic microbead conjugated mAb can be performed using either an AutoMacs instrument or manually with appropriate selection columns (Miltenyi). This sequential magnetic sorting procedure results in the isolation of highly pure CD123 expressing CD304+ pDC and CD11c expressing CD1c+ mDC (Fig. 1). pDC and mDC are then cultured in complete media supplemented with IL-3 and GM-CSF, respectively. These rare subsets of DC isolated from blood display an immature phenotype that is consistent with the established literature (Ziegler-Heitbrock et al., 2010). Important to note is that although pDC may be isolated with anti-CD303 mAb, ligation of this receptor with the currently available clones (Miltenyi) attenuates type-1 IFN production (Dzionek et al., 2001), TLR9 induced phenotypic maturation, and optimal antigen presentation (Jahn et al., 2010).
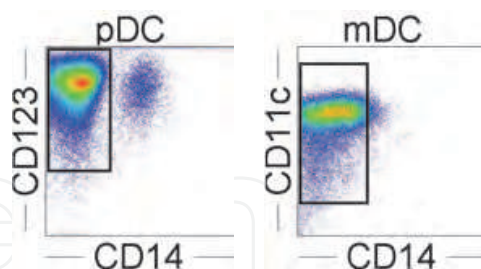


Fig. 1. Phenotype and purity of human pDC and mDC sorted from blood.

pDC and mDC were positively selected on an AutoMacs after staining with anti-CD304 and anti-CD1c mAb conjugated directly to magnetic microbeads, respectively (Miltenyi). Freshly isolated pDC or mDC were stained with anti-CD123 or anti-CD11c, respectively, and anti-CD14 mAbs (BD Biosciences). Surface expression was evaluated using flow cytometry (BD FACS Calibur) and data was analyzed with FlowJo software (Treestar).

**2.3.2 Differentiation of monocyte derived DC**
Due to the rarity of DC subsets in blood and skin an alternative method was developed to more readily study DC (Sallusto & Lanzavecchia, 1994). This method to *in vitro* generate

MDDC from monocytes significantly accelerated investigations of human DC function. Here, primary monocytes are isolated from PBMC fractions. Highly pure CD14+ monocytes are obtained either by collection of plastic-adherent cells or treatment of PBMC with RosetteSep CD14+ enrichment kit (Stem Cell Technologies) (Adams et al., 2009, Lambert et al., 2009). Subsequent culture of monocytes with recombinant human interleukin (IL)-4 and granulocyte macrophage-colony stimulating factor (GM-CSF) at optimal concentrations over 6 days induces monocytes to differentiate into MDDC that display CD1a, DC-SIGN, HLA-DR, but lack CD14 (Fig. 2).
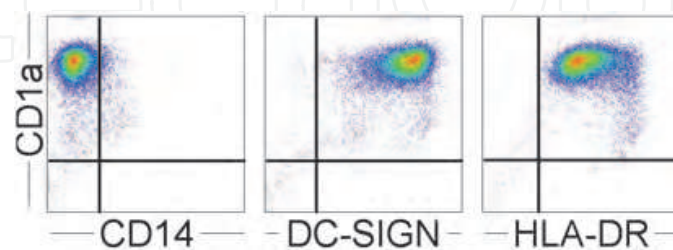


Fig. 2. Phenotype of human DC differentiated from primary monocytes with GM-CSF and IL-4 over six days.

Human PBMC were treated with RosetteSep CD14+ enrichment kit to isolate monocytes, which were then cultured for 6 days in the presence of IL-4 (4 ng/ml) and GM-CSF (4 ng/ml). On day 6, the cells were washed, stained with anti-CD14, anti-DC-SIGN, anti-HLA-DR, and anti-CD1a mAbs (BD Biosciences). Surface expression was evaluated using flow cytometry and data was analyzed with FlowJo software.

### 2.3.3 Isolation of skin DC subsets

We have recently described methods to isolate DC subsets from healthy skin tissue obtained after reconstructive plastic surgery (Bond et al., 2009). These methods employ a skin graft mesher (Zimmer) that mechanically expands skin in a net-like fashion and increases the accessibility of dispase, an enzyme that separates the dermal and epidermal layers, to penetrate the tissue. After dispase treatment, these layers can then be physically teased apart with forceps. This step is followed by incubation with collagenase, which enzymatically disrupts the collagen fibers and thereby the tissue integrity. After sequential filtering steps, this method results in single cell suspensions enriched for dDC and LC from the dermis and epidermis, respectively. In an alternative method to isolate skin DC, the separated layers are incubated with collagenase and GM-CSF, which induces the cells to migrate from the tissue and into the media. Suspensions of the cells typically consist of a higher percentage of DC, but which may display a more mature phenotype compared to DC isolated with collagenase alone. Regardless of maturation state, the harvested LC are identified by uniform and high expression of HLA-DR, CD1a and Langerin. dDC express HLA-DR, but exhibit differential expression of CD1a and CD14 (Bond et al., 2009). These techniques allow for efficient isolation of significant numbers of skin DC useful for investigations of rAd infection.

## 3. Recombinant adenovirus vectors

### 3.1 Background

The family of human Adenoviruses (*Adenoviridae*) consists of at least 50 different subtypes divided into seven species and causes a diverse array of acute human diseases (Arnberg,

2009). The virion has an icosohedral non-enveloped capsid containing fibers spikes protruding from each vertice that encapsulates a double stranded linear DNA genome. The complete high resolution structure of the 150 megadalton Ad viron has recently been solved (Liu et al., 2010, Reddy et al., 2010) and provides critical insights into the virology of Ad. The genome organization and capsid structure are relatively conserved amongst Ad species, but receptor usage, cellular and tissue tropism, and activation of immune cells differs. Recombinant Adenoviruses (rAd), rendered replication incompetent by removal of viral early genes (e.g. E1, E3, and E4), have steadily gained prominence as vectors in various gene therapy and vaccine applications (Liu, 2010, Patterson et al., 2009). The use of rAd as gene delivery vehicles is driven largely by the extensive characterization of Ad virology and the ability to produce high titers of replication incompetent virus that encode for relatively large foreign gene inserts. Transduction of many cell types by rAd leads to transcription of the inserted transgene and high production of its encoded protein, especially when the transgene is under control of an optimized promoter element. Moreover, replication incompetent rAd vectors have proven safe in both pre-clinical toxicology and clinical trials (Catanzaro et al., 2006, Sheets et al., 2008). rAd type 5 (rAd5) of species C has been used most widely, but due to various limitations such as common pre-existing antibody mediated immunity, alternative Ad species (e.g. B) are now being investigated and employed (Abbink et al., 2007). Thus, investigation of these alternative Ad species, which are often less well characterized compared to rAd5 in terms of their specific receptor usage and ability to transduce different cells, is highly warranted and will hopefully expand their usefulness in gene therapy and vaccination.

### 3.2 Generation of recombinant adenovirus vectors

Replication incompetent rAd vectors can be efficiently generated in mammalian packing cells lines and are the type in common use (He et al., 1998). These vectors are rendered replication incompetent by genetic deletion of early genes, which are transcribed early in the virus life cycle and are required for viral replication. Numerous packaging cell lines, such as PER.C6 or 293-ORF6, have been developed that provide deleted early genes *in trans*. rAd5 and rAd35 vectors have capacity for foreign transgenes of up to 7.5 kb under control of a CMV promoter (McVey et al., 2010). This type of promoter has been found to be the most active in human DC (Papagatsias et al., 2008). However, these authors noted that promoter type strongly affected transgene expression, and promoter activity was dependent on cell type. Thus, cell lines may neither accurately represent promoter activity nor predict gene expression in primary immune cells. Viral expression cassettes typically also include SV40 polyadenylation signals to further enhance expression of the transgene. Transgenes encoding fluorescent proteins can be used to follow viral infection. These current methods result in the generation of high viral titre stocks with severely limited viral replication.

### 3.3 Receptor usage
### 3.3.1 Primary cellular attachment receptors

Ad use a variety of cellular attachment receptors that are determined both by cell type and the virus species (reviewed by (Arnberg, 2009)). Furthermore, receptor usage may be substantially different depending on the host species; such as between human and mice. Therefore, for the purposes of this chapter, we will focus on the receptors expressed by human DC that have been or may be implicated in rAd infection. Table II provides an

overview of described and potential receptors on human DC for selected Ad types. It is well established that species B Ad35 requires the complement regulatory protein CD46 to attach to and infect a variety of human cells (Gaggar et al., 2003). The trimeric fiber knob protein mediates high affinity and avidity binding of rAd35 to a region within the extracellular short consensus repeats (SCR)1 and 2 of CD46 (Nemerow et al., 2009, Wang et al., 2007, Wang et al., 2008). In fact, all species B rAd probably use CD46 except types 3 and 7 (Marttila et al., 2005). We have previously confirmed these findings by showing that rAd35 requires CD46 to infect pDC and mDC (Lore et al., 2007). Using anti-CD46 mAb directed against the known binding regions of the rAd35 knobs we demonstrated that CD46 attachment was required for rAd35 infection. CD46 is ubiquitously expressed on all nucleated cells and it is therefore likely that rAd35 infects or at least binds to a range of cells. In addition to its role as a complement regulatory protein, CD46 regulates immune cell function through putative signalling domains within its cytoplasmic tails (Kemper & Atkinson, 2007, Wang et al., 2000). Thus, CD46 using rAd vectors, such as rAd35, may modulate immune cells through receptor interactions. For example, CD46 engagement drives the differentiation of CD4+ T cells to a regulatory phenotype (Kemper et al., 2003).

Contrary to rAd35, the receptors used by the species C Ad5 to infect human DC are less clear. While the coxsackie-adenovirus receptor (CAR) is the described receptor for rAd5 on epithelial cells (Bergelson et al., 1997), blood DC were found to not express this receptor at levels detectable by flow cytometry (Lore et al., 2007). However, we have found that CAR plays a minor role in mediating rAd5 infection of skin DC, which express CAR (Adams et al., 2009). Thus, rAd5 may infect DC, especially blood DC, in a CAR-independent manner. To this end, several CAR-independent pathways for rAd5 infection have been suggested. Lactoferrin (Lf), an iron-binding protein present in abundance at mucosal sites and in many bodily fluids, was shown to facilitate epithelial cell infection by species C Ad (Johansson et al., 2007). We expanded on this report and found that Lf strongly enhanced rAd5 infection of all tested blood and skin DC subsets (Adams et al., 2009). Of particular interest in the application of rAd5 as a gene therapy or vaccine vector was the mechanism by which Lf facilitated infection. Lf species with high mannose type *N*-linked glycans mediated rAd5 infection via binding to DC-SIGN. As mentioned earlier, this receptor is expressed by both MDDC and a subset of skin resident dDC. Thus, Lf represents a mechanism to mediate rAd5 infection of CAR- human DC and may provide a means to enhance the infection of DC both *in vitro* and *in vivo*. Coagulation factors also play a critical role in mediating *in vivo* tropism of rAd5 vectors, especially after intravenous administration (Kalyuzhniy et al., 2008, Waddington et al., 2008). High affinity Ad5 hexon protein interactions with coagulation factor X (FX) mediate liver tropism through high efficiency transduction of hepatocytes in mice. These studies illustrate that cellular tropism may be determined by binding events that occur independent of the classical Ad knob-receptor interactions. It is currently unknown to what extent these soluble factors mediate infection of human DC *in vivo*, but this will be important to determine in future studies. In murine DC, a region within the rAd5 fiber-shaft facilitates infection in a heparin dependent manner (Cheng et al., 2007). It will be critical to determine whether this receptor usage also exists in DC. These authors also found that rAd5 mutants with ablated CAR binding retained their ability to infect murine DC, which supports our earlier findings that Ad5 infects human DC, albeit to a lesser extent than rAd35, in the absence of CAR.

| Adenovirus Type | Species | Receptors or mediators | Receptor expressed on DC | Reference |
|---|---|---|---|---|
| **Ad5** | C | CAR | Blood: no skin: yes | Bergelson et al.,1997; Lore et al., 2007 Adams et al., 2009 |
| | | Lactoferrin | yes | Johansson et al., 2007; Adams et al., 2009 |
| | | FX | n.d. | Kalyuzhniy et al., 2008; Waddington et al., 2008 |
| | | Heparin | n.d. | Cheng et al., 2007 |
| **Ad35** | B(2) | CD46 | yes | Gaggar et al., 2003; Marttila et al., 2005; Lore et al., 2007 |
| **Ad37** | D | GD1a glycan | n.d. | Nilsson et al., 2011 |
| **Ad3** | B(1) | Desmoglein | n.d | Wang et al., 2011 |
| | | CD80/CD86 | n.d. | Short et al., 2004; Short et al., 2006 |

n.d.: not determined

Table 2. Definitive and potential receptors on human DC for select Ad types.

Finally, increased vector transduction of DC has been tested by genetically modifying rAd vectors to target DC expressing CD40 (Korokhov et al., 2005b) and DC-SIGN (Korokhov et al., 2005a, Maguire et al., 2006). Targeting DC in this manner led to greater transduction efficiency of DC by retargeted rAd vectors compared to unmodified vectors. These reports are reminiscent of how Lf also enhanced infection through DC-SIGN (Adams et al., 2009). In conclusion, rAd vectors may be retargeted through genetic modification of the capsid structure or other soluble proteins to more efficiently infect DC, but it remains to be determined how effective such strategies are *in vivo*.

### 3.3.2 Secondary cellular receptors

A secondary interaction with cellular $\alpha v/\beta 3$ and $\alpha v/\beta 5$ integrins and RGD motifs of the Ad penton bases facilitates membrane penetration and internalization of Ad particles (Wickham et al., 1993). $\alpha v\beta 5$ integrins may even be sufficient to allow rAd infection when CAR is not present (Lyle & McCormick, 2010). However, mutant rAd with ablated integrin binding retained their ability to infect murine DC, which indicates that such interactions are not necessary on DC (Cheng et al., 2007). It will be important to further elucidate the role of integrins in mediating rAd infection of DC, particularly since the expression may differ between DC subsets and host species. $\beta_3$ integrins displayed by mouse macrophages are the major initiators of innate immune response towards Ad vectors *in vivo* (Di Paolo et al., 2009). In that study, binding of RGD motifs to integrins induce IL-1$\alpha$ independent of membrane penetration. This report highlights how Ad interactions with receptors may, in addition to mediating cellular attachment, influence immunity independent of infection (Shayakhmetov et al., 2010).

### 3.3.3 Other potential cellular attachment receptors on DC

The co-stimulatory receptors, CD80 and CD86, involved in the antigen presentation process have been implicated as receptors for Ad3 (Short et al., 2004, Short et al., 2006). These findings are relevant for DC since they display these markers whereas most other cells do not. As will be discussed in more detail in **section 5.1**, surface CD80 and CD86 levels increase on DC during phenotypic maturation. Whether CD80 and CD86 can act as candidate receptors for Ad3 on DC, needs to be confirmed. A recent report demonstrates

that Ad3 binds the desmoglein receptor (Wang et al., 2011), although it is unknown if this receptor is expressed on DC and can facilitate infection. GD1a glycan was recently identified as the receptor for Ad37, although again the relevance of this receptor for DC infection has not yet been studied (Nilsson et al., 2011). Taken together, these data highlight the importance of understanding how rAd vectors used in gene therapy interact with immune cells. In particular, certain interactions with DC may have positive or negative consequences on immunity generated towards the rAd vector.

## 4. Susceptibility of DC to rAd infection

### 4.1 Methods for testing DC susceptibility *in vitro*

As previously discussed, there is significant complexity in the receptor usage of rAd vectors derived from different species or types, which likely results in vast differences in their ability to infect DC. What particular cells are infected with rAd after vector delivery is largely unknown. Whether expression of vector transgenes in all or specific cells of the heterogeneic DC population is desired or not may also depend on the specific gene therapy or vaccine application. It is thus important to determine the susceptibility of primary human DC subsets to rAd. We have developed methods to monitor rAd infection in DC (Adams et al., 2009, Lore et al., 2007). In these assays, freshly isolated DC are exposed to rAd vectors encoding green fluorescence protein (GFP) reporter transgene. Following receptor binding and penetration of the cellular membrane, the virus traffics to and enters the nucleus where replication occurs. Since the vectors are optimized for expression of the transgene, GFP may be expressed in susceptible cells. GFP expression can then be used as a surrogate marker of productive rAd infection. We have included examples here to demonstrate how the method is performed and how it can be used to compare the capacity of different rAd types to infect MDDC (Fig. 3), mDC and pDC (Fig. 4*A*), and LC and dDC (Fig. 4*B*). In these experiments, the DC were exposed to different inocula of rAd types 35 or rAd5, or rAd26. After 24 h, the cells were stained for surface markers to examine phenotype simultaneously with GFP by
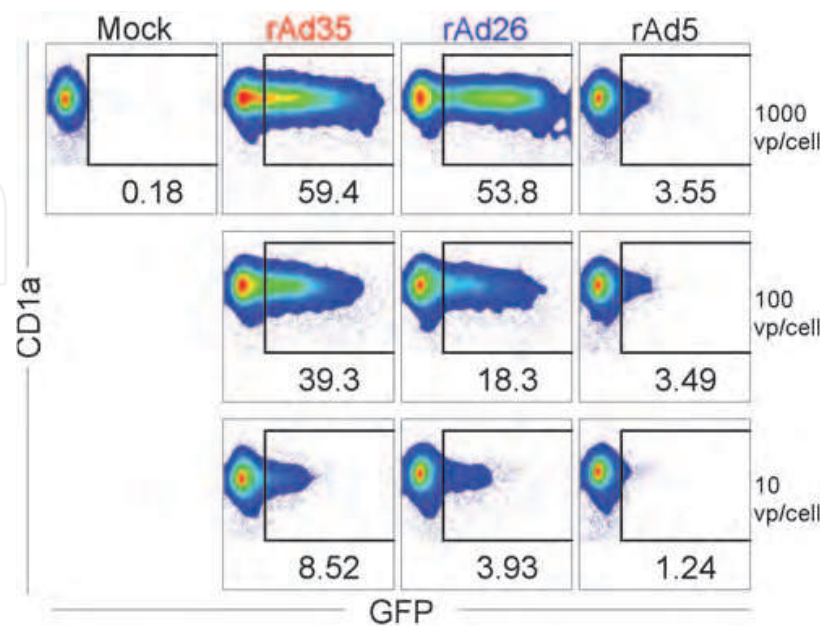


Fig. 3. Flow cytometry analysis of GFP transgene expression in human MDDC.

flow cytometry. GFP+ cells can be detected at earlier time points (≥ 8 hours), but the level of infection usually peaks around 24 hours. We have previously optimized these methods in pDC and mDC with similar results as presented here (Lore et al., 2007). Here, we confirmed these findings in all the mentioned DC (Fig. 3-4). In **section 4.2** of this chapter we will discuss the major differences observed between the susceptibility of DC subsets to different rAd species.

MDDC were exposed to rAd types 35, 26, and 5 encoding GFP at the indicated inocula (virus particles (vp) per cell). After 24 hours, the cells were washed and stained with directly conjugated anti-CD1a and anti-CD14 mAbs (BD Biosciences). Expression of surface markers and GFP was evaluated using flow cytometry and data was analyzed with FlowJo software.
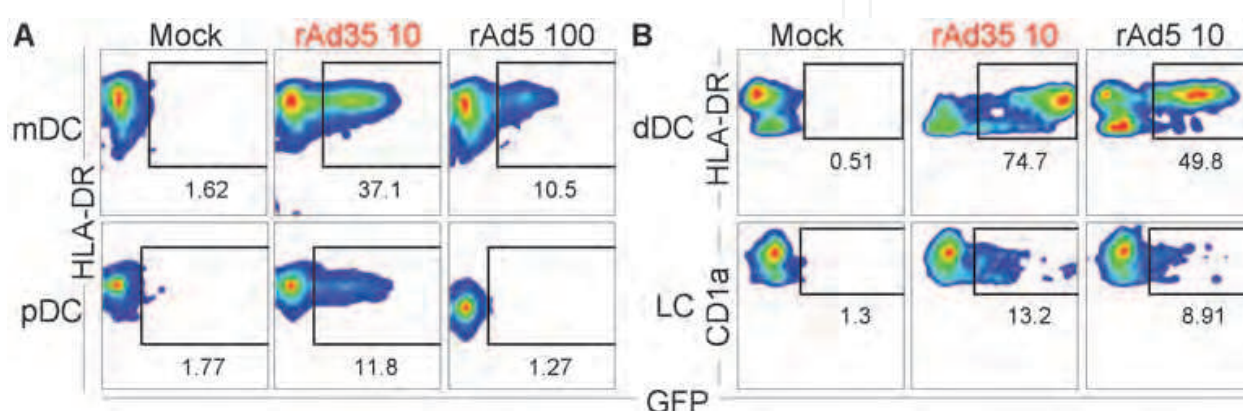


Fig. 4. Flow cytometry analysis of GFP transgene expression in human blood and skin DC subsets.

Freshly isolated (A) mDC and pDC or (B) dDC and LC were exposed to rAd35 or 5 encoding GFP at the indicated inocula (infectious virus particles (ip) per cell). After 24 hours, the cells were washed and stained with directly conjugated anti-CD1a or anti-HLA-DR mAbs (BD Biosciences). Expression of surface marker and GFP was evaluated using flow cytometry and data was analyzed with FlowJo software.

### 4.2 Notable differences in DC infection between rAd species

There may be important implications for gene delivery vehicles that differentially target DC. We have reported previously on the capacity of rAd vectors to infect primary human DC and MDDC (Adams et al., 2009, Lore et al., 2007). In these studies we have compared the species C rAd5 and species B rAd35, which are widely used as gene delivery vehicles. As discussed in **section 3.3**, a major difference between these viruses is their receptor usage. rAd35 uses CD46 as a primary attachment receptor, while rAd5 uses CAR to infect CAR+ cells. A flow cytometric analysis of surface marker expression revealed that pDC, mDC, and MDDC express high levels CD46, but have undetectable levels of CAR (Adams et al., 2009, Lore et al., 2007). Moreover, exposure of rAd35 encoding GFP led to a greater frequency of GFP+ pDC and mDC when compared to rAd5 (Fig. 4*A*). These differences have been observed by others as well (Ophorst et al., 2004, Rea et al., 2001) and are in agreement with data presented here that rAd35 infects MDDC (Fig. 3) and the cutaneous dDC and LC (Fig. 4*B*) more efficiently than rAd5. Others have also found that rAd35 infects skin emigrating DC more efficiently than rAd5 (de Gruijl et al., 2006). Here, we also show new data that species D rAd26 infected MDDC to about the same degree as

rAd35 (Fig. 3). On this note, the specific receptor used by rAd26 for infection is still controversial and there are diverging reports implicating either CD46 (Abbink et al., 2007) or CAR (Chen et al., 2010). rAd5 infection of DC occurred in the absence of CAR expression and neutralizing anti-CAR mAb had no effect on infection (Lore et al., 2007). Unlike blood DC, subsets of cutaneous DC display CAR and blocking CAR has a noticeable but incomplete reduction of rAd5 infection (Adams et al., 2009). We also show here that dDC were substantially more susceptible to both rAd5 and 35 infection than donor matched LC (Fig. 4*B*). Another report found that LC were more susceptible to rAd infection compared to dDC when using skin DC differentiated from CD34+ haematopoietic stem cells *in vitro* (Rozis et al., 2005). These differing studies highlight the complexity in comparing data generated using different sources of DC. It is critical to perform detailed characterizations of the phenotypes and functions of the DC in each culture system to be able to relate it to how accurately they represent DC *in vivo*. The level of maturation should be carefully monitored since it may substantially affect DC susceptibility to rAd infection. Finally, even though rAd5 infects DC to a lesser extent than rAd35, it was recently shown that CD11c+ DC were indispensable for generating strong transgene specific CD8+ T cell responses in mice (Lindsay et al., 2010). This shows that DC recognition of rAd vectors plays a crucial role in mediating immunity and that it may be beneficial in gene therapy to retarget rAd to not infect DC in order to minimize insert specific immunity.

## 5. rAd induced activation of DC

### 5.1 Phenotypic maturation

As mentioned earlier in this chapter, phenotypic maturation is an important differentiation step in which DC convert from an immature resting state to an activated state with increased capacity to process and present foreign antigen. Maturation licenses DC to activate naïve T cells through expression of co-stimulatory molecules concurrently with presentation of foreign peptides on MHC molecules. Mature DC upregulate activating members of the B7 family (CD80 and CD86) that provide co-stimulation through CD28 engagement and optimally activate naïve T cells. DC also upregulate MHC class II (HLA-DR) and CD40 that activates both T and B cells through CD40L. There are also many more molecules that positively and negatively regulate DC mediated activation of T cells that are outside the scope of this chapter. Flow cytometry analysis of these surface markers is the most common and instructive method to assess maturation as it quantifies the change in expression on the surface of DC, which is indicative of the strength by which DC can activate T cells. We have found that different rAd types have vastly different capacities to induce phenotypic maturation of DC. For example, the species B rAd35 was found to induce maturation of primary human DC subsets, while rAd5 was not (Lore et al., 2007). In fact, while very high doses of rAd5 did not induce differentiation, a dose of only a few rAd35 particles per DC induced strong maturation. rAd35 induced upregulation of the maturation markers CD80, CD83, CD86, HLA-DR, and CD40 (Lore et al., 2007)(Adams and Loré, unpublished data). The maturation caused by rAd35 is comparable to that induced by strong maturation stimuli such as the TLR4 ligand lipopolysaccharide and the TLR7/8 using imidazoquinolines. While others have found rAd5 activates DC (Philpott et al., 2004), there may be important differences in the source of DC and viral dose between studies. Receptor usage may be linked to the capacity of different rAd types to induce maturation. Although

the mechanisms of cellular entry may differ between Ad species and cell type (Hall et al., 2010), it is likely that viral nucleic acids could signal through endosomal or cytosolic expressed PRR and thereby initiate DC maturation. It is currently unclear why or how certain Ad species induce maturation while others do not. However, one potential explanation may be that species C and B Ads have different kinetics of endosomal retention and escape to the cytosol following receptor mediated endocytosis, which thereby affect PRR recognition in these compartments (Miyazawa et al., 2001). *In vivo*, maturation of mDC induced by rAd vectors was dependent on type-1 IFN signalling (Hensley et al., 2005), indicating that phenotypic maturation of DC may be induced directly through infection or facilitated indirectly through cytokine production. However, it is currently unknown what PRR are responsible for rAd mediated DC maturation.

## 5.2 Cytokine induction

In addition to phenotypic maturation, DC also produce numerous cytokines in response to foreign antigen exposure. In this regard, DC subsets differ in the specific cytokines they produce (Table I). Whereas mDC secrete IL-12p70, pDC possess a unique ability to rapidly secrete abundant type-1 interferons (IFN$\alpha$/$\beta$) following viral infection (Swiecki et al., 2010). Like many other viruses Ads can potently induce systemic IFN$\alpha$/$\beta$ *in vivo*. We have previously used three methods to measure cytokines levels in DC: (i) enzyme-linked immunosorbent assay (ELISA), (ii) intracellular cytokine staining with flow cytometry, and (iii) quantitative RT-PCR (Douagi et al., 2009, Lore et al., 2007). DC cytokine production may also be measured *in situ* (Lore et al., 1998, Lore et al., 2001), but this method has not been combined with rAd infection. There are benefits and drawbacks to each of these techniques, which is why the appropriate method should be selected depending on the study aim. ELISA is highly sensitive and useful when the purity of the sorted cells is high, as is the case with sorted primary DC. This method quantifies secreted cytokines, but does not measure production on a per cell basis. Intracellular staining does allow for such assessment however. In this method the use of pharmacological inhibitors of protein secretion (e.g. Brefeldin A) enables detection of cytokines by causing their accumulation within the cells in which they are produced. Subsequent fixation and saponin-mediated cell membrane permeabilization enables staining of intracellular accumulated cytokines and detection by flow cytometry. This method is particularly useful for detecting cytokines in unsorted cell populations or when measuring GFP expression simultaneously (Fig. 5). Since several common pharmacological agents, such as monensin and chloroquine, may interfere with rAd infection or PRR signalling (Adams and Loré, unpublished data), they should be tested rigorously to avoid unwanted effects on DC function.
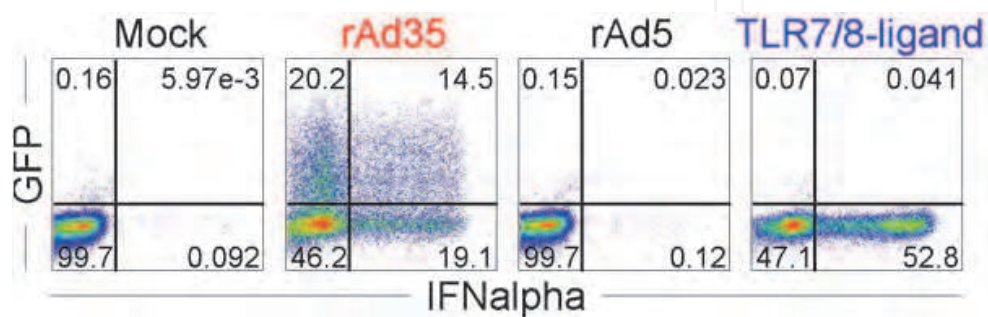


Fig. 5. Simultaneous detection of rAd derived GFP and intracellular IFN$\alpha$.

pDC were exposed to rAd35-GFP, rAd5-GFP (100 ip/cell), or a TLR7/8-ligand (1 μg/ml) for 8 hours with Brefeldin A present for the last 7 hours. The cells were then washed, fixed and permeabilized with BD cytofix/cytoperm kit, and stained for anti-CD123 (BD) and anti-IFNα (Interferon Source). GFP expression was evaluated simultaneously with IFNα by flow cytometry (BD FACS LSR II) and data was analyzed with FlowJo software.

Intracellular IFNα, accumulated during the last 7 hours of stimulation by Brefeldin A, can be readily detected in pDC exposed to rAd35 (Lore et al., 2007). To add to our previous study we show here the simultaneous detection of the frequencies of infected (GFP+) and IFNα producing cells (Fig. 5). rAd35 again induced almost as much IFNα as the positive control, a TLR7/8-ligand, whereas rAd5 neither infected nor induced IFNα in pDC. We also observed four distinct groups of pDC following rAd35 exposure: GFP-, GFP+, GFP+/IFNα+, or IFNα+ (Fig. 5). Since DC may be infected with rAd even though no GFP is detected, this may explain why PDC make IFNα in the absence of GFP expression. Differential kinetics of GFP and IFNα production may also partly explain the observed expression pattern. Nevertheless, the strong induction of IFNα by pDC is an important parameter to study in the context of rAd infection. Type-1 IFN induction in PBMC was shown to be a feature unique to CD46 using Ads (Iacobelli-Martinez & Nemerow, 2007). In that study, IFNα/β production was dependent on endosomal TLR9 signalling. While rAd35 induces IFNα/β in pDC *in vitro*, there may be multiple sources of systemic IFNα/β, especially for non-CD46 using Ads. For example, murine splenic mDC are the major source of IFNα/β *in vivo* following inoculation of Ad3 or Ad5, independent of TLR and cytosolic nucleic acid receptor (RIG-I like) signalling (Fejer et al., 2008). Moreover, virus associated RNA synthesized by RNA polymerase III may also contribute to systemic type-1 IFN production after rAd immunization (Yamaguchi et al., 2010). Nevertheless, potent transgene-specific CD8+ T cell responses are mounted in the absence of intact type-1 IFN signalling (Hensley et al., 2005). IFNα likely has beneficial effects for vaccination in driving adaptive immunity, whereas in gene therapy antiviral properties of IFNα may blunt rAd mediated gene delivery.

RT-PCR is an alternative and sensitive method to quantify cytokine gene transcription (Douagi et al., 2009). However, this method requires highly pure populations of sorted cells and does not allow for cytokine measurement on a per cell basis. In addition, it is important to consider that detection of RNA may not correlate with protein translation and functional cytokine secretion.

## 5.3 Antigen presentation

When rAd are used in either gene therapy or vaccine vector applications, it is crucial to determine the immune responses to the transgene. Following vaccination the goal is to induce strong transgene immunity, while for gene therapy such immune responses may blunt the intended effect of the transgene. As such, we have previously studied *in vitro* the capacity of rAd vectors to activate transgene specific memory T cells (Lore et al., 2007). To be able to measure antigen-presentation of the transgene we developed rAd5 or rAd35 vectors encoding the immunodominant pp65 antigen of CMV. These rAd encoding pp65 were exposed to freshly isolated pDC or mDC for 24 hours to allow for sufficient time for transgene presentation and the DC were then added to autologous sorted CD4+ or CD8+ T cells from donors with known pre-existing T cell responses to CMV pp65. We found that rAd exposed DC were able to activate antigen (pp65)-specific memory T cells equivalently

to antigen matched overlapping pp65 peptide pools. Importantly, rAd35 vectors more efficiently activated memory T cells compared to rAd5. While infected DC likely display rAd-derived peptide on class I to activate CD8 T cells, the mechanisms for class II presentation to activate CD4+ T cells are less clear. Nevertheless, these studies indicate that rAd5 and rAd35 exposed DC are able to present Ad encoded antigen and stimulate antigen specific T cells. Future studies should also evaluate how rAd vectors influence DC priming of naive T cells. These findings have important implications in clinical applications and depend on whether immune responses towards the transgene are desired or not (Zaiss et al., 2009).

## 6. Conclusions

In this chapter we have reviewed the basic concepts relating to the infection of primary human DC. Studying the interactions between clinically relevant rAd vectors and multiple subsets of pAPC is instructive for guiding the use of these delivery vectors *in vivo*. While studies in rodents, such as mice, may offer clues to how rAd vectors are recognized *in vivo*, there are notable biological differences between humans and mice (Mestas & Hughes, 2004). While receptors for viral nucleic acid may be similar in these species, the expression patterns within DC subsets are quite different. Additionally, the primary viral attachment receptors for human rAd species are likely different between their natural human hosts and mice; for example, mice do not express the species B receptor CD46. This likely has a significant impact on tissue and cellular tropism as well as innate viral recognition. For these reasons, it is crucial to study the interaction of rAd on immune cells from the host species (i.e. humans) with which the viruses co-evolved. To this end, isolating phenotypically immature primary human DC from both blood and skin tissue provides highly relevant cells with which to study the interactions between innate immune cells and different recombinant rAd species. Using GFP reporter transgenes, the susceptibility of different DC subsets can be monitored *in vitro*, as can the subsequent induction of DC activation (i.e. phenotypic maturation and cytokine production). Following infection and induction of maturation, DC become specialized to activate antigen-specific lymphocytes, which can also be tested *in vitro*. While it may be important to exploit the induction of innate immune responses to drive development of transgene specific adaptive immunity in a vaccine setting, the opposite is likely desired for locally targeted gene therapy. In both clinical applications it is evermore crucial to gain a more complete understanding of the human immune response raised against rAd vectors.

## 7. Acknowledgments

## 8. References

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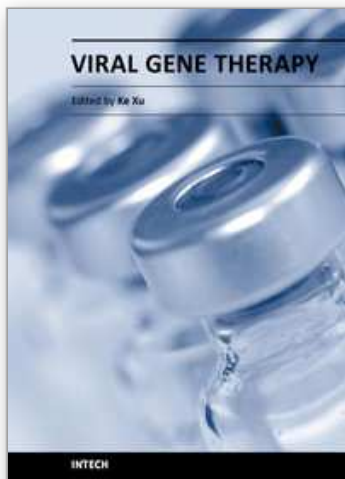
**Viral Gene Therapy**

Edited by Dr. Ke Xu

The development of technologies that allow targeting of specific cells has progressed substantially in recent years for several types of vectors, particularly viral vectors, which have been used in 70% of gene therapy clinical trials. Particular viruses have been selected as gene delivery vehicles because of their capacities to carry foreign genes and their ability to efficiently deliver these genes associated with efficient gene expression. This book is designed to present the most recent advances in viral gene therapy

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William Adams and Karin Loré (2011). Recombinant Adenovirus Vector Infection of Human Dendritic Cells, Viral Gene Therapy, Dr. Ke Xu (Ed.), ISBN: 978-953-307-539-6, InTech, Available from: http://www.intechopen.com/books/viral-gene-therapy/recombinant-adenovirus-vector-infection-of-human-dendritic-cells

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