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REGULATION OF HUMAN DENDRITIC CELLS AND T CELLS BY ADENOVIRUS VECTORS TYPES 5 AND 35: IMPLICATIONS FOR VACCINE DESIGN

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nothing is more fatal to the progress of the human mind than to presume that our views of science are ultimate, that our triumphs are complete, that there are no mysteries in nature, and that there are no new worlds to conquer

Humphry Davy

ABSTRACT

Following viral infection or vaccination dendritic cells (DC) perform an intricate series of roles at the interface of innate and adaptive immunity. Peripheral DC recognition of pathogen associated molecular patterns initiates signaling cascades leading to morphological and phenotypic maturation. The differentiation to a mature phenotype licenses DCs to efficiently prime T- and B-lymphocytes. Thus, DCs shape early innate immune responses that limit viral replication and initiate the generation of protective and adaptive immunological memory.

In this thesis, we began by studying the interaction of human primary DCs with human adenovirus (AdV). While the causative agent of a variety of human diseases, AdVs are also a valuable research tool for probing virological, immunological, and cellular mechanisms of nature. Recombinant human AdVs (rAdV), rendered replication incompetent and thus unable to cause disease, have gained prominence as gene delivery vehicles in multiple vaccine trials. In light of the clinical importance of AdV vectors, we employed a reductionist approach to study mechanisms of virus-mediated regulation of human DC function. Since DCs activate adaptive immunity, we extended our investigations to the impact of rAdV on the activation of T-lymphocytes. These studies are particularly relevant since the induction of potent T-cell responses is one objective of rAdV based vaccine vectors.

In assessing the interaction of rAdV with primary human blood myeloid and plasmacytoid DC subsets, we found that activation of these cells was dependent on rAdV type. rAdV-35 more efficiently infected DCs than rAdV-5, and matured blood DCs and strongly induced interferon-α in plasmacytoid DCs. Infection by rAdV-35 was dependent on the receptor CD46, whereas the receptor for rAdV-5 was less clear. We then showed that lactoferrin facilitated rAdV-5 infection of multiple DC subsets in a similar manner to epithelial cells. rAdV-exposed DCs were able to process and present rAdV encoded transgenes and activate polyfunctional memory T cells, which indicated that rAdV infected DCs retained their antigen presentation capacity. However, it remained unclear from these studies whether rAdV affected the activation of naive T cells, which is an important step for vaccination. To this end, rAdV-35 was found to strongly inhibit activation of naive CD4+ T cells through binding of its cellular attachment receptor, CD46. Attenuated activation was characterized by lower proliferation and IL-2 production, as well as deficient NF-κB nuclear translocation. Further studies showed that cross-linking with CD46 monoclonal antibodies and recombinant trimeric rAdV-35 knob proteins was sufficient to cause similar suppression as the whole virus, substantiating the role of CD46 in regulating CD4+ T-cell function.

Our findings provide insights into the mechanisms by which host immune cells respond to rAdV and also how the virus may act to modulate host cell function. These findings may also guide the development of rAdVs as vaccine vectors.

ORIGINAL PAPERS

I. Karin Loré, **William C. Adams**, Menzo J. Havenga, Melissa L. Precopio, Lennart Holterman, Jaap Goudsmit, Richard A. Koup. Myeloid and plasmacytoid dendritic cells are susceptible to recombinant adenovirus vectors and stimulate polyfunctional memory T-cell responses.
Journal of Immunology. 2007 Aug 1; 179 (3), 1721-1729.

II. **William C. Adams**, Emily Bond, Menzo J. Havenga, Lennart Holterman, Jaap Goudsmit, Gunilla B. Karlsson Hedestam, Richard A. Koup, Karin Loré. Adenovirus serotype 5 infects human dendritic cells via a coxsackievirus-adenovirus receptor-independent receptor pathway mediated by lactoferrin and DC-SIGN.
Journal of General Virology. 2009 Jul; 90 (7), 1600-1610.

III. **William C. Adams**, Cornelia Gujer, Gerald M. McInerney, Jason G. D. Gall, Constantinos Petrovas, Gunilla B. Karlsson Hedestam, Richard A. Koup, Karin Loré. Adenovirus type-35 vectors block human $CD4^+$ T-cell activation via CD46 ligation.
Proceedings of the National Academy of Sciences USA. 2011 May 3; 108 (18), 7499-7504.

IV. **William C. Adams**, Cornelia Gujer, Ronald J. Berenson, Jason G. D. Gall, Gunilla B. Karlsson Hedestam, André Lieber, Richard A. Koup, Karin Loré. Cross-linking CD46 with trimeric adenovirus type-35 knob proteins attenuates CD4+ T cell function.
Manuscript

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PREFACE

This thesis has been divided into two main sections. In the first I will introduce basic concepts of viral immunity with particular focus on our current knowledge in humans. It is my intention that this introduction will be accessible to readers whom are outside this specific field. The historical rationale for using adenoviruses as tools in research and as vaccine vectors will be discussed in order to provide a greater contextual significance to the work in this thesis. The aims of the thesis will then be presented. Finally, a broad overview of the origin, definition, phenotype, and function of dendritic cells and T cells will be presented followed by basic adenovirus virology and vector generation.

In the second section I will cover the materials and methods used throughout the papers in the thesis. Then, the results from papers I-IV will be presented and discussed together in order to demonstrate how the findings are related. Studies performed here used a reductionist approach that we hope provides instruction for understanding human immune cell function and guidance for using recombinant adenoviruses as vaccine vectors in humans. The thesis will conclude with remarks on these topics and on future directions, followed by reprints of the original papers.

William C. Adams   May 16th, 2011

ABBREVIATIONS

AA	amino acids
Ab	antibody
AdV	human adenovirus
BDCA	blood dendritic cell antigen
CAR	coxsackievirus-adenovirus receptor
CCR	c-c chemokine receptor
CD	cluster of differentiation
CMV	cytomegalovirus
CTL	cytotoxic T-lymphocyte
CTLA-4	cytotoxic T-lymphocyte antigen-4
cyt	cytoplasmic tail (as in CD46)
DAMP	danger associated molecular pattern
DC	dendritic cell
dDC	interstitial dermal dendritic cell
DC-SIGN	DC-specific intercellular adhesion molecule-3-grabbing non-integrin
DNA	deoxyribonucleic acid
ds	double stranded (as in DNA)
ELISA	enzyme linked immunosorbent assay
GFP	green fluorescence protein
GM-CSF	granulocyte macrophage-colony stimulating factor
HHV-6	human herpes virus-6
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
IFN	interferon
Ig	immunoglobulin
IκBα	nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α
IL	interleukin
ip	infectious virus particle
IRF	interferon regulatory factor
ITAM	immunoreceptor tyrosine-based activation motif
LC	Langerhans cell

Lf	lactoferrin
LPS	lipopolysaccharide
TLR	toll like receptor
mAb	monoclonal antibody
mDC	myeloid dendritic cell
MDDC	monocyte derived dendritic cell
mip	macrophage inflammatory protein
MV	measles virus
NF-κB	nuclear factor κ-light-chain enhancer of activated B cells
NHP	non-human-primate
NK cell	natural killer cell
OL	overlapping (as in peptide)
PAMP	pathogen associated molecular patterns
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PD	programmed death
pDC	plasmacytoid dendritic cell
PRR	pathogen recognition receptor
rAdV	replication incompetent recombinant adenovirus
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RLR	retinoic acid inducible–gene I (RIG-I)-like receptor
RT-PCR	real-time polymerase chain reaction
SCR	short consensus repeat of CD46
ss	single stranded (as in RNA)
TCR	T-cell receptor
Tf	transferrin
T_h	helper T-cell
TNF	tumor necrosis factor
VA-RNA	virus associated ribonucleic acids
vp	virus particle

1 INTRODUCTION

1.1 HUMAN IMMUNOLOGY AND VACCINATION

Organisms face continuous challenge throughout most anatomical sites from self-antigen, cancers, commensal bacteria, latent viruses, and external pathogens. Survival depends on finely balancing the generation of tolerance to self-antigen and commensal flora and the generation of protective immunity to acute and chronic pathogen infections. Vertebrates of the kingdom *Animalia*, such as ourselves, have evolved a complex three-limbed, interconnected immunological system, which in general terms includes: complement, and innate and adaptive immunity. These three limbs together have a remarkable capacity to confront immense pathogen diversity in nature. Commensal bacteria (1) and chronic or latent viruses (2) also play many well defined and as yet undefined roles in regulating tolerance and immunity. The evolutionarily ancient complement system serves as a primary host defense that eliminates microbial invaders by a "hub-like network" of canonical and alternative pathways (3). Amplification of these pathways from the normal steady-state sampling leads to the formation of lytic pores, termed terminal complement complex, on marked host or microbial cells that causes their destruction. The numerous cell types of the more recently evolved second limb, the innate immune system, recognize pathogen expressed chemical signatures by germ line-encoded receptors and work in concert with complement to provide rapid and immediate responses and clearance of antigen (4). Cells of the innate immune system, namely dendritic cells (DC), orchestrate direct antimicrobial responses by type-1 interferons (IFN-I) and tolerance when appropriate. Perhaps most significantly the innate system in concert with complement initiate and regulate adaptive immune responses, which can provide long-lasting immunological memory with humoral antibody (Ab) (B-cell) and cellular (T-cell) responses. T cells function by either directly killing infected cells or by supporting B cells that can produce antibodies that neutralize extracellular pathogens. However, a potential cost of adaptive immunity is developing autoimmune disease or allergy. The three limbs of immunity, which are coevolving with each other and with commensal and external microbes, are thus linked by a complex web of interactions that together enables an organism to protect against pathogens while limiting damage to self.

In 1796 Edward Jenner found that inoculation with cowpox virus protected individuals against subsequent human smallpox infection. Vaccines are now known to exploit adaptive immunological memory in order to provide individual and herd protection against subsequent pathogen challenge (5-7). Administration of live attenuated virus, killed virus, or virus-like-particles (VLP) that do not cause disease can prime the immune system and generate protective cellular and humoral memory. While the correlate of protection for most approved vaccines is Ab titers (humoral immunity), the induction of cellular T-cell responses is nonetheless important. Vaccination exemplifies the adaptability and specificity of the host immune response. The live-attenuated yellow fever virus 17D (YFV) vaccine potently primes the adaptive immune system and provides durable protection of the host upon wild-type YFV challenge (8). Yet, the traditional empiric vaccine approach used to make vaccines like that against YFV is largely inadequate for developing vaccines against pathogens such as HIV-1 (the

causative agent of AIDS), *Plasmodium falciparum* (causing Malaria), and *Mycobacterium tuberculosis* (TB) for which there are no efficacious vaccines (9). Thus, there is a strong need to develop novel strategies to generate vaccine induced immunity.

1.2 ADENOVIRUSES IN BASIC AND CLINICAL RESEARCH

Viruses are a remarkably diverse Order of ancient obligate intracellular pathogens that cause a variety of human diseases. They are classified as obligate because their existence depends on a host cell. Human adenoviruses (proper nomenclature is HAdV; AdV is used here) of the family *Adenoviridae* and genus *Mastadenovirus* refers to at least 50 types subdivided into 6 distinct species that share similar capsid structures and genome organization. They cause numerous respiratory, ocular, and gastrointestinal diseases in humans. However, AdVs have also been widely used in research to uncover biochemical and cellular mechanisms of Nature. Perhaps most noteworthy is the discovery of alternative splicing of AdV derived RNA transcripts in infected cells by two independent groups (10, 11). These groups observed that viral RNA was not as expected transcribed in a collinear manner with its genomic template (genes). In early gene therapy trials employing replication incompetent recombinant adenoviruses (rAdV) strong immune responses (humoral and cellular) were generated towards genomic backbone encoded transgenes (12, 13). These experiments suggested that rAdVs may be effective vehicles (i.e. vectors) to efficiently deliver and induce immunity to encoded pathogen genes. At the present time, rAdV vectors continue to be the most widely used viral vectors in pre-clinical and clinical gene therapy (14, 15) and vaccine trials for a wide range of human pathogens (16, 17). However, a greater understanding of AdVs in the context of basic human immunology is essential for providing insights into natural host-pathogen interactions and for potentially enhancing their efficacy as vaccine vectors.

2 AIMS OF THESIS

The overall aim of this thesis was to study the interactions of human DCs and T cells with human rAdV vectors. We focused on species C AdV-5 and species B AdV-35. These particular AdVs were studied because they are used as viral vectors in numerous clinical vaccine trials for diseases associated with HIV-1, *Plasmodium falciparum*, and *Mycobacterium tuberculosis*. The aims were as follows:

- To study the capacity of rAdV-5 and rAdV-35 to infect primary human myeloid and plasmacytoid DCs from blood, and induce phenotypic maturation and cytokine production in these cells.

- To study receptor usage of rAdV-5 and rAdV-35 on primary human DCs.

- To study the ability of rAdV exposed human DCs to process and present rAdV-encoded antigen and activate autologous antigen-specific memory T cells.

- To evaluate effects of rAdV vectors on the activation of naive CD4+ T cells.

- To study mechanisms of how rAdV-35 may regulate T-cell function via binding to its receptor CD46.

3 DENDRITIC CELLS

3.1 DEFINITION AND GENERAL FUNCTION

The term dendritic cell (DC) was first used to describe populations of non-adherent cells morphologically distinct from macrophages in murine spleens and lymph nodes (18, 19). From the Greek word déndron meaning tree, dendritic describes a branched morphology of membrane processes extending outward from the cell. The discovery of DCs provided the first clues to how lymphocytes were activated with antigen, which had been a significant unanswered question in immunology (20-22). These DCs turned out to be remarkably similar to the cells Paul Langerhans observed over a century before.

The identification of DCs has led to a vast number of studies that have begun to reveal the roles of these cells in the immune system. During the steady state DCs are mainly circulating in the periphery with an immature phenotype and high endocytic capacity that allow them to internalize antigen. A diverse array of cytosolic and endosomally expressed pathogen recognition receptors (PRR) also enables DCs to recognize and respond to specific and normally conserved viral nucleic acid signatures of extracellular and intracellular pathogens. PRR engagement can then initiate downstream signaling cascades that lead to DC activation, which is characterized by upregulation of spleen or secondary lymphoid organ homing receptors (e.g. CCR7), phenotypic maturation and production of cytokines such as IFN-I (23). Together, these events license and support DCs to activate antigen-naive T cells. DCs normally migrate from the periphery and activate T cells in secondary lymphoid organs. However, skin DCs may also transport HSV-1 antigens from the periphery to lymph node resident DCs, which in turn can activate CD8+ T cells (24). The morphological and phenotypic changes of matured DCs allow them to activate lymphocytes in an antigen specific manner that may be up to one-hundred times stronger than other leukocytes (25). To activate T cells, DCs load processed peptides on major histocompatibility complex (MHC) (in humans termed human leukocyte antigen (HLA)) that bind cognate αβ-T-cell receptors (TCR) expressed on T cells. TCR and co-stimulatory signals provided by mature DCs lead to activation of cytotoxic CD8+ T cells (cytotoxic T lymphocyte or CTL) and helper CD4+ T cells (T_h). Although DCs induce T cells to proliferate, DCs themselves do not appear to proliferate. While most non-hematopoietic and hematopoietic cells present endogenous antigen on MHC-I, DCs are termed professional antigen presenting cells (pAPCs) because they can also present exogenous foreign peptides on MHC-II. It has been proposed that MHC-II presentation in mature DCs occurs efficiently because peptide bound MHC (pMHC)-II are efficiently transported in polarized endosomes towards the immune synapse where TCR binding occurs (26, 27).

Attenuation of allograft rejection in mice treated with anti-DC mAbs provided evidence that DCs could activate T cells (28) and it is still commonly accepted that DCs are crucial in mediating viral immunity (29). However, only recently have studies – where specific DC subsets were depleted in mice before pathogen challenge – provided more definitive evidence for the role of DCs in controlling infection. Diphtheria toxin receptor (DTR) mediated depletion of CD11c+ cells demonstrated that DCs were

essential for priming T-cell responses against *Listeria monocytogenes* (30). Specific DTR ablation of BDCA-2 expressing plasmacytoid DCs (pDC) led to attenuated IFN-I and increased viral loads after challenge with a DNA murine cytomegalovirus (MCMV) (31). Early replication of the RNA Vesicular stomatitis virus (VSV), which like MCMV activates pDCs, was also increased when pDCs were absent. Interestingly, pDC mediated activation of NK cells and CD8+ T cells were shown to be important for controlling MCMV and VSV, respectively. To this end, DCs can shape the activation of innate immune cells in a multitude of ways (32). In summary, DCs are now widely regarded for their central role in initiating immune responses towards foreign antigen and linking innate and adaptive limbs of the immune system (33). The diversity of DC function may be at least partially traced to the presence of several distinct DC subsets in blood and tissue.

3.2 HUMAN DC SUBSETS

3.2.1 Blood subsets: similarities and differences

The assortment of human DCs in blood and other tissues is both well and poorly described. Human blood DCs may be separated into plasmacytoid DCs (pDCs) and two types of myeloid DCs (mDCs) based on unique expression of blood DC antigens (BDCA) (34-37). pDCs co-express CD303 (BDCA-2) and CD304 (BDCA-4), whereas one subset of mDCs displays CD1c (BDCA-1). DCs also express MHC-II (HLA-DR) (Table I). Immature pDCs have a round and non-dendritic morphology, but have been classified as DCs due to their ability to mature and activate naive T cells (38). pDCs also display, though not uniquely, the IL-3 receptor-α (CD123). mDCs share CD1c expression with a subset of B cells and CD11c expression with monocytes. While most mDCs are CD14-, a small frequency are CD14+ (37). The CD14+ to CD14- mDC ratio changes with certain toll-like-receptor (TLR)-ligand stimulations, although the reasons are poorly understood (W.C. Adams, unpublished data). A separate mDC subset expressing CD141 (BDCA-3) is notably proficient at loading and presenting exogenous foreign peptides on MHC-I in a process termed cross-presentation (39-42). Cross-presentation describes the observation that immunization with soluble proteins or viruses that do not infect DCs leads to the induction of CD8+ T-cell responses (reviewed by (43)). Unless noted otherwise mDC will hereafter refer to CD1c+ mDCs. It is important to remark here that the current ternary division of blood DCs may oversimplify the actual subset heterogeneity.

mDCs and pDCs share several classical DC features, such as efficient uptake of antigen, expression of multiple PRRs, and the ability to mature, migrate and activate naive T cells. But they differ in specific ways. First, mDCs are more efficient APCs when observed activating autologous T cells (44-46). Their repertoire of TLRs is distinct as well with mDCs expressing TLR1-8 and 10, and pDCs having TLR7 and 9. mDCs specialize in producing T_h1 driving IL-12p70 and pDCs in the rapid and copious production of anti-viral IFNα/β (47-51) (Table I). pDC derived IFN-I and IL-6 also facilitate the formation of Ab-producing plasma cells after influenza exposure (52). Also, pDCs assist TLR-ligand induced activation of naive B cells through IFN-I, while mDCs do not seem to be as effective in this respect (53-55). It has been suggested that pDCs may be more potent cross-presenting cells than CD1c+ mDCs, but how pDCs

compare to canonical CD141+ mDCs has not yet been studied thoroughly (56).

Before concluding this section it is important to compare human blood DC subsets with other mammalian vertebrates. In NHPs homologous mDCs and pDCs can be identified and isolated from blood (57, 58). In regard to mice, blood DCs may be subdivided into three types: pDCs that make high levels of IFNα, and two subsets of mDCs that are similar to the CD1c+ and CD141+ mDCs in humans (37).

	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
Blood Subsets	Plasmacytoid DC (pDC)	CD303+ (BDCA-2) CD304+ (BDCA-4) CD123+ (IL-3Ra) 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
Cutaneous Subsets	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 epidermal skin layer	RPMI media 10 % fetal calf sera

Table I. Human DC subsets

Adapted from (59) and used with approval from publisher (intech.org)

3.2.2 In vitro derived DC subset

IL-4 and GM-CSF differentiate blood monocytes into a myeloid DC surrogate termed monocyte derived DC (MDDC) (60). MDDCs lose CD14 expression and gain CD1a and DC-SIGN (Table I). They also express MHC-II and are more potent pAPCs than monocytes. It is currently unknown whether MDDCs represent a single primary DC subset, but they may at least partly mimic skin resident interstitial dermal DCs (dDC) as they produce similar cytokines and express DC-SIGN (61). The ability to generate DCs from blood monocytes in vitro suggests that circulating monocytes have a certain level of plasticity in their differentiation program or fate. MDDCs are notable inducers of IL-12p70 in response to TLR-ligands (62), which indicates a role for these cells in driving T_h1 type responses (Table I).

3.2.3 Cutaneous subsets

In the steady state DCs are dispersed throughout peripheral tissue including the skin. These cells also have a dendritic morphology and may be considered more differentiated than blood mDCs. Cutaneous DCs are normally divided into two subsets

based on the tissue in which they reside under steady state conditions: interstitial dDCs resident in the dermal layer, and Langerhans cells (LC) resident in the epidermal layer (29, 63). Both subsets express MHC-II and likely have a myeloid lineage. LCs display Langerin and CD1a, while the dDC population is more diverse based on expression of DC-SIGN, CD1a and CD14 (61, 64) (Table I). It is plausible that the dDCs defined here actually represents multiple unique subsets. The role each of these skin DCs play in detecting viral infection and initiating immune responses likely depends on both the route of challenge and the nature of the particular virus (35). It has been shown that dDCs, in particular the CD14+ subset, initiate humoral immunity (i.e. Ab-producing B cells) and LCs specialize in mediating cellular immunity (i.e. cytotoxic CD8+ T cells) (61). The notion that Langerhans cells potently induce T cells to proliferate has been appreciated for some time (65). pDCs are not normally found in the skin, but may migrate to the skin during inflammation and once there mediate immunity to viral infection and autoimmune diseases such as psoriasis (66, 67). While LCs and dDCs are found in human breast skin (64), the local and global distribution of these cells in this and other skin locations is largely unknown in mice or primates. Understanding differential local site distribution may provide insights into peripheral tolerance or immunity. These descriptive studies will be critical to perform in the future.

3.3 LIFE HISTORIES

Blood DC subsets arise from bone-marrow derived hematopoietic precursor cells, although their development is substantially less well defined that lymphocytes (68). Based on morphology and surface marker expression mDCs are thought to arise from a common myeloid precursor cell and pDCs from a lymphoid precursor (69). The expression of CD13 and CD33 on mDCs associates these cells with a myeloid lineage (37). The phenotype of pDCs circulating in blood together with the apparent lack of any pre-pDC subset suggests that these cells are fully developed in the bone marrow. DCs also differ in their life cycles (38, 70). mDCs enter the blood upon exit from the bone marrow and then migrate to and sample peripheral tissues. Circulating mDCs appear to remain plastic as incubation of these cells with GM-CSF and IL-4 induces the cells to differentiate further into phenotypically distinct subsets (71). The tissue environment likely plays a significant role in driving mDC differentiation. mDCs may then leave the peripheral tissue either constitutively (tolerance) or after activation by foreign antigen and migrate through the afferent lymphatics to the spleen or lymph nodes to activate T cells. In contrast, pDCs are scarce in peripheral tissue during the steady state but tend to migrate to sites of infection or inflammation. One clue that suggests blood DCs have not encountered antigen is that most isolated display an immature phenotype (44-46, 72). Both the frequency and function of circulating blood DCs has also been shown to be negatively affected by chronic HIV-1 infection (73, 74). These findings provide caution for studying DCs in humans since most are colonized by numerous latent viruses that may cause similar effects. Much remains to be learned about the life histories of blood and skin resident DCs.

3.4 ACTIVATION

Activation licenses DCs to induce tolerance or viral immunity. As has been discussed above, DCs located at peripheral sites (non-lymphoid tissue) in the steady state are

normally of an immature phenotype. These cells have high capacity to sample the extracellular environment and take-up antigen. Uptake of foreign or self-antigen alone may induce migration to lymphoid tissues. However, these cells will be in a quiescent state and will more likely induce a state of T-cell tolerance to the antigen. Because DCs can present peptides on MHC-I and MHC-II, tolerance will be regulated by both CD8+ and CD4+ T cells, respectively. Tolerance may take the form of ablation of these T cells (anergy) or induction of regulatory T cells (Treg). Conversely, when antigen is in the presence of a so-called 'danger signal' DCs undergo a maturation process in which upregulation of co-stimulatory markers licenses DCs to induce immunity (rather than tolerance) by activating naive T cells (29, 70). TCR and co-stimulatory signaling provided by mature DCs is commonly termed the two-signal model and is essential for controlling T-cell activation (75, 76). Thus, induction of DC maturation is an important checkpoint for driving either tolerance or immunity. Danger signals, often termed pathogen associated molecular patterns (PAMP), may be microbial products such as, bacterial cell wall components like LPS, nucleic acids (dsDNA or ssRNA), or CpG-DNA motifs. However, commensal microbes also express PAMPs so the usage of 'pathogen' is semantically ambiguous. Components from damaged cells or tissue that are released upon necrotic cell death are termed damage associated molecular patterns (DAMP). There are a number of well and poorly defined PRRs that sense foreign derived components. TLRs, cytosolic retinoic acid inducible–gene I (RIG-I)-like receptors (RLR), and the inflammasome are a few examples (reviewed in (77)). The licensing of DCs to activate lymphocytes is illustrated by the findings that TLR-ligands adjuvant protein-based vaccines in vivo to induce potent immunity to the immunized protein (78-80). DCs also make numerous cytokines such as, TNF, IFN-I, IL-1, IL-12, and IL-6 that drive innate immune responses and shape adaptive immune responses (32).

Mature DCs may upregulate activating members of the B7 family (CD80 and CD86) that provide co-stimulation to optimally activate naive T cells through engagement of CD28. While DCs provide early co-stimulation to T cells via B7-CD28 signaling axis, B7 may also bind CTLA-4 on T cells later after activation (48 hours) (81). At this time-point T cells convert from expressing CD28 to expressing CTLA-4 on their surface in a process that dampens further activation of the T-cell. DCs also upregulate MHC-II (HLA-DR) as well as CD40 that activates T cells through CD40-ligand binding. DCs may express other inhibitory receptors to control activation of T cells, such as the programmed death (PD)-1 ligands, PD-L1 and PD-L2. The role of the PD1 : PD-L1 axis is not as well defined, although the expression of PD-1 on HIV-1 specific memory CD8+ T cells is correlated with an exhausted phenotype, increased apoptosis, and reduced control of infection (82). Transcription factor expression may also influence DC activation of T cells. For example, DC expression of the transcription factor Foxo3 acts as one factor that can limit clonal T-cell expansion (83). How AdV can induce DC activation will be discussed in the chapter on AdVs.

4 T CELLS

4.1 DEFINITION, LIFE HISTORIES, AND FUNCTION

DCs are specialized to sense and interpret innate signals in order to activate and shape adaptive CD4+ and CD8+ T-cell responses. T cells begin life in the thymus as CD4-/CD8- thymocytes expressing a diverse repertoire of αβ-TCRs generated by somatic gene recombination. Intricate negative and positive clonal selection processes generate mature naive T cells expressing either CD4 or CD8 that exit the thymus lacking TCR reactivity to self. In the periphery multiple mechanisms, such as promotion of naive T-cell survival by presentation of self-pMHC and production of IL-7, regulate homeostatic maintenance of non-self reactive T cells with requisite functionality and diversity (84). Foxo1 has been suggested to be an integral transcription factor in regulating peripheral T-cell homeostasis by controlling IL-7 signaling and CCR7-mediated homing (85). Notably, αβ-TCRs are unlike B-cell receptors (BCR) that undergo affinity maturation in peripheral secondary lymphoid organs, because TCR affinity for pMHC appears to be set in the thymus and to remain static in the periphery. Inherited allelic variation in the HLA repertoire (also termed HLA haplotype) further increases the diversity of potential antigen recognition and responses.

After infection or vaccination, αβ-TCRs recognize cognate DC-displayed pMHC in a largely stochastic manner due to the rarity of αβ-TCR clones and the size of the animal. These interactions are further complicated by the low affinity and degenerate binding of pMHCs to TCRs. TCR recognition of pMHC is also restricted (86, 87), in the sense that pMHC-Is have a greater affinity for TCRs expressed on CD8+ T cells, whereas pMHC-IIs have higher affinity for αβ-TCRs expressed on CD4+ T cells. However, multiple mechanisms act to enhance the probability of successful encounters including, but not limited to, (i) the morphology of DCs that allows for simultaneous interactions with numerous T cells, (ii) low affinity integrin binding partners, such as lymphocyte function-associated antigen-1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1), that facilitate DC and T-cell attachment, and (iii) continuous recirculation of naive T cells in blood, peripheral tissue, lymphatic vessels, and secondary lymphoid organs where antigen becomes constrained in space and (iv) a network of stromal fibroblastic reticular cells (FRC) enhance the frequency of DC and T-cell contacts (88). CD4 also stabilizes the contact by binding MHC. Once the pMHC recognizes its cognate αβ-TCR, T-cell activation may be initiated. Together, these and numerous other molecules proximal to the TCR-MHC complex form the immune synapse. As mentioned previously, T-cell activation signals are transmitted through ITAM-containing CD3 side-chains in proximity to the TCR and quantitatively enhanced by CD28 co-stimulation. Subsequent downstream intracellular signaling pathways converge to activate three main transcription factors namely, nuclear factor κ-light-chain enhancer of activated B cells (NF-κB), nuclear factor of activated T cells (NFAT), and activator protein-1 (AP-1) that drive proliferation and cytokine production (89, 90). In this way, TCR-activation at the polarized synapse on the naive T-cell leads to a clonal burst (i.e. proliferation) and induces other functions specific to each T-cell subset. While activation of naive T cells most likely occurs in lymphoid tissues, memory T cells may be activated in lymphoid and peripheral non-lymphoid tissues near the site of certain

infections like HSV-1 (91). In general terms, activated CD8+ T cells are termed CTLs because they can kill (i.e. induce apoptosis of) infected (intracellular microbes) or cancerous targets cells. CD4+ T cells provide critical 'help' to various cells, including CD8+ T cells (92). This help in concert with IL-2 provide signals to facilitate optimal priming of CD8+ T-cell responses that can establish durable cellular immunity (7). Thus, the induction of immunological cellular memory depends largely on priming of CD4+ T-cell helper (T_h) responses (93, 94). These functions make CD4+ T-cell responses crucial for vaccination (7), which is why we have focused on this subset in this thesis. Numerous intrinsic and extrinsic signals regulate CD4+ T-cell activation and differentiation, and for reasons discussed later, we have narrowed our studies to intrinsic regulation by CD46. The role of this receptor in modulating T-cell function will also be discussed in detail.

In conjunction with antigen-specific activation, inherently plastic naive CD4+ T cells differentiate into at least four known peripheral effector lineages (95). Seminal work by Coffman and colleagues first described two terminally differentiated T_h1 and T_h2 effector subsets (96). T_h1 cells are induced by IL-12p70, express IFNγ and the transcription factor T-bet, and control intracellular pathogens like viruses (92). T_h2 cells express IL-4 and trans-acting T-cell specific transcription factor GATA-3, and control external pathogens like worms via humoral immunity. Potential drawbacks of these responses may be the induction of autoimmune-induced pathology and allergy, respectively. Still, these effector cells are important components of an adaptive immune response. As an example, the induction of polyfunctional T_h1 responses correlates with vaccine protection against *Leishmania major* in mice (97). As often the case in biology the T_h1/T_h2 paradigm is likely too simplistic. More recently, additional effector CD4+ T-cell lineages have been identified including induced regulatory T cells (Treg) and T_h17 cells, which express forkhead box P3 (FoxP3) and RORγt transcription factors, respectively (98). Tregs keep T_h1 and T_h2 cells in check, whereas T_h17 cells control extracellular pathogens at mucosal surfaces by producing IL-17 and IL-22. CD4+ T cells also generate long-lived effector and central memory subsets that persist and are maintained after viral clearance. A final subset, follicular helper T cells (T_{FH}), is present in lymph node associated germinal centers and directs B-cell Ab responses via IL-4 signaling (99). It is not entirely clear how these effector subsets arise, especially given that TCR-clone specificity of the original antigen-activated CD4+ T-cell ostensibly needs to be preserved. The prevailing "one cell-multiple fates" model is supported by an expanding body of evidence (100). Asymmetric T-cell division upon initial activation results in both effector and memory T cells during the first division and provides one cellular mechanism (101, 102). The finding that transplantation of a single naive CD4+ T-cell clone into a recipient mouse was sufficient to induce multiple T-cell fates also supports this model (103). DC signaling may also drive these fate decisions, though there is much to learn regarding the precise mechanisms at work (104). The concept of fate determination are discussed here because it will be critical to more accurately define the activation and differentiation steps in the life of CD4+ T cells in order to design the next generation of cellular and Ab mediated vaccines. While certain vaccine adjuvants have tendencies to drive particular CD4+ T-cell effector fates (78), many molecular and cellular determinants must still be elucidated.

4.2 CD46: DEFINITION AND FUNCTION

4.2.1 A complement and viral receptor

Complement plays multifaceted roles in rapid destruction of microbial invaders and in shaping innate and adaptive immune responses (3, 105-108). An important requirement of complement is protection of healthy host cells from opsonization and elimination. Several complement regulatory proteins, such as decay accelerating factor (DAF or CD55), protectin (CD59), and membrane cofactor protein (MCP or CD46), are displayed on cellular membranes and control how complement components distinguish between healthy cells and foreign or apoptotic cells. As such, most nucleated cells, including all immune cells in peripheral blood, express CD46 (44). The extracellular structure of CD46 contains four short consensus repeats (SCR) (Figure 1). CD46 serves as a cofactor for the cleavage of complement proteins C3b and C4b. While wild-type mice do not express CD46, transgenic mice expressing human CD46 have been generated and facilitated the study of CD46 function in vivo (109).

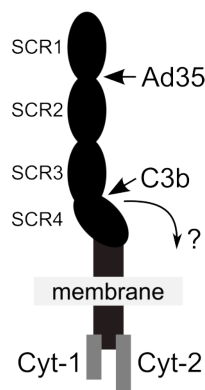

Figure 1. Schematic of the structure of CD46 and binding location of AdV-35 and C3b.

CD46 has been termed the *pathogen magnet* as at least seven human pathogens use CD46 as a primary attachment receptor, including AdV-35 (110). Upon engagement several CD46-using pathogens reduce CD46 expression: AdV-35 (72, 111), AdV-11p (112), Measles virus (MV) (113, 114), Human Herpes Virus-6 (HHV-6) (115), *Neisseria gonorrhoeae* (116), and *Streptococcus pyogenes* (117, 118). Downregulation of CD46 leads to increased sensitivity to complement mediated lysis, indicating that this regulatory protein plays an essential role in protecting healthy host cells from complement elimination (114, 119). In lymphoid cells, CD46 internalization does not occur constitutively but is induced when the receptor is engaged, whereas in myeloid cells downregulation of CD46 may be constitutive (120). CD46 contains a cytoplasmic Tyr-Arg-Tyr-Leu membrane trafficking motif that mediates internalization (121). In summary, receptor downregulation appears to be conserved amongst CD46-using pathogens. The capacity of surface CD46 to internalize upon ligation may also indicate intrinsic signaling potential.

4.2.2 A regulator of T cells

Emerging evidence is defining CD46 as an important regulator of T-cell function (122-

124). It was originally observed that engagement of CD46 by either MV or recombinant C3b protein leads to lower LPS-induced IL-12 production in monocytes (125). The authors offered this finding as one mechanism to explain MV-mediated immune-suppression. The data also implied that CD46 might be linked to downstream signaling pathways in immune cells. After the discovery that CD46 regulated IL-12 production, Wang et al. found that CD46 was able to transmit extracellular signals to the cytoplasm through its two cytoplasmic tails (cyt) (126). This finding provided much of the impetus for studying the function of CD46 on immune cells. Further supporting the role of CD46 signaling in regulating innate immune cells, it was shown that CD46 engagement blocked IL-12 in macrophages (127) and enhanced IFNγ-induced nitric oxide production in macrophages (128). Alternative slicing of CD46 generates four isoforms with each expressing one of two different cytoplasmic tails. The cyt-1 isoform is 16 amino acids (AA) and the cyt-2 isoform is 22 AA in length (Figure 1). The cyt-1 isoform of CD46 contains a putative tyrosine phosphorylation site for protein kinase C and casein kinase 2, whereas the cyt-2 isoform is tyrosine-phosphorylated by src kinases (in particular, Lck) in T cells (126). We have found using RT-PCR analysis that cyt-1 and cyt-2 are expressed at similar ratios in peripheral lymphocytes including, CD4+ T cells (W.C. Adams, unpublished data).

Numerous reports in the literature attribute both negative and positive regulatory properties of CD46 on TCR-dependent activation of CD4+ T cells. Initially, Marie et al. generated transgenic mice expressing human CD46 to study the role of CD46 in mediating T-cell activation in vivo (109). In this report, mice were generated that expressed one or both of the cyt isoforms. The effect of CD46 engagement on T-cell activation was dependent on cyt expression. Importantly, when both cyt tails were expressed, which more closely resembles expression in human CD4+ T cells, IL-2 but not IFNγ production was reduced. We have confirmed that CD46 engagement by either mAbs or rAdV-35 blocks IL-2 and not IFNγ in human CD4+ T cells (72). Nuclear translocation of NF-κB, a crucial factor for IL-2 gene transcription (129), was also inhibited by CD46 engagement (72). CD46 also induces expression of negative regulators of IL-2 transcription: inducible cAMP early repressor/cAMP response element modulator (ICER/CREM) (130, 131). However, it is unclear how this may interfere with early IL-2 production since ICER/CREM was expressed days after activation and correlated temporally with a switch from IL-2 production to IL-10 in type-1 regulatory T cells (Tr1) (discussed below). CD46 may also regulate T-cell proliferation as was shown initially by Marie and colleagues (109). Similarly, CD46 engagement causes abortive proliferation as a result of defective akt/surviving signaling pathways in CD4+ T cells (132). The effector functions of T-cell subset displaying γ/δ-TCRs seems to also be inhibited by engagement of CD46 (130).While these effects of CD46 seem to mainly downregulate T-cell function, this may not always be the case. For example, CD46 ligation has been shown to increase proliferation (133) and IL-2 and IFNγ production (134). These differential effects may be driven by cyt expression, as cyt-1 expression promotes T-cell activation, while cyt-2 causes inhibition of T-cell activation (135). The apparent discrepancies in these data are not well understood. However, as discussed by Meiffren et al., the discrepancies may be due to the strength of the provided CD3/CD28 signal (132). Different CD46 mAb clones may also induce markedly different signaling; whereas a SCR1 targeting mAb blocked proliferation, a SCR2 mAb had no effect (72).

CD46 engagement has been implicated in driving the induction of Tr1 cells. Stimulation of CD4+ T cells with anti-CD3 and CD46 mAbs with exogenous IL-2 causes the induction of IL-10 producing Tr1 cells, which can suppress bystander T-cell proliferation (136). These results were confirmed with more relevant CD46 binders C3b and *S. pyogenes* M protein (137). Numerous functions have been attributed to these CD46-induced Tr1 cells. First, the cells express Granzyme A and may kill autologous target T cells, monocytes, and DCs in a perforin dependent manner (138). Second, they can attenuate *mycobacterium*-specific memory T-cell responses, but surprisingly this suppression occurs independent of IL-10 (139). Third, while CD46-induced Tr1 cells reduce T-cell activation and induce IL-10, they still allow for the maturation of DCs (140). And fourth, these Tr1 cells support B-cell Ab responses in an IL-10 dependent manner without enhancing B-cell proliferation (141). Thus, this current evidence suggests that CD46-induced regulatory T-cell responses may serve to downmodulate T_h1 responses (142). Whether CD46-induced Tr1 cells are terminally differentiated effectors cells analogous to T_h1 or T_h2 cells has not been rigorously tested. In addition how these cells compare to canonical FoxP3+ induced-Tregs is also not known.

The restriction of CD46 expression to primates constrains investigations in vivo. Yet certain human diseases have been helpful as CD46 is associated with dysregulated immune responses in patients with autoimmune disease. CD4+ T cells from patients with multiple sclerosis (MS) have a diminished capacity to make IL-10 after CD46, but not CD28, stimulation (143). Similarly, patients with rheumatoid arthritis (RA) show a defective switch from IL-2 to IL-10 producing Tr1 cells induced by CD3 and CD46 engagement in the presence of exogenous IL-2 (130). These two studies suggest that MS and RA autoimmune diseases may provide suitable models for studying CD46 in vivo together with human CD46-transgenic mice.

While most of the CD46 discussion thus far has focused on CD4+ T cells, CD46 engagement also modulates CD8+ T cells in unique ways that merit discussion here. CD46 blocks CD3/CD28 induced IFNγ and also interferes with polarization of the immune synapse and recruitment of CD3 (144). A similar effect was seen for NK cells as CD46 ligation negatively impacted the recruitment of perforin and their ability to kill target cells (144). This report raised an important question regarding CD46 signaling: how does cis- versus trans- engagement of CD46 affect downstream signaling? These authors found that only soluble CD46 ligands caused these effects with the anti-CD3/CD28 mAbs immobilized onto a bead, which helps to explain the inefficient polarization. A follow-up report provides a potential mechanism. In this model, CD46 can compete for lipid rafts to alter the T-cell polarity towards the site of CD46 ligation (145). Still, more needs to be learned about the effects of CD46 on T-cell polarization, particularly with respect to CD4+ T cells and how improper polarization may affect T-cell activation by DCs (146). Whether CD46 may also control fate decisions should be elucidated.

An ever-expanding body of literature is more accurately defining the roles of CD46 in regulating innate and adaptive immune cell function. It is particularly interesting since these studies have collectively illustrated how (i) complement regulates the other facets

of the immune system and (ii) how pathogens may have hijacked CD46 signaling networks to regulate host immune responses. However, it is important to note that there remains significant lack of clarity about how complement factors versus pathogen binding may impact T-cell function. Cardone et al. have recently found that activated CD4+ T cells may be a significant source of C3b, which may indicate that these cells can regulate their own function via CD46 (130). Much of the work studying this receptor has been done using a variety of mAbs and how they replicate natural or foreign ligand binding is not entirely clear. Since mice do not express a CD46 homologue, an emphasis should be placed on performing these studies in humans and NHPs. The temporal aspects of CD46 engagement and subsequent signaling may also play a significant role in the type of effect caused. In summary, the currently available literature is helping to paint the complex picture of the role of CD46 in immune regulation.

5 ADENOVIRUS

AdVs have been extensively studied since the 1950s when they were isolated from human adenoids (or pharyngeal tonsils) (147). As mentioned, the human AdV genus contains at least 50 different types divided into six species (A through F) and cause numerous acute human diseases (148, 149). Species B may be divided into two species based on receptor usage (referred to as B1 and B2) (150). Classification into serotypes was traditionally accomplished by testing their sensitivity to neutralization by different human antisera, and division into species by their capacity to agglutinate erythrocytes from different species. AdVs are now mainly classified based on sequence data and phylogenetic analysis. AdV species may be generally correlated with clinical disease. In this thesis, we have performed a comparative analysis between AdV-5 (species C) and AdV-35 (species B). In this section the basic virological and clinical applications of AdVs will be discussed.

5.1 STRUCTURE

The AdV virion has an icosahedral non-enveloped capsid with fiber spikes protruding from each vertice that encapsulates a double stranded linear DNA genome (Figure 2A-B). The complete high resolution structure of the AdV virion, which has a mass of ~150 mega Daltons, a diameter of ~90 nm/~900 Ångströms, and contains ~$1x10^6$ AA, has recently been solved using cryo-electron microscopy and x-ray crystallography (151-153) and provides critical insights into the virology of AdV. The genome organization and capsid structure are relatively conserved amongst AdV species, but receptor usage, cellular and tissue tropism, and activation of immune cells differs. There is a strong relationship between the AdV capsid structure and its function in mediating steps in the virus life cycle (149). AdV particles are especially stable due to the capsid structure and absence of lipid envelope, as is exemplified by the particle's retention of infectivity after multiple freeze-thaw cycles. The stability of AdV particles also largely determines cell-cell spread, host-to-host transmission, and tissue tropism. The viral structural components can be categorized as major, minor, and genomic core associated proteins. These basic building blocks of the AdV capsid will now be discussed.

5.1.1 Major proteins

The complex icosahedral capsid contains three groups of major proteins: (i) two-hundred-forty trimeric hexons that form the 20 pseudo-equilateral triangular capsid facets, (ii) twelve pentameric penton-bases that form at each capsid vertice, and (iii) twelve trimeric fibers that are anchored in the penton base pentamers and protrude from the vertice (Figure 2A-B). There are four different hexon proteins that form in groups of nine (GON) on the planar face and in groups of six (GOS) surrounding the penton base. Hexon proteins contain hypervariable regions (HVR) exposed on the outer-face and may represent the primary neutralizing targets**.** This was shown experimentally when chimeric rAdV-5 vectors with HVRs from other serotypes evaded AdV-5 neutralizing Abs (154). The pentameric penton base contains a central pore in which the fiber is positioned non-covalently. In solving the high resolution structure, Reddy et

al. observed that the penton base was highly flexible and allowed for different pore sizes in order to accommodate different fibers (153). These data shed light on how the chimeric vectors used in this thesis remained stable. Finally, the penton base also contains RGD loop motifs that interact with cellular integrins to mediate AdV entry via endocytosis (155, 156). The fiber protein is a complex polypeptide that consists of three parts: (i) a tail, (ii) a shaft, and (iii) a knob. The shaft has three intertwined proteins and the length varies between AdV species. For example, the AdV-5 shaft is nearly twice the length of AdV-35. The trimeric knob polypeptide that is located at the C-terminus of the shaft mediates binding to the receptor, such as the binding between AdV-35 and CD46 (157). It is thought that most of the virus-cell interactions occur via the major capsid proteins.

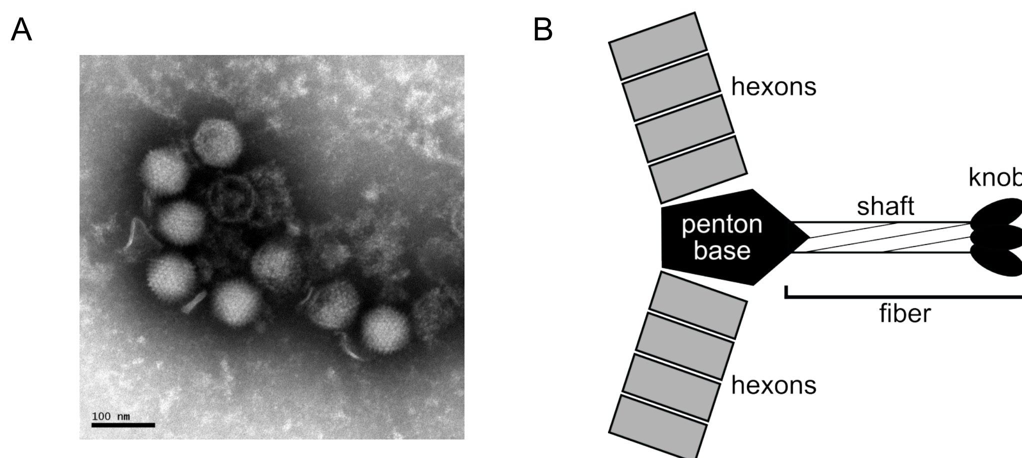

Figure 2. (A) Electron micrograph of rAdV-5 particles used in these studies (courtesy of Kjell-Olof Hedlund; Swedish Institute for Communicable Disease Control). **(B)** Schematic of major AdV capsid proteins and structure.

5.1.2 Minor and genomic core proteins

In addition to the three groups of major proteins, the capsid also contains a number of minor proteins: (i) IIIa, (ii) VI, (iii) VIII, and (iv) IX. These minor proteins have also been termed 'cement proteins' for their function in stabilizing the assembled major proteins (151-153). IIIa is located on the inner capsid surface and acts to support penton bases and GOSs. There are at least two-hundred copies of protein VI that may be located within the hexon trimers. At least one-hundred-twenty VIII proteins are located on the inner capsid surface and serve to support GONs, but also link GONs and GOSs together. Finally, a complex network of IX proteins laying within the space between hexons support the capsid and may help orchestrate the final virion assembly. Other functions relating to cell binding have been associated with these minor proteins. For example, a specific motif within protein VI mediates trafficking of the virus particle to the nucleus by supporting microtubule-dependent movement (158). Protein VI as also been implicated in the later life cycle of AdV by mediating lysis of the endosomal membranes (159). Finally, the genomic core contains a further five proteins associated with the DNA genome: (i) V, (ii) VII, (iii) μ, (iv) IVa2, and (v) a terminal protein. The final protein within the genomic core is termed the 23K virion protease and is not associated with the viral nucleic acid.

5.2 VIRAL LIFECYCLE

The AdV lifecycle typifies that of most viruses in that it may be separated into the following three phases: (i) virus entry (attachment, penetration, and uncoating), (ii) genome replication (transcription and translation), and (iii) virus release (virion assembly, maturation, and exit). However, it is important to note here that the details may differ depending both on cell type and AdV species. For this reason, one must be careful to extrapolate cell line data to DCs that are non-dividing and non-adherent. In the first step, the AdV knob binds with high affinity to a primary attachment receptor. Attachment to this receptor as well as interactions between the penton base and cellular integrins initiate penetration of the plasma membrane and subsequent clathrin-mediated endocytosis (155, 160). The ensuing fusion of clathrin-coated pits with endosomes enables AdV particles to uncoat and then escape the endosome concurrent with their acidification. As endosomes are generally considered extracellular, this entry into the cytoplasm marks entry into the cell. The virus particle containing its nucleic acid (linear dsDNA) then binds the microtubule associated molecular dynein motor, which facilitates retrograde transport along microtubules to the nuclear membrane (161). Formation of nuclear membrane pore complex facilitates entry of the nucleic acid into the nucleus where replication occurs (162). The AdV nucleic acid replicative cycle is generally divided into early and late phases based on replication of early and late genes, respectively. Synthesis of both early and late AdV mRNA transcripts is performed by the host cell RNA polymerase II, which is known to occur since α-amanitin enzyme blocks mRNA synthesis (163). The use of host cell machinery makes AdV genomic replication less prone to errors and subsequent mutation. Deletion of AdV early genes strongly attenuates replication, which indicates the important of these genes in the AdV life cycle. The late phase of transcription includes synthesis of the AdV structural proteins, which will be discussed at greater length in the next section. Mature virions incorporating the genome are then released from the plasma membrane through lysis. In regards to the kinetics of the AdV lifecycle, AdV entry occurs very rapidly (minutes to hours), while DNA replication begins hours later.

5.3 ATTACHMENT RECEPTORS

Receptor binding provides the initial mechanism of viral attachment to cells. AdVs use a variety of cellular attachment receptors that are determined both by cell type and AdV type (reviewed by (148)). Receptor usage may also depend on the host species – such as between human and mice. Therefore, in this thesis we will focus on the receptors expressed by human DCs that have been or may potentially be implicated in rAdV infection.

5.3.1 AdV-35 receptors

It is well established that species B AdV-35 uses the complement regulatory protein CD46 to attach to and infect multiple human cells (157, 164, 165). Due to the ubiquitous expression of CD46 on numerous cell types rAdV-35 may infect or at least bind to a wide range of cells. CD46 may also be a suitable receptor since it is endocytosed upon ligation, which would give the virus a means to gain entry into the cell (120). For these reasons in addition to the immuno-modulatory properties of CD46,

it is perhaps not surprising that several human pathogens have evolved to hijack this receptor for primary attachment (110). The trimeric fiber knob protein mediates high affinity and avidity binding of AdV-35 to a region within the extracellular SCR1 and 2 domains of CD46 (157, 165, 166). AdV-35 uses CD46 to infect primary pDCs and mDCs (44). In fact, all species B AdVs probably use CD46 except types 3 and 7 (167). Not only do species B AdVs bind CD46, but they may also dramatically affect CD46 conformation like has been shown for AdV-11 (168).

5.3.2 AdV-5 receptors

In contrast to the well defined case of AdV-35, receptor(s) used by the species C AdV-5 are less clear. This is particularly apparent with respect to DCs. The coxsackievirus-adenovirus receptor (CAR) is the described receptor for rAdV-5 on epithelial cells (169-172). Lack of expression of the tight junction protein CAR on the apical side of polarized epithelial cells may make it difficult for rAdV to access this receptor. For AdV to infect epithelial cells via CAR in vivo would ostensibly require the breakdown of the epithelial barrier. Although this scenario remains plausible, CAR-independent infection has been noted in epithelial cells (173), hepatocytes (174), fibroblasts (175, 176) and primary DCs (44, 177-179). rAdV-5 mutants with ablated CAR binding also retain their ability to infect murine DCs (177), which supports our reports that AdV-5 infects human blood DCs in the absence of CAR expression (44, 180). Johansson et al. isolated the iron binding protein lactoferrin (Lf) as the component from tear fluid that facilitates species C AdV infection of ocular epithelial cells in vitro (173). Lf also enhances rAdV-5 infection of primary human blood and skin DC subsets (180). High affinity interactions between AdV-5 hexon proteins with coagulation factor X (FX) also may enable efficient transduction of hepatocytes and mediate liver tropism (181, 182). FX and FIX also enhance AdV-5 binding to and transduction of epithelia cells in a heparin dependent manner (183). It is currently unclear to what extent these soluble factors mediate infection of human DCs in vivo. Receptor usage may be dependent on the route of inoculation, so it may not be surprising that AdV vectors bind coagulation factors with artificial intravenous administration. These studies also illustrate that cellular tropism may be determined by binding events that occur independent of the classical AdV knob-receptor interactions. For example, a Lys-Lys-Thr-Lys (KKTK) AA motif within the rAdV-5 fiber-shaft facilitates murine DC infection in a heparin dependent manner (177). This motif however does not facilitate AdV-5 infection of liver cells in vivo (184). Whether this receptor usage also exists in human DCs should be analyzed.

5.3.3 Other AdV receptors on DCs

The co-stimulatory receptors, CD80 and CD86, involved in the antigen presentation process have been suggested as receptors for AdV-3 (185, 186). These findings are relevant here since DCs display these markers whereas most other cells do not. As discussed earlier, surface CD80 and CD86 levels increase on DCs during phenotypic maturation. The usage of CD80 and CD86 by AdV-3 to infect DCs still needs to be confirmed experimentally. However recent evidence suggests that these are not the receptors, but rather that AdV-3, -7, -11, and -14 bind desmoglein-2 with high affinity to infect epithelial cells (187). It is unknown if this receptor is expressed on human DCs

and facilitates infection, but this report highlights how receptor usage may differ between cell types since AdV-11 also binds CD46 (168). A novel receptor for AdV-37 has also been identified as sialic acid binding residues in the AdV-37 knob mediate binding to GD1a glycans (188). In light of this finding and the observation that AdV-37 derived knob proteins enhance AdV-5 infection of myeloid APCs it will be interesting to determine whether this receptor is relevant for mediating infection of human DCs (189).

5.3.4 Secondary AdV receptors

A secondary interaction with cellular αv/β3 and αv/β5 integrins and Arg-Gly-Asp (RGD) motifs of the AdV penton bases facilitates membrane penetration and internalization of AdV particles (156). While RGD motifs are not required for cell attachment they seem to be essential for efficient entry (190). It has been suggested that αv/β5 integrins may even be sufficient to allow rAdV infection when CAR is not present (191), but this finding should be confirmed on human DCs. However, mutant rAdVs with ablated integrin binding retain their ability to infect murine DCs, which indicates that such interactions are not essential on DCs (177). It will be important to further elucidate the role of integrins in mediating rAdV infection of DCs, particularly since the expression may differ between DCs subsets and host species.

5.3.5 Genetic retargeting of AdV to DCs

Retargeting rAdV to use unnatural receptors to infect specific cell types has also been studied. This has been accomplished by genetic modification of the capsid structure or addition of soluble proteins. As an example, increased vector transduction of DCs has been tested by genetically modifying rAdV vectors to bind CD40 (192) or DC-SIGN (193, 194). Targeting DCs in this manner led to greater transduction efficiency of DCs by retargeted rAdV vectors compared to unmodified vectors. These reports are reminiscent of how Lf also enhanced infection through DC-SIGN (180). AdV particles modified to express the hexon-derived RGD motif also had enhanced infectivity of mouse DCs (195). These studies indirectly demonstrate that importance of receptor usage in determining cellular tropism of AdVs and may have clinical applications.

5.4 INNATE IMMUNE RECOGNITION OF ADENOVIRUS

5.4.1 Viral nucleic acid recognition

We have reported that rAdV-35 induces DC maturation comparable to LPS or TLR7/8-ligands (44, 72) and cytokines, such as IFN-I, in pDCs (44). These findings together indicate that DCs may sense and respond to AdV infection.

As such, how might innate immune cells recognize AdV? Numerous PRRs have been implicated in the recognition of viral or bacterial dsDNA (196). In the TLR family, the endosomally expressed TLR9 binds dsDNA and induces IFN-I. Longer endosomal retention time and complexing with interferon regulatory factor-7 (IRF-7) of TLR9 ligands in pDCs are proposed mechanisms for why these cells are particularly efficient at producing IFN-I (197, 198). The endosomal location of TLR9 may be one

mechanism that allows this receptor to distinguish between host and pathogen associated DNA (199). The rationale being that host DNA would not gain entry to the endosome, whereas viral derived DNA could. In human innate immune cells TLR9 expression is thought to be restricted to pDCs. To that end, the induction of IFN-I in pDCs by other DNA viruses, namely HSV-1 (200) and HSV-2 (201), is dependent on TLR9 signaling. CD46-using AdVs also induce IFN-I in human PBMCs through endosomal TLR9 (202, 203), which makes sense given that AdV enters endosomes during entry. Genomic content may also influence the capacity of AdVs to activate immune cells. For example, the greater frequency of immunosuppressive unmethylated CpG motifs in the AdV-5 genome has been shown to be one factor that makes this virus less stimulatory in human PBMCs (204). It would be interesting to follow-up on this analysis with other species of AdV.

Other PRRs may enable cells that do not express TLR9 to sense AdV. In fact, TLR-dependent and TLR-independent pathways mediate the recognition of pathogen associated DNA and initiation of immunity (196, 205). Data from the dsDNA HSV-1 has shown that IFN-I is induced by TLR-dependent and TLR-independent mechanisms in mice (200), which suggests that DCs may not be the only mediators of innate responses to dsDNA viruses. AdV induction of IFN-I in pDCs was dependent on TLR signaling, whereas in other cell types IFN-I was independent of TLRs (206). Splenic mDCs were shown to be the major source of IFN-I in vivo following AdV-3 or AdV-5 immunization (207). These authors also found that IFN-I induction occurred independent of TLR and cytosolic nucleic acid receptor recognition. While IFN-I required the IRF-7, IRF-3 was not required. Another group also observed that IFN-I was not dependent on TLR signaling, but required IRF-3 (208). Myeloid differentiation primary response gene (MyD88), a universal TLR signaling adaptor protein, was recently found to be partially involved in the induction of CD8+ T-cell responses by AdV immunization (209). Since in this study TLRs – including TLR3-4 which can signal independent of MyD88 – were not found to be required, the significance of this finding is not entirely clear. Whether, as the authors assert, this finding implies that multiple innate signaling pathways are thus induced by AdV remains to be shown. Another study has also confirmed that TLRs, as well as inflammasome activation, do not play a substantial role in driving CD8+ T-cell responses in AdV infection (210). However, transgene-specific CD8+ T-cell responses are mounted in the absence of intact IFN-I signaling, which suggests that IFN-Is do not play a critical role in mediating cellular responses (211).

Potential cytosolic DNA receptors have also been identified, which include DNA-dependent activator of IRF (DAI) and absent in melanoma 2 (AIM2) (212, 213). Sensing by DAI leads to the induction of IFN-I, whereas the inflammasome associated AIM2 leads mostly to IL-1 production. Other evidence also indicates that AdV DNA and membrane penetration can activate the inflammasome to induce pro-inflammatory cytokine (IL-1β) responses (214, 215). The role of these cytosolic dsDNA sensors remains controversial and more work will need to be done to confirm the reported findings. A central component of cytosolic recognition may be the family of high-mobility group box (HMGB1-3) proteins that bind a diverse array of pathogen associated nucleic acids, including dsDNA (216). HMGB-1 knockout mice have defective cytosolic receptor and TLR recognition of DNA. Mouse embryonic

fibroblasts from these mice failed to make IFN-I upon B-DNA, HSV-1, VSV, or CpG ODN stimulation, indicating that HMGB is essential for nucleic acid recognition. It is not currently known how HMGB binds nucleic acids and affects PRR signaling through cytosolic receptors. While PRRs sense viral infection directly, another possible scenario is that DCs respond to necrotic neighboring cells and the danger signals they release (i.e. DAMPs). Since some DNA viruses like AdVs are lytic viruses, it is plausible that these pathways may play a significant role in mediating immunity.

5.4.2 Alternative innate AdV recognition

In addition to viral genome, viral nucleic acid transcripts and capsid proteins may also induce innate immune responses. For example, virus associated RNA (VA-RNA) intermediates synthesized by RNA polymerase III may also contribute to systemic IFN-I production after AdV immunization (217, 218). Interestingly, the presence of VA-RNA correlated specifically with a second wave of IFN-I production after infection. Pathogen attachment to cells has been implicated in the recognition of AdVs. Binding of AdV penton base RGD motifs to β_3-integrins induced IL-1α independent of membrane penetration (219). In this way, mouse macrophages act as major initiators of innate immune response towards AdV vectors in vivo. Furthermore, interactions between AdV-37 RGD motifs and cellular integrins have been shown to facilitate the recruitment of leukocytes in keratitis by inducing chemokine production (220). These reports highlight how AdV interactions with receptors may facilitate infection and triggering of innate immune responses independent of cell infection (221).

A better understanding of innate immune recognition of AdVs in vivo is required. Unnatural tropism of human AdV in mice may add an additional layer of complexity in translating these findings to human. Recognition of AdV may also be dramatically different in different subsets of DCs depending on PRR repertoires. DC receptors and signaling pathways leading to phenotypic maturation seem to be substantially less well defined compared to those leading to IFN-I production.

5.5 GENERATION OF REPLICATION INCOMPETENT VECTORS

Replication incompetent rAdV vectors, deleted of earlier genes (e.g. E1, E3, and/or E4), can be efficiently generated in mammalian packaging cells lines (222). PER.C6 or 293-ORF6 provide deleted early genes *in trans* that produce rAdV-5 and rAdV-35 vectors with a capacity for foreign transgenes of up to 7.5 kb under control of a CMV promoter (223). Optimized promoter elements further enhance transgene expression. A thorough analysis of promoters of different origins found CMV promoters to be the most active in human DCs (224). However, these authors noted that promoter type strongly affected transgene expression and that promoter activity was dependent on cell type. Cell lines may thus 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 (e.g. green fluorescent protein or GFP) can be used to follow viral infection. These current methods result in the generation of high viral titer stocks with severely reduced viral replication.

5.6 IN USE AS VACCINE VECTORS

5.6.1 Background

rAdVs continue to steadily gain prominence as they are the most widely used vectors in gene therapy and vaccine trials (14, 17, 225). Their use as vaccine vectors was driven to a large degree by studies showing that rAdVs induce higher a magnitude of insert specific cellular and humoral responses (i.e. immunogenicity) in NHPs compared to other viral vectors (226, 227). A variety of 'prime-boost' regimens have been tested to further enhance immunogenicity. Such regimens are termed (i) homologous when multiple administrations are given with the same rAdV or (ii) heterologous when multiple administrations are given of different rAdV types, or rAdV with DNA plasmids. The advantage of rAdVs also lies in their extensive characterization and in the ability to produce high titers of replication incompetent virus encoding relatively large foreign gene inserts. AdV are also naturally adjuvanted unlike DNA plasmids or protein subunit vaccines and transduce many cell types, which leads to extensive transgene expression in vivo. In addition, rAdV vectors have been shown to be safe in both pre-clinical toxicology (228, 229) and clinical trials (230-233). Finally, AdV genes have been found to not integrate in the host genome and AdV particles are cleared after three months (229).

The use of rAdVs in NHP and human trials has yielded insights into the potential usefulness of these viruses as vaccine vectors. It is important to remark here that most of these studies have been carried out in the context of generating HIV immunity. Clearly, the correlates of HIV protection are deeply complicated by numerous viral (e.g. diversity and evasion strategies) and host (e.g. HLA haplotype and restriction factor expression) factors. While the primary goal of rAdV vaccines for HIV is to generate HIV-specific CTL responses, it is still largely unknown whether this is an effective correlate of viral protection or control. For these reasons, the discussion here will not focus on the controversial failures or successes of these studies, but rather on what these studies illustrate about inducing adaptive immune responses by rAdV vectors. The setbacks in using rAdVs for HIV should not necessarily act as a deterrent in using rAdV based vectors for HIV or other diseases. This is mainly because the failure of rAdVs in this setting may mostly reflect the general difficulty of eliciting responses that translate into protective immunity against HIV-1 (i.e. targeting conserved epitopes). Moreover, rAdV immunization safely induced insert-specific CD8+ T-cell responses in the majority of human vaccine recipients (231), which is an important minimum – though difficult to achieve – requirement of any potential vaccine. It was also recently shown that rAdV immunization can induce protective neutralizing anti-influenza hemagglutinin Abs when given as a boost to a DNA plasmid prime (234).

5.6.2 Lessons from iterative trials

Effective rAdV induced cellular immunity (CD8+ T-cell mediated) has been demonstrated in the literature. rAdV-5 vectors encoding immuno-dominant HIV proteins effectively controlled simian–human immunodeficiency virus (SHIV) infection in NHPs (235). Similarly, effective anti-Ebola virus immunity was also

generated with rAdV-5 vectors encoding immuno-dominant Ebola glycoprotein (236, 237). However, in subsequent pre-clinical trials using a more difficult to control simian immunodeficiency virus (SIV) challenge model, the anti-SIV immunity generated by rAdV immunization was substantially less effective (238). Other vectors, namely rAdV-26 and rAdV-35, have also been shown to provide partial and complete protection, respectively, against Ebola challenge (239). rAdV-26 priming of rAdV-5 immunization also seemed to enhance the induction of anti-SIV immunity (240). However, a general criticism of these SIV protection studies is that the challenge virus was not stringently heterologous to the virus genes used in the rAdV vaccine. Whether this is informative for rAdV immunization or SIV pathogenesis is not clear.

A recent phase III trial (Merck-STEP) demonstrated that rAdV-5 did not induce efficacious anti-HIV-immunity (241), although this study has offered insights into potential advantages and disadvantages of using rAdVs as vaccine vectors. It is poorly understood whether this lack of efficacy was due to insufficient quality of the immune response towards diverse circulating virus or other complex virus and host factors. One explanation may have to do with the central memory CD8+ T-cell responses induced by rAdV-5, since replicating CMV vector-induced effector memory CD8+ T cells have been shown to be powerfully protective against heterologous SIV challenge (242, 243). A surprising finding of the STEP trial was that the rAdV-5 vaccinated group appeared to have an increased acquisition rate compared to the unvaccinated group, although acquisition was likely confounded by factors such as circumcision, seropositive HSV-2 status, and AdV-specific Ab titers. There have been a few hypotheses put forth to explain these results. First, AdV vectors may have induced HIV-1 specific memory CD4+ T cells that were preferentially infected by HIV-1 (244, 245), although this is difficult to test directly in vivo. The induction of these T cells remains a potential issue with any HIV vaccine since the virus is CD4-tropic. In analyzing samples taken from human vaccinees, immunization with rAdV-5 vectors did not lead to anti-AdV-5 cellular immunity that correlated with pre-existing Ab titers (246, 247). This was confirmed using cells from a different clinical trial employing rAdV-5 vectors (248). These studies suggest that rAdV does not induce HIV-susceptible activated T cells. Another hypothesis is that complexes of AdV-5 with Abs facilitate the activation of AdV-5 specific memory T cells (249), with the implication that the vaccine increases HIV acquisition because there are more activated target cells present for HIV to infect. Alternatively, rAdV was shown to preferentially induce AdV-specific memory CD4+ T cells expressing CCR9 and $\alpha V/\beta 7$ integrins in T cells in vitro from AdV seropositive individuals compared to seronegative individuals (244). The memory T cells here were also more susceptible to HIV infection in vitro. The mucosal homing phenotype may also suggest that these AdV-specific T cells could then facilitate dissemination of HIV-1 in the gut. These analyses were however limited to T cells collected from blood, so the phenotype and antigen specificity of gut-resident lymphocytes, where much of the HIV pathogenesis occurs, may be different. Nonetheless, such studies are important to carry out so that the potential of viral vectors to cause increased acquisition of the target pathogen is not repeated. It will also be important to further investigate alternative AdV species, which are often even less well characterized compared to rAdV-5 in terms of their specific receptor usage and ability to transduce different cells.

Several limitations of rAdVs have been reported with the primary being a potential for high prevalence of pre-existing neutralizing Ab immunity, notably to AdV-5 (250). Since these Abs were found to attenuate immunogenicity of AdV-5 in mice, vectors derived from alternative AdV types with lower sero-prevalence or that were not attenuated by pre-existing AdV-5 immunity have been tested (251, 252). Of those, it was found that AdV-35 based vectors were the most efficient at circumventing pre-existing immunity (251, 253). However, the role of natural infection induced Abs – or even rAdV immunization induced Abs – in blunting AdV vector immunogenicity remains controversial because it is unclear how well the Ab titers in these mouse experiments correlate with circulating titers found in the population. Mice might be poor indicators of AdV vector immunogenicity in humans since mice are an unnatural host for human derived AdVs. As an example, vectors based on rAdV-35 have shown weaker immunogenicity than rAdV-5 vectors in NHPs (227, 254, 255). More recent data from mice also demonstrate that vectors based on rAdV-35 and rAdV-26 are measurably less immunogenic than rAdV-5 based vectors (256). These authors also showed that AdV-26 has a higher sero-prevalence than previously realized, which suggests that current data on AdV seroprevalence may need to be reevaluated. Data from ongoing rAdV-35 trials may indicate useful NHPs are for predicting immunogenicity in humans (225). A phase 1 trial using rAdV-35 to vaccinate against TB recently reported that a single administration of rAdV-35 induced detectable CD4+ and CD8+ TB-specific T-cell responses, a homologous rAdV-35 prime-boost regimen induced lower cellular responses and had no detectable boosting effect (230). While anti-AdV-35 Abs from the prime immunization may be attenuating the boost, it may also be plausible that AdV-35 vectors are immunosuppressive (72). There may be other methods to circumvent pre-existing immunity. For flu, aerosol delivery of AdV particles to the lungs of NHPs generated effective flu-specific immune responses in the presence of pre-existing anti-AdV immunity (257). Future studies should continue to study these limitations and develop means to overcome them.

6 MATERIALS AND METHODS

6.1 ISOLATION OF HUMAN PRIMARY CELLS

6.1.1 Isolation of primary human DCs

The experiments performed in this thesis and the accompanying papers were approved by the ethical review boards at Karolinska Institutet and US National Institutes of Health. We and others have developed methods to sort significant numbers of highly pure immature human DCs from blood (44-46, 54, 72, 180). pDCs and mDCs were sequentially separated based on differential expression of the BDCA markers, with mDCs having CD1c (BDCA-1) and pDCs having CD304 (BDCA-4) (34, 35). First, DCs and monocytes were enriched from total PBMC by either (i) aphaeresis of donor leukocytes and counterflow centrifugation elutriation to separate monocytes and lymphocytes based on cell size and sedimentation density (45), or (ii) by treatment with RosetteSep CD14+ enrichment kit (258). Such methods resulted in a fraction of cells highly enriched for monocytes and DCs. pDCs were then positively selected by staining with anti-CD304 monoclonal antibodies (mAb) directly conjugated to magnetic microbeads (Miltenyi). B cells expressing CD1c were depleted by staining with anti-CD19 mAbs microbeads (Miltenyi). mDCs were thereafter positively selected with mAbs against CD1c. Cell separation based on magnetic microbead conjugated mAb was performed on an AutoMacs instrument. Sequential magnetic sorting resulted in highly pure and phenotypically immature CD123 expressing CD304+ pDCs and CD11c expressing CD1c+ mDCs. These phenotypes were consistent with the established literature (37). pDCs and mDCs were then cultured in complete media (RPMI; 10% fetal calf serum; penicillin and streptomycin) supplemented with IL-3 and GM-CSF, respectively. Although pDCs may be isolated with anti-CD303 mAb, ligation of this receptor may ablate IFN-I production (259), phenotypic maturation, and optimal antigen presentation (260).

6.1.2 In vitro differentiation of DCs (MDDC)

The rarity of DCs in human blood and other tissues make the isolation of significant numbers of DCs challenging and thus present a significant roadblock in DC research. As such, primary monocytes may be differentiated into DCs in vitro (60). Highly pure monocytes (>90% CD14+) were obtained either by collection of plastic-adherent cells or by treating PBMCs with RosetteSep CD14+ enrichment kit (Stem Cell Technologies) (180, 258). Isolated monocytes were then subsequently cultured for 6 days in complete media supplemented with suitable concentrations of recombinant human IL-4 and GM-CSF.

6.1.3 Isolation of cutaneous DCs

For **paper II** we used methods developed by Bond et al. to isolate cutaneous DCs subsets from healthy skin tissue obtained after reconstructive plastic surgery (64). A skin graft mesher (Zimmer) was used to mechanically expand skin in a net-like fashion in order to increase the activity of dispase, an enzyme that separates the dermal and epidermal layers. The layers were then pulled apart with forceps and incubated with

collagenase, which enzymatically disrupts the collagen fibers and the integrity of the tissue. After filtering through 75 μM pores, single cell suspensions were enriched of dDCs and LCs from the dermis and epidermis, respectively. Alternatively, the separated layers were incubated with collagenase and GM-CSF, which induced the cells to migrate from the tissue into the media. While these suspensions typically consisted of a higher percentage of DCs, the DCs present normally displayed a more mature phenotype compared to DCs isolated with collagenase alone.

6.1.4 Isolation of total and naive human T cells

In **paper I**, CD4+ and CD8+ T cells were isolated by depletion of CD19+ B cells and CD56+ NK cells from donor elutriation fractions enriched for lymphocytes (44). Lymphocytes were stained with anti-CD19 and anti-CD56 mAb conjugated to magnetic microbeads and depleted on AutoMacs columns (Miltenyi). An extensive flow cytometry panel was used to exclude dead cells and CD14+ and CD19+ cells from analysis of antigen specific memory T-cell responses. In **papers III-IV** we developed an alternative method to isolate CD4+ and CD8+ T cells from PBMCs (72). Buffy coats were treated with RosetteSep CD4+ or CD8+ T-cell enrichment kits (StemCell Technologies) and separated on density gradient centrifugation (Ficoll Paque, GE). The resulting cell fractions were at least 90 % CD3+ and CD4+ or CD8+ as determined by flow cytometry. These fractions contained a mixed population of naive and memory T cells, based on differential expression of CD45RA. The main contaminating population was CD19+ and each T-cell sort contained either CD4+ or CD8+ subsets. To sort the naive subset, total CD4+ or CD8+ T cells were treated with anti-CD45RO mAb conjugated to magnetic microbeads. The labeled cells were sensitively depleted on AutoMacs columns to yield populations of naive T cells that were at least 90 % CD3+, CD4+ or CD8+, CD45RA+, and CCR7+. This phenotype fits the standard definition of naive T cells. These cells were also shown to make mostly IL-2 (not IFNγ) and have a high capacity to proliferate upon stimulation, which are functions consistent with naive CD4+ T cells. The purity of all isolations was determined by flow cytometry before proceeding with subsequent experiments.

6.2 ADENOVIRUS SUSCEPTIBILITY AND ACTIVATION OF DCs

In this thesis we used methods to monitor rAdV infection of human DCs in vitro. rAdV vectors with genomic backbones encoding GFP transgenes under control of a CMV promoter and polyadenylation signals were used in these assays. First, freshly isolated and immature DCs in complete media were exposed to rAdV-5-GFP or rAdV-35-GFP. After 24 h incubation GFP expression was measured by flow cytometry. Although GFP expression was highest at 24 h in susceptible cells, GFP was also readily detectable after 8 h. We also assessed GFP in rAdV exposed PBMCs in a similar manner. Co-staining for various surface markers allowed for the detection of GFP in different cell types with the PBMCs fraction. DC activation (or phenotypic maturation) was also measured after stimulation. Following exposure to rAdV-GFP or TLR-ligands, DCs were stained with mAbs against anti-CD80, CD83, CD86, CD40, or HLA-DR. Expression was then measured by flow cytometry.

6.3 EX VIVO ACTIVATION OF HUMAN T CELLS

In **papers I, III, and IV** several methods were used to stimulate primary human T cells. In **paper I**, we activated CD4+ and CD8+ T cells specific to the cytomegalovirus pp65 antigen and measured recall responses to antigen. The responses were measured by intracellular staining for three cytokines (IL-2, IFNγ, TNF), a chemokine (Mip-1β), and a marker of degranulation (CD107a). Expression was detected by flow cytometry. Analysis of these five functions represents the majority of CMV specific T cells in the blood specific to pp65. To stimulate the recall responses to pp65 antigen, T cells were treated by either of two different, but complementary, methods: (i) with overlapping pp65 peptide pools that were compatible with both MHC-I and MHC-II and could thus activate CD8+ and CD4+ T cells, or (ii) with autologous DCs that had been infected with rAdV encoding for pp65 for 24 h previously (Figure 4; page 33). Both type 5 and 35 rAdV encoding pp65 or empty, which acted as a control, were used. Both methods of activation were able to effectively activate memory T-cell responses in vitro. SEB was used as a control to show whether the intracellular straining worked correctly.

In **papers III and IV**, we used three additional methods to activate sorted human T cells: (i) allogeneic DCs (Figure 5; page 34), (ii) plate immobilized anti-CD3 and anti-CD28 mAb (Figure 6; page 35), or (iii) PMA and ionomycin. These conditions were shown to activate sorted total, memory, and naive subsets of both CD4+ and CD8+ T cells, where activation was assessed by measuring proliferation, cytokine (IL-2, IFNγ, TNF) and chemokine (Mip-1β) production, and nuclear translocation of NF-κB/degradation of IκBα. Since methods (i) and (ii) activate T cells in a TCR dependent manner, we alternatively stimulated T cells with PMA and ionomycin, which activated T cells downstream of the TCR. The allogeneic reaction exploits the inherent cross-reactivity of CD4+ T-cell expressed α/β-T-cell receptors (TCR) for peptide-bound MHC-IIs expressed on allogeneic DCs (261). Proliferation was measured by CFSE dilution or ^{3}H-thymidine incorporation. Flow cytometry was used to measure cytokine levels, while the activation of NF-κB pathways was measured by Western blot. In these experiments, the nuclear and cytoplasmic contents were fractionated after stimulation by treatment with nuclear and cytoplasmic extraction kit NE-PER (Pierce).

6.4 FLOW CYTOMETRY

In **papers I-IV** flow cytometry was used extensively use to assess multiple functions of primary human DCs and T cells including, surface phenotype, DC maturation, T-cell activation, and the intracellular accumulation normally secreted proteins. In all experiments using flow cytometry, cells were stained with mAb directly conjugated to fluorescent dyes (e.g. FITC, PE, PerCP, APC, Alexa647, etc.) in 5 ml polystyrene round bottom tubes (Becton Dickinson). To measure surface expression of proteins, cells were washed in PBS-, centrifuged, blotted, and stained with mAbs. After 20 min incubate at 4 °C, cells were washed again in PBS- and immediately run on the flow cytometer. In the instance that the cells could not be run immediately, cells were fixed with 0.5 % paraformaldehyde and stored at 4 °C. Flow cytometry was also used to detect intracellular GFP expression.

We also used flow cytometry to detect apoptotic, necrotic, or dead cells for two

particular reasons: (i) exclusion of these cells from the analysis, and (ii) measurement of viability. Dead cells were excluded from analysis by staining with either 7-AAD (eBioscience) or LIVE/DEAD fixable dead cell stains (Invitrogen), the latter of which was compatible with fixation and permeabilization procedures discussed below and described in (262). In **papers III and IV** we measured 7-AAD in combination with Annexin-V in order to measure T-cell viability (or survival) over a time-course of stimulation with different CD46 ligands. Co-staining with 7-AAD and Annexin-V enabled us to distinguish between necrotic/late apoptotic cells (7-AAD+/Annexin-V+), early apoptotic cells (7-AAD-/Annexin-V+), and live cells (7-AAD-/Annexin-V-). Prior to cell permeabilization when measuring intracellular cytokine we used green or violet LIVE/DEAD fixable dead cell dyes that bind amine groups. Due to the greater concentration of protein within cells compared to the plasma membrane, dead cells with porous plasma membranes display a greater fluorescence than live cells.

In **paper I** we used previously developed methods to simultaneously measure intracellular accumulation of three cytokines (IL-2, IFNγ, TNF), a chemokine (Mip-1β), and a marker of degranulation (CD107a) by flow cytometry (262-265). Protein accumulation was caused by treatment of cells during the stimulation time with two pharmacological compounds that inhibit protein secretory pathways: Brefeldin A and monensin. Briefly, following stimulation of T cells (with exogenous peptide or rAdV-exposed autologous DCs) T cells were washed with PBS and stained with mAb against surface antigen. The mAb specific for CD107a was present during the course of the stimulation (6 h). After the surface staining, cells were treated with the cytofix/cytoperm kit (Becton Dickinson) and subsequently stained with mAb for the intracellular proteins. mAb were incubated for 20 min at room temperature in all these experiments. Before collecting cells on the flow cytometer, cells were washed one final time with cytofix/cytoperm wash buffer and fixed with 1 % paraformaldehyde. In **papers III and IV**, we used similar methods to detect intracellular IL-2, TNF, IFNγ, and Mip-1β following anti-CD3 and anti-CD28 or PMA/ionomycin stimulation.

For **paper I**, we modified the above methods for intracellular cytokine staining in T cells in order to be able to measure IFNα production by pDCs (266). Following stimulation with TLR-ligands (CpG for TLR9, a TLR7/8-ligand) or rAdVs, pDCs were treated with the cytofix/cytoperm kit (Becton Dickinson) and subsequently stained with a mAb against multiple subtypes of IFNα (PBL/Interferon Source). This mAb was commercially available directly conjugated to FITC meaning that we could not use it in combination with GFP. However, we subsequently conjugated the mAb to Alexa647 allowing for the simultaneous detection of GFP and IFNα in pDCs (59). ELISA was used to confirm flow cytometry data for IFNα.

7 RESULTS AND DISCUSSION

At the onset of this thesis, there were both well and poorly understood aspects of how rAdV vectors interacted with primary human DCs and T cells. With the wide use of these vectors in clinical vaccine trials for a host of human pathogens (14, 17), we recognized a need to more thoroughly understand these interaction. Since that time, we and others have provided several unique insights into how rAdV vectors gain entry into DCs and how DCs may then respond to rAdVs. rAdV vectors may also affect T-cell activation in both DC-independent and dependent ways. In this section, we will summarize and discuss the results presented in **papers I-IV**.

7.1 ADENOVIRUS INFECTION AND RECEPTOR USAGE IN DCs

As discussed in the introduction, DCs are potent professional antigen presenting cells (pAPC) that possess a unique ability to prime adaptive immune responses (33, 35). After rAdV immunization, DCs are likely to detect rAdV vectors in the periphery and may therefore play a significant role in regulating immunity towards the vector itself and/or encoded transgenes. In **paper I**, we first addressed the susceptibility of primary immature mDCs and pDCs, freshly isolated from human blood, to two different rAdV types: species C AdV-5 and species B AdV-35. The DCs were exposed to rAdV vectors encoding a GFP transgene for 24 h at which time the frequency of GFP+ cells was measured by flow cytometry. It was found that rAdV-35 infected both mDCs and pDCs about ten times more efficiently than rAdV-5. Moreover, mDCs were consistently more susceptible than donor-matched pDCs. Infection of pDCs by rAdV-5 was nearly undetectable except at very high viral doses. These finding in mDCs and pDCs confirmed earlier reports in other DC types (255, 267). Following rAdV-5 immunization in mice mDCs were found to be more susceptible to infection than pDCs (210). Skin DCs were also found to be more efficiently infected by rAdV-35 than rAdV-5 (268). rAdVs pseudotyped with species B fibers to mediate CD46 binding infected PBMCs more efficiently as well (269). We found a similar pattern of susceptibility in **papers II and III** using MDDCs. Although no donor-matched experiments were performed, we found that mDCs were more susceptible to rAdV infection than MDDCs. Data in **paper II** and presented in (59) show that cutaneous DCs (LCs and dDCs) are also susceptible to both rAdV-5 and rAdV-35 infection. Others have also found that cutaneous DCs are susceptible to rAdV infection (178). Recently, it was shown that murine CD11c+ DCs become infected with rAdV-5 in vivo and are necessary to generate CD8+ T cell responses against rAdV encoded genes (210). This study demonstrates a significant role for rAdV-5 infected DCs in mediating immunogenicity, although it remains plausible that DC acquisition and presentation of AdV-encoded proteins from infected non-hematopoietic cells (e.g. fibroblasts) may also be necessary for generating immunity. Future studies should examine the in vivo contributions of direct-presentation and cross-presentation in generating CD4+ and CD8+ T-cell responses to rAdV-encoded antigens. In light of the reports in the literature on the topic of vaccine targeting to DCs, it is important to clarify here that our studies have not directly addressed whether efficient rAdV infection of DCs is beneficial or not for generating vaccine induced T-cell responses. However, susceptibility of DCs to AdV may still influence innate recognition and responses.

We continued in **paper I** by assessing receptor usage of these two rAdV vectors on human DCs. As described above, rAdV-35 uses the complement regulatory protein CD46 as its primary attachment receptor on a host of human cell types (157, 164, 165). The trimeric fiber knob protein mediates high affinity and avidity binding of rAdV-35 to a region within the extracellular SCR1 and 2 domains of (164, 166, 270-272). In contrast, the receptor usage of AdV-5 is less well defined. CAR is the described primary receptor for rAdV-5 on epithelial cells (169-172). However, AdV-5 may infect cells independent of CAR (44, 173, 175-179, 273). As such, we began by measuring surface CD46 and CAR expression on immune cells in human peripheral blood. We found that mDCs and pDCs, in addition to T cells, B cells, and monocytes, expressed high levels of surface CD46. In contrast, CAR expression was undetectable by flow cytometry. To test whether AdV-35 required CD46 binding to infect DCs, we treated DCs with rAdV-35 or rAdV-5 encoding GFP in the presence or absence of two mAbs: clone 13/42 bound within the SCR1 domain and clone M177 bound within the SCR2 domain (270, 274) (Figure 1). We found that both mAbs significantly blocked rAdV-35, but not rAdV-5, infection of mDCs and pDCs. In contrast, the one available anti-CAR mAb that has been described to block rAdV-5 binding (275) had no effect on either rAdV-5 or rAdV-35 infection. We subsequently performed these experiments in CAR expressing cells lines and found a similar effect (W.C. Adams, unpublished data). In **paper IV** we also showed that trimeric rAdV-35 knob proteins were efficient at blocking rAdV-35 infection of MDDCs, supporting the fact that the knob region of the capsid is required for infection. Taken together, these data indicate that rAdV-35 infection of mDCs and pDCs is dependent on knob-CD46 interactions whereas the receptor for rAdV-5 was less clear, but seemed to be independent of CAR binding. In **paper II**, rAdV-5 was found to infect CAR-expressing cutaneous DCs in a partially CAR-dependent manner. The expression of CAR on tissue resident DCs may not be surprising since gut resident DCs also express an extensive repertoire of other tight junction proteins (276). We then followed-up on the finding that a majority of rAdV-5 infection in human primary DCs occurred independent of CAR. A report showing that the iron-binding protein lactoferrin (Lf) facilitated epithelial cell infection by species C AdV (173) prompted us to test this receptor use in DCs. Lf is a highly relevant protein since Lf exists at mucosal tissues and in many body fluids (milk, tear fluid, saliva, nasal secretions and vaginal mucus, and serum), which makes it plausible that rAdV-5 contacts Lf in the periphery (277, 278). Lf concentrations increase during the acute phase of many infections. Lf also directly simulates DCs and modulates their migration, cell activation and induction of T-cell responses (279-281). In support of the findings reported by Johansson et al. (173), we reported in **paper II** that Lf strongly enhanced rAdV-5 infection of all tested blood and skin DC subsets (180). A related iron-binding protein, Transferrin (Tf), did not cause a similar effect. DC activation by Lf was also not the cause of increased infectivity in our experiments. While others have found that Lf inhibits infection of HIV-1 (282), Semliki Forest virus (SFV) (283), human Papillomavirus (284) and AdV-2 (285, 286), we found no evidence that Lf negatively affected rAdV-5 infection of human DCs (287). We did however observe that Lf blocked SFV infection of DCs (W.C. Adams, unpublished data). The receptor for human Lf remains unclear on DCs or other cell types (288, 289). In **paper II** we additionally compared Lf of human and bovine origin. While Lf from these species have similar AA sequences and tertiary protein structures (290), BLf has four sites for potential N-linked glycosylation, of which two or three are occupied by high-mannose-

type oligosaccharides and natural HLf has two N-acetyl-lactosaminic-type oligosaccharides (291-293). In comparing Lf from these species we observed that bovine Lf had a significantly greater effect than human Lf on rAdV-5 infection. The positive effect of bovine Lf on rAdV-5 infection was ablated on MDDCs in the presence of mannan or anti-DC-SIGN mAb or when the Lf was treated with the enzyme EndoH to remove N-linked glycans. From these data we suggested that bovine Lf might be a useful tool for increasing rAdV-5 transduction in vitro or in vivo. Also, it is plausible that there are numerous Lf species expressing different sugar moieties that may interact with other carbohydrate receptors to facilitate AdV infection of DCs.

7.2 PHENOTYPIC MATURATION OF DCS BY ADENOVIRUS

As discussed in the introduction to this thesis, DCs have the capacity to undergo phenotypic maturation upon recognition of foreign antigen or nucleic acid. Thus, in **papers I and III** we evaluated the ability of rAdV-5 and rAdV-35 to activate human mDCs and pDCs. For these experiments freshly isolated and phenotypically immature DCs were exposed to a range of doses of rAdV-5 or rAdV-35 for 24 hours. Flow cytometry was then used to measure surface expression of panel of markers of maturation: CD80, CD83, CD86, CD40, and HLA-DR. Even at lower virus doses (10 ip/cell) rAdV-35 was found to mature mDCs, pDCs, and MDDCs to a degree that was equal to positive controls like LPS, CpG, or a TLR7/8-ligand. While others have found AdV-5 can mature DCs (294-297), there may be substantial differences in the source of DCs and viral doses between studies. Receptor usage may be linked to the capacity of different rAdV types to induce maturation. Although the mechanisms of cellular entry may differ between AdV species and cell type (14), it is likely that viral nucleic acids could signal through endosomal or cytosolic expressed PRRs and thereby initiate DC maturation. One potential explanation for why or how certain AdV species induce maturation while others do not may be that species C and B AdVs have different kinetics of endosomal retention and escape to the cytosol following receptor mediated endocytosis (298). PRR recognition in these compartments may thus be affected by intracellular trafficking kinetics of different AdVs. In vivo, maturation of mDCs induced by rAdV vectors was dependent on IFN-I signaling (211), indicating that phenotypic maturation of DCs may be induced directly through infection or facilitated indirectly through cytokine production. What PRRs are responsible for rAdV mediated DC maturation remains largely unknown.

7.3 ADENOVIRUS INDUCTION OF IFNα

A second crucial function of DCs is their ability to produce cytokines that orchestrate innate and adaptive immune responses. As mentioned in the introduction, pDCs produce IFN-I in response to infection by DNA viruses (31). Thus, we hypothesized that rAdVs may induce IFN-I and that this induction may occur through TLR9 expressed in pDCs. To address our hypothesis, we exposed pDCs to rAdV-5 or rAdV-35 for 2 to 24 hours. IFNα was measured in the supernatants by ELISA or Brefeldin A was added to the cultures to allow for intracellular accumulation of the cytokine. These cells were then fixed and permeabilized and stained for intracellular IFNα. In both assays rAdV-35, but not rAdV-5, was a potent inducer of IFNα in pDCs. We measured production of this cytokine at levels nearly comparable to the positive controls, CpG C

and TLR7/8-ligand. IFNα was found at detectable but very low levels in mDC cultures treated with rAdVs, although it could not be ruled out that this IFNα was from the few contaminating pDCs. Concurrently with our study, other groups found that IFN-I induction in PBMCs was shown to be a feature unique to CD46-using AdVs (202, 203). IFNα/β production was dependent on endosomal TLR9 signaling, as it was blocked by inhibitors of endosomal acidification (e.g chloroquine). Yet, based on the current model whereby the AdV nucleic acids remains contained within the capsid structure while trafficking to the nuclear membrane, it is difficult to understand how pDCs could sense protected nucleic acids. One explanation may be that intracellular viral trafficking is stochastic. That is, some AdV particles disintegrate before reaching the nuclear membrane and expose their nucleic acids that can activate PRRs, while others complete their trafficking to the nucleus and undergo replication. As discussed in the introduction, there may be additional sources of systemic IFNα/β, especially for non-CD46-using AdVs. Finally, it will be interesting to further study how CD46 engagement may affect production of other cytokines. We are currently studying whether AdVs can modulate DC function because others have reported that CD46 engagement by MV (125) and CD46-using AdVs (299) blocks IL-12 production in myeloid lineage APCs. Our preliminary results with CD46-ligation substantiate these reports, however we have observed that AdV-35 infection enhances LPS-induced IL-12 in a synergistic manner (W.C. Adams, unpublished data).

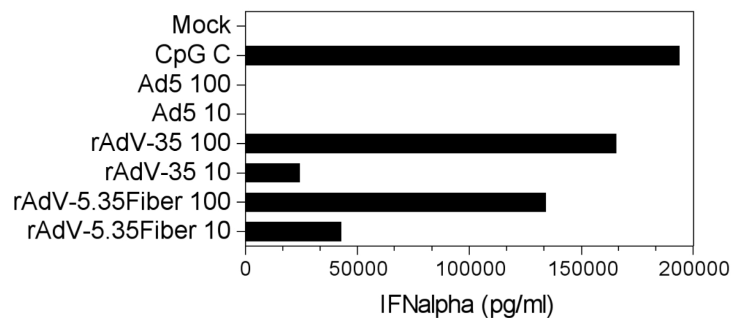

Figure 3. Requirement of CD46 to induce IFNα in pDCs. Freshly isolated pDCs were exposed CpG C, rAdV-35, rAdV-5.35Fiber, and rAdV-5 for 18 hours. IFNα was measured in the supernatants by ELISA.

Related to the induction of IFNα we had a lingering question: Would rAdV-5 induce IFNα in pDCs if it was modified to use CD46 for entry? The corollary of this question being, does IFNα induction require a chemical signature present specifically in the nucleic acid content of rAdV-35? To attempt to answer these questions we exposed freshly isolated pDCs to CpG C, rAdV-5, rAdV-35, or rAdV-5 with fibers from rAdV-35 (rAdV-5-35Fiber) encoding GFP and measured infection and IFNα in the supernatants by ELISA. First, we found that the 35-fiber enabled rAdV-5 to infect pDCs like rAdV-35, whereas the wild-type rAdV-5 was not able to infect pDCs as we have shown before (data not shown). Interestingly, addition of the 35-fiber enabled rAdV-5 to induce IFNα like rAdV-35, even though wild-type rAdV-5 could not (Figure 3). These data show that entry pathways associated with CD46 mediate IFNα production and that rAdV-5 probably does not induce IFNα because it does not infect pDCs, but not because its genome cannot replicate or activate PRRs in pDCs. Also,

CD46 engagement alone is not sufficient to induce detectable cytokine production (W.C. Adams, unpublished data).

7.4 DC PRESENTATION OF RECOMBINANT ADENOVIRUS-ENCODED ANTIGEN TO T CELLS

Due to inherent differences between rAdV-5 and rAdV-35 in their capacity to infect, mature, and induce cytokine production in human primary DCs, we next assessed whether these vectors might differentially affect T-cell activation. In **paper I** to address this question we setup an autologous DC-T-cell co-culture system to measure CMV recall responses. As explained in the material and methods section of this thesis and outlined in Figure 4, mDCs or pDCs were exposed to rAdV vectors encoding the immune-dominant pp65 gene of CMV. After 24 hours, the infected cells were washed and transferred at a 1:10 ratio to sorted autologous CD4+ and CD8+ T cells from donors with detectable CMV responses. The rAdV-infected DCs were compared to overlapping pp65 peptide mixes, which were used as the positive control. After 6 hours of co-culture T cells were analyzed for intracellular expression of three cytokines (IL-2, IFNγ, TNF), a chemokine (Mip-1β), and a marker of degranulation (CD107a). Expression of one or several of these 5 functions represents the majority of the antigen specific memory T-cell response against latent CMV infection in healthy donors.

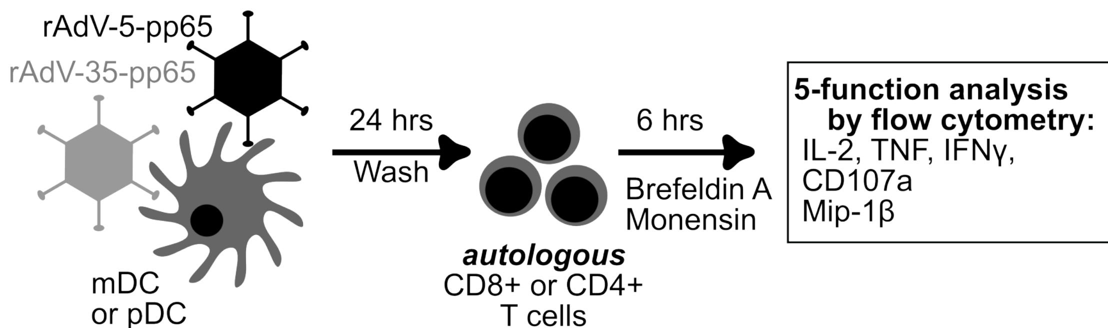

Figure 4. Schematic of autologous DC:T-cell co-culture.

We found that rAdV-pp65 exposed DCs activated a frequency of memory CD4+ and CD8+ T cells comparably to the overlapping peptide mix. rAdV-35 exposed DCs were more efficient than rAdV-5 and mDCs were more potent than pDCs. Taken together, these data suggest that rAdV-infected DCs retained a potent capacity to process and present antigen to activate T cells. While infected DCs likely displayed rAdV-derived peptide on MHC-I to activate CD8 T cells, the mechanisms for MHC-II presentation to activate CD4+ T cells were less clear. Since we did not detect significant cell death or expect secretion of endogenously produced protein (i.e. pp65), it is plausible that some form of cross-presentation may explain how CD4+ T cells became activated. As such it may be interesting in the future to compare the BDCA-3+ mDCs subset in this system.

7.5 REGULATION OF NAIVE T-CELL ACTIVATION BY ADENOVIRUS

While these studies were instructive in demonstrating how efficient rAdV-infected primary DCs were at activating memory T cells, we were presented with another

question: do rAdV vectors affect the activation of naive T cells? We sought to address this question in **paper III** because it was potentially more relevant for understanding the immunogenicity of these vectors in a vaccination setting. Initially, we set up allogeneic DC:T-cell co-cultures (Figure 5). DCs were exposed to rAdV-5 or rAdV-35 for 24 hours before being added at a 1:10 ratio to sorted naive CD4+ T cells. Another critical difference between these co-cultures and those used in **paper I** was that the DCs were not washed and thus free virus was available to affect T-cell activation.

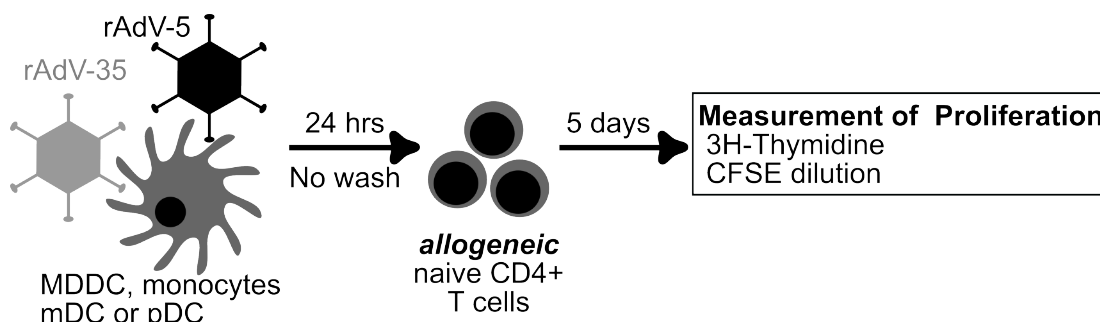

Figure 5. Schematic of allogeneic DC:T-cell co-culture.

We observed inhibition of naive CD4+ T-cell proliferation when rAdV-35, but not rAdV-5, was present. This occurred even when the frequency of infected DCs was controlled for and was not due to downregulation of MHC-II or measurable DC death. In fact, we had already shown in **paper I** that rAdV infected DCs maintained pAPC function. While a report had shown that rAdV-5 negatively affected T-cell proliferation (300), we did not observe this in our experiments. One explanation for the differences in these findings may be that these authors used higher doses of rAdV than we have in our studies. MV, another CD46 using human pathogen, also inhibited allogeneic-DC induced proliferation of naive CD4+ T cells (301). These findings are consistent with the immunosuppressive properties of MV (302, 303).

7.6 IMPACT OF CD46 ENGAGEMENT ON T-CELL ACTIVATION

Based on our observations of selective inhibition of T-cell proliferation by rAdV-35 and that supernatants from infected DCs were sufficient to inhibit proliferation, we hypothesized that rAdV-35 might be directly impacting naive CD4+ T-cell proliferation via binding its receptor CD46. We addressed this hypothesis primarily in **paper III**, and followed up on these studies in **paper IV** using alternative CD46 ligands. First though we sought to establish whether rAdV-35 could bind CD46 on T cells. To do this we assessed the ability of rAdV-35 and the CD46 mAbs (clones 13/42 and M177) to induce receptor downregulation, a feature of CD46 ligation. We found that rAdV-35 and CD46 mAbs caused receptor downregulation, which indirectly showed that binding with CD46 occurred. This finding has precedent in that other CD46-using pathogens cause a similar effect (111-118). As mentioned, a membrane trafficking motif within the CD46 cytoplasmic domain (121) facilitates induced CD46 downregulation in lymphoid cells (120).

The basis for our hypothesis that rAdV-35 may directly affect T-cell activation was discussed in the section on CD46 function. Briefly, we were intrigued by four reports

on CD46 in particular: (i) CD46 engagement had signaling capacity in monocytes that led to reduced IL-12 (125), (ii) CD46 was a regulator of naive CD4+ T-cell proliferation and cytokine production (109), (iii) CD46 drove CD4+ T cells towards an IL-10 producing regulatory phenotype (136), and (iv) CD46 engagement induced abortive proliferation of CD4+ T cells (132). In our studies we compared rAdV-35 to rAdV-5 and used two mAbs (previously discussed clones 13/42 and M177) to mimic rAdV-35 binding. We also used rAdV-35 mutant viruses with ablated CD46 binding. In these assays, sorted naive CD4+ T cells were stimulated with plate-immobilized anti-CD3 and anti-CD28 mAbs (Figure 6). As noted in **paper III**, we selected to include CD28 co-stimulation for two reasons. First, naive T cells required this signal to become activated. And second, we had observed that rAdV-35 infection led to upregulated expression CD80 and CD86 on DCs, which are the natural ligands of CD28. This is an important distinction between our work and others, where CD28 co-stimulation was not included.

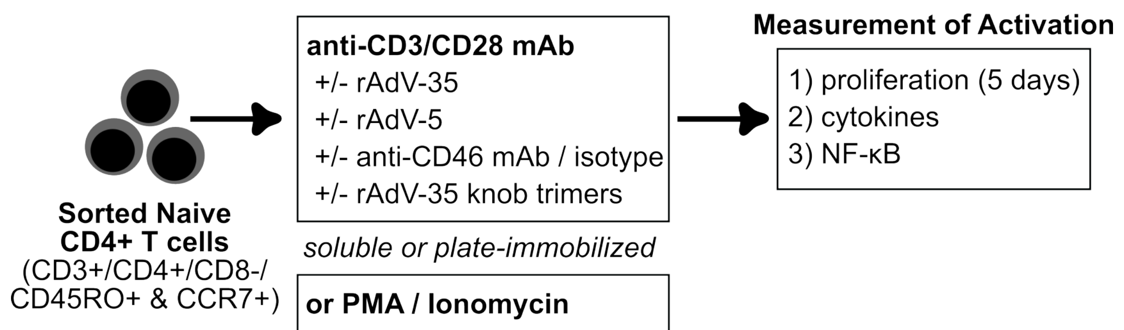

Figure 6. Schematic of anti-CD3/CD28 mAb stimulation of naive CD4+ T cells.

We found that CD46 ligation by either mAb (clones 13/42, but not M177) or whole rAdV-35 particles efficiently blocked proliferation of naive CD4+ T cells (72). As controls, rAdV-5 and mutant rAdV-35 vectors with ablated CD46 binding had no effect on T-cell proliferation. In **paper IV**, we followed up on these findings using a panel of recombinant trimeric rAdV-35 knob proteins that had increasing affinity for CD46 (165). We used a wild-type knob (35K) that bound CD46 with a K_D=14.64 nM, a higher affinity mutant (35K++), and a CD46-binding deficient mutant (35K279). The mutants were generated from an *E. coli* screening library. 35K++ contained two AA substitutions (Asp to Gly and Thr to Ala) at positions 207 and 245, respectively, and bound CD46 with 23.2-fold higher affinity (K_D=0.63 nM). 35K279 was constructed with a single Arg to Cys substitution at position 279 that completely ablates CD46 binding. The CD46 binding knob proteins induced downregulation of CD46 on T cells like rAdV-35 and anti-CD46 mAbs. In support of our previous findings (72), we found that CD46 engagement by 35K and 35K++ reduced proliferation in naive CD4+ T cells. However, plate immobilization of the knob proteins was required – potentially due to increased avidity for the receptor – since soluble proteins had no effect. The control 35K279 protein had no effect on proliferation, suggesting that non-specific steric hindrance caused by the presence of knob proteins was the cause of reduced activation.

We observed differential effects of rAdV-35 on naive CD4+ and CD8+ T-cell proliferation in **paper III**. In the case of CD8+ T cells, we observed no effect with

rAdV-35. The clone 13/42 anti-CD46 mAb blocked proliferation in both T-cell subsets. However, the effects of clone 13/42 on CD8+ T cells varied noticeably between donors. We hypothesized that different thresholds of activation may be one explanation. To this end, we performed additional experiments in which the strength of CD3/CD28 signaling was titrated (Figure 7). As CD3/CD28 signals increased, clone 13/42 still inhibited CD4+ T-cell proliferation whereas it had less of an effect on CD8+ T-cells.

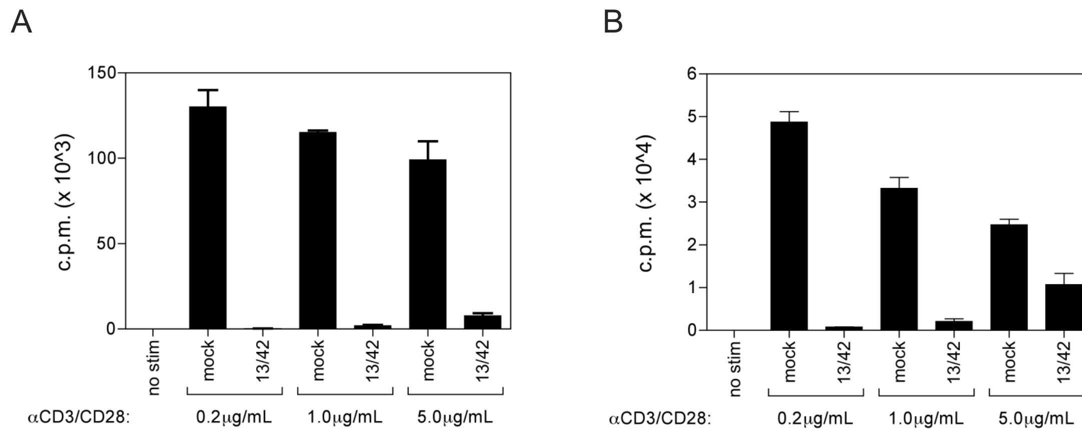

Figure 7. Effect of anti-CD46 mAb clone 13/42 on naive **(A)** CD4+ and **(B)** CD8+ T-cell proliferation induced by a range of anti-CD3/CD28 concentrations. Proliferation was measured on day 5 in triplicate by ^{3}H-thymidine incorporation (mean ± SD).

In **papers III and IV**, we next assessed the impact of CD46 ligation on early cytokine production. We analyzed three relevant cytokines (IL-2, TNF, IFNγ) and one chemokine (Mip-1β) made by CD4+ T cells upon CD3/CD28 activation. We chose to analyze IL-2 since it represents a major helper function of CD4+ T cells, is essential for T-cell growth (304), and has been shown to be modulated by CD46 (109). IFNγ production was also analyzed since it had been reported that CD46 ligation blocked IFNγ in CD8+ T cells (144). An important inflammatory cytokine, TNF, and a chemokine that recruits CD8+ T cells to DC:CD4+ T-cell conjugates in lymph nodes, Mip-1β (305) were also monitored. While the total CD4+ T-cell population made all four of these functions in response to CD3/CD28 stimulation, sorted naive CD4+ T cells made mainly IL-2, but only modest TNF and IFNγ, and undetectable levels of Mip-1β. rAdV-5 had no significant effect on cytokine production. Instead, CD46 ligation – by either mAb or rAdV-35 – led to strongly reduced IL-2 and TNF, but had a more modest or no effect on IFNγ and Mip-1β. In **paper IV**, we confirmed these findings by showing that CD46 engagement with either the 35K or 35K++ trimeric knob proteins significantly blocked IL-2 production in sorted total and naive CD4+ T cells. As expected, the 35K279 mutant knob protein with deficient CD46 binding had no effect. Since the IL-2 gene is a major target of NF-κB transcription factor activity (129), in **paper III** we assessed NF-κB activation in total CD4+ T cells. Nuclear translocation of the p65 subunit and cytosolic degradation of its regulatory component IκBα were measured by western blot. CD46 ligation led to deficient nuclear translocation of p65 and IκBα was not degraded, which together indicate deficient activation of this transcription factor pathway. This may provide a general mechanism to explain the reduced proliferation and IL-2 in these cells. In regards to CD46 driving IL-10 production by Tr1 cells, we have not addressed that possibility for rAdV-35 for

two reason. First, IL-10 is induced by CD3/CD46 stimulation in the absence of CD28 co-stimulation and the presence of exogenous IL-2. We argue that in viral infection CD28 signaling would likely be present and IL-2 is actually reduced in our hands. Second, we have found that IL-10 can be induced in CD4+ T cells with polyclonal CD3 and CD28 stimulation (W.C. Adams, unpublished data). This IL-10 production is likely a result of polyclonal activation rather than activation or differentiation of certain T-cell subsets, so it is unclear to us whether CD46 ligation really induces T-cell differentiation as has been proposed.

The potential implications of CD46 engagement by rAdV vectors are numerous. Firstly, CD46 downregulation has been shown to make cells more sensitive to autologous complement mediated lysis (119). Thus, T cells may be less protected from autologous complement killing after infection or vaccination with rAdV-35. This may negatively impact the activation of naive CD4+ T cells and thereby helper T-cell responses. Helper CD4+ T-cell responses are essential for optimal generation of cellular (CD8+ T cells) and humoral (B cells) memory (92, 94, 306-308). Proliferation and IL-2 production are both important functions of helper T cells, so it is plausible that blocking these functions would further curtail helper T-cell responses. We speculated in **paper III** that these apparent immune-suppressive effects of rAdV-35 may partially explain why these vectors are less immunogenic in non-human-primate preclinical trials (227, 254, 255). Homologous rAdV-35 prime-boost vaccination in humans also showed lower immunogenicity and no boosting compared a single immunization (230), although it was unclear from this study whether this result was due to AdV-35 Abs from the prime or some immunosuppressive effect of rAdV-35 vectors. The in vivo setting may be more complex than involving CD46 interactions alone. The extent to which this T-cell inhibition occurs in vivo is currently unknown, but our findings raise important questions about the spatio-temporal aspects governing CD46 modulation of T-cell activation. To this end, we have observed that no inhibition occurs with CD46 mAbs if they are added 15 minutes prior to CD3/CD28 activation (W.C. Adams, unpublished data). AdVs were able to still block activation in this setting, but these observations should be further investigated. It is also currently unknown how or where rAdV vectors may contact DCs or T cells in vivo after infection or vaccination. Interaction with T cells in the periphery or lymph nodes, which would require trafficking through lymphatic vessels, are plausible scenarios. Intravital AdV-tracking studies may be useful to elucidate such questions. It must also be remembered that after infection or vaccination CD46 signals would originate from both natural complement activation (e.g. C3b) as well as from the CD46-using pathogen itself. To my knowledge, no studies have analyzed how these signals would interact with or counteract each other. Understanding the effects of these dual signals shall help elucidate the in vivo roles of CD46.

Another further area of interest relating to the function of CD46 is the downstream signaling cascades. As discussed in the introduction, CD46 exists as four isoforms that express one of two cytoplasmic tails (cyt-1 and cyt-2) (126). Marie et al. first addressed these questions in transgenic mice expressing human CD46 and found profound differences between the signaling transmitted through these cytoplasmic tails (109). It has been suggested that cyt-1 expression promotes T-cell activation, but cyt-2 causes inhibition of T-cell activation (135). Our analysis of cyt-1 and cyt-2 mRNA revealed

that peripheral CD4+ T cells expressed an even ratio of these cytoplasmic tails (W.C. Adams, unpublished data). These findings raise tantalizing questions such as: (i) Is there competition between the cytoplasmic tails for kinases and adaptor molecules? (ii) Do the cytoplasmic tails compete with other co-stimulatory molecules and affect their function? (iii) Do the cytoplasmic tails play different roles at different time points of T-cell activation? (iv) Does CD46 engagement cause physical or steric interference with formation of the immune synapse? (v) And do different CD46 ligands transmit different signals? Using siRNA (143, 309) it may be possible to knockdown different cytoplasmic domains in primary T cells as a way to being answering questions (i-iii). Our observation that CD3/CD28/CD46 treatment was equivalent in strength to CD3 alone indirectly suggests that CD46 may be out-competing CD28 for signaling kinases or adaptor signaling molecules (W.C. Adams, unpublished data). Regarding question (iv), engagement of CD46 may misdirect formation of lipid rafts and microtubule organizing centers away from the immune synapse and toward the sites of CD46 (144, 145). Binding of the large AdV particle might also interfere sterically with DC:T-cell contacts, although we find this possibility unlikely since we have seen similar inhibitory effects with the smaller mAbs and trimeric knob proteins. In relation to question (v), we have already observed that while SCR1 targeting mAbs block proliferation and IL-2, SCR2 targeting mAbs only block IL-2. These differences may be due to inherent properties of the mAb, but may also suggest that binding affinity and avidity may influence CD46 signaling activity. It will also be interesting in the future to study whether the natural ligand of CD46, C3b, recapitulates the effects of AdV-35 we have reported in our studies or if it has completely different effects. Since C3b binds between the SCR3 and 4 domains, the effects may indeed be very different with the natural ligand. Induction of autophagy by CD46 occurred specifically through the cyt-1 domain (309). Since autophagy occurs constitutively in lymphoid cells, a possible induction of autophagy in T cells may also play a role in affecting T-cell function. Whether this occurs is unknown, but it would be important to analyze. In conclusion, further dissection of downstream CD46 signaling is required in order to more fully understand how CD46 imparts both negative and positive regulatory effects on T cells.

In summary, the findings on the interactions of AdV-35 and CD46 illustrate how different vaccine components may affect naive CD4+ T-cell activation. This activation is both indirectly and directly related to the differentiation of effector and memory T-cell fates, which will ultimately determine vaccine efficacy. More still needs to be learned about the ways that AdV-35 suppressed naive CD4+ T cells impact the quality of adaptive immune responses generated towards encoded antigens.

8 CONCLUSIONS

At the onset of this thesis we aimed to study how rAdVs interact with human immune cells in order to better understand how these vectors may be inducing immune responses during vaccination. We realized an opportunity to study AdV in the context of human primary immune cells, as much of the published work used cell lines, in vitro differentiated DCs, or mice. As outlined in the introduction, there are numerous well defined differences between in vitro differentiated DCs and primary DCs. Furthermore, murine DC subsets differ to some degree and immunization studies in these animals may not accurately predict vaccine efficacy in vivo for human diseases like HIV-1. Ultimately, mice and humans diverged at least 65 million years ago, have vastly different body sizes and basal heart rates, and have evolved in separate ecological niches (310). A specific example is that mice do not seem to express a homologue of CD46, the receptor for AdV-35. In studying innate immune cell susceptibility to and recognition of AdVs we focused our efforts on primary human pDCs and mDCs. Because antigen presentation is a major function of these pAPCs, we became interested in how rAdVs influence DC-mediated activation of naive and memory T cells. As was discussed at length throughout the thesis, DCs and T cells play major roles in the generation of both innate and adaptive immune responses, respectively, after viral infection or vaccination. The work presented in this thesis provides evidence that substantiate the following four general conclusions:

- rAdV-35 infects and matures human mDCs and pDCs more efficiently than rAdV-5 and also induces IFNα in pDCs.
- rAdV-35 infection of mDCs and pDCs is dependent on CD46, whereas CAR is not implicated in rAdV-5 infection of these cells. Rather, lactoferrin enhances rAdV-5 infection in several human DC subsets.
- rAdV-infected DCs can activate autologous transgene-specific polyfunctional memory T cells, indicating that infected DCs retain pAPC capacities.
- rAdV-35 attenuates activation of naive CD4+ T cells via engagement of its receptor CD46. Reduced IL-2 and proliferation caused by rAdV-35 can be mimicked by anti-CD46 mAbs and trimeric AdV-35 knob proteins.

9 FUTURE DIRECTIONS

Current vaccines provide potent individual and herd protection against human pathogens. In fact, the vaccine for AdV represents a prime example. Oral AdV vaccination induces effective immunity against subsequent natural exposure to AdV-4 and AdV-7 (311) and has been used extensively to significantly reduce acute respiratory disease-associated morbidity in US military recruits (312). This vaccine and the previously mentioned YFV vaccine induce potent and pathogen-specific adaptive immunity. Vaccines are effective prophylactic treatments on a herd basis also because they are cost-effective and minimally invasive. As such, vaccines continue to represent the optimal solution to confronting present and emerging human pathogens. The new era of vaccine development, where past empirical methods may not work for these pathogens, will require a vastly increased understanding of the human immune system. Continuing from this thesis, it may be of interest to further elucidate what role (if any) infection of DCs plays in the generation of cellular responses in vivo. For example, are naive T cells activated by direct or cross-presentation of rAdV derived antigen? We must also seek to understand the cellular and molecular determinants governing lymphocyte differentiation and fate (e.g. effector vs. memory T cells) after viral infection or vaccination. That is to ask: can vaccines be designed or tailored to induce immunological memory with the requisite specificity and potency to induce suitable correlates of protection? The specificity and potency relate to both effector cell function and neutralizing Ab responses. Viral vectors, despite significant setbacks, continue to represent a powerful and immunogenic pathogen-gene delivery vehicle. Whether viral vectors can induce the necessary immune specificity from encoded pathogen genes – after all, a DNA AdV is quite different from a RNA retrovirus – remains to be determined. As reported in thesis, receptor binding by a viral vector may dysregulate T-cell response. Generating specific neutralizing Abs and protective T-cell responses depends not simply on gene delivery, but also – and potentially more so – on the complex activation of complement and the innate immune responses that shape the formation of adaptive immunity. That is, the quality of the memory response is paramount to the quantity in determining vaccine efficacy (313). Both basic research and iterative vaccination trials will be essential to answering these questions and designing vaccines against pathogens for which there is currently none (314). As demonstrated in this thesis and by many others before and concurrently, AdVs will nevertheless continue to be vital tools in helping to reveal mechanisms of immune system regulation and an instrumental part in understanding human immunology.

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