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ADAPTER BASED STRATEGIES FOR ADENOVIRUS VECTOR RETARGETING TO T LYMPHOCYTES

by

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A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2013

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ADAPTER BASED STRATEGIES FOR ADENOVIRUS VECTOR RETARGETING TO T LYMPHOCYTES

MATTHEW S. BEATTY

MOLECULAR AND CELLULAR PATHOLOGY

ABSTRACT

Adenoviruses are the most commonly used gene therapy vector for cancer therapy clinical trials. While adenovirus has shown a great track record in a variety of cancer therapeutics it has not progressed as a vector system for the modification of T lymphocytes. One of the major roadblocks towards utilizing adenovirus for T cell therapy is the lack of coxsackie virus and adenovirus receptor on the cell surface of T cell lineages.

Exploitation of alternative receptors has allowed adenovirus vectors to be utilized in a variety of cell types that native adenovirus type 5 cannot infect. Thus, retargeting adenovirus to an alternative receptor expressed on T cells should allow for increased infectivity and full utilization of adenovirus as a gene therapy vector. There are a variety of retargeting strategies employed to retarget adenovirus to alternative cell types. Of the strategies available, we utilized the adapter-based strategy to retarget adenovirus to T cells. This strategy has a variety of advantages including its rapid development, lack of interference with adenovirus biology and assembly, and the ability to utilize secreted biological ligands.

Within, we describe how resistance to adenovirus infection observed in T cells can be overcome utilizing a bi-specific adapter, sCAR-mIL-2, which retargets adenovirus to the murine interleukin 2 receptor. This adapter shows excellent ability to promote adenovirus infection in a murine T cell line, an increase was also observed in isolated

primary murine T cells. These results show that alternative targeting strategies can bypass native biology and allow for the utilization of adenovirus in genetically modifying T cells for therapy.

ACKNOWLEDGMENTS

I would like to thank my mentor, Dr. David T. Curiel for his guidance and support. I would also like to thank my committee members, Drs. Joel Glasgow, Zdenek Hel, John Mountz, and Gene Siegal. I would also like to thank Dr. Alexander Pereboev. Dr. Pereboev was instrumental in the planning and early stages of developing the sCAR-mIL-2 and sCAR-hIL-2 adapters. Dr. Pereboev passed away before this work was completed, he will be greatly missed. Lastly, I would like to thank Drs. Hideyo Ugai, and Igor Dmitriev, as well as past and present members in Dr. Curiel's lab for their contributions, guidance, and friendship. I would also like to thank my wife, Kendal, my family, and my friends for their constant love and support.

TABLE OF CONTENTS

Page

ABSTRACT....iii

AKNOWLEDGMENTS....v

LIST OF TABLES....viii

LIST OF FIGURES....ix

CHAPTER I: INTRODUCTION....1

Adenovirus....1
Adenovirus Structure and Replication....2
Adenovirus Fiber....4
Coxsackie Virus and Adenovirus Receptor....5
T Lymphocytes....5
$CD4^+$ T Cells....5
$CD8^+$ T cells....7
Interleukin 2 Pathway....8
Interleukin 2....8
Interleukin 2 Receptor....9
IL-2 in Activated T Cells....10
IL-2 in Regulatory T Cells....11

CHAPTER II: ADENOVIRUS STRATEGIES FOR TISSUE SPECIFIC TARGETING....17

CHAPTER III: AUGMENTED ADENOVIRUS TRANSDUCTION OF MURINE T LYMPHOCYTES UTILIZING A BI-SPECIFIC PROTEIN TARGETING MURINE INTERLEUKIN 2 RECEPTOR....59

CHAPTER IV: ADDITIONAL STUDIES....89

Murine T Cell Targeting....89
SCAR-mIL-2 Enhancement of Ad5-mediated Gene Transfer to Murine T Cells....89
Receptor Analysis of Primary Murine T Lymphocytes....90

TABLE OF CONTENTS (CONTINUED)

Page

SCAR-mIL-2 Mediated Transduction of Isolated Splenocytes 90
Development of a Human T Cell Targeting Adapter....................91
SCAR-hIL-2 Mediated CTLL-2 Proliferation....................92

SUMMARY AND GENERAL DISCUSSION....................98

LIST OF REFERENCES....................104

APPENDIX:

A INSTITUTIONAL REVIEW BOARD FOR HUMAN USE DESIGNATION OF NOT HUMAN SUBJECTS RESEARCH....................115

B ANIMAL STUDIES COMMITTEE APPROVAL OF PROTOCOL FOR EXPERIMENTS URILIZING ANIMALS....................117

LIST OF TABLES

Table *Page*

CHAPTER I: INTRODUCTION

1 Classification of human adenovirus serotypes.. 1

LIST OF FIGURES

Figure *Page*

CHAPTER I: INTRODUCTION

1 Structure of adenovirus and the adenovirus proteins 13

2 The pathway of human adenovirus serotype 5 (HAdV-5) infection..........................14

3 Transcription map of the adenovirus genome..15

4 Interleukin 2 structure 16

CHAPTER II: ADENOVIRUS STRATEGIES FOR TISSUE SPECIFIC TARGETING

1 Adenovirus capsid structure...43

2 Transductional re-targeting modifications 44

CHAPTER III: AUGMENTED ADENOVIRUS TRANSDUCTION OF MURINE T LYMPHOCYTES UTILIZING A BI-SPECIFIC PROTEIN TARGETING MURINE INTERLEUKIN 2 RECEPTOR

1 Schema for adapter based retargeting strategy for adenovirus vectors......................79

2 Western blot analysis of sCAR-mIL-2 adapter protein 80

3 Analysis of sCAR-mIL-2 binding to Ad5 knob and murine IL-2R...........................81

4 CTLL-2 receptor analysis by flow cytometry...82

5 SCAR-mIL-2 promotes increased adenovirus transduction of
CTLL-2 cells...83

6 SCAR-mIL-2 promotes increased adenovirus transduction of naïve
and activated primary murine T lymphocytes 84

LIST OF FIGURES (CONTINUED)

Figure *Page*

CHAPTER IV: ADDITIONAL STUDIES

1 SCAR-mIL-2 conjugated AdUP-GFP provides increased transduction of CTLL-2 cells..94

2 Receptor analysis of primary murine T cells 95

3 SCAR-mIL-2 promotes increased adenovirus transduction of naïve and ConA treated splenocytes...96

4 Analysis of sCAR-hIL-2 binding to murine IL-2R...97

CHAPTER I

INTRODUCTION

Adenovirus

Among gene therapy based clinical trials, adenovirus is still the most widely used vector technology (1) (http://www.wiley.co.uk/genmed/clinical/). These human adenoviruses, of the genus Mastadenovirus, were first isolated from human adenoids in the 1950s (2). Currently, at least 57 serotypes (Table I) of human adenoviruses (HAdVs) have been identified and grouped into seven species (A-G) (3-5).

Table I. Classification of human adenovirus serotypes

Species	Serotypes	Sites of Infection	Receptor(s)
A	12, 18, 31	Gastrointestinal tract	CAR
B	3, 7, 11, 14, 16, 21, 34, 35, 50, 55	Lung, Urinary tract	CD46, CD80, CD86
C	1, 2, 5, 6, 57	Upper respiratory tract	CAR
D	8, 9, 10, 13, 15, 17, 19, 20, 22, 23, 24, 25, 26, 27, 28, 29, 30, 32, 33, 36, 37, 38, 39, 42, 43, 44, 45, 47, 48, 49, 51, 53, 54, 56	Eye, Gastrointestinal tract	CAR, Sialic Acid, CD46
E	4	Respiratory tract	CAR
F	40, 41	Gastrointestinal tract	CAR
G	52		

Adenovirus structure and replication

The adenovirus is composed of an icosahedral capsid that is about 920 angstroms in diameter (6). This non-enveloped virion is composed of multiple copies of both major and minor structural proteins. The three major proteins include hexon (II), penton base (III), and fiber (IV). The minor proteins encompass proteins IIIa, VI, VIII, and IX (6, 7). The adenovirus genome is composed of 30-40 kb of linear, double-strand DNA within the capsids core. This DNA is complexed with five additional proteins (V, VII, mu, Iva2, and terminal protein) that together makeup the genome core (8) (Figure 1).

The process of HAdV infection (Figure 2) is initiated through receptor-mediated interactions with the host cell. Most of the HAdV serotypes bind to the coxsackie virus and adenovirus receptor (CAR). Roelvink et al. showed that adenovirus from species A, C, D, E, and F all bound to CAR (9). Species B HAdVs and HAdV-37, however, have been reported to utilize CD46 (10-15) as a cellular attachment receptor. Additionally, adenoviruses HAdV-8, -19 and -37 of species D have been reported to utilize α(2-3)-linked sialic acid as a cellular receptor (16-19). Following initial binding to the primary receptor, HAdVs require a secondary interaction of the arg-gly-asp (RGD) motif on the viral penton base with αvβ3 and αvβ5 integrins (20). This binding promotes virus internalization through clathrin mediated endocytosis (21). Acidification of the endosome leads to a partial disassembly of the capsid proteins and a release of fiber, penton base and protein VI (22, 23), facilitating escape of viral particles into the cytoplasm. Once in the cytoplasm, viral particles interact with motor proteins, such as dynein, to travel along microtubules to the nucleus (24-27). Subsequently, viral capsid uncoating through

interactions with nuclear pore proteins and kenesin-1 allows for the viral genome to be imported into nucleus (28).

The Ad genome is comprised of two groups of genes that control replication. The early transcription unit (E1A, E1B, E2, E3 and E4) is transcribed during the early phase of viral replication, while the major late unit (L1 to L5) is transcribed during the late phase of viral replication (Figure 3). The E1A protein is responsible for a variety of early transcript regulation in addition to regulating host cell gene expression and proliferation. E1A, binding to the retinoblastoma (Rb) protein, releases E2F which then activates viral and cellular gene expression (29). Additionally, E1A also regulates cell division through interactions with both NF-κB and p53. E1B binds to p53, Bax, other cellular proteins to inhibits apoptosis (29). The E2 transcription unit transcribes viral proteins responsible for viral DNA replication. These include DNA polymerase (Ad Pol), preterminal protein (pTP) and DNA binding protein (DBP). Proteins in E3 function to interfere with host immune responses (30). Lastly, the E4 transcription unit is responsible for down regulation of cellular protein synthesis, inhibition of apoptosis, viral DNA replication, viral mRNA transport, and mRNA splicing (31).

The major late promoter (MLP) is responsible for the production of the late transcription unit. This group of genes includes the adenoviral structural proteins that comprise the capsid and aid in assembly. The major-late pre-mRNA sequences contain a tripartite leader sequence (TPL) at the 5’ end. This leader is responsible for efficient translation of these proteins (32). Following translation, the adenoviral capsid proteins are imported into the nucleus where viral assembly occurs.

Adenovirus fiber

Attachment of adenovirus to its host cell is achieved through binding of the adenoviral fiber protein to its cognate receptor. Harisse et al. first analyzed the adenovirus fiber sequence (33), as being comprised of three domains: tail, shaft, and knob. The tail domain is responsible for attachment of the fiber protein to the viral capsid through interactions with penton base protein. This region is also responsible for targeting of fiber to the nucleus during assembly.

The shaft domain is comprised of a number of repeated sequence motifs containing two β-strands. These repeats form a highly stable triple-beta spiral. A wide variation of shaft lengths is found among adenovirus serotypes (34). The shaft domain is also responsible for the flexibility seen in the fibers of some adenovirus serotypes. Located in the third repeat of the shaft is a four amino acid insertion. Serotypes from species A, C, and E possess this insertion, providing flexibility, while inflexible fibers from certain species D adenoviruses lack this insertion (35).

The knob domain is responsible for primary receptor binding. X-ray crystallography studies have shown that the knob domain is comprised of two anti-parallel β-sheets. Within these sheets are six loop structures: AB, CD, DG, GH, HI, and IJ (36, 37). Of these loops, loop AB was determined, by mutagenesis studies (38) and crystal structure (39), to be responsible for CAR binding. In addition to receptor binding, the knob domain also contains a trimerization motif that is crucial for tertiary fiber structure. The trimerization of the fiber occurs without additional proteins and is required for docking with penton base (40-43).

Coxsackie virus and adenovirus receptor

The coxsackie virus and adenovirus receptor (CAR) is utilized by a wide variety of HAdV serotypes from species A, C, D, E, and F (9). CAR was first identified as a receptor for HAdV serotypes 2 and 5 by Bergelson et al. (44) Although CAR has a predicted molecular weight based on amino acid sequence of approximately 38kDa, CAR has been shown to migrate on sodium dodecyl sulfate (SDS) polyacrylamide gels at a molecular weight of approximately 46 kDa (45). This is likely a product of post-translational glycosylation.

CAR is a transmembrane protein, comprised of a cytoplasmic domain of 107 amino acids and an extracellular domain of 216 amino acids. This extracellular domain includes two immunoglobulin-like domains, D1 and D2, of which HAdV binds to D1 through residues in the AB loop of the knob domain (38, 39). Due to the trimerized structure of HAdV fiber, multiple CAR molecules can bind to each fiber. When bound in such fashion this interaction is shown to have a high affinity (20).

Biologically, CAR is found in tight junctions of polarized epithelial cells (46, 47). CAR distribution in humans has been shown to be highest in the brain, heart, pancreas, prostate, small intestine, and testis, with lower amounts in the liver and lung (48). CAR is also found in normal tissue of mice. However, the distribution varies with the highest amounts found in the heart, kidney, liver, and lung, and lower amounts in the brain (48).

T Lymphocytes

CD4^{+} T cells

T lymphocytes of the $CD4^+$ lineage play an important role in immune protection. This T cell lineage coordinates an immune response through aiding B cells in antibody production, inducing macrophages, recruiting neutrophils, eosinophils, and basophils, and modulating immune responses through the production of cytokines and chemokines. The work of classifying $CD4^+$ T cells began when Mosmann et al. subdivided $CD4^+$ T cells into those that made interferon-gamma (IFN-γ) and those that produced interleukin (IL)-4 (49).

$CD4^+$ T cells were originally classified into Th1 and Th2 cells, with further classifications developed later. Th1 cells play a critical role in mediating immune responses against intracellular pathogens (50, 51) and are responsible for induction of some autoimmune diseases. Of the cytokines produced, IFN-γ, lymphotoxin α (LTα), and IL-2 are crucial to their role. IFN-γ plays a role in promoting macrophage activation through binding to IFN-γ receptors on the cell surface of macrohphages (52). IL-2 is important for both the development of $CD4^+$ memory T cells (53) and the stimulation of $CD8^+$ T cells (54). Additionally, Th1 cells are also distinguished by their expression of IL-12 receptor. IL-12 receptor is upregulated by T Cell Receptor (TCR) activation and IFN-γ (55, 56).

While Th1 cells are responsible for intracellular pathogens, Th2 cells mediate immune responses against extracellular parasites. These cells have also been found to be important in the induction of asthma and other allergy based diseases (50, 51). Th2 cells express a wide variety of cytokines including IL-4, IL-5, IL-10, IL-13, and IL-25. IL-4 acts as a positive feedback cytokine and is responsible for Th2 differentiation (57). IL-4 also aids the IgE class switching in B cells.

Th17 T cells. A relatively new lineage of Th cells, labeled Th17, are responsible for immune responses against extracellular bacteria and fungi (58) and have been linked to many autoimmune diseases. This T cell lineage differs from the typical Th1/Th2 lineages in that it does not express the "classical" helper T cell cytokines and can be inhibited by cytokines such as IL-4 and IFN-γ. These cells are induced in the presence of IL-6 and TGF-β. Identifying characteristics of this lineage include expression of IL-21, IL-17, IL-22, and other cytokines. In addition, the gene RORγt has been identified as a master regulator of Th17 cells (59, 60).

Regulatory T cells. Regulatory T cells play an important role in regards to maintaining self-tolerance and regulating immune response (61) through a variety of mechanisms. This ability to balance the immune response makes Treg cells an interesting target for therapeutic manipulation. When Treg cells are increased their suppression of immune response may be beneficial in treating autoimmune diseases. On the other hand, depletion of Treg cells may promote increased immunity against cancer and chronic infectious diseases. These cells have been found to express high amounts of CD25, a subunit of the IL-2 receptor (61), and are reported to be transcriptionally regulated by Foxp3 (62, 63).

$CD8^+$ T cells

$CD8^+$ T cells are responsible for providing protection against intracellular pathogens that utilize the host-cell cytoplasm in order to evade inactivation by host antibodies. This T cell subset is responsible for cell-mediated lysis of target cells and was

first elucidated from tumor transplant models and allogeneic lymphocyte cultures (64-66). Post infection, naïve $CD8^+$ T cells are primed by antigen-presenting cells (APCs), such as macrophages and dendritic cells, in secondary lymphoid regions (67, 68). Following activation, $CD8^+$ T cells go through an expansion of up to 500,000 fold (69). To achieve this expansion $CD8^+$ T cells utilize a variety of signals, including TCR, costimulatory, and inflammatory cytokines (70, 71). Upon activation and expansion $CD8^+$ T cells are able to migrate to peripheral tissues through up regulation of CXCR3. Expression of CXCL3 allows $CD8^+$ T cells to respond to inflammatory signals from CXCL9 and CXCL10.

Interleukin 2 Pathway

The effects of interleukin 2 were first discovered when Morgan et al. (72) and Gilles et al. (73) described the proliferative effect of culture from activated T cells on antigen-activated T cells. Following studies showed that this effect was in fact due to a single molecule and was assigned the nomenclature interleukin 2 (74).

Interleukin 2

IL-2 is located on chromosome 4 in humans and chromosome 3 in mice. Similar to other cytokines, IL-2 is transcriptionally controlled by multiple factors. Upon interaction of the T cell antigen receptor with MHC/antigen complex on the surface of antigen presenting cells, a multitude of signals are triggered that lead to transcription of IL-2 and other cytokines (75). The T cell antigen receptor triggers transcription factors NFAT (76, 77), NF-κB (78), and AP-1 (79) through a phospholipase C (PLC)γ-

dependent pathway. For optimal expression, signals from other co-stimulatory receptors such as CD-28 are needed (80). IL-2 is also controlled at the mRNA level. Within the IL-2 message are several AU-rich elements that target the transcript for degradation. This is characteristic of a variety of cytokines such as IL-6, GM-CSF, and IL-3 (81).

IL-2 is structurally similar to other type 1 cytokines in that it is composed of four α-helical bundles in an up-up-down-down orientation (Figure 4). IL-2 contains an essential disulphide bond which provides structural stability. In addition to its translated structure, IL-2 also shows signs of O-linked glycosylation. The functional properties of this glycosylation are not known but it is not required for biological activity (82).

Interleukin 2 receptor

The IL-2 receptor is made up of three subunits: IL-2 Rα (CD25), IL-2 Rβ (CD122) and the common gamma chain (CD132). The gamma chain (γc) subunit is a commonly expressed subunit. It is found in virtually all hematopoietic cell types and is used by the IL-4, IL-7, IL-9, IL-15, and IL-21 receptors (83, 84). CD122 is expressed on only a few immune cell types including NK cells, NKT cells, memory $CD8^+$ cells, and $Foxp3^+$ T regulatory cells. Its expression, however, is induced on antigen activated T cells. While IL-2 has been shown to bind to these two subunits, it is unlikely that this binding is biologically relevant as mouse models that only express these two subunits have the same phenotype as IL-2 knockout models (85, 86). While most cells do not express CD25, activation of naïve T cells causes a transient expression of CD25 in a two-step process. First, TCR and costimulatory signals cause CD25 expression to be induced

(87). Following this, IL-2 then promotes increased levels of expression through a positive feedback loop.

All 3 subunits are required for the high-affinity IL-2 receptor. First IL-2 binds to CD25. This interaction then causes IL-2 and CD25 to associate with CD122 and γc. Once this quaternary complex forms, IL-2 induces signaling through the cytoplasmic tails of CD122 and γc. These signals lead to an activation of mitogen-activated protein kinase (MAPK), phosphotiylinositol 3-kinase (PI3K), and Stat 5 and 3 pathways. MAPK and PI3K pathways promote cell growth and survival (83, 88) while Stat pathway activation regulates genes important in T cell growth and function. Once IL-2 binds to the high affinity receptor it is rapidly internalized, having a half-life of ten to twenty minutes. While IL-2, CD122, and γc are targeted for lysosomal degradation, CD25 is recycled back to the cell surface (89-92).

IL-2 in activated T cells

In vitro studies have shown that IL-2 signaling enhances the initial response of T cells, the number of memory T cells that develop, and the ability to recall proliferation (93-95). On the other hand, studies in which IL-2 receptor pathway deficient mice are challenged have shown that an effective immune response can be mounted without IL-2 signaling in vivo (96-99). However, some model systems have shown that specific immune responses are lower compared to wild type mice. This may indicate that IL-2 does contribute to immune response but works in concert with other inflammatory cytokines such as IL-12 and IL-4 (100, 101). Recent studies have also shown that IL-2 may play a more important role in memory responses. IL-2 deficient $CD4^+$ T cells will

readily respond to antigen-pulsed dendritic cells but have low survivability and lower numbers of them transition to a memory phenotype (102, 103).

IL-2 in regulatory T cells

While most T cells can secrete their own IL-2, regulatory T cells lack the ability. In this aspect Foxp3 and other regulatory proteins bind the IL-2 promoter and contribute to the transcriptional repression of IL-2 (104, 105). At the same time, Foxp3 is also responsible for regulating expression of regulatory T cell markers such as CD25, cytotoxic T lymphocyte-associated antigen (CTLA-4), and glucocorticoid-induced tumor necrosis factor receptor (GITR) (104, 105). Paracrine IL-2 is important for these developments in that IL-2 signaling increases Foxp3 expression (106).

Natural regulatory T cells develop in the thymus and require their own development steps before migrating to peripheral immune tissues (107). During this process paracrine IL-2 is needed for developing Treg cells. IL-2 and IL-2 R deficient mouse models have shown two-fold decreases in the number of Foxp3 thymocytes (106, 108, 109). In addition, anti-IL-2 treatment has shown to reduce the number of thymic Treg cells in wild type mice (110). IL-2 is also important in the development of induced Treg cells. Naïve T cells can become induced Treg cells upon activation of their TCR when in the presence of TGF-β and IL-2 (111, 112). In this setting IL-2 plays a two-part role; IL-2 induces Foxp3 and promotes T cell growth (113).

IL-2 helps to maintain Treg cells that have migrated to the peripheral immune compartments. IL-2 and IL-2 R deficient mouse models show up to ten-fold less Treg cells when compared with total $CD4^+$ T cells (106, 114). In addition, Tregs from IL-2

deficient mice transferred into IL-2 expressing mice can suppress spontaneous EAE (115). Lastly, IL-2 R deficient Treg cells have reduced proliferation (106, 116). These experiments show that IL-2 signaling provides needed signals for maintaining the number of peripheral Tregs.

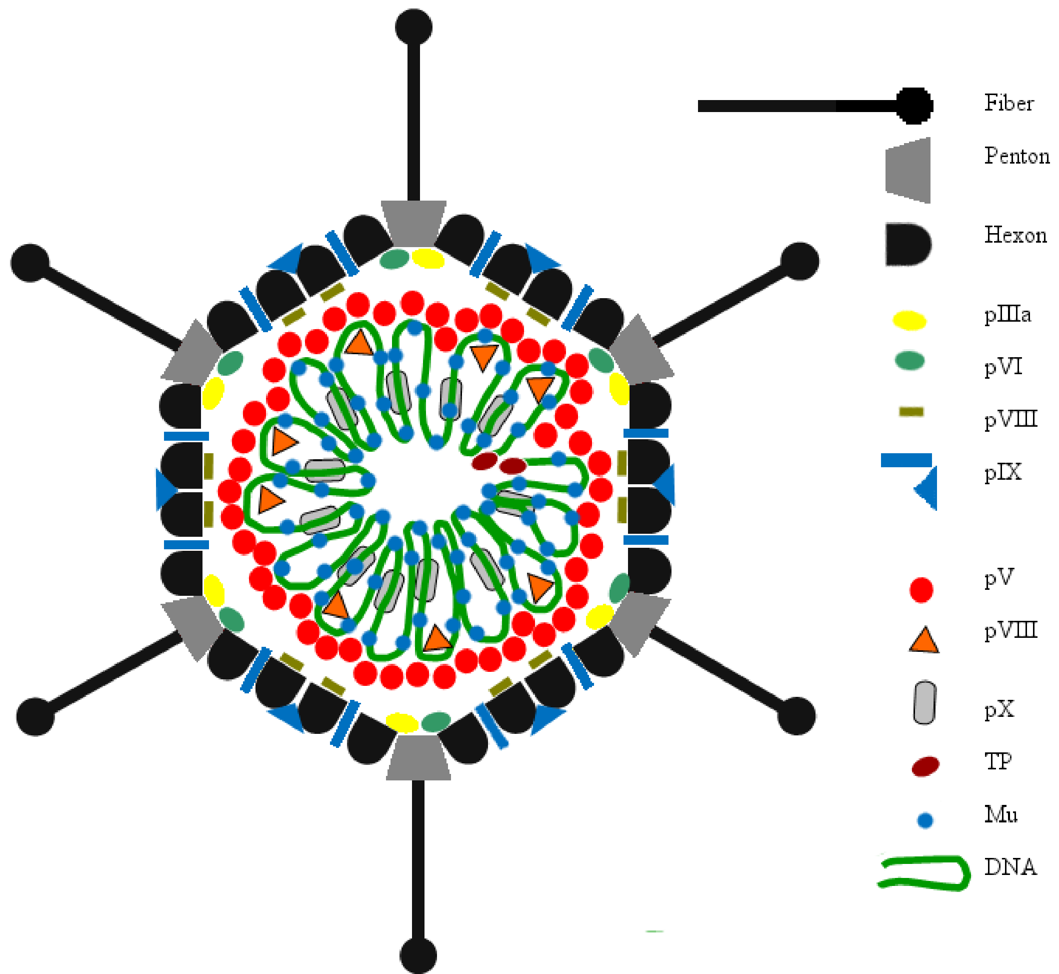

Figure 1: Structure of the adenovirus and the adenovirus proteins. Adenovirus is nonenveloped icosohedral capsid containing a linear, double-stranded, DNA genome. The virion is comprised of multiple copies of major proteins, hexon (II), penton base (III), and fiber (IV), and minor proteins, VI, VIII, IX, and IIIa. The adenovirus genome in the interior capsid core is associated with terminal protein (TP), V, VII, and mu.

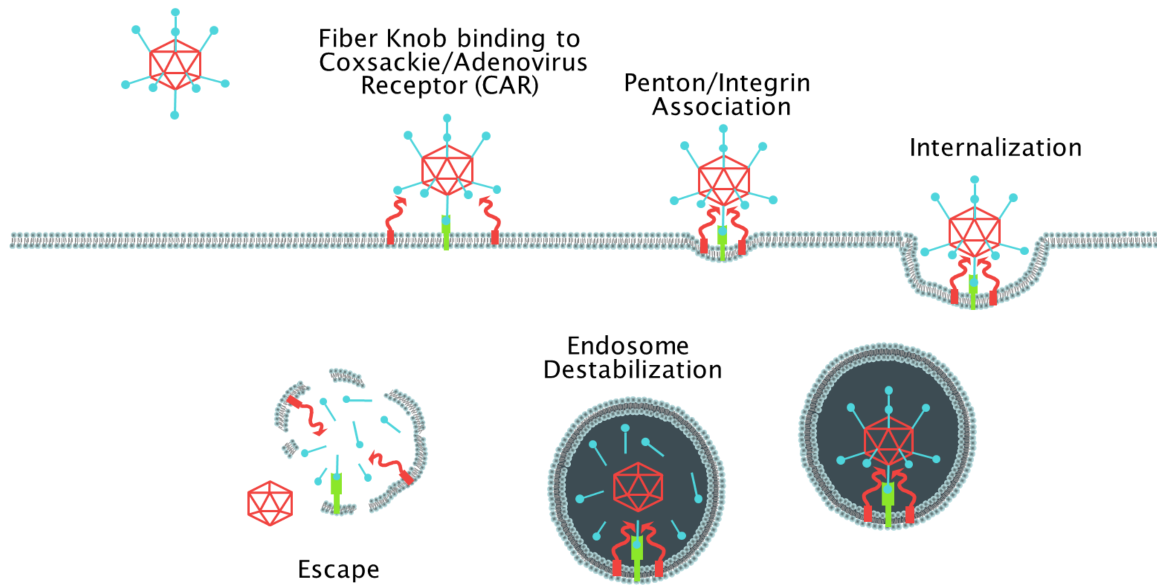

Figure 2: The pathway of human adenovirus serotype 5 (HAdV-5) infection. Adenovirus infection is initiated through binding of the fiber knob domain to cellular CAR. Adenovirus then interacts with cellular integrins through an Arg-Gly-Asp domain within penton base. This subsequent interaction triggers internalization through clathrin-mediated endocytosis. Following endocytosis, the viral capsid disassembles during endosome acidification, leading to viral escape. Upon escape, adenovirus is transported to the nucleus where the viral genome is released and imported into the nucleus.

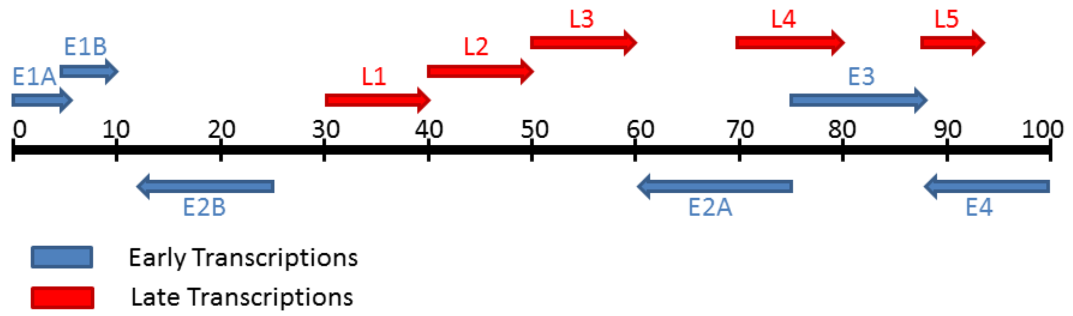

Figure 3: Transcription map of the adenovirus genome. Early transcription unites (E1-E4) are labeled in Blue. The Late transcription units (L1-L5) are labeled in red.

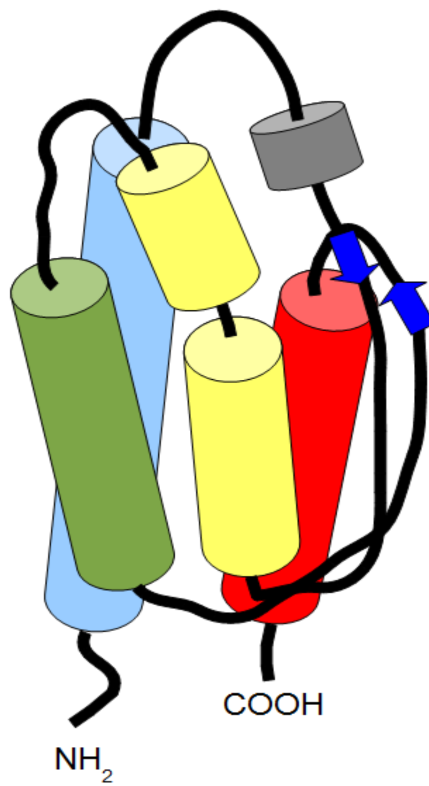

Figure 4: Interleukin 2 structure. The structure of interleukin 2 is comprised of 4 alpha-helical domains in an Up-Up-Down-Down orientation. These domains are color coded in blue, yellow, green, and red.

CHAPTER II: ADENOVIRUS STRATEGIES FOR TISSUE SPECIFIC TARGETING

by

MATTHEW S. BEATTY AND DAVID T. CURIEL

Advances in Cancer Research **115**:39-67, 2012

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Format adapted for dissertation

Abstract

Cancer gene therapy approaches have benefited greatly from the utilization of molecular-based therapeutics. Of these adenovirus-based interventions hold much promise as a platform for targeted therapeutic delivery to tumors. However, a barrier to this progression is the lack of native adenovirus receptor expression on a variety of cancer types. As such, any adenovirus-based cancer therapy must take into consideration re-targeting the vector to non-native cellular surface receptors. Predicated upon the knowledge gained in native adenovirus biology, several strategies to transductionally re-target adenovirus have emerged. Here-in, we describe the biological hurdles as well as strategies utilized in adenovirus transductional targeting; covering the progress of both adapter-based and genetic manipulation based targeting. Additionally we discuss recent translation of these targeting strategies into a clinical setting.

The development of rationally designed cancer interventions has followed the progress of the molecular understanding of cancer development and progression. To this end, gene therapy has endeavored multiple strategies for molecular targeted therapeutics. Of these strategies, adenovirus (Ad)-based vectors have been used prevalently, especially in the field of cancer. Ad vectors entail many characteristics that make it an ideal choice compared to other vectors. Biologically, Ad is able to efficiently transduce a variety of both dividing and quiescent cell types *in vitro* and *in vivo*. Additionally, the amenability to genetic modification, large genetic payload capacity, and the ability to produce high titers of good manufacturing practice quality are all factors that favor the use of Ad-based vectors as cancer therapeutics. Of importance, Ad-based vectors have shown an impressive safety record in the pre-clinical and clinical setting. However, despite the safety profile and pre-clinical efficacy these vectors have failed to achieve therapeutic efficacy in the clinical setting. Thus efforts have been refocused on basic vector design, especially on maximizing gene delivery by specifically transducing the target cell population. Achievements in Ad transductional targeting technology have steadily progressed and show great promise as a therapeutic for cancer treatment.

Adenovirus Biology

The most common adenovirus used in gene therapy, human adenovirus (Ad) serotype 5, is a member of the Adenoviridae family. This family is comprised of 51 Ad serotypes originally classified by their ability to be neutralized by animal antisera. These serotypes are then divided into six species based upon hemagglutination properties, oncogenicity, and genomic structure (1). In addition, there are some correlations between

species and tissue target and clinical presentation. Of importance to current Ad-based gene therapy vectors are species B, C, and D. These Ad species comprise the serotypes most commonly used in gene therapy.

Adenovirus Capsid Structure

The human Ad is a 36 kilobase double-stranded DNA virus encapsulated by an icosahedral protein particle (Figure 1). Progress in X-ray crystallography and cryo-electron microscopy has furthered our understanding of the structural components of the Ad capsid (2-4). Three major proteins comprise most of the Ad capsid. Hexon is a homotrimeric protein found in 720 copies and comprises most of the Ad capsid, playing a structural role as the main coating protein. The Ad hexon is highly conserved among human serotypes; however, hexon contains 9 surface exposed hypervariable regions (HVR1-HVR9) which contain serotype variation (5). Penton forms a homo-pentamer which makes up the penton base. This pentameric structure is located at each of the 12 vertices and plays a structural role, interacting with each of the Ad capsid 12 fibers. In addition, penton contains an Arg-Gly-Asp (RGD) motif which is responsible for virion internalization. Fiber is found as a homo-trimer at each vertice and non-covalently binds to its corresponding penton base via its N-terminus. Fiber is composed of a shaft domain and a globular knob domain found at the distal tip which plays a role as the primary cellular attachment site (6). In addition to the major capsid proteins, the Ad capsid is comprised of an assortment of minor proteins: IIIa, VI, VIII, and IX. Of note, polypeptide IX, which has gained favor as an alternative locus for targeting incorporation (discussed later), is a structural protein that is believed to help stabilize the Ad capsid (7). Four

monomers of pIX interact to form a 4 helix bundle with a surface exposed C-terminus (4, 8).

Adenovirus Entry Biology

Ad entry biology is comprised of two distinct steps: attachment of the virus to its primary receptor subsequently followed by molecular interactions that lead to internalization of the virus. Initially, high-affinity interactions occur when the fiber knob domain binds to its cognate primary receptor. In general, for serotypes from species A, C, E, and F this receptor is the coxsackie and adenovirus receptor (CAR), while serotypes from species B and D tend to utilize alternative receptors. CAR is a 46 kilo Dalton protein that is a member of the immunoglobulin superfamily and is involved in the formation of tight junctions (9, 10). Once the Ad virion has attached, cellular integrins including αvβ3, αvβ5 (11), αvβ1 (12), α3β1, and α5β1 (13) interact with RGD motifs in the penton base. This interaction induces cellular responses that lead to cytoskeleton alterations which aid in internalization (14, 15). Ultimately, virus internalization occurs via clathrin-coated vesicles and the Ad virion is transported to the endosome (16). Upon endosomal acidification the Ad virion disassembles and is released into the cytoplasm where it ultimately travels to the infected cells nucleus for viral replication.

Transductional Targeting of Adenovirus Vectors

Knowledge gained from studies concerning native adenovirus entry biology has predicated understanding of findings that non-CAR expressing cancer cells are refractory to Ad infection and gene delivery. Thus, if target cancer cells exhibit low levels of CAR

we are left with a scenario where by high-CAR expressing non-target cells are effectively transduced while low-CAR cancer cells show poor transduction. Higher expression of CAR also appears to be growth inhibitory in some cancers (17). In summary, targeting CAR appears to be strategically incompatible with Ad-based cancer therapeutics.

Bio-distribution of Ad, although effected by CAR distribution, is not solely determined by expression profiles *in vivo* (18). Systemic administration of Ad, intravenously, results in the majority of transduction occurring in the liver followed by the spleen, heart, lung, and kidneys of mice. This profile however, does not correlate with the highest levels of CAR expression (19). This is especially true in regards to liver transduction which absorbs the vast majority of systemic Ad vector via hepatic kupffer cell uptake (20) and hepatocyte transduction, potentially resulting in liver toxicity. Due to the toxicity issues regarding liver transduction, this biological interaction *in vivo* has been given great scrutiny.

Early strategies to re-target Ad to non-CAR pathways were initially thought to also de-target the liver, as the initial hypothesis was that liver transduction was CAR and integrin dependent. However, studies that ablated CAR and integrin binding in the Ad capsid had little effect on bio-distribution profiles (21, 22). Thus, Ad liver tropism was shown to be linked to a novel pathway. Following initial studies implicating motifs in the fiber shaft (23-25), *Shayakhmetov et al.* reported a major role for fiber interactions with blood coagulation factors and complement component C4 binding protein in hepatocyte and kupffer cell transduction. Modification of the Ad5 fiber to ablate this interaction resulted in a 50-fold decrease in liver transduction along with reduced levels of liver toxicity. Analysis determined that this *in vivo* tropism was due to Ad associating with

hepatocellular heparin sulfate proteoglycan and low density lipoprotein receptor related protein (26).

More recent studies however, have shown that fiber structure and motifs do not play a role in liver sequestration and have elucidated hexon interactions with blood coagulation factors as the major pathway directing hepatocyte transduction by systemic delivery of Ad. *Kalyuzhniy et al.* and *Waddington et al.* defined the specific interaction between blood coagulation factor X (FX) and hexon. In addition, utilizing structural studies, FX was shown to interact with hypervariable regions 3, 5, and 7 of hexon. Both groups showed that this interaction could be inhibited by mutated forms of hexon or by pharmacological methods involving warfarin or snake venom protein X-bp (27, 28). Additionally, *Waddington et al.* elucidated that different serotypes of Ad interact with FX with different affinities, some to the point of not binding at all. Of note, those Ad serotypes that did not bind FX were all from species D. Following this, candidate viruses from low (Ad35) and non-binding (Ad26 and Ad48) groups were examined *in vivo* by intravenous injection with or without X-bp protein. These viruses showed a lack of liver transduction (27).

From this work, several groups have reported success with genetic manipulations of hexon to ablate liver sequestration. These strategies fall into two different categories. From the original studies, hexon mutations blocking the FX/hexon interaction have been utilized and shown to drastically reduce liver sequestration and transduction. This strategy was further developed by *Alba et al*., identifying the exact amino acids in hypervariable regions 5 and 7 responsible for FX binding. Altering either of these points resulted in a drastic decrease in FX binding and FX mediated gene transduction (29).

These FX ablated Ad vectors also possessed altered biodistribution, with decreased liver transduction and greater vector accumulation in the spleen, especially following macrophage depletion (30). Additionally, since different serotypes have different affinities for FX, some labs have reported that either whole hexon swaps or hypervariable region swaps with lesser binding serotypes have also been successful in preventing liver uptake. *Short et al.* utilized the swapping of Ad5 hexon with that of Ad3 hexon. This modification was shown to block FX binding to Ad virions by surface plasmon resonance (SPR) analysis and prevent FX-mediated gene transduction *in vitro*. Interestingly, ablation of FX binding in an oncolytic Ad vector provided increased tumor killing and prolonged viral replication in a skov3.ip1 subcutaneous flank tumor model (31).

In summary, primary biology determined by *in vitro* experimentation as well as host interactions outside of this primary pathway play a role in the over all *in vivo* bio-distribution of Ad. It is clear that alternative targets for cell transduction must be explored as a wide variety of target cells including cancer are not amenable to CAR targeted Ad vectors. Two distinct approaches have been utilized to transductionally target Ad-based vectors: 1) adapter based and 2) genetically capsid modification. While these strategies show great efficacy in re-targeting Ad-based therapeutics, it is also clear that liver de-targeting must also be considered in any Ad vector as these external biological forces will effect overall target cell transduction *in vivo.* Thus, any Ad-based therapeutic must entail both de-targeting strategies and re-targeting strategies in order to reach its full therapeutic efficacy.

Adenovirus Transductional Targeting: Adapter Based Strategies

The adapter based strategy (Figure 2A) involves the use of a molecular bridge to re-target the adenovirus from its native primary receptor to a different cell surface receptor. This function is performed due to the bi-specific nature of adapter molecules. One end of the adapter binds specifically to the Ad capsid while the alternative end re-directs Ad away from its native CAR binding and interacts with the alternative cellular receptor. Of note, this process does not impede with Ad infection as entry biology is determined by a two-step process, with binding being a separate mechanism from internalization. Conceptually, the most elegant location for conjugating an adapter molecule to the Ad virion is the fiber knob domain. This interaction allows for the re-targeting of Ad to alternative receptors while also de-targeting Ad from its primary receptor CAR. As such, within this re-targeting strategy, the majority of adapters interact with the Ad knob domain. However, alternative capsid sites have been used as adapter interaction locales and have shown to be effective in re-targeting Ad.

FAB Antibody Adapters

The first *in vitro* manifestations of the adapter strategy were accomplished by conjugating an anti-knob FAB antibody to a re-targeting ligand. This strategy was first shown by chemically conjugating an anti-knob FAB antibody to folate. Conjugated to Ad virions, this adapter showed CAR independent targeting to cancer cells over expressing the folate receptor (32). FAB antibody adapters against fibroblast growth factor 2 (FGF2) receptor have also been widely used and shown to retarget Ad-based vectors to a variety of cancers including pancreatic cancer (33), melanoma (34), Kaposi's sarcoma (35),

ovarian cancer (36), and head and neck cancer (37, 38). In all, a wide variety of alternative cancer relevant targeting ligands have been conjugated to anti-knob FAB antibodies and used to re-target adenovirus vectors to cellular receptors such as CD40 (39), epidermal growth factor (EGF) receptor (40), epithelial cell adhesion molecule (EPCAM) (41, 42), prostate-specific membrane antigen (42), and Tag-72 (43), resulting in similar transductional gains over un-targeted Ad. Full utility of this strategy was progressed when *Reynolds et al.* employed an anti-knob FAB antibody chemically conjugated to monoclonal antibody (9B9) against angiotensin-converting enzyme (ACE) (44). This cellular marker is found in the pulmonary epithelium and up regulated in various pulmonary diseases. Of note, this study showed a 20 fold-increase in lung gene expression while also reducing liver expression by 83% when compared to an un-targeted Ad vector. More recently an anti-knob FAB antibody fused to FGF2 showed increased transduction efficiency in a mouse model of head and neck carcinoma. The targeted Ad, expressing a mutant Rad50 protein, also demonstrated an increase in tumor suppression compared to un-targeted Ad when combined with cisplatinum, resulting in greater DNA double strand breaks and reduced angiogenesis (38).

Recombinant Fusion Adapters

Studies have shown FAB antibody based adapters to be effective in providing a proof-of-principle strategy for re-targeting Ad vectors. However, the production of these molecules has been hampered by the randomness of chemical conjugation, producing heterogeneous populations of molecules. As such, strategies that utilize single component molecules could be advantageous. Thus, more recent efforts have focused on the

development of single component fusion proteins that can be easily expressed and whose population is genetically homogenous. Building on the use of anti-knob antibodies, *Haisma et al.* developed a recombinant fusion protein composed of a single-chain antibody (scFv) against Ad fiber knob domain fused to a scFv against EGF receptor. This adapter could be easily purified without loss of function and increased adenovirus gene transfer compared to un-targeted Ad in EGF receptor expressing cell lines (45). Later, *Haisma et al.* showed selective targeting to a variety of angiogenesis related markers including $\alpha v\beta 3$ integrins, vascular endothelial growth factor (VEGF) receptor 2, and the angiopoietin receptor TIE-2. These adapters re-targeted Ad to both mouse (H5V) and human (HUVEC) endothelial cell lines. In addition, they showed *in vivo* tumor specific targeting to a subcutaneous C26 colon carcinoma (46). These scFv diabodies have also shown efficacy in a variety of studies re-targeting Ad to cellular targets such as EGF receptor (47, 48), EPCAM (49), human epidermal growth factor receptor 2 (HER2/neu), carcinoembryonic antigen (CEA), endoglin (CD105) (50), and high molecular weight melanoma antigen (51).

In addition to scFv diabody based adapters, a novel adapter utilizing a truncated, soluble portion of CAR (sCAR) fused to a targeting molecule was developed. Utilizing the first sCAR based adapters, *Dmitriev et al.* created sCAR adapters fused to either an anti-CD40 antibody or EGF (52). With this, they demonstrated in several EGF over-expressing cancer cell lines a 9-fold increase in gene expression when compared to non-targeted Ad vectors. In addition to Ad targeting specificity, *Dmitriev et al.* questioned the stability of these adapters when complexed with Ad virions. They showed through comparing Ad/sCAR-EGF and Ad/sCAR-EGF purified through gel filtration that there

was no difference in targeting profile, confirming the stability of these complexes. Similarly to scFv diabody based adapters, studies have shown this strategy to be efficacious in retargeting Ad vectors utilizing a wide variety of targeting ligands against EGF receptor (53), urokinase-type plasminogen activator receptor (UPAR) (53), CEA (54, 55), HER2/neu (56), CD40 (57), and high-affinity Fcgamma receptor I (CD64) (58).

Kashentseva et al. furthered this strategy by incorporating a novel trimerization domain (59). This trimeric adapter contained sCAR fused to a HER2/neu specific scFv and displayed increased affinity for the Ad fiber knob. In addition, the adapter augmented gene transduction 17-fold in HER2/neu positive breast and ovarian cancer cell lines. *Kim et al.* additionally reported that adapter trimerization provided drastic increases (100-fold) in gene transduction over its identical monomeric adapter (60). These studies have proven the targeting efficacy and stability of adapter virus complexes and have paved the way for these complexes to be utilized in a clinical setting, were complex stability could affect targeting efficacy and safety.

Alternative Adapter Binding Locales

In addition to bi-specific adapters that utilize the adenovirus fiber knob domain for presentation, penton base and hexon have also been explored as sites for adapter based re-targeting. *Li et al.* constructed a bi-specific adapter molecule composed of an anti-penton monoclonal antibody fused to tumor necrosis factor α (TNF-α), insulin-like growth factor 1 (IGF-1), and EFG (61). These adapters when complexed with Ad provided increased gene transduction in M21-L12 melanoma cells. The use of hexon as a re-targeting local is predicated on newly identified *in vivo* biology regarding adenovirus

interaction with blood coagulation factors. As previously described, upon entering circulation Ad hexon interacts with FX. *Chen et al.* utilized this interaction to create a novel re-targeting ligand by fusing the GLA domain of FX to a scFv against HER2/neu (62). When conjugated to Ad, this novel adapter promoted increased transduction of HER2/neu positive cells versus cells lacking HER2/neu expression. They additionally showed that this strategy could be used to target other cell surface receptors such as EGF receptor and ATP-binding cassette protein G2 (ABCG2). Since this technology utilizes the same binding interaction that leads to liver transduction, gains in re-targeting Ad to an alternative receptor also yield gains in de-targeting the liver.

Adapters Utilizing Genetically Modified Ad Capsid

Alternative to bi-specific adapters, approaches that genetically modify the Ad virion to bind re-targeting ligands have been developed. The benefit of this strategy is that a variety of off the shelf, commercially available ligands can be used without any additional modification. This strategy is best portrayed by incorporation of the immunoglobulin (Ig) binding domain of Staphylococcus aureus protein A into the fiber HI-loop or C-terminus. This fiber modified Ad vector is thus able to bind a wide variety of targeting moieties that contain the Fc region of Ig. Most recently, *Takahashi et al.* utilized this technology to screen antibody libraries against the prostate cancer cell line LNCaP (63). Identifying an antibody against neural adhesion molecule 2, they showed that this antibody when conjugated to Ad increase gene transduction in prostate and breast cancer cell lines. A variety of other targets including CD40 (64), mesothelin (65), EGF2 receptor, HER2/neu, CA242 antigen, and PSMA (66) have been explored.

Utilizing the same concept, a biotin acceptor peptide (BAP) has been inserted into fiber (67, 68). During virus production, BAP is biotinylated by the endogenous biotin ligase in 239 cells. Once purified, this virus can be conjugated to biotin labeled ligand. PIX has also been explored as a site for BAP insertion. *Campos et al.* fused BAP to the terminus of pIX and compared targeting to BAP inserted in fiber. Of interest, using an anti-CD71 (transferin receptor) antibody as ligand, BAP fused to pIX failed to retarget Ad vectors unlike BAP inserted into fiber. However, when pIX-BAP Ad was retargeting with biotinylated transferin transduction was successful (69).

Adenovirus Secretion of Adapter

Although, the adapter strategy for re-targeting Ad vectors allows for efficient and specific re-targeting it is reliant upon a two part system. The Ad vector and the adapter molecule are produced separately and are conjugated before being introduced to their cellular target. However, genetic incorporation of targeting ligands is biologically incompatible with a variety of ligands utilized in adapter based strategies, such as scFvs. This is due to the fact that most scFv molecules must be processed through the ER in order to fold and function correctly while Ad assembly takes place in the cytosol. Bridging this gap between cytosolically unstable targeting ligands and single-component Ad vector retargeting have been reports of Ad vectors that secrete their own adapter. With this, single component Ad vectors can utilize targeting ligands which are not biologically compatible with viral assembly. In addition, while adapter conjugated Ad vectors provide efficient targeting this strategy only allows for a single round of targeting. Any viral progeny will regress to their native cellular receptor CAR. As a

result, adenoviruses that secrete their own adapter have targeting advantages over traditional adapter strategies in regards to replicating Ad vectors.

A novel method for achieving this was explored by Glasgow et al. This strategy utilizes a unique leucine zipper based biding motif derived from vittelogenin gene binding protein (VBP) to allow for adapter conjugation. One zipper domain was incorporated into a knob-less fiber while its corresponding zipper domain was fused to the re-targeting ligand and secreted. Utilizing a scFv against CD40, *Glasgow et al.* showed that these novel structures could be incorporated into the Ad capsid and corresponding secreted adapter. Most importantly, upon completion of the Ad native life cycle and Ad release, crude viral lysate was shown to have CD40 specific targeting, thus showing that the virus could interact and bind with its cognate adapter in the inter-cellular space (70).

Overall, adapter based Ad targeting studies have provided vast evidence that native Ad tropism can be re-targeted to alternative cellular receptors and increase gene transfer in non-CAR expressing cells *in vitro*. Adapter targeted vectors have also shown to be efficacious in *in vivo* studies, showing great stability and transductional efficacy. With further development of expression systems and rigorous analysis of the stability and kinetics of vector adapter complexes, adapter based strategies could progress to clinical translation.

Adenovirus Transductional Targeting: Genetically Incorporated Strategies

With greater understanding of Ad virion structure, genetic manipulation of capsid proteins has yielded great strides in Ad targeting. Conceptually, genetic manipulation of

the Ad capsid to incorporate novel targeting ligands could yield a multitude of targeting strategies. However, any genetic manipulation must function within the structural and biological constraints of the native Ad capsid. Based on this, most genetic manipulation of the Ad capsid have focused on the fiber, since this domain is the primary determinant of native Ad tropism and can be modified without interfering with capsid assembly.

Chimeric Adenovirus

Although Ad5's primary receptor is CAR and thus non-amenable to a variety of cancer therapeutic strategies, other serotypes of Ad do not use CAR as their primary binding receptor. As such, one genetic strategy for re-targeting Ad, termed pseudotyping, developed as a mechanism to utilize non-CAR targeting serotypes while not abandoning the vast knowledge of Ad5 biology (Figure 2B). These pseudotyped virions have shown great transductional efficacy in a variety of Ad5 refractory cell types such as ovarian carcinoma (71, 72), prostate cancer (73), breast cancer (74), colon carcinoma (75), glioblastoma (76) and others. With pseudotyped Ad vectors, the fiber knob domain or the entire fiber is genetically replaced with its structural counterpart from a different human serotype that recognizes an alternative cellular surface receptor. These alternative serotypes are primarily developed from species B and species D adenoviruses. Species B viruses have been shown to interact with a variety of non-CAR receptors including CD46 (77), CD80, and CD86 (78). Additionally, subgroup D serotypes have been shown to interact with CD46 and the glycoprotein component α(2-3)-linked sialic acid (79). With the great progress developed in pseudotyped Ad vectors, the strategy was also expanded to include the insertion of fiber elements from non-human Ad serotypes. This strategy,

termed xenotyping, has yielded a variety of non-CAR targeted Ad vectors including vectors with fiber elements from avian, bovine, canine, murine, and porcine Ad vectors (80-84). Of note, most of the receptor targets for these vectors are undetermined as of today. However, the fiber element from porcine Ad serotype 4 has recently been shown to interact with glycan chains containing repeats of n-acetyllactosamine though evidence that this interaction leads to cellular uptake is unknown (85). Bovine Ad serotype 4 fiber has also been elucidated recently. This non-human Ad was shown to interact with two different immunoevasion molecules of the B7 family of proteins, B7-1 and B7-H1, in murine leukemia cells. Of interest, this fiber requires both interactions in order to result in cellular uptake (86). In addition to non-human Ads, structurally similar binding domains from other virus species have also been incorporated into the Ad fiber. This was first shown by the incorporation of the fiber like σ1 reovirus attachment protein into the Ad fiber. This allowed for effective transduction of target cells expressing junctional adhesion molecule (87).

Peptide Targeted Adenovirus

Although Ad pseudotyping has shown great success, it is predicated by the discovery of novel non-CAR targeting Ads. As such, development of re-targeting Ad vectors has progressed into rationally designed targeted Ads. Meticulous structural studies of the knob domain of fiber have yielded two separate locations within the knob that can be exploited for genetic peptide presentation without disrupting fiber function, the C-terminus and a region termed the HI loop (Figure 2C). Conceptually, the C-terminus is an ideal location for peptide insertion. Successful genetic insertions of an

integrin binding RGD motif or poly-lysine peptides have yielded positive *in vitro* and *in vivo* results (88). However, other peptide insertions have shown no effect possibly due to steric hindrances. Structural studies using a genetically inserted FLAG tag into the HI loop, an exposed loop structure connecting β sheets H and I in the Ad knob domain, showed proof of principle evidence that this location is structurally amenable to peptide insertion (89). In fact, further studies have shown that this location can handle peptides insertions of up to 100 amino acids without detriment to fiber function (90). As such, *Dmitriev et al.* inserted an integrin- binding RGD motif into this location and showed that this virus, AdlucRGD, has enhanced transductional efficacy and gene delivery in ovarian cancer cell lines and primary tumors versus non-targeted Ad (91, 92). This tropism expanded adenovirus has been utilized widely in the field and shown to be efficacious in gene delivery to wide variety of cancers including ovarian ((93), cervical (94), colon (95), melanoma (96) and others.

Progressing beyond the tropism expansion seen in RGD and poly-lysine motif insertions, several groups have inserted cellular specific targeting peptides into the HI loop. These peptides developed by traditional phage display biopanning or more novel strategies utilizing peptide incorporated Ad libraries (97-99) have been proven to be highly specific and generally amenable to Ad insertion. *Nicklin et al* showed that the vascular endothelial cell targeting peptide, SY-GYLPLP, provided increased transduction in a variety of cancer cell lines (100). In addition peptides have been inserted to target a variety of cancers including head and neck (101), medullary thyroid carcinoma (102), glioma (103), and renal cell carcinoma (104). In addition to classical target specific short peptides, *Myhre et al.* inserted an Affibody, a small antibody mimetic, into the HI loop

and showed HER2/neu and Taq polymerase specific targeting (105). They also showed that HER2/neu specific, Affibody targeted oncolytic Ad provided increased transduction and killing in prostate cancer cells *in vitro* and increased survival time while decreasing serum prostate specific antigen in an orthotopic mouse model of prostate cancer (106).

"Knob-less" Targeted Adenovirus

While peptide insertion has shown to be a successful strategy in re-targeting Ad, structural conflicts have emerged from fiber knob modifications. As such, a platform by which a wider variety of targeting ligands could be utilized would be a rational goal in further progressing Ad re-targeting. The observation that Ads lacking various portions of their knob domain could be rescued lead to the concept of utilizing a knob-less fiber as a platform for ligand presentation (Figure 2B). Limiting this concept however was the fact that the knob domain contained the trimerization domain for the fiber that is required for fiber function and insertion into the Ad capsid. Overcoming this structural problem, a foreign trimerization domain, the foldon domain of T4 fibritin, was fused to the native Ad fiber shaft to replace that which was lost by deletion of the knob domain (107). *Krasnykh et al.* replaced the fiber and knob with the bacteriaphage T4 fibritin and showed that this platform could present a 6 histadine (6-His) motif inserted into the C-terminus (108). This vector showed a 100 fold increase in gene expression in cells expressing an artificial 6-His binding receptor. Variant "de-knobing" strategies have also been explored by *Magnussen et al*, demonstrating that an RGD motif could target integrin expressing cells (109). Further, labs have progressed towards larger peptide displays such as small peptides and Affibodies. *Belousova et al.* incorporated a HER2/neu specific Affibody

into a knob-less fiber, showing that the novel Affibody technology was compatible with knobless fiber platforms. This HER2/neu targeted vector also showed increased gene delivery in HER2/neu expressing cancer cells (110). The knob-less Ad platform provides the ability to move beyond small ligands and into the use of proteins as targeting ligands. Previously this would be a very problematic strategy as large protein insertions are much more likely to interfere with native Ad assembly and function. Notably, trimeric CD40 has been fused to this fiber providing evidence that this platform can be amenable to large protein ligands. The CD40 incorporated Ad provided CD40 specific gene delivery *in vivo* following systemic delivery (111).

Alternative Capsid Locations

Although fiber is the most developed capsid protein for re-targeting Ad, the difficulty of incorporating ligands into the Ad capsid has furthered the development of alternative locales. Potentially, alternative sites could provide increased presentation of the targeting ligand through increased copy number per virion and could also allow for multiple targeting ligands to be utilized on the same capsid. To date a variety of alternative sites have been proposed and explored including hexon, polypeptide IX (pIX), and pIIIa (Figure 2C). The first two have been shown to be compatible with ligand presentation while the latter was shown incompatible due its current structural location within the capsid (112).

Hexon is the most abundant protein in the Ad capsid and as such is an ideal candidate for ligand incorporation. The potential 720 copies of hexon could allow for a "coating" of the Ad capsid in any incorporated ligand. Although most of the hexon

sequence is highly conserved among serotypes, nine hypervariable regions are found within the hexon and have solvent exposed loops. As such, these loops lay in an ideal location for modification. *Vigne et al.* genetically modified hypervariable region 5 (HVR5) and inserted an integrin binding RGD domain (113). This RGD motif had no effect on hexon structure or capsid stability but increased CAR independent transduction of vascular smooth muscle cells. Further HVR's 2, 3, and 5-7 were found to be amenable to insertion of a 6 histadine (6-His) motif (114). In addition, anti-6His antibodies recognized Ad vectors with 6-His inserted into HVR's 2 and 5. The rescue of Ad vectors with peptides inserted in various hypervariable regions provides us with a potential platform for various downstream targeting applications.

More recently, pIX has developed as a practical platform for the presentation of targeting ligands. Polypeptide IX is a small protein that plays the role of a cement protein, helping to stabilize hexon interactions. Found in 240 copies within each virion, pIX provides drastically increased ligand presentation over fiber modifications. Structural studies and the observation that the C-terminus of pIX may be solvent exposed lead to several groups exploring the concept of pIX presented ligands. The first reported targeting ligand incorporated into the terminus of pIX was presented by *Dmitriev et al.* By incorporating poly-lysine or FLAG motifs, they showed CAR independent transduction via interactions with heparin sulfate chains on the target cell surface (115). Furthering development of pIX as a targeting local, *Vellinga et al.* fused varying sized α-helical linkers to the terminus of pIX and used these linkers to present integrin binding RGD motifs (116). Of note, longer linker length corresponded with increased gene delivery in CAR negative endothelial cells. Relatively large proteins fused to pIX have

also been explored. Incorporation of hyper-stable scFv against β-glactosidase fused to pIX showed that the scFv retained its binding affinity to β-glactosidase (117). However, this antibody has no targeting applications and the availability of hyper-stable scFv's is limiting. *Poulin et al.* attempted to incorporate a scFv against a mutant form of the EGF receptor (EGFRvIII) fused to pIX but the scFv failed to fold properly, resulting in a lack of targeting (118). As a consequence, they attempted to route the pIX-scFv through the endoplasmic reticulum (ER) but biological incompatibility between the cytoplasmically assembled Ad and the ER routed pIX resulted in low levels of incorporation and thus a lack of targeting. However, they were able to incorporate a single domain antibody (AFAI) against CD66c (carcinoembryonic antigen-related cell adhesion molecule family 6 [CEACAM6]). This ligand provided CD66c specific binding and transduction of A549 non-small-cell lung carcinoma cell line. *De Vrij et al.* showed that large single-chain T-cell receptors could also be attached to pIX (119). They fused pIX to a single-chain T-cell receptor against the CT antigen melanoma-associated antigen A1 (MAGE-A1). This vector specifically transduced melanoma cell lines. In addition, transduction was shown to correlate with the levels of MAGE-A1 peptide within the cells. However, lack of or down-regulation of HLA-A1 molecules can drastically reduce the transduction efficiency of this vector. As a whole, studies have shown that pIX is a flexible platform for the display of both small and large targeting ligands.

Novel Transductional Strategies

Although Ad-based therapeutics has progressed greatly, the vast majority of targeting strategies have relied upon a single cellular surface receptor for the target. This

strategy has been shown to work remarkably well for homogenous cell line populations. However, some target cells such as cancer are not comprised of a homogenous population. As such, targeting a single cellular surface receptor may lead to a selected population that is resistant to further therapy. Thus, any molecular based therapy should take this into account when designing a targeting strategy. Several groups have begun developing Ad targeting strategies that utilize multiple ligands within the same virion. One of the first vectors reported was an Ad5 containing both an RGD motif and a poly-lysine ligand (120). This vector could thus target both cell surface integrins and heparin sulfate proteoglycans. Following that, *Borovjagin et al.* inserted an integrin binding RGD motif into the C-terminus of a chimeric Ad fiber composed of the shaft domain of Ad5 and the knob domain of Ad3 (121). This vector showed 55-fold increase in gene transduction of bladder cancer cell lines. Utilizing adapter based re-targeting, *Grill et al.* combined an adapter composed of a scFv against EGF receptor with genetically incorporated RGD motif in the HI loop of fiber knob (48). This virus was shown to be able to target both EGF receptor and cell surface integrins on primary glioma cells and spheroids.

Although these strategies provide insight into the efficacy of dual targeting over single, they are limited in their ability to insert multiple ligands within the same fiber. Using multiple fibers within the same Ad virion would by-pass this restriction and provides a platform for the utilization of multiple complex targeting ligands. *Pereboeva et al.* first showed that this strategy was feasible by generating an Ad vector incorporating both the wild type fiber and a knob-less fiber fibritin presenting a 6-His motif (122). Utilizing this mosaic vector, they showed both CAR and artificial 6-His receptor specific

gene transduction, though in high CAR expressing cells no additional gain was seen from the 6-His containing fiber fibritin. The combination of an Ad5/3 chimeric fiber with a fiber containing the reovirus σ-1 protein in the same Ad virion has also shown to provide an increase in infectivity enhancement of ovarian cancer cell lines and primary ovarian cancer tissue slices (123). *Murakami et al.* additionally provided evidence that with the correct genetic construct, equal expression and incorporation of the two distinct fibers can be accomplished (124). This vector, containing both Ad5 and Ad3 fiber, provided both CAR and CD46 specific gene transduction. Of interest, they showed that this vector could target two distinct cells, PC-3 cells expressing CD46 and Cho-CAR cells expressing CAR, in a mixed culture experiment.

Adenovirus Targeting: Recent Clinical Developments

Although Ad-based therapeutics have shown great promise in pre-clinical studies, in the clinical setting therapeutic efficacy of Ad vectors has not followed. Although ample evidence concludes that lack of CAR expression upon cancer cells drastically limits Ad-based therapeutic efficacy, the vast majority of clinical trials utilizing Ad rely upon native CAR based transduction. One hurdle limiting the translation of Ad-based targeting strategies has been the additional complexity of adapter/Ad conjugates. Since these strategies are two-component systems, they entail additional production complexity and scrutiny in regards to safety before being approved for clinical use. As such, the few clinical trials to date utilizing targeting have relied upon genetically inserted targeting ligands.

Recently, clinical studies involving Ad-based therapies utilizing genetically incorporated integrin binding RGD motifs in the fiber knob have been reported. *Kimball et al*. recently finished a phase I clinical trial examining the therapeutic efficacy and maximum tolerated dose of a tropism-modified, infectivity enhanced conditionally replicative adenovirus (CRAd), Ad5-Δ24-RGD, in patients with malignant gynecologic diseases (125, 126). Following treatment, of the 21 patients 71% had stable disease while 29% still showed disease progression after 1 month of follow-up. Of note, 7 patients did show a decrease in CA-121 levels, with 4 of these being a greater than 20% decrease. Although no patients showed regression of disease in this study, toxicity associated with therapy was limited to grade 1/2 fever, fatigue, and abdominal pain. A similarly sized trial reported by *Nokisalmi et al*. analyzed an integrin targeted CRAd, ICOVIR-7, in patients with a variety of solid tumors (127). Similarly, this trial saw only mild to moderate treatment-related side effects. Of note, 9 of 17 evaluable patients showed evidence of anti-tumor activity, with 1 patient showing partial response and 2 patients with minor responses. This strategy has also shown similar safety and therapeutic outcomes when combined with expression of granulocyte-macrophage colony factor (GMCSF), an immune stimulatory molecule (128). Progression of targeted Ad-based cancer therapies in the clinical setting has lead to new studies utilizing chimeric Ad-based vectors replacing the Ad5 knob domain with that of the Ad3 knob (Ad5/3) (129). *Pesonen et al*. showed similar safety in a trial of 18 patients with varying solid tumors (130). Of these patients 61% showed evidence of anti-tumor activity.

In addition to these CRAd-based trials, *Matthews et al.* reported plans for and are currently finishing another phase I clinical trial involving Ad5.SSTR/TK.RGD (131).

This Ad-based therapeutic utilizes a genetically incorporated RGD ligand to target the expression of a therapeutic suicide gene, herpes simplex virus thymidine kinase (TK), and an imaging motif, somatostatin receptor type 2 (SSTR), for viral tracking via nuclear imaging.

In all, translation of targeted Ad-based therapeutics to the clinical setting has shown a solid safety record similar to their un-targeted counterparts. Although some levels of anti-tumor activity and therapeutic response have been noted, the response rate is still far below that required for therapeutic usage. This emphasizes the need for further targeting trials and the utilization of additional strategies involving Ad virion/host interactions such as liver de-targeting and immune evasion.

Concluding remarks

Adenovirus based vectors are a widely used therapeutic platform for gene delivery. They are especially prominent in the field of cancer gene therapy where shorter gene expression times are not an issue. However, biological hurdles stand between native Ad-based vectors and their full utilization as a therapeutically effective cancer treatment platform. Of these hurdles, effective gene transduction of cancer cells drastically limits potential of Ad-based vectors. Early clinical trials highlighted this issue by reporting Ad vectors safe but therapeutically non-efficacious. In this regard, studies have clearly illustrated the case for increased transduction of target cells leading to increased therapeutic efficacy. To this end, Ad-based vectors utilizing cancer specific targeting should continue to be progressed and examined in stringent models of cancer with the goal of full therapeutic efficacy in the clinical setting.

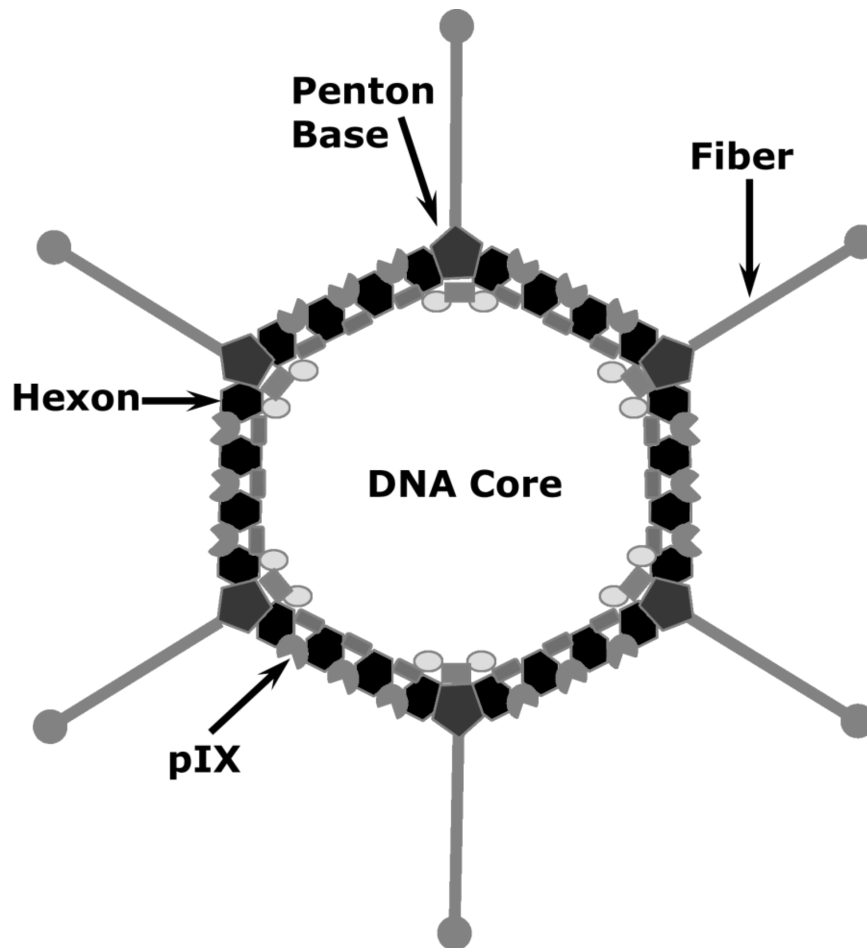

Figure 1: Adenovirus capsid structure: The adenovirus capsid structure is composed of both major capsid proteins and minor proteins. Labeled here are capsid proteins hexon, penton base, fiber, and polypeptide IX (pIX). These capsid structures have all been utilized as locales for re-targeting Ad strategies.

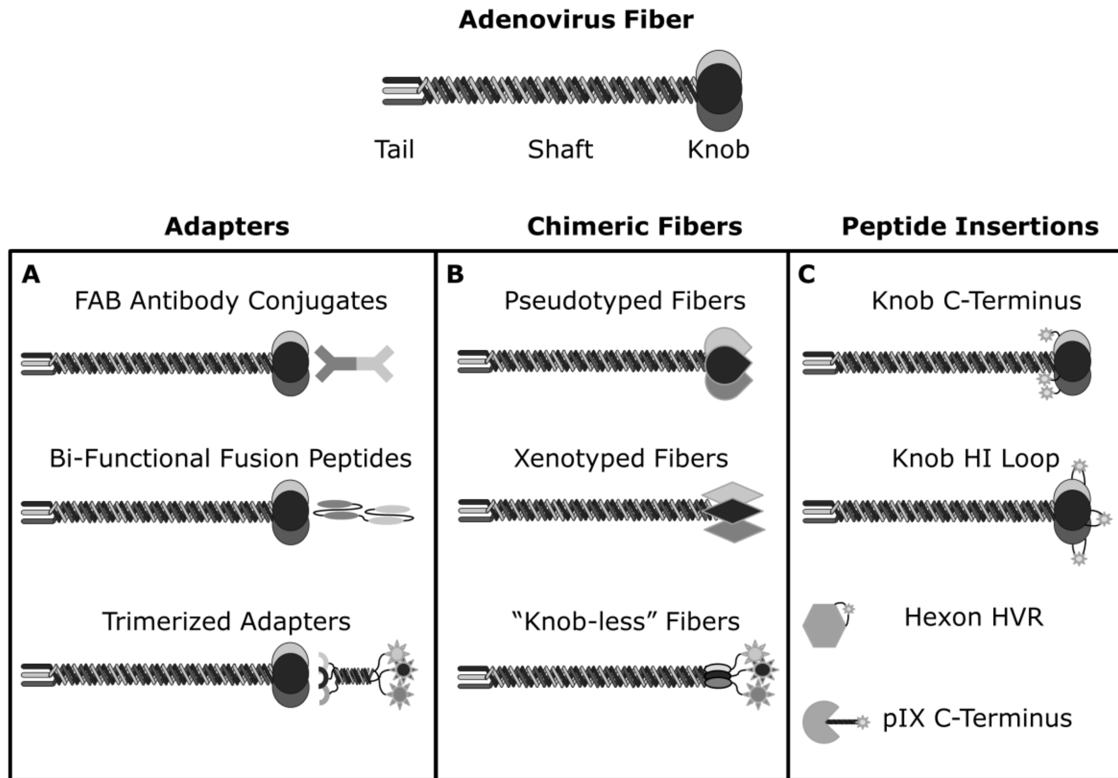

Figure 2: Transductional re-targeting modifications: Adenoviral vectors can be re-targeted to specific cell surface receptors using a variety of strategies: A) Heterologous retargeting ligands (adapters) composed of a variety of ligands including FAB antibodies, single-chain antibodies, and biological ligands. B) Chimeric Ad's composed of fiber / knob domains from alternative serotypes and "knob-less" fiber modifications. C) Genetic incorporation of peptides into fiber, hexon, and pIX.

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CHAPTER III: AUGMENTED ADENOVIRUS TRANSDUCTION OF MURINE T LYMPHOCYTES UTILIZING A BI-SPECIFIC PROTEIN TARGETING MURINE INTERLEUKIN 2 RECEPTOR

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Accepted into *Cancer Gene Therapy*

Format adapted for dissertation

ABSTRACT

Adenoviruses are currently used in a variety of bench and bedside applications. However, their employment in gene delivery to lymphocyte lineages is hampered by the lack of coxsackie virus and adenovirus receptor on the cell surface. Exploitation of an alternative receptor on the surface of T lymphocytes can allow for utilization of adenovirus in a variety of T lymphocyte based diseases and therapies. Here, we describe how resistance to infection can be overcome by the utilization of a bi-specific fusion protein, sCAR-mIL-2, that retargets adenovirus to the murine interleukin 2 receptor. Infection of a murine T cell line, CTLL-2, with a sCAR-mIL-2/Adenovirus conjugate provided a 9-fold increase in both green fluorescence protein positive cells and luciferase expression. In addition, this increase in infection was also seen in isolated primary murine T lymphocytes. In this context, the sCAR-mIL-2 adapter provided a 4-fold gene transduction increase in activated primary murine T lymphocytes. Our results show that recombinant sCAR-mIL-2 fusion protein promotes interleukin 2 receptor targeted gene transfer to murine T lymphocytes and that alternative targeting can abrogate their native resistance to infection.

ABBREVIATIONS

adenovirus type 5 (Ad5); coxsackie virus and adenovirus receptor (CAR); fetal bovine serum (FBS); green fluorescence protein (GFP); human embryonic kidney (HEK); interleukin 2 receptor (IL-2R); murine interleukin 2 (mIL-2); ubiquitin c promoter (UP)

KEYWORDS

adenovirus; bi-specific adapter; gene transfer; interleukin 2; T lymphocyte; targeting

The utilization of genetically modified T lymphocytes has gained much attention in treating a variety of disorders including autoimmune disorders, cancer, infectious disease, and transplant rejection (1-3). Current vector strategies rely upon lentivirus and retrovirus vectors for genetic delivery. These strategies, however, are hampered by variety of vector-related issues. Ineffective transduction (4), low viral titers, and their reliance upon host cell-cycle progression (5) all impede the full utilization of these vectors. In addition, such vectors raise biosafety concerns such as the occurrence of insertional oncogenesis (6, 7) and dysregulation (8) due to vector integration. In principle, adenoviruses type 5-based vectors (Ad5) retain many properties that could be utilized in genetically modifying T cells. However, full utilization is confounded biologically by the lack of coxsackie virus and adenovirus receptor (CAR) expression in T cells (9, 10).

To surmount CAR deficiency, multiple strategies have been endeavored to reroute adenoviral into non-CAR pathways. Most of these strategies have included genetically modifying Ad5 to retarget new cellular receptors such as CD46 and αvβ3 and αvβ5 integrins (11-15). In addition, strategies involving the use adapter molecules like bi-specific antibody conjugates against CD3 (16), and the use of non-genetic methods of transduction using lipofectamine (17) have been endeavored. However, the gains from these approaches have not achieved practical levels of utility.

With this in mind, we endeavored to reroute adenovirus into a CAR independent pathway that would provide enhanced transduction of T lymphocytes. Of the possible T lymphocyte specific targets, interleukin 2 receptor (IL-2R) represented a potentially useful candidate. As an Ad5 retargeting receptor, IL-2R is T lymphocyte specific and

highly expressed in therapeutic T lymphocyte populations such as CD4^{+}Foxp3^{+} regulatory T lymphocytes and activated CD4^{+} and CD8^{+} T lymphocytes (18). Here, we explored a novel strategy utilizing a bi-specific protein/adapter composed of soluble CAR fused to murine interleukin 2 (mIL-2) to target adenovirus to murine T lymphocytes. We show that this adapter remains biologically active and does not lose its binding potential to IL-2R. More importantly, this adapter promotes improved levels of gene transduction in both murine T cell cultures and primary murine T lymphocytes. These data indicate that alternative targeting strategies to T lymphocytes can yield enhanced levels of gene delivery and, as such, Ad5 may be a novel tool in T lymphocyte-based treatments of disease.

MATERIALS AND METHODS

Animals

C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME, USA) between the ages of 6 to 10 weeks old were used in this study. All methods were approved by the Institutional Animal Care and Use Committee (IACUC) at Washington University at St.Louis and performed according to their and the National Institutes of Health guidelines.

Cell Lines

Human embryonic kidney (HEK) 293 (19), HEK293T, CTLL-2 (ATCC, Manassas, VA, USA), and HEK293A (Invitrogen, Grand Island, NY, USA) cells were used in this study. HEK293, HEK293T, and HEK293A cells were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham, (Sigma-Aldrich, St. Louis, MO,

USA) containing 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Mediatech Inc., Manassas, VA, USA). CTLL-2 cells were cultured in RPMI 1640 (Invitrogen) containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml beta-mercaptoethanol, and 10 U/ml recombinant human interleukin 2 (eBioscience, San Diego, CA, USA). All cells were propagated at 37 °C in a 5% CO_2 atmosphere. The cell lines infected with the Ad5 vectors were maintained using the corresponding cell culture medium but containing 2% instead of 10% FBS.

Primary T Lymphocytes

Primary T lymphocytes were isolated from harvested spleens of C57BL/6J mice. A single cell suspension of splenocytes was made by straining cells through a 70 μm nylon mesh screen. Primary T lymphocytes were then purified by magnetic bead isolation using a Pan T Cell Isolation Kit II (Miltenyi Biotec Inc., Auburn, CA, USA) according to the manufacturer's directions. Isolated primary T lymphocytes were then immediately used for infection studies or incubated using a T Cell Activation/Expansion Kit (Miltenyi Biotec Inc.).

Construction of Recombinant Adenovirus Plasmids

Ad5GL plasmid, encoding an adenovirus expressing both green fluorescence protein (GFP) and firefly luciferase under CMV promoters, was constructed as previously described (20). The adenovirus shuttle plasmid, pSH-UP-UP-GFP (R) (21), contains an ubiquitin c promoter (UP) driving expression of GFP and a second ubiquitin c promoter

with a multiple cloning site downstream. This plasmid was then digested with NotI and the larger fragment re-ligated, creating a shuttle plasmid with only one ubiquitin c promoter driving GFP, pShUP-GFP.

Adenoviral Vectors

The recombinant adenoviral plasmids were linearized by PacI and transfected into HEK293 cells using Lipofectamine 2000 reagent (Invitrogen). All vectors propagated in HEK293 cells were purified by two rounds of CsCl gradient ultracentrifugation (22). CsCl was removed by dialysis against PBS (pH 7.4) containing 10% glycerol. The Ad5 vectors were stored at −80 °C prior to use. The infectious titer (plaque forming units [pfu]/ml) of purified Ad5 vectors was determined by triplicate $TCID_{50}$ assays using HEK293A cells, as described elsewhere (22). The physical titer (viral particles [vp]/ml) were determined by Maizel's method with a conversion factor of 1.1×10^{12} vp/ml per absorbance unit at 260 nm (23).

Lentiviral vectors

The lentiviral vector used in the study to obtain purified sCAR-mIL-2 was constructed as described previously (24). The resulting lentiviral vector contained an internal myeloproliferative sarcoma virus enhancer with the negative control region deleted promoter (MND) (25) expressing sCAR-mIL-2 (the ectodomain of human CAR fused to mIL-2 via a short, 6HIS tag containing peptide linker). sCAR-mIL-2 is fused to puromycin N-acetyl-transferase gene via an internal ribosomal entry site (26) and is followed by the central polypurine tract/central termination sequence. The virus was

generated as described by Zielske et al (24). Briefly, HEK293T cells were triple transfected with the packaging vector (pCMVdeltaR8.91), the VSV-G pseudotyping vector (pMD.G), and the pLVmnd-sCAR-mIL-2 transfer vector at a mass ratio of 3 : 1 : 3, using Lipofectamine 2000 reagent according to the manufacturer's instructions. Virus produced 24–48 hours after transfection was harvested in Dulbecco's Modified Eagle Medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% FBS and 2 mM L-glutamine. Virus-enriched media was filtered through 0.45 μm syringe filter units (Millipore, Billerica, MA, USA) and stored at −80°C.

Production of stable sCAR-mIL-2 expressing cells

HEK293 cells were infected with the lentivirus, LVmnd-sCAR-mIL-2. Following infection, the cells were distributed in a 96-well plate and grown in medium supplemented with 3 µg/ml puromycin until about 2/3 of the well surface was covered. The supernatants from the wells were then tested for sCAR-mIL-2 production by dot-blot assay using rabbit antibodies against CAR produced in the laboratory. A sCAR-mIL-2 expressing clone was expanded for future analysis.

Purification of the sCAR-mIL-2 adapter protein

The medium from stable sCAR-mIL-2-expressing cells was collected and proteins precipitated by addition of an equal volume of cold-saturated ammonium sulfate (Sigma-Aldrich). Following centrifugation the precipitate was dissolved in 1/20 the original volume of phosphate buffered saline (PBS) and then dialyzed against PBS. The sCAR-mIL-2 protein was then purified from the dialyzed solution by immobilized metal-affinity

chromatography using cobalt-immobilized TALON affinity resin (Clontech, Mountain View, CA, USA) followed by dialysis against PBS.

Western blot analysis of sCAR-mIL-2

Aliquots of purified sCAR-mIL-2 equal to 500 ng were denatured by boiling in laemmli sample buffer (Bio-Rad laboratories Inc., Hercules, CA, USA) at 95 °C for 10 min. The protein concentration was determined using the DC protein assay kit (Bio-Rad Laboratories Inc.) according to the manufacturer's instructions. The proteins were separated by electrophoresis in sodium dodecyl sulfate 10% polyacrylamide gels. The separated viral proteins were transferred onto a polyvinylidene difluoride membrane. Subsequently, western blot analysis was performed using a rat monoclonal anti-mIL-2 antibody S4B6 (BD Biosciences, San Jose, CA, USA) or rabbit polyclonal anti-CAR antibody followed by a horseradish peroxidase-conjugated secondary anti-mouse or anti-rabbit antibody (Dako North America Inc., Carpinteria, CA, USA). The blot was developed using an ECL Plus Western Blotting Detection Kit (Amersham Biosciences, Pittsburgh, PA, USA). Pre-stained protein ladder of Kaleidoscope Standards (Bio-Rad Laboratories Inc.) was used in the stained gel and the western blot. Pre-stained protein ladder of Kaleidoscope Standards was used for identification of the sCAR-mIL-2 protein in western blots.

ELISA assay

The binding specificity of the sCAR-mIL-2 adapter protein to the Ad5 recombinant knob (27) was tested by ELISA. Recombinant Ad5 fiber knob or BSA was

diluted in 0.2M sodium carbonate/bicarbonate buffer, absorbed in triplicate on a 96-well plate at 300 ng/well, and incubated at 4°C overnight. The plate was then washed three times in wash buffer (0.1M phosphate, 0.15M sodium chloride, pH7.2 containing 0.05% Tween 20) and incubated in blocking buffer (5% Non-fat milk in wash buffer) at 4°C overnight. Blocking buffer was then removed, 2-fold dilutions of sCAR-mIL-2 was added, and incubated for 1 hour at room temperature. Following incubation, the plate was washed 3 times, rabbit polyclonal anti-CAR antibody added, and incubated for 1 hour at room temperature. Plate was then washed three times, anti-rabbit-HRP (Bio-Rad Laboratories Inc.) added, and incubated at room temperature for 1 hour. Following this incubation, the plate was washed six times, Sigma Fast o-phenylenediamine dihydrochloride substrate (Sigma-Aldrich) added, and developed according to manufacturer's directions. Absorbance was measured at 492nm.

In Vitro Proliferation Assay

In vitro proliferation assays using CTLL-2 cells we performed as previously described (28, 29). CTLL-2 cells were plated in flat bottom 96-well tissue culture plates at $1x10^4$ cells per well in quadruplicate. Varying concentrations of either purified sCAR-mIL-2 or recombinant mIL-2 (eBioscience) were added to each well and incubated at 37 °C in a 5% CO_2 atmosphere for 48 hours. 10 µL of WST-1 reagent (Clontech) was added to each well and incubated for 4 hours. Plates were then read at absorbance 450 nm with a reference wavelength of 690 nm according to manufacturer's instructions.

In vitro proliferation blocking assays were performed in a similar fashion to the proliferation assays previously described. $1x10^4$ CTLL-2 cells were plated in 96 well flat

bottom tissue culture plates in quadruplicate and incubated with varying concentrations of rat monoclonal anti-murine CD25 antibody PC61 (BD Biosciences) or rat monoclonal IgG control antibody (BD Biosciences) for 30 minutes at 37 °C in a 5% CO_2 atmosphere. Cells were then incubated with 28 ng/ml of sCAR-mIL-2, a concentration that gave roughly 85% maximum proliferation, and incubated for 48 hours. 10 μL of WST-1 reagent was added to each well and incubated for 4 hours. Plates were then read at absorbance 450 nm with a reference wavelength of 690 nm according to manufacturer's instructions.

Gene transfer assays

CTLL-2 or primary murine T cells were distributed at a density of $5x10^5$ cells/well in a 24-well plate. Indicated amounts of sCAR-mIL-2 were incubated with $2.5x10^8$ vp (500 vp/cell) of Ad5GL (CTLL-2 cells) or Ad5UP-GFP (primary murine T lymphocytes) for 30 min at 37°C. The sCAR-mIL-2/Ad5 complexes were then transferred to the cells and incubated for 1 h at 37°C followed by virus removal and medium change to growth medium supplemented with recombinant human IL-2. Twenty four (CTLL-2 cells) or forty eight (primary murine T lymphocytes) hours post infection the cells measured for luc and/or GFP expression.

Flow Cytometry

Expression of CAR and CD25 on CTLL-2 cells were analyzed by flow cytometry. CTLL-2 cells were stained for CAR using an anti-CAR antibody RmcB conjugated to biotin. Cells were then incubated with streptavidin labeled with Alexa Flour 647

(Invitrogen). A mouse IgG-biotin (Invitrogen) followed by streptavidin-Alexa Flour 647 and streptavidin-Alexa Flour 647 alone was used as isotype controls. CTLL-2 cells were stained for CD25 using anti-CD25 PC61 conjugated to APC (BD Biosciences). Rat IgG1-APC (BD Biosciences) was used as an isotype control.

Mock, Ad5GL, and sCAR-mIL-2 conjugated Ad5GL transduced CTLL-2 cells were tested for GFP expression. Cells were harvested 24 hrs post infection, washed three times in FACS buffer and analyzed for GFP fluorescence by flow cytometry. Data was represented as percentage of cells expressing GFP protein.

Mock, Ad5UP-GFP, and sCAR-mIL-2-conjugated Ad5UP-GFP transduced naive and activated murine T lymphocytes were tested for CD25 and GFP expression. Cells were harvested 48 hours post infection, washed twice with FACS buffer and stained for CD25 using anti-CD25-APC antibody or rat IgG1-APC control for 1 hr at 4°C. Following incubation, the cells were washed three times in FACS buffer and CD25 and GFP expression were analyzed. Data was represented as percentage of cells positive for CD25 and/or GFP expression.

All data was acquired by flow cytometry using BD FACSAria Special Edition (BD Biosciences) and analyzed by FlowJo software version 10.0.4 (Tree Star, Inc., Ashland, OR, USA).

RESULTS

Generation of sCAR-mIL-2 adapter

Previous work has shown that Ad5 is a poor candidate for gene delivery to T cells due mainly to a lack of the Ad5 receptor, CAR. Therefore, retargeting adenovirus to a

cell surface protein that is highly expressed, such as the IL-2R, should provide for increased gene transduction. Our lab has previously shown that utilizing a bi-specific protein/adapter allows for efficient retargeting of Ad5 (30-34). To this end our laboratory has developed the use of bi-specific fusion proteins that bind adenovirus fiber knob and our target of interest, in this case murine IL-2R. These adapters once conjugated to Ad5 have shown dramatically increased gene delivery to otherwise non-infectable cell types (Figure 1B). The aim of the current study was to generate a recombinant fusion protein capable of affecting association between the native CAR binding of the Ad5 fiber and IL-2R. We assembled the cDNA coding for the recombinant fusion protein, sCAR-mIL-2 (Figure 1A), consisting of the ectodomain of human CAR fused to murine interleukin 2 (mIL-2) via a short peptide linker, preceded by a six-histidine (6His) detection/purification tag, into the lentivirus plasmid pLVmnd-sCAR-mIL-2 and confirmed its construction by digest analysis and DNA sequencing. We then used this plasmid to generate lentivirus in HEK293T cells. Virus laden media was then used to make stable HEK293 cell lines producing sCAR-mIL-2 as a secreted protein. We selected for high expressing cells using puromycin selection, expanded the selected culture, and purified the fusion protein by immobilized metal-affinity chromatography from the culture medium.

Characterization of sCAR-mIL-2 adapter

To analyze sCAR-mIL-2 we performed a western blot assay on boiled samples of the purified protein. Probing the membranes with antibodies against CAR and mIL-2 (Figure 2) showed that all portions of the recombinant protein were detected. The

electrophoretic mobility of boiled sCAR-mIL-2 showed a molecular weight of approximately 58 kDa. This is higher than the predicted size of 44.6 kDa, but could be explained by glycosylation in mammalian cells, as has been noted previously (34).

Further, we analyzed our sCAR-mIL-2 fusion protein for retention of its individual components binding characteristics. To verify the sCAR portions binding to Ad5 knob, we performed an ELISA assay. Following immobilization of recombinant Ad5 knob or control protein BSA, varying concentrations of sCAR-mIL-2 were incubated and then probed with an anti-mIL-2 antibody (Figure 3A). These results show that our sCAR-mIL-2 fusion protein still retains efficient binding to the Ad5 knob. We then examined binding of sCAR-mIL-2 to murine IL-2R using a CTLL-2 cell proliferation assay. CTLL-2 cells are an ideal tool for analyzing interactions between IL-2 and its cognate receptor, IL-2R. These cells express high concentrations of IL-2R and are dependent upon signaling through IL-2R for survival and proliferation. This assay showed strong proliferation of CTLL-2 cells when presented with recombinant mIL-2 and sCAR-mIL-2 (Figure 3B). This response was also shown to be blockable following incubation of CTLL-2 cells with an anti-murine CD25 antibody (Figure 3C). This shows that our sCAR-mIL-2 fusion protein is not inducing proliferation through an alternative receptor but exploiting the target, IL-2R. Together, these results indicate that the sCAR-mIL-2 adapter protein was produced in the correct conformation and retains all Ad5 knob and murine IL-2R binding affinity.

SCAR-mIL-2 enhancement of Ad5-mediated gene transfer to murine T cell line

We next tested the capacity of sCAR-mIL-2 to target Ad5 vectors to cells expressing murine IL-2R in gene transfer experiments using the murine T cell line, CTLL-2. These cells were verified for positive expression of CD25 and a lack of CAR expression by flow cytometry (Figure 4). We incubated Ad5GL vector encoding firefly luciferase and GFP under the CMV promoter with different amounts of sCAR-mIL-2 and then used these complexes to transduce CTLL-2 cells. The sCAR-mIL-2 noticeably augmented GFP expression in transduced CTLL-2 cells (Figure 5A). Approximately 87% of CTLL-2 cells treated with sCAR-mIL-2-conjugated Ad5GL exhibited green fluorescence as compared to less than 9% for untargeted Ad5GL as measured by flow cytometry. Secondly, sCAR-mIL-2 enhanced luciferase gene expression in CTLL-2 cells with the Ad5GL vector roughly 9 fold when compared to Ad5GL alone (Figure 5B). These results show that Ad5GL complexed with the sCAR-mIL-2 adapter protein is extremely efficient at transducing murine IL-2R expressing CTLL-2 cells.

sCAR-mIL-2 mediated Ad transduction of primary murine T lymphocytes

To next analyze sCAR-mIL-2-mediated transduction of T lymphocytes, we decided to further analyze our sCAR-mIL-2 adapter in primary murine T lymphocytes as primary cells are frequently more difficult to infect than cell lines. We harvested primary murine T lymphocytes from spleens of C57BL/6J mice. Following isolation, we analyzed the ability of sCAR-mIL-2 to augment gene transduction of an Ad5 expressing GFP under the ubiquitin c promoter, Ad5UP-GFP. Forty-eight hours post infection, 1.4% of CD25 positive T lymphocytes showed GFP expression when infected with sCAR-mIL-2-conjugated Ad5UP-GFP compared to 0.046% with unconjugated Ad5UP-GFP, a 30-fold

enrichment in GFP expressing cells (Figure 6). Considering that only a small portion of these cells initially expressed CD25 we decided to analyze the ability of sCAR-mIL-2 mediated transduction in an activated T lymphocyte population. Following activation, we infected these activated murine T lymphocytes as we did their naive counterparts. These cells showed increased percentages of GFP positive cells following transduction with Ad5UP-GFP (1.2%) or sCAR-mIL-2-conjugated Ad5UP-GFP (5.1%), with sCAR-mIL-2 mediating a 4-fold increase in GFP positive cells (Figure 6).

DISCUSSION

Despite promising new therapies utilizing genetically modified T lymphocytes, the tools used to efficiently deliver genes to T lymphocytes have been limited. In this study we have shown that, through the utilization of an adapter based targeting strategy, Ad5 can be retargeted to the IL-2R on the cell surface of murine T lymphocytes. This strategy was initially chosen because it allows for a rapid exploration of targeting ligands that are not biologically compatible with adenovirus biology. Most secreted biological ligands are not compatible with adenovirus biology because they are folded and transported through the Golgi apparatus while adenovirus assembly occurs in the nucleus and cytoplasm. The adapter strategy allows construction of targeting moieties that

maintain their binding properties and allow for retargeting of adenovirus after conjugation (35).

Initial testing and analysis of our sCAR-mIL-2 adapter was performed in CTLL-2 murine T cell lines. This cell line was initially chosen because it is a gold standard for studying IL-2 interactions with its cognate receptor. This cell line, under stimulation from IL-2 signaling, proliferates in a dose-dependent manner (36, 37). As such we could utilize this cell line to analyze how binding to IL-2Receptor was affected in our sCAR-mIL-2 adapter. This experiment showed that sCAR-mIL-2 adapter was still effective in stimulating CTLL-2 proliferation (Figure 3B) and was IL-2R specific (Figure 3C). Interestingly, our data showed that when molecular weight was taken into consideration, our sCAR-mIL-2 adapter stimulated CTLL-2 cells just as effectively as recombinant mIL-2. These results regarding binding efficiency of sCAR-mIL-2 show that the adapter based strategy for retargeting adenovirus is amenable to cytokine based targeting.

The CTLL-2 cell line was also an ideal cell line for initial analysis of sCAR-mIL-2 mediated adenovirus transduction. The cell line showed no expression of CAR on its surface but showed very high levels of expression of CD25, a subunit of the high affinity IL-2R (Figure 4). Corresponding with this receptor expression, sCAR-mIL-2 mediated a roughly 10 fold increase in GFP expressing cells. Of note, both the luciferase and GFP expression reduce slightly at higher concentrations of sCAR-mIL-2. This could be due to saturation of available Ad5 fiber binding sites and thus excess sCAR-mIL-2 would compete with virus for available mIL-2R sites on the cell surface.

Following the encouraging results in CTLL-2 cells, we studied the ability of sCAR-mIL-2 adapter to mediate Ad5 gene transduction in primary murine T lymphocytes. An important difference at this stage was the utilization of a ubiquitin c promoter (21) in place of the CMV promoter analyzed in our CTLL-2 data. Wan et al. (38) and Hurez et al. (10) have both shown that within primary T cells CMV promoter expression is dependent on the activation status of the cell. We felt that this artifact may limit or inhibit our ability to analyze gene expression in primary T lymphocytes. For this reason we used the ubiquitin c promoter, which both of these groups have shown to provide good gene expression in both naive and activated T lymphocytes. Following primary T lymphocyte isolation from C57BL/6J mice, we transduced naive cells with adenovirus, Ad5UP-GFP, utilizing the same protocol developed for CTLL-2 cells. This experiment showed a modest increase of GFP expressing cells (1.6%) compared to Ad5UP-GFP alone (0.043%). Considering that small percentage of these T lymphocytes expressed CD25 before transduction, we were fairly optimistic in regards to this outcome. To increase our potential pool of targets we activated isolated primary murine T lymphocytes. Under these conditions, our sCAR-mIL-2 adapter showed a modest 4.6 fold increase in the percentage of GFP positive CD25 positive T lymphocytes.

Previous studies have also shown success in improving gene transduction in primary T lymphocytes. Wickham et al. showed that specifically targeting T cells was possible through the use of a chemically conjugated bi-specific antibody against CD3. This strategy allowed for increased transduction of Jurkat, Molt-3, and resting human T cells (16). Yotnda et al. applied a panel of fiber modified adenoviruses including type 5, type 11, type 35, RGD, and varying poly-lysine chains to variety of human T cell lines.

They showed that many of these non-CAR targeting moieties can increase adenovirus delivered GFP expression (13). They further showed that Ad5/35 could be utilized to infect EBV-specific cytotoxic T cells with a transduction efficiency of roughly 51-58% as measured by GFP expression (39). Schroers et al. has also shown that Ad5/35 can be utilized for increased transduction of Jurkat and PM-1 cell lines, and primary CD3+ T cells (12). Most recently Sengupta et al. utilized an Ad5 targeted to cell surface integrins through incorporation of an RGD motif into Ad5 fiber knob. Their study showed a 3-fold increase in luciferase gene transfer and a 2.6-fold increase in hexon in ex-vivo infected primary mouse T lymphocytes (15).

A variety of issues could be hampering gene transduction efficiency of our sCAR-mIL-2 adapter strategy and leading to the variation seen between the initial CTLL-2 model and our later primary murine T lymphocyte data. Colin et al. showed that adenovirus type 5 may have post-internalization defects in HSB-2 T cell line. These defects lead to normal levels of infection but drastically lower levels of gene transduction as the Ad5 was unable to escape the endosomal compartment (40). They hypothesized that the virus was being internalized through an alternative, clathrin-independent method, and that the virus was not able to promote escape in this pathway. Interestingly IL-2 has been shown to internalize in a clathrin-independent method as well (41). It is a possibility that our sCAR-mIL-2 conjugated Ad5 vector is being internalized through the IL-2Receptors pathway instead of through the virus's native pathway that utilizes integrin binding. It may be that this alternative internalization is not amenable to endosomal escape. If this indeed is the case, then alternative T lymphocyte targets may abrogate this issue. Alternative to post-internalization pathways, it may be possible to increase the

transduction efficiency through modification of our sCAR-mIL-2 adapter. We have previously shown that the utilization of a trimerization domain within the adapter allows for increased binding of the adapter with adenovirus and leads to further increases in gene transfer (32, 34, 42).

This study along with other recent studies analyzing adenovirus transduction of T lymphocytes show that although native human adenovirus is lacking transduction affinity, alternative targeting strategies can bypass this roadblock. We have demonstrated that new recombinant sCAR-mIL-2 fusion protein promotes IL-2R targeted gene transfer to murine T lymphocytes. These findings have broad application for the study of T cell biology and genetic modification of T cells for therapeutic use.

ACKNOWLEDGMENTS

I would like to thank Dr. Alexander Pereboev. Dr. Pereboev was instrumental in the planning and early stages of developing the sCAR-mIL-2. Dr. Pereboev passed away before this work was completed, he will be greatly missed. The adenovirus shuttle plasmid, pSH-UP-UP-GFP (R) was kindly provided by Dr. J. DeGregori (University of Colorado School of Medicine, Denver, CO, USA). The plasmids for the self-inactivating lentiviral vector were kindly provided by Dr. Justin Roth (University of Alabama at Birmingham, Birmingham, AL, USA). The rabbit polyclonal anti-CAR antibody was a kind gift of Dr. Hideyo Ugai (Washington University in St. Louis, St. Louis, MO, USA).

DISCLOSURE/DUALITY OF INTEREST

The authors declare no duality of interest.

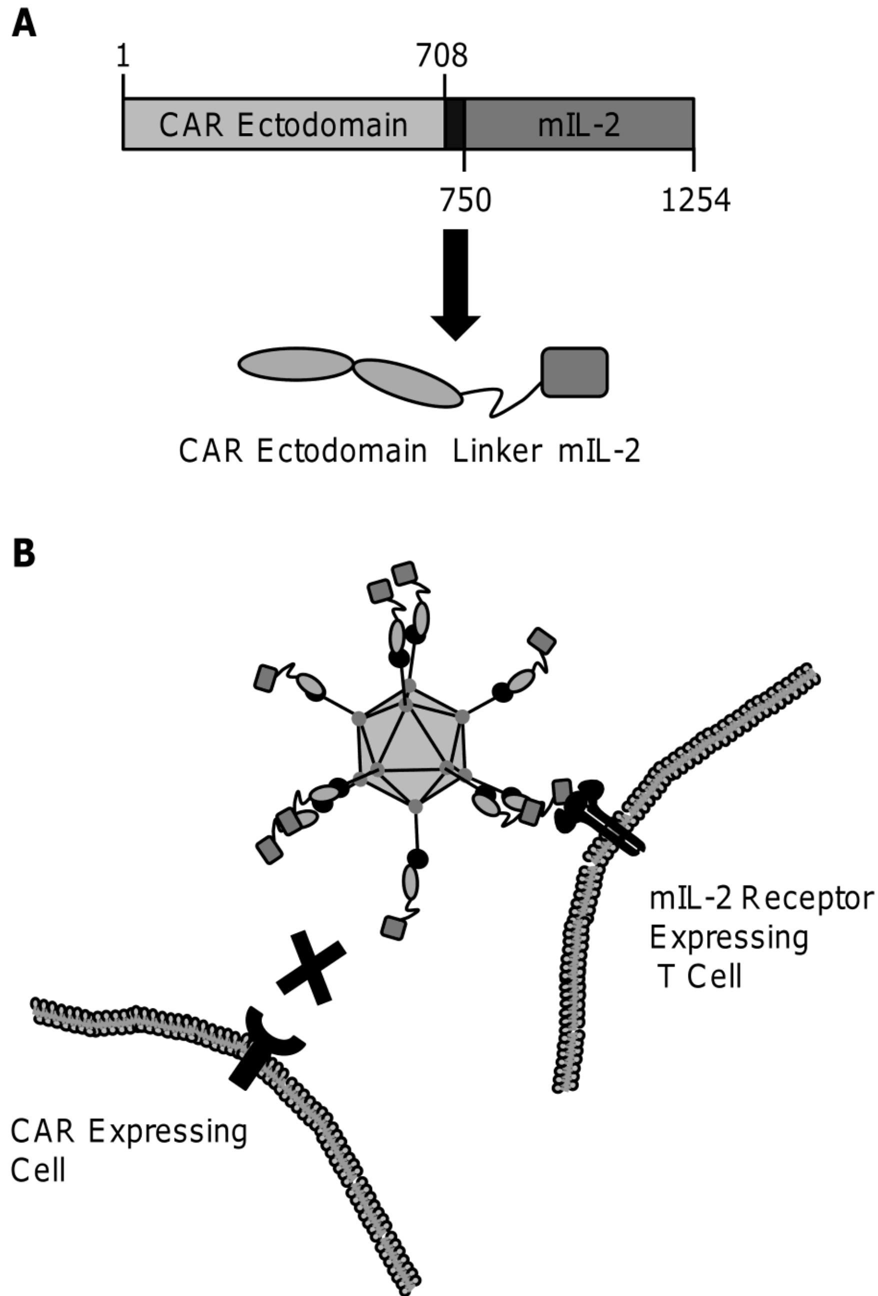


Figure 1: Schema for adapter based retargeting strategy for adenovirus vectors. Utilization of bi-specific fusion proteins allows for retargeting to a wide variety of cell surface exposed proteins. (A) The sCAR-mIL-2 adapter is composed of the exodomain of CAR fused to murine IL-2 through a short flexible linker containing a 6HIS tag. Scale is in nucleotides. (B) Once the adapter has been purified, adapter can be incubated with any Ad5 vector containing native fiber to retarget the virus to a non-CAR pathway, IL-2R.

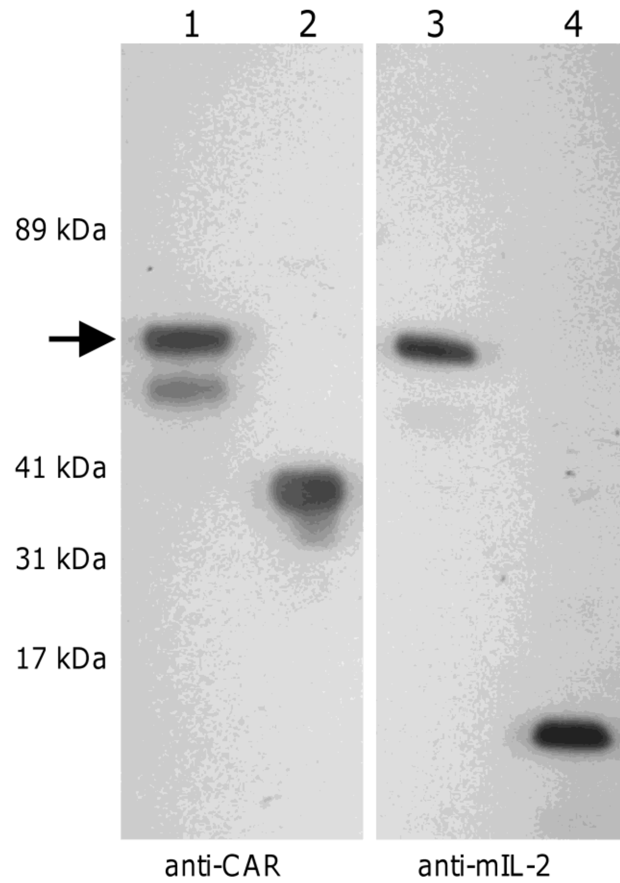


Figure 2: Western blot analysis of sCAR-mIL-2 adapter protein. Purified sCAR-mIL-2 was analyzed by western blot analysis for both sCAR (Lane 1) and mIL-2 (Lane 3) portions of the protein. Purified sCAR-6HIS (Lane 2) and recombinant mIL-2 (Lane 4) were used as positive control proteins.

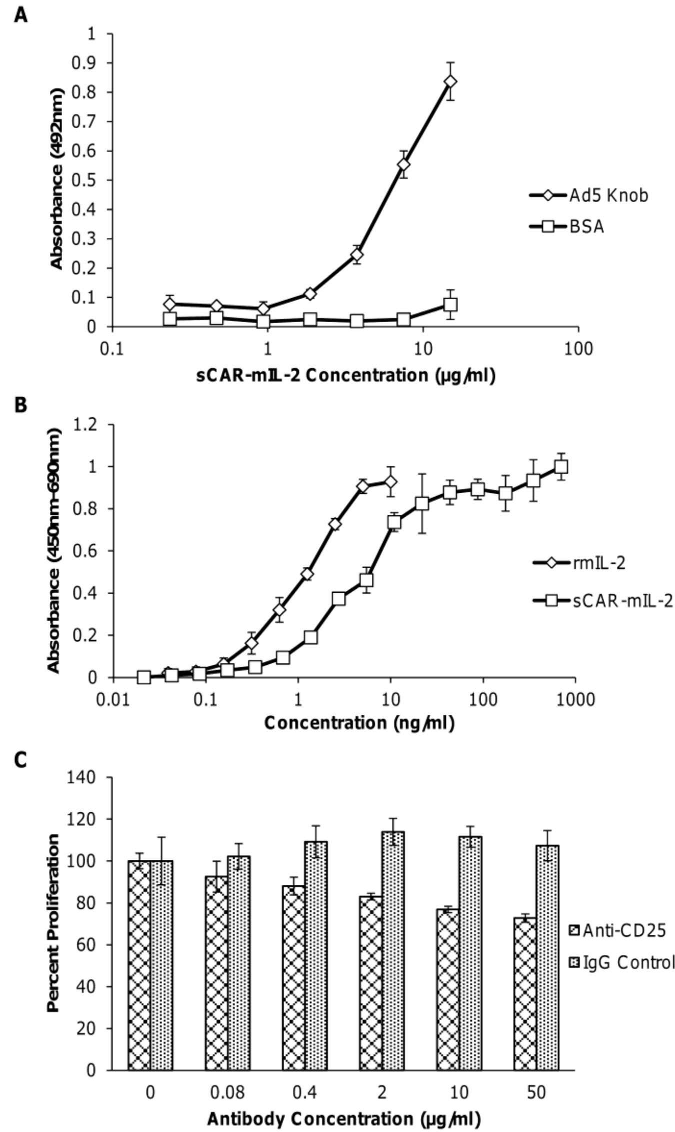


Figure 3: Analysis of sCAR-mIL-2 binding to Ad5 knob and murine IL-2R. (A) Purified sCAR-mIL-2 was analyzed for binding to recombinant Ad5 knob (diamond) or control protein, BSA (square), at varying concentrations by ELISA assay. (B) sCAR-mIL-2 affinity to murine IL-2R was analyzed by induced proliferation of CTLL-2 cells. CTLL-2 cells were incubated with varying concentrations of either sCAR-mIL-2 (square) or recombinant mIL-2 (diamond) for 48 hours. (C) sCAR-mIL-2 specificity to murine IL-2R was analyzed by blocking murine IL-2R. CTLL-2 cells were incubated with anti-mCD25 antibody (hash) or control IgG (dot) for 30 min before addition of sCAR-mIL-2 and incubated for 48 hours. After 48 hours, WST-1 reagent was added for 4 hours and cells were analyzed for proliferation.

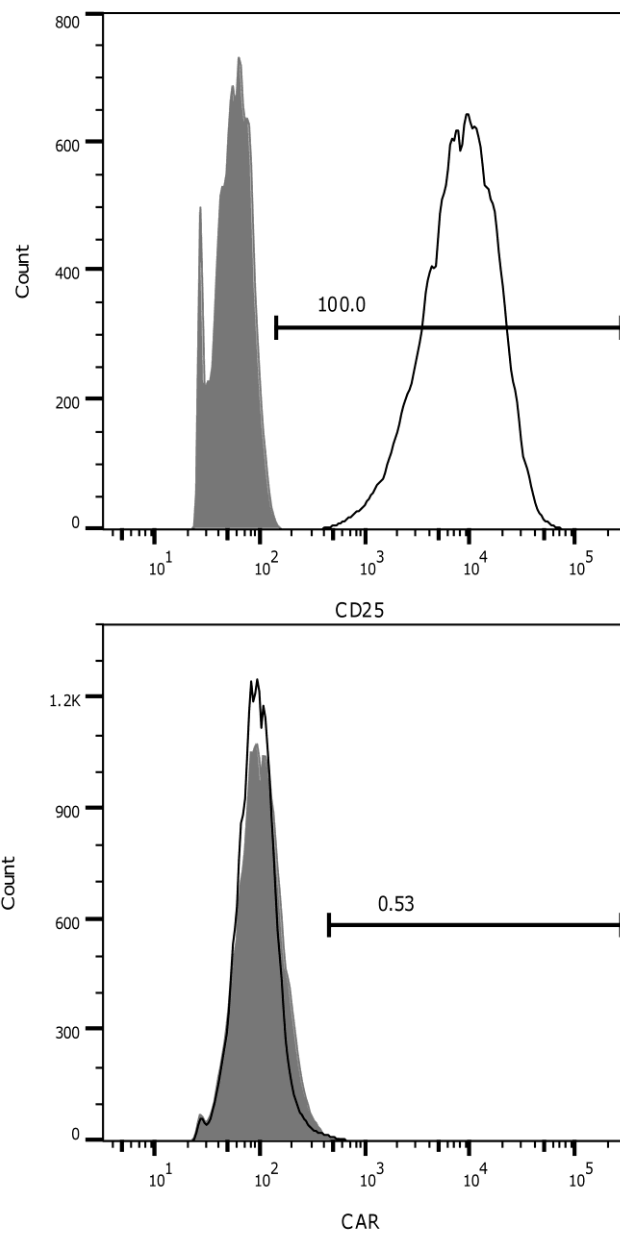


Figure 4: CTLL-2 receptor analysis by flow cytometry. CTLL-2 cells were analyzed for CAR and CD25 expression (black line) compared to isotype controls (shaded grey).

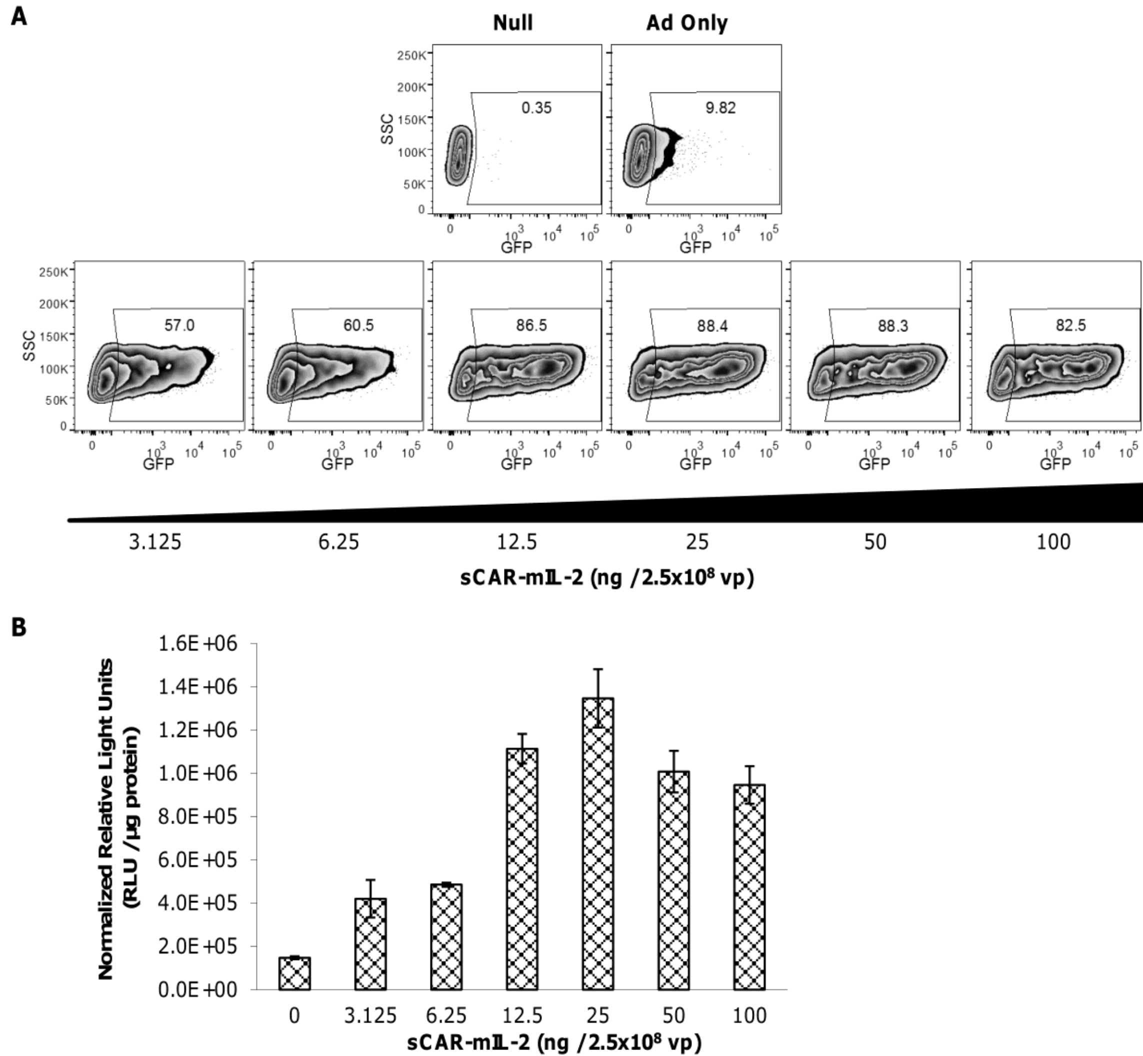
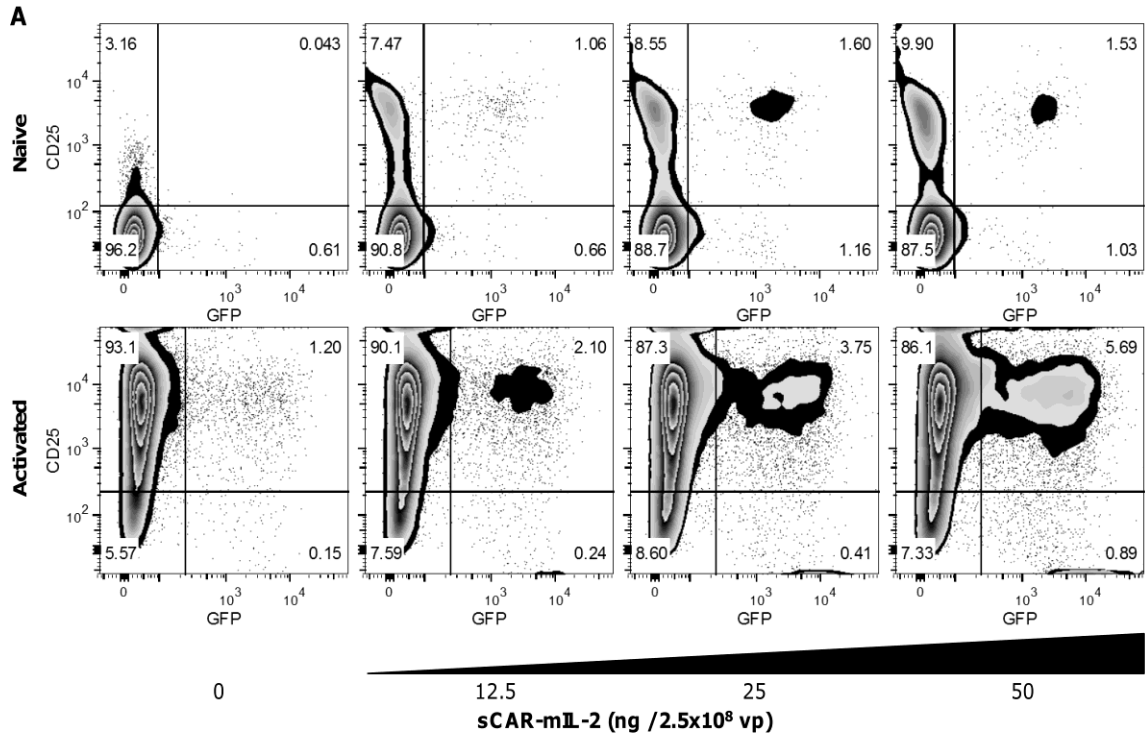


Figure 5: SCAR-mIL-2 promotes increased adenovirus transduction of CTLL-2 cells. Varying amounts of sCAR-mIL-2 were incubated with Ad5GL. Following incubation, CTLL-2 cells were infected with no virus, Ad5GL, or Ad5GL conjugated with sCAR-mIL-2 at 500 vp/cell. Twenty four hours post-infection, cells were measured for GFP expression (A) and luciferase activity (B).



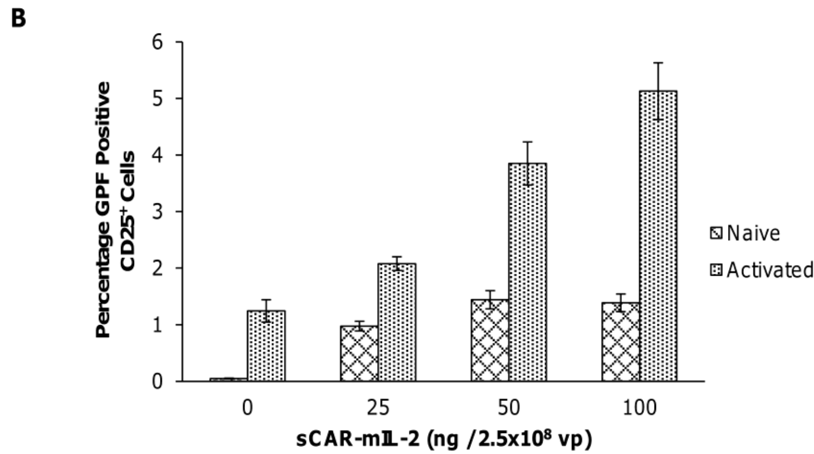


Figure 6: SCAR-mIL-2 promotes increased adenovirus transduction of naïve and activated primary murine T lymphocytes. Varying amounts of sCAR-mIL-2 were incubated with Ad5UP-GFP. Following incubation, naive or activated primary murine T lymphocytes were infected with no virus, Ad5UP-GFP, or Ad5UP-GFP conjugated with sCAR-mIL-2 at 500 vp/cell. Forty eight hours post-infection, cells were measured for GFP and CD25 expression by flow cytometry.

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CHAPTER IV

ADDITIONAL STUDIES

Murine T cell targeting

In addition to the data presented in the accepted article comprising chapter 3, additional experiments were conducted. While these experiments were not included in the published article, they do expand upon the studies performed and further elucidate the role of sCAR-mIL-2 mediated adenovirus infection.

SCAR-mIL-2 enhancement of Ad5-mediated gene transfer to murine T cell line

In addition to using the ubiquitin C promoter in primary murine T cells, we analyzed this promoter's activity in CTLL-2 cells (Figure 1). For this study, we conjugated varying amounts of sCAR-mIL-2 to AdUP-GFP. After incubating the adenovirus with sCAR-mIL-2 for 30 minutes, we infected CTLL-2 cells at 200 virus particle per cell for 1 hour. Following infection, the cells were washed and given fresh growth media supplemented with recombinant murine IL-2. Twenty-four hours post infection, we analyzed the cells by flow cytometry for GFP expression. Utilizing AdUP-GFP alone, we were able to transduce 21.2% of the cell population. However, with the addition of sCAR-mIL-2, 40.2% of the cells expressed GFP. The percentage of GFP positive cells increase with increasing amounts of sCAR-mIL-2 to a maximum gene transduction of 58.8%. Concentrations beyond this point decreased the transduction efficiency.

Receptor analysis of primary murine T lymphocytes

Following isolation of murine primary mouse T lymphocytes, we analyzed the cells for their purity. For this analysis we labeled the cells with fluorescent antibodies against CD4 and CD8 cell markers and measured the percentage of each population by flow cytometry (Figure 2A). We also analyzed the cells for expression of CD25, a subunit of the high affinity IL-2 receptor (Figure 2B). Analysis of freshly isolated primary murine T lymphocytes showed that 5.99% of these cells were $CD25^+$. Following activation, as described in chapter 3, we analyzed the percentage of $CD25^+$ cells. Post-activation, 95.1% of the cultured primary T cells were $CD25^+$ (Figure 2C).

SCAR-mIL-2 mediated gene transduction of isolated splenocytes

In addition to analyzing sCAR-mIL-2 mediated gene transduction in primary T lymphocytes, we wanted to study sCAR-mIL-2 in a heterogeneous cell population. For this study we choose to infect isolated murine splenocytes, as it provided a mix of immune cells found in close proximity with T lymphocytes and would help us elucidate how efficiently sCAR-mIL-2 could target IL-2 receptor positive cells. We harvested and pooled spleens from 8 week old, female, C57BL/6J mice and dissociated them into a single cell suspension of splenocytes. Half of the splenocytes were activated via the addition of concanavalin A (ConA) to the media for 12 hours, while the other half were not. We then infected these naïve or activated splenocytes with either AdUP-GFP or AdUP-GFP conjugated to varying amounts of sCAR-mIL-2 at 500 virus particles per

cell. Twenty-four hours post infection, cells were analyzed by flow cytometry for CD4, CD8, CD25, and GFP expression.

After infection with AdUP-GFP alone, 0.45% of naïve splenocytes were $CD25^+$, GFP^+. However, AdUP-GFP conjugated to sCAR-mIL-2 showed an increase in gene transduction, with 1.29% of naïve splenocytes being $CD25^+$, GFP^+ (Figure 3A). After infection with AdUP-GFP alone, 0.82% of ConA treated splenocytes were $CD25^+$, GFP^+. SCAR-mIL-2 mediated an increase in gene transduction, with 4.15% of ConA treated splenocytes expressing both CD25 and GFP. This was a 4.8 fold increase in the percentage of GFP positive cells.

Additionally, we analyzed the transduction of both CD4 and CD8 expressing T cells within the isolated splenocytes. Within the naïve $CD4^+$ T cells, 3.81% were GFP^+. In addition, if we looked at those cells which were $CD4^+$ GFP^+, 53.2% were $CD25^+$. Within the activated $CD4^+$ T cells, the percentage of transduction increased to 7.26% and of those GFP^+ cells 88.3% were $CD25^+$ (Figure 3B). The $CD8^+$ population showed lower transduction, with only 1.9% of activated $CD8^+$ T cells expressing GFP. However, of those $CD8^+$ T cells transduced, 93.2% were $CD25^+$ (Figure 3C). These experiments further support that sCAR-mIL-2 can mediate selective gene transduction of IL-2 receptor positive cells within a mixed culture.

Development of a human T cell targeting adapter

An issue with utilization use of sCAR-mIL-2 is that it is restricted to cells expressing mouse IL-2 receptor. Understanding this issue, we developed a human IL-2 version of our targeting adapter. Utilizing human IL-2 should enable us to analyze

targeting in both mouse and human models (117,118). The adapter, sCAR-hIL-2, will also be translatable to clinical applications.

SCAR-hIL-2 was produced in a similar manner to the previous adapter described. The adapter consists of the ectodomain of human CAR fused to human interleukin 2 (hIL-2) via a short peptide linker containing a 6His tag. Following insertion into a lentivirus expression plasmid, pLVmnd-sCAR-hIL-2 was confirmed by digest analysis and DNA sequencing. We used this plasmid to generate lentivirus in HEK293T cells. Virus laden media was then used to make stable HEK293 cell lines producing sCAR-hIL-2 as a secreted protein. We selected for high expressing cells using puromycin selection, expanded the selected culture, and purified the fusion protein by immobilized metal-affinity chromatography from the culture medium.

SCAR-hIL-2 mediated CTLL-2 proliferation

Following purification, we analyzed sCAR-hIL-2 for its ability to bind to IL-2R. Since human IL-2 can bind to and signal through mouse IL-2R, we utilized the CTLL-2 cell line. As described previously, CTLL-2 cells express IL-2R and proliferate in response to IL-2 signaling. As, such they are an excellent model for studying IL-2 interactions. CTLL-2 cells were plated in flat bottom 96-well tissue culture plates at $1x10^4$ cells per well in quadruplicate. Varying concentrations of either purified sCAR-hIL-2 or recombinant hIL-2 were added to each well and incubated at 37 °C in a 5% CO_2 atmosphere for 48 hours. 10 µL of WST-1 reagent was added to each well and incubated for 4 hours. Plates were then read at an absorbance of 450 nm, with a reference wavelength of 690 nm, according to manufacturer’s instructions. Analysis of this

experiment showed that the CTLL-2 cells proliferate well in response to recombinant hIL-2. In addition, sCAR-hIL-2 promotes roughly the same amount of proliferation in CTLL-2 cells (Figure 4A). This data shows that sCAR-hIL-2, like sCAR-mIL-2, retains its native binding affinity to IL-2R.

Additionally, we analyzed whether this proliferation was in response to IL-2R signaling. In vitro proliferation blocking assays were performed in a similar fashion to the proliferation assays previously described. $1x10^4$ CTLL-2 cells were plated in 96 well flat bottom tissue culture plates in quadruplicate and incubated with varying concentrations of rat monoclonal anti-murine CD25 antibody PC61 or rat monoclonal IgG control antibody for 30 minutes at 37 °C in a 5% CO_2 atmosphere. SCAR-hIL-2 was then added to the media at a concentration that gave roughly 85% maximum proliferation and incubated for 48 hours. 10 μL of WST-1 reagent was added to each well and incubated for 4 hours. Plates were then read at an absorbance 450 nm, with a reference wavelength of 690 nm, according to manufacturer's instructions. This blocking study showed that as we increase the amount of anti-CD25 antibody we see a dose dependent decrease in the amount of proliferation seen. The addition of a control antibody had no effect on proliferation (Figure 4B). Interestingly, the percent of proliferation blocked is greater than that seen with sCAR-mIL-2.

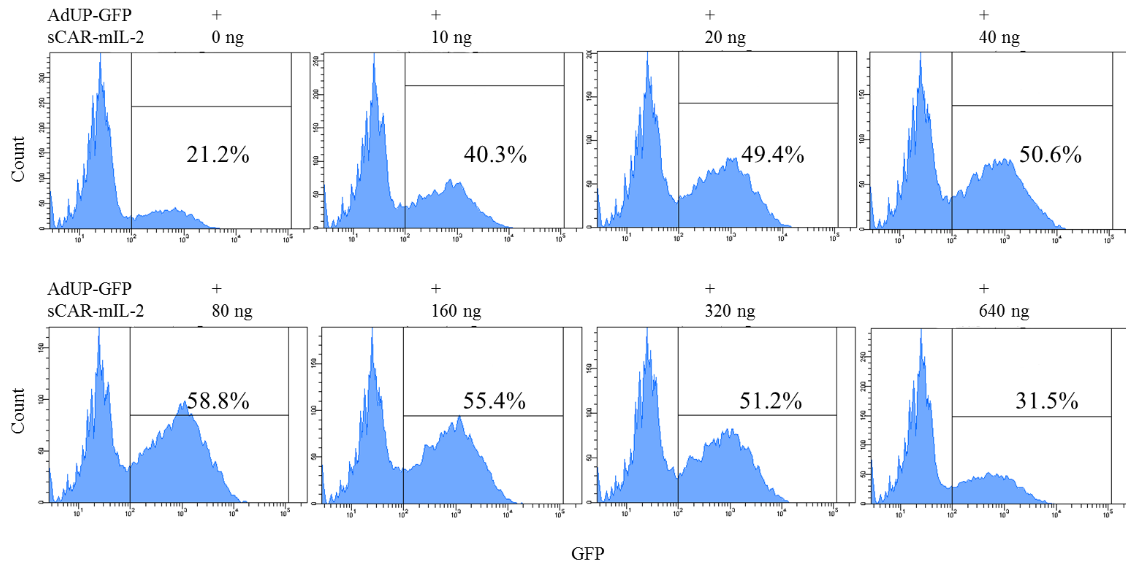

Figure 1: SCAR-mIL-2 conjugated AdUP-GFP provides increased transduction of CTLL-2 cells. Varying amounts of sCAR-mIL-2 were incubated with AdUP-GFP. Following incubation, CTLL-2 cells were infected with no virus, AdUP-GFP, or AdUP-GFP conjugated with sCAR-mIL-2 at 500 vp/cell. Twenty four hours post-infection, cells were measured for GFP expression.

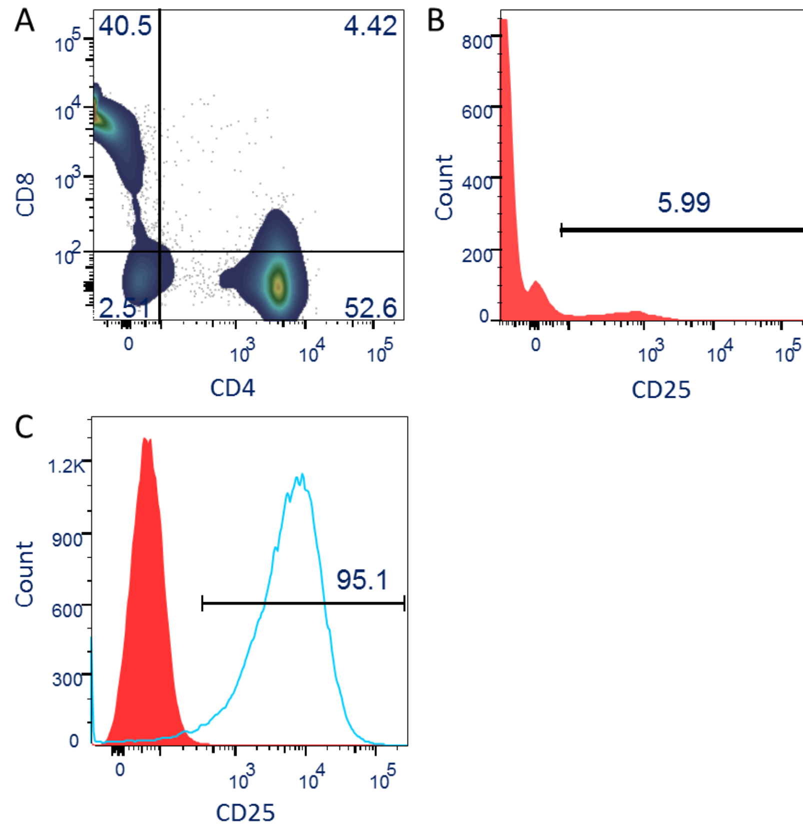

Figure 2: Receptor analysis of primary murine T cells. Following purification, primary T cells were analyzed by flow cytometry for (A) CD4, CD8, and (B) CD25 expression. (C) Post-activation, primary cells were reanalyzed by flow cytometry for CD25 expression.

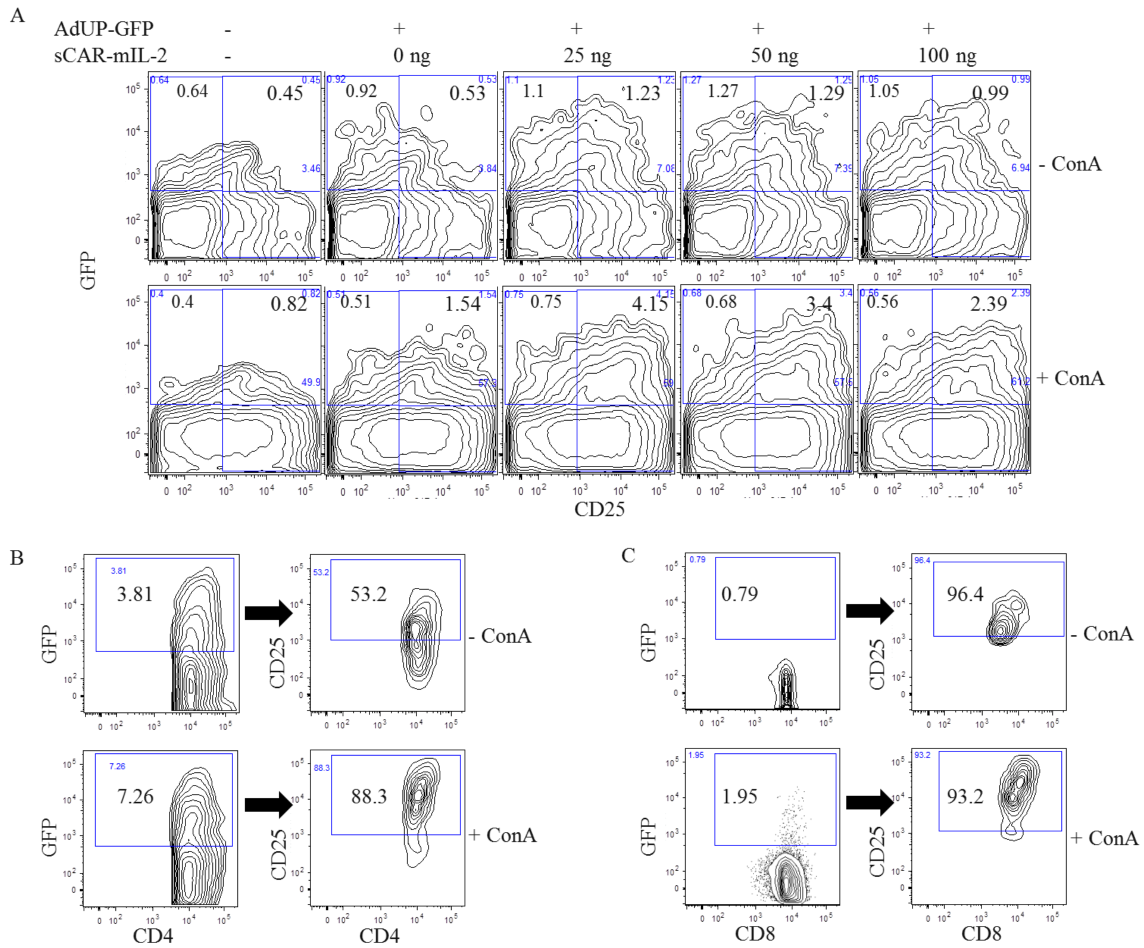

Figure 3: SCAR-mIL-2 promotes increased adenovirus transduction of naïve and ConA treated splenocytes. Varying amounts of sCAR-mIL-2 were incubated with AdUP-GFP. Following incubation, naive or ConA treated splenocytes were infected with no virus, AdUP-GFP, or AdUP-GFP conjugated with sCAR-mIL-2 at 500 vp/cell. (A) Twenty-four hours post-infection, cells were measured for GFP and CD25 expression by flow cytometry. (B) $CD4^+$ and (C) $CD8^+$ cell populations from splenocytes treated with AdUP-GFP and 25ng of sCAR-mIL-2 were analyzed for GFP expression. Those GFP^+ cells were further analyzed for CD25 expression.

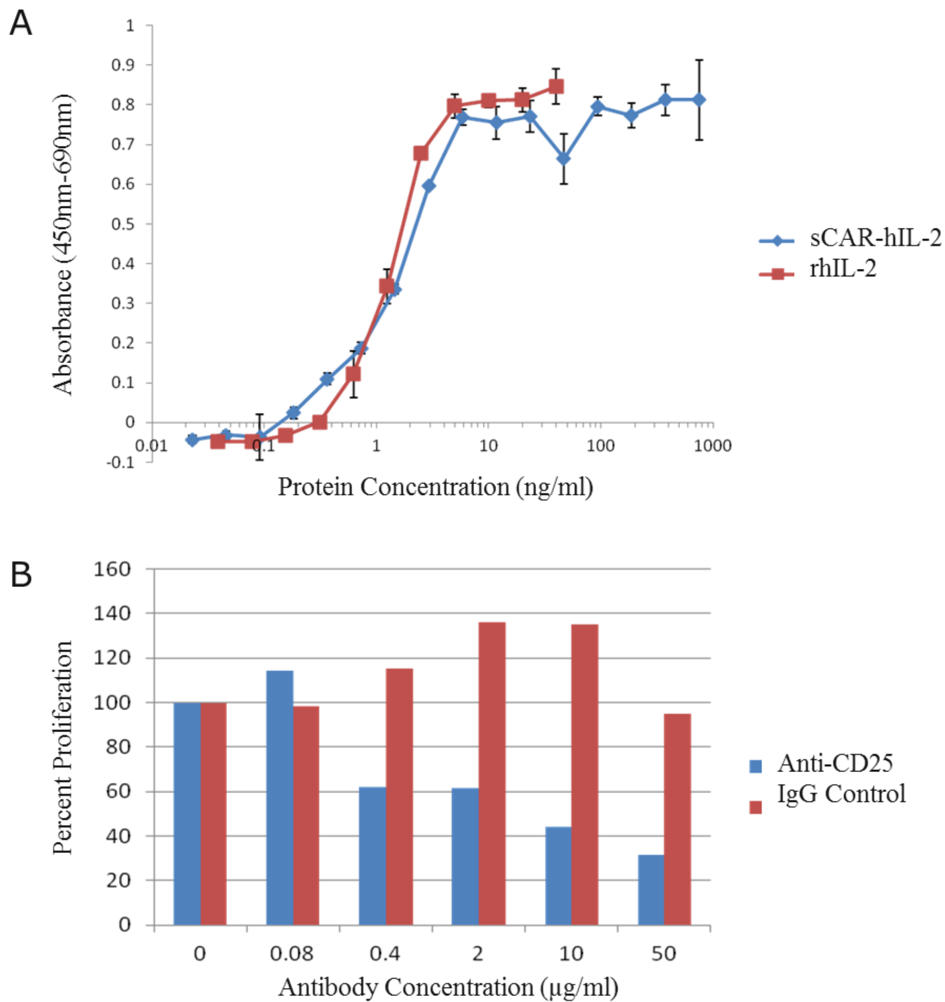

Figure 4: Analysis of sCAR-hIL-2 binding to murine IL-2R. (A) SCAR-hIL-2 affinity to murine IL-2R was analyzed by induced proliferation of CTLL-2 cells. CTLL-2 cells were incubated with varying concentrations of either sCAR-hIL-2 (blue) or recombinant hIL-2 (red) for 48 hours. (B) SCAR-hIL-2 specificity to murine IL-2R was analyzed by blocking murine IL-2R. CTLL-2 cells were incubated with anti-mCD25 antibody (blue) or control IgG (red) for 30 min before addition of sCAR-hIL-2 and incubated for 48 hours. After 48 hours, WST-1 reagent was added for 4 hours and cells were analyzed for proliferation.

SUMMARY AND GENERAL DISCUSSION

Genetically modified T cells are being used in a variety of experimental treatments for a multitude of diseases including autoimmune disorders, cancer, infectious disease, and transplant rejection (119-121). Currently the ability to genetically modify T cells is reliant upon only a few vector technologies, the most common being retrovirus and lentivirus vectors. However, issues with these vector technologies hamper their full utilization (124-126).

Adenovirus vectors have been utilized in more clinical trials than other vectors and their use has been shown to be safe. However, one obstacle with utilizing adenovirus type 5 is native tropism. As explained in chapter 1, human adenovirus type 5 utilizes the coxsackie virus and adenovirus receptor (CAR) as a cellular receptor (9). The effectiveness of adenovirus therapies thus depends upon the expression of CAR. This tropism is useful for a variety of therapeutic application but T cell lineages show low to no CAR expression (127, 128). Considering the lack of CAR expression in T lymphocytes, we looked at alternative targets to replace the native cellular receptor. Interleukin 2 receptor (IL-2R) embodied a potentially advantageous candidate. IL-2R is highly expressed in activated $CD4^+$ and $CD8^+$ T lymphocyte as well as $CD4^+Foxp3^+$ regulatory T lymphocytes (129), all of which are potential therapeutic targets for gene therapy interventions.

The review reported in chapter 2 discusses a variety of strategies that have been developed to circumvent CAR deficiency. Of the strategies available, a genetic retargeting strategy has a lot of advantages. However, any incorporated ligand must be compatible with adenovirus biology. As such, targeting ligands can fail to incorporate due to size restrictions, incorrect folding of the final fiber protein, and other issues regarding the interaction of two proteins that have evolved separately. In addition, adenovirus biology restricts the types of proteins that can be utilized as targeting ligands. Since adenovirus replicate and assemble in the nucleus, all ligands must be compatible with this reducing environment. An alternative strategy utilizes a separate bi-specific protein that bridges the gap between the adenovirus fiber knob domain and an alternative cellular receptor. This strategy relies upon a recombinant protein comprised of a soluble CAR fused to a flexible linker which is in turn fused to a targeting ligand (130,131). Of importance to our strategy of utilizing IL-2 as a targeting ligand, the adapter strategy is compatible with ligands that rely upon disulphide bonds for proper folding and function.

The studies presented in chapters 3 and 4 demonstrate that adenovirus can be retargeted to T lymphocytes through a bi-specific adapter. Though few reports have been published on the topic, previous studies have shown success in improving adenovirus mediated gene transduction in T cells. Wickham et al. utilized a chemically conjugated bi-specific antibody against CD3 and showed that an increase in transduction of Jurkat, Molt-3, and resting human T cells (132). Other groups have studied a variety of fiber modified adenoviruses including type 5, type 11, type 35, RGD, and varying poly-lysine chains in a panel of human T cell lines and EBV-specific cytotoxic T cells. (133,134). Schroers et al. also studied Ad5/35 and demonstrated that this virus had increased

transduction of Jurkat and PM-1 cell lines, and primary CD3$^+$ T cells (135). Most recently, Ad5-RGD, an Ad5 targeted to cell surface integrins through incorporation of an RGD motif into Ad5 fiber knob, was shown to provide a 3-fold increase in luciferase gene transfer and a 2.6-fold increase in hexon in ex vivo infected primary mouse T lymphocytes (136).

In our study we developed a recombinant protein comprised of a soluble CAR fused to murine interleukin 2 through a flexible linker. As shown, this adapter retained the original binding characteristics of its constituents when fused together. We then analyzed its ability to increase adenovirus infectivity of T lymphocytes. The adenovirus vector, Ad5-GL, which expresses both GFP and luciferase under CMV promoters, was chosen for this experiment. This vector allowed us to analyze both the percentage of cells infected and the amount of recombinant protein expressed. For our initial study we utilized the CTLL-2 murine T cell line. This cell line is easier to maintain than primary cells and had the appropriate surface receptor profile (CD25$^+$, CAR$^-$) for analyzing our sCAR-mIL-2 adapter. The sCAR-mIL-2 adapter provided a 10-fold increase in adenovirus mediated luciferase and GFP gene expression, with 88% of CTLL-2 cells expressing GFP.

Though the CTLL-2 cell line is a useful analog for T cell studies, cell lines can carry a variety of cellular differences when compared to their primary counterparts. Progressing into primary T lymphocytes, we decided to change the promoter used to drive gene expression. This decision was made on previous studies showing that gene expression through the CMV promoter is influenced by activation status of the cells. As such, we chose to utilize the ubiquitin C promoter to drive our gene expression. Studies

have shown that the ubiquitin promoter has excellent gene expression profiles in T lymphocytes and does not respond to activation status of the cells (128,137). Following the development of our AdUP-GFP vector we analyzed this vector in CTLL-2 cells. In this experiment we conjugated AdUP-GFP with varying amounts of sCAR-mIL-2 and infected CTLL-2 cells. In this study, sCAR-mIL-2 provided an increase in gene transduction. Of note, the percentage of cells transduced by AdUP-GFP is much greater than the percentages seen using our previous Ad5-GL vector. The difference in GFP expression is probably due to the varying promoter strengths in T cells and helps support our decision to switch from CMV to ubiquitin c promoters. This infection is not CAR mediated since these cells were negative for CAR expression based on flow cytometry. Interestingly, Colin et al. described similar results, demonstrating CAR independent endocytosis in certain T cell lines (138).

Naïve and activated primary T lymphocytes were also infected with adenovirus conjugated to sCAR-mIL-2. In naïve T cells, sACR-mIL-2 mediated a 37-fold increase in gene transduction, infecting 15.7% of $CD25^+$ T cells. In activated T cells, sCAR-mIL-2 mediated a 4.6-fold increase in gene transduction, infecting 6.2% of $CD25^+$ T cells. While the fold-increase in gene expression was lower in activated T cells, the overall percentage of transduced T cells was higher, compared to the naïve counterpart. This is expected since the pool of targetable cells was much larger. However, this data hints at the possibility that there may be differences in the infectivity of naïve versus activated T cells. Overall, this study showed that a retargeting strategy utilizing a recombinant adapter approach can increase adenovirus mediated gene transduction in T lymphocytes.

In addition to purified primary T cell infection studies we analyzed the ability of sCAR-mIL-2 to mediate infection of IL-2R positive cells in a mixed culture. In the non-activated splenocytes, sCAR-mIL-2 conjugated Ad transduced 17.4% of $CD25^+$ cells, with 1.29% of all cells being GFP^+ $CD25^+$. In ConA treated splenocytes, sCAR-mIL-2 conjugated Ad transduced 7% of $CD25^+$ cells, with 4.15% of all cells being GFP^+ $CD25^+$. Interestingly, the percentages of $CD25^+$ cells transduced was very similar to the same experiment performed in purified primary mouse T cells, as described in chapter 3. Also seen is the same decrease in the percentage of transduced $CD25^+$ cells after activation. This pattern shows that even in a mixed culture infection, the activation status of the T cells may be interfering with Ad infection.

Future studies will focus on two aspects of the reported studies. Targeting activated T lymphocytes resulted in a small but consistent increase in transduction efficiency. Of note, some studies have shown that adenovirus may fail to escape the endosome when internalized through an alternative, clathrin-independent method (138). The variation in infectivity between the CTLL-2 cell line and activated primary cells may be explained by variations in this internalization pathway. As such, targeting a receptor that utilizes a different internalization pathway may yield increased transduction efficiency. In addition, we may target a non-internalizing receptor and rely up the native adenovirus internalization pathway.

Secondly, the fold change in transduction efficiency between naïve and activated T lymphocytes was intriguing. In non-activated primary T cells, the increase in transduction efficiency was 37-fold. However, the fold increase in transduction decreased to 4.6-fold in activated primary T cells. Since the majority of $CD25^+$ T cells in a naïve

population are regulatory T cells, this may point to a preferential infectivity of this cell population. Further studies on infectivity of purified regulatory T cells should be progressed, as an adenovirus vector targeting regulatory T cells would have great potential in a variety of autoimmune and cancer therapies.

Lastly, we are developing the use of an IL-2 receptor targeted adenovirus for human therapies. Initial studies were progressed using a mouse IL-2 receptor targeted adenovirus. Unfortunately mouse IL-2 does not bind to the human IL-2 receptor with high affinity (117,118). To remedy this issue we developed a human IL-2R targeting adapter, sCAR-hIL-2. This adapter was shown to retain its IL-2 receptor binding in CTLL-2 cells. Though more studies are needed to fully analyze sCAR-hIL-2, our goal is to develop this adapter into a reagent usable in regulatory T cell and adoptive T cell therapies.

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APPENDIX A

INSTITUTIONAL REVIEW BOARD FOR HUMAN USE DESIGNATION OF NOT HUMAN SUBJECTS RESEARCH

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Review Board for Human Use

DATE: 8/17/10

MEMORANDUM

TO: Matthew Beatty
Principal Investigator

FROM: Sheila Moore, CIP
Director, UAB OIRB

RE: Request for Determination—Human Subjects Research
IRB Protocol #N100813001 – Adapter Based Strategies for Adenovirus Vector Retargeting to T Lymphocytes

An IRB Member has reviewed your application for Designation of Not Human Subjects Research for above referenced proposal.

The reviewer has determined that this proposal is **not** subject to FDA regulations and is **not** Human Subjects Research. Note that any changes to the project should be resubmitted to the Office of the IRB for determination.

SM/cro

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APPENDIX B

ANIMAL STUDIES COMMITTEE APPROVAL OF PROTOCOL FOR EXPERIMENTS URILIZING ANIMALS

Animal Studies Committee

Animal Welfare Assurance # A-3381-01
May 20, 2013

To: David Curiel, MD, Ph.D. Campus Box 8224
From: Dana R. Abendschein, Ph.D., Chairman
Subject: Approval of Protocol for Experiments Utilizing Animals

Agency/Title: NATIONAL INSTITUTES OF HEALTH
Monitoring of Advanced Virotherapy for Ovarian Cancer

The Animal Studies Committee of Washington University has reviewed this protocol for the use of animals in conjunction with the research project named above. Federal laws and guidelines require that institutional animal care and use committees review ongoing projects annually. For the first two years after initial approval of this protocol, you will be asked to submit an annual progress report describing any changes in this protocol.

Approval Date: 02/11/2011 **Approval No. 20100254**
Expiration Date: 02/11/2014
Amendment (#1, Year 3) approved 05/20/2013
SPECIES:
Mouse

It is your responsibility to see that all persons who use animals under your direction understand and follow the approved protocol. Should it become necessary to make substantial changes in this protocol, you must submit a new protocol. **Failure to comply with these provisions can result in suspension of the research.**

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