2017-12-01

Capacity of Human Immunodeficiency Virus Targeting Chimeric Antigen Receptor T Cells to Eliminate Follicular Dendritic Cells Bearing Human Immunodeficiency Virus Immune Complexes

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A dissertation submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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ABSTRACT

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An important obstacle to a functional cure for HIV/AIDS is the persistence of viral reservoirs found throughout the body in various cells and tissues. Reservoirs can be latently infected cells, or in the case of follicular dendritic cells (FDC), non-infected cells that trap infectious virus on their surface through immune complexes (HIV-IC). Although several strategies have been employed to target and eliminate viral reservoirs, they are short-lived and ineffective. In an attempt to provide a long-term approach, chimeric antigen receptor T (CAR-T) cells were designed to eliminate native HIV on FDCs. Although effective at eliminating HIV-infected cells, and halting spreading infection, their ability to eliminate the viral reservoir found on (FDCs) remains unclear. We used a novel second-generation CAR-T cell expressing domains 1 and 2 of CD4 followed by the mannose binding lectin (MBL) to allow recognition of native HIV envelope (Env) to determine the capacity to respond to the viral reservoir found on FDCs. We employed a novel fluorescent lysis assay, the Carboxyfluorescein succinimidyl ester (CFSE) release assay, as well as flow cytometric based assays to detect functional CAR-T activation through IFN-γ production and CD107a (i.e., LAMP1) membrane accumulation to test cytolytic capacity and functional activation of CD4-MBL CAR-T cells, respectively. We demonstrated their efficacy at eliminating HIV-infected cells or cells expressing gp160. However, these CAR-T cells were unable to lyse cells bearing surface bound HIV-IC. We found that failed lysis was not a unique feature of a resistant target, but a limitation in the CAR-T recognition elements. CAR-T cells were inactive in the presence of free HIV or in the presence of concentrated, immobilized virus. Further experiments determined that in addition to gp120 recognition by the CAR-T, the adhesion molecule ICAM-1 was necessary for efficient CAR-T cell killing of HIV-infected cells. CAR-T cell activity and killing were inhibited in the presence of ICAM-1 blocking antibody. These results suggest that other factors, such as adhesion molecules, play a vital role in CAR-T responses to HIV-infected cells. In addition, our findings highlighted the necessity to consider all models of HIV reservoirs, including FDCs, when evaluating therapeutic efficacy.

Key words: HIV, chimeric antigen receptor, follicular dendritic cells

ACKNOWLEDGEMENTS

This work could not have been accomplished without the invaluable contributions of several people. I would like to genuinely thank Dr. Gregory F. Burton for his patience and insight in helping me in this journey. I also would like to thank my graduate committee members Drs. Barry Willardson, Bradford Berges, Josh Andersen, and Steven Graves who provided unique insight and timely direction during the course of my degree. I want to thank the members of the Burton lab that assisted me by completing projects, discussing ideas, and solidifying theories. I am thankful for our collaborators Drs. Edward Berger, Elizabeth Connick, and Pamela Skinner for their assistance and guidance during our collaboration.

I am grateful to BYU and the Chemistry and Biochemistry department for allowing me to pursue my degree here. I also thank the staff members Peggy Erickson, Sue Mortensen, Anna Kennington, and Janet Fonoimoana whose willingness to assist in every possible way did not go unnoticed.

I am indebted to my family who assisted me in countless ways to achieve this goal. I am grateful to my parents who taught me integrity. Their example is manifest in all of the work conducted herein. My wife Chelsea demonstrated profound love, support, and dedication while I pursued my graduate degree, and she humbly took on several added responsibilities to help our three children Kaleb, Lexie, and Noah. I am grateful to my children for their love and understanding. They have only known me as a student.

I am grateful to my Father in Heaven who has assisted me in several ways along this path. His gentle nudges throughout this journey made this possible.

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LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
BCMA	B cell maturation antigen
CAR	Chimeric antigen receptor
CCR5	C-C motif chemokine receptor 5
CD	Cluster of differentiation
CR2	Complement receptor 2
CRS	Cytokine release syndrome
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCL12	C-X-C motif chemokine ligand 12
CXCL13	C-X-C motif chemokine ligand 13
CXCR4	C-X-C motif chemokine receptor 4
CXCR5	C-X-C motif chemokine receptor 5
DNA	Deoxyribonucleic acid
Env	Envelope protein (HIV)
ESCRT	Endosomal-sorting complexes required for transport
FcγRIIb	Fc gamma receptor type IIb
FDC	Follicular dendritic cells
FITC	Fluorescein isothiocyanate
Gag	group specific antigen- p55
gp	glycoprotein

HIV	Human Immunodeficiency Virus, Type 1
HLA	Human leukocyte antigen
ICAM-1	Intercellular adhesion molecule 1
ICOS	Inducible co-stimulatory molecule
IFN-γ	Interferon gamma
IgSF	Immunoglobulin superfamily
IL-	Interleukin
IN	Integrase protein (HIV)
IS	Immune synapse
LAMP-1	Lysosomal associated membrane protein 1
LFA-1	Lymphocyte function associated antigen 1
LTi	Lymphoid tissue inducer
LTR	Long terminal repeat (HIV)
LTαβ2	Lymphotoxin alpha 1 beta 2
LTβR	Lymphotoxin beta receptor
MA	Matrix protein (HIV)
MBL	Mannose binding lectin
Mfge8	Milk fat globule epidermal growth factor 8
MHC	Major histocompatibility complex
NC	Nucleocapsid protein (HIV)
Nef	Negative regulatory factor protein (HIV)
NF-κB	Nuclear factor of kappa light chain enhancer of activated B cells
NFAT	Nuclear factor of activated T cells

p24	Capsid protein (HIV)
PBMC	peripheral blood mononuclear cells
PD-1	Program death receptor 1
PDGFRβ	Platelet-derived growth factor receptor beta
PKC	Protein kinase c
PR	Protease protein (HIV)
PV	Pemphigus vulgaris
RNA	Ribonucleic acids
scFv	single chain variable fragment
SMAC	supramolecular activation cluster
TCR	T cell receptor
TFH	T follicular helper
TNFR1	Tumor necrosis factor receptor 1
TNFRSF	Tumor necrosis factor receptor superfamily
TNP	2,4,6-trinitrophenyl
Vif	Viral infectivity factor protein (HIV)
β2M	β-2 microglobulin

Chapter 1 Literature review

1.1 Human Immunodeficiency Virus

1.1.1 Structure of mature virus

Human Immunodeficiency Virus, type 1 (HIV) is a retrovirus from the lentivirus family [1-3]. The mature virion consists of glycosylated envelope (Env) receptors gp120/gp41 [4] embedded in a lipid bilayer derived from the producing cell during the viral budding process [5]. The virion has a diameter of approximately 120 nm. Internally, major structural proteins derived from the group specific antigen (*gag*) gene consist of the matrix (MA), p17, capsid (p24), nucleocapsid (NC), and p6 proteins [6-8]. Gag (p55) exists initially as one polypeptide in the immature virion and assists in viral assembly. The HIV *pol gene encodes enzymes needed for infection and replication including integrase (IN), protease (PR) and Reverse Transcriptase (RT).* The HIV protease cleaves Gag p55 into the four proteins mentioned above [6]. MA protein associates with the lipid bilayer and provides structural integrity to the virus particle [9]. The core protein p24 forms the internal shell around viral RNA [10, 11]. NC assists in binding viral RNA [12]. HIV p6 binds viral accessory proteins, and is also required for budding [13, 14]. Within the shell of the capsid protein are two identical viral RNA copies [15, 16], the tRNA primer [17], and viral accessory proteins, viral protein R (Vpr) [18], protease (PR) [19, 20], integrase (IN) [21], viral infectivity factor (Vif) [22], and negative regulatory factor (Nef) [23].

1.1.2 Infection

Initial studies into HIV entry found that this virus targets the CD4 receptor on host cells [24, 25]. Later it was determined that CXC- and CC- chemokine receptors CXCR4 [26] or CCR5 [27] were also necessary for HIV infection. Upon interaction of the HIV receptor gp120 with

CD4, a conformational change occurs [28] that induces the interaction with the co-receptor [29]. Upon interaction with the co-receptor, gp41 inserts an ectodomain into the lipid bilayer of the target cell [30, 31]. A conformational change occurs in gp41 which allows for fusion between the virion and the cell [32]. The capsid penetrates into the cell, and within the cytosol, full-length viral RNA is reverse transcribed and carried into the nucleus where it is integrated into the host genome by IN [33].

1.1.3 Replication

Replication is defined as transcription and translation of virally encoded genes/proteins leading to the generation of progeny virions. HIV utilizes a number of host transcription factors [34, 35], as well as viral factors that enhance viral replication [36, 37].

As Gag accumulates within the cell, it interacts with the host membrane [38, 39]. This process is ordered and initiates viral budding [6, 40]. Because uncleaved Gag not only accumulates at the intracellular plasma membrane but also contains binding sites for viral accessory proteins and viral RNA, viral assembly is Gag dependent [41, 42]. Env can also interact with Gag as well [43, 44], although this is not a requirement for Env incorporation [45]. Once budding is initiated, the host's endosomal-sorting complexes required for transport (ESCRT) pathway completes the budding process [46, 47]. Interestingly, this is the same pathway utilized by T cells to release T cell receptors within an immunological synapse [48].

1.1.4 HIV transmission

To enhance infection of budding virions, cell-cell transmission occurs [49], termed virological synapses [50]. It was shown that static cultures contained more infected cells than

cultures that were gently shaken [51]. This same study also demonstrated that very few cells were infected with HIV when separated from HIV-infected cells via a Transwell™ membrane; however, the removal of the membrane permitted infection of nearly all the cells [51].

The virological synapse closely resembles the immune synapse as described in section 1.4. Virological synapses assemble in a way that causes CD4 and the co-receptors CXCR4 and CCR5 to accumulate in a centralized location [52]. Instead of peptide-MHC complex recognition, the T cell recognizes viral gp120. Intriguingly, CD4 and the co-receptors CXCR4 and CCR5 are found within both the immune synapse and the virological synapse [53]. In addition to receptors for HIV recognition, adhesion molecules play a role in the virological synapse [54, 55]. Adhesion molecules, such as lymphocyte function associated antigen 1 (LFA-1), and intercellular adhesion molecule 1 (ICAM-1) form a ring around the concentrated CD4, co-receptor center [52]. This ring may assist in the transfer of HIV particles into acceptor cells through endocytotic vesicles [56-58], similar to those found with the immune synapse [48]. The virological synapse may also assist in infection with multiple HIV particles [59]. In addition to viral transmission through the formation of the virological synapse, HIV can also be transmitted from cells bearing surface-bound, native HIV, such as follicular dendritic cells (FDCs) [60].

1.2 Follicular Dendritic Cells (FDCs)

FDCs are unique cells found in secondary lymphoid tissues. Within these lymphoid tissues, FDCs are located in specialized compartments called follicles. When these follicular compartments become activated, they are referred to as germinal centers (GC) [61]. The main purpose of the GC is to provide an environment that allows for the humoral immune response to develop upon antigen recognition. FDCs contribute to this process through a number of key factors

including antigen trapping [62], providing survival and activation signals to surrounding B and T cells [63, 64], and through antigen-independent mechanisms [65].

1.2.1 Development and maturation

FDCs arise from perivascular precursor cells found throughout the body [66]. The precursor cells express platelet-derived growth factor receptor β (PDGFRβ) and milk fat globule epidermal growth factor 8 (Mfge8). Moreover, these precursor cells can exist outside secondary lymphoid tissue. The precursors differentiate into pre-FDCs via lymphotoxin alpha 1 beta 2 (LTαβ2) produced by B cells and Lymphoid Tissue Inducer (LTi) generating cells. Additional signaling from B cells, through tumor necrosis factor receptor 1 (TNFR1) and lymphotoxin beta receptor (LTβR) on the pre-FDCs, induces further differentiation into mature FDCs. Fully differentiated FDCs retain TNFR1 and LTβR, but no longer express PDGFRβ. Other factors may also contribute to maturation of FDCs, as signaling through TNFR1 and LTβR *in vitro* did not recapitulate the mature phenotype. However, depletion of FDCs is quickly achieved by blocking signaling through LTβR [67].

1.2.2 Characteristic phenotype

Mature FDCs, as the name implies, express intricate dendritic processes that can be characterized as thin and elongated, beaded, or an intermediate form [68]. The dendritic processes assist in the presentation of native antigen to B cells [69]. In addition to the dendritic processes, Chen et al., also demonstrated the structure of FDCs in mice [70]. Using transmission electron microscopy, they found that FDCs contain irregular nuclei and minimal intracellular organelles. They also found that FDCs were able to retain trapped antigen for long periods, but did not

internalize it. Another striking feature of FDCs is that they are post-mitotic and resistant to irradiation

1.2.3 Immune complex trapping

Injection of antigen(s) into immune animals results in the almost spontaneous generation of immune complexes comprised of the injected antigen, and specific antibody and/or complement proteins [72]. FDCs express receptors that recognize immune complexes through antibody and complement proteins bound to native antigen. These include: type II Fc gamma receptor, (FcγRIIb or CD32), Fcε Receptor II or CD23, and Complement Receptors 1 (CD35) and 2 long isoform (CD21L). Each plays an important role in antigen retention on FDCs as well as assisting in the GC reaction, the series of events following antigen trapping on FDCs.

Although CD32 is considered a low-affinity receptor for antigen binding, FDCs are capable of retaining antigen for many months [62, 71, 72]. This retention is thought to occur through multi-point attachment of immune complexes to the FDC [73]. Thus, because the immune complexed antigen consists of several antibodies, one antigen may be bound to FDCs via engagement of several CD32 and/or complement (i.e., CD21) receptors. In addition, FDC-CD32 receptor signaling induces additional expression of CD32 that may further enhance long-term antigen trapping. Long-term antigen retention on FDCs persists for many months and even years. [62, 71]

1.2.4 FDCs and HIV

During early stages of HIV infection, HIV is found within GCs [74]. In untreated individuals, as many as 5×10^{10} virions can be found bound to FDCs [75]. HIV capitalizes on the

unique nature of the GC to assist in disease progression. Because of strict selection requirements for cell admission, very few CD8 effector cells are able to enter the GC [76]. As a result of limited entry, HIV-specific cytotoxic T cells, are secluded from the GC. Although $CD8^+$ T cells have limited access, HIV-permissive $CD4^+$ T cells are actively recruited within the GC. Thus, the GC serves as a sanctuary site for ongoing HIV disease progression.

HIV trapping on FDCs also plays an important role in viral pathogenesis. Work done by Heath, et. al., showed that HIV can be bound to FDCs with both neutralizing and non-neutralizing antibodies [60]. The neutralizing antibodies, in the absence of FDCs, prevented HIV infection of T cells. However, when the virus was trapped on FDCs with neutralizing antibodies, it remained infectious [60]. Even the addition of a thousand-fold excess of neutralizing antibody could not prevent infection of $CD4^+$ T cells. Later studies showed that virus bound to FDCs remained infectious for at least one month *in vitro* [72, 77], and at least 22 months in HIV-infected patients [71]. Based on these results, it appears that antibody plays an important role in HIV trapping on FDCs.

However, contradictory studies have shown that other mechanisms appear to be involved in HIV trapping on FDCs [78]. For example, work done by Ho et al., [79] showed that complement binding is the major contributor to HIV trapping on FDCs. Using a mouse model, they showed that mice lacking CR2, or pretreatment of wild-type mice with blocking antibodies to CR2 were able to decrease HIV binding to FDCs. Recent work showed that FDCs are able to trap HIV through complement and retain virus in recycling endosomes [78]. These endosomes were always associated with transferrin and were dependent on CD21. If decoy soluble CD21 was used, virus was eliminated from the cycling endosomes. Although several studies have attempted to determine which receptor is more important, each methodology used to isolate FDCs, or use of

cell lines, may uniquely contribute to the observed results. In addition, each lab utilized different experimental parameters to determine the importance of the receptors.

Whatever the mechanism of virus trapping, FDCs serve as a major reservoir for HIV. Work done by Keele et al., [71] showed that FDCs fulfill the criteria of true virus reservoirs and thus should be so classified. To be a reservoir a cell must maintain replication-competent forms of the virus and persist despite anti-retroviral treatment [80]. Keele's work showed that virus on FDCs remained infectious, as they were able to transfer infection by co-culture of FDCs with T cells [71]. Sequencing results also demonstrated the diversity of virus isolated from FDCs, including several drug-resistant mutants. In this same study, HIV-infected peripheral blood mononuclear cells (PBMCs), harvested at different time points (months) prior to death, contained virus that was found on FDCs obtained at autopsy some 22 months later [71].

In addition to HIV binding, FDCs contribute to disease progression through other mechanisms [60, 71, 78, 81-83]. For example, FDCs are able to recruit permissive $CD4^+$ T cells, called GC T cells or T follicular helper TFH, by secretion of the chemokine (C-X-C motif) ligand 13 (CXCL13) [81]. CXCL13 is a major factor for activated T cell trafficking into GCs, as only chemokine (C-X-C motif) receptor 5 (CXCR5) expressing T cells gain access to GCs [61]. In addition, FDCs are able to promote CXCR4 expression on GC T cells [81]; although the mechanism remains unclear, GC T cells have increased expression of CXCR4 [81]. As noted earlier, CXCR4 is important because it is a coreceptor for HIV infection in some strains of virus [26]. When GC T cells are activated, and have increased expression of CXCR4, X4 virus infection is heightened while R5 virus infection remains unchanged. Interestingly, CXCR4 is the chemokine receptor for (C-X-C motif) ligand 12 (CXCL12), which is not readily expressed in the light zone of GCs where FDCs reside [84]. Reason would suggest then that GC T cells would migrate out of

the GCs towards higher concentrations of CXCL12, such as found in the dark zone of GCs, or other anatomical regions. However, FDCs also induce expression of Regulators of G protein signaling (RGS) proteins 13 and 16 in GC T cells [82]. The expression of RGS13 and RGS16 effectively inhibit CXCL12 mediated migration, while having no effect on CXCL13 mediated migration required for access to GCs. These FDC-mediated events prevent GC T cell migration away from the FDC network that bears infectious HIV particles. As such, FDCs are able to contribute to HIV pathogenesis by: trapping infectious HIV on their surface for long periods of time, promoting permissive cell migration to the FDC reservoir, increasing coreceptor expression required for HIV infection, and inhibiting GC T cell migration from this environment.

Because of the large contribution of FDCs in HIV pathogenesis, they are an important target for cure strategies. However, because FDCs are not infected by HIV, but trap native HIV on their surface, a mechanism that targets native surface bound viral antigen(s) is necessary for elimination of the HIV reservoir on FDCs. One possibility for targeting FDC-trapped HIV and its antigens is through the design of chimeric antigen receptors (CARs) on lymphocytes capable of cytoxicity.

1.3 Chimeric Antigen Receptor T cells

Chimeric Antigen Receptor T (CAR-T) cells are T cells that express genetically engineered receptors [85]. Originally conceived and developed in the late 1980s [86], chimeric receptors were designed to bypass conventional requirements for T cell recognition and activation. Because T cells require antigen to be processed and presented as peptide fragments in the context of MHC molecules, experiments were designed to determine whether T cells could be engineered to recognize native antigen. This was accomplished by replacing the variable region of the T cell

receptor (TCR) with the variable region of an immunoglobulin gene targeting the organic molecule 2,4,6-trinitrophenyl (TNP). They showed that by switching the peptide recognition regions of the TCR with anti-TNP IgG, they were able to redirect T cells to target and lyse cells bearing TNP. In that same year, Becker et al. [87], produced an *in vivo* mouse model of a CAR targeting digoxin. They showed that their CAR-T cells developed and circulated as conventional T cells while maintaining specificity to the organic molecule digoxin.

Since the original experiments, others have enhanced CAR function with new features. Unlike the original constructs that simply replaced the variable regions of the TCR, these strategies completely omit the TCR all together [88]. Multiple revisions have resulted in the implementation of a nomenclature of 1st, 2nd, or 3rd generation CAR T cell designs. All generations of CARs contain an extracellular recognition domain, a transmembrane domain, and intracellular signaling proteins [89]. The major difference between the various generations is the incorporation of different intracellular signaling proteins. 1st generation CARs contain the CD3ζ signal molecule [86], 2nd generation CARs contain CD3ζ followed by one other co-stimulatory signaling protein (e.g., CD28) [90, 91], while 3rd generation CARs contain CD3ζ with multiple co-stimulatory signaling domains [92, 93], with co-stimulatory proteins including CD28 [94-96], 4-1BB [97, 98], ICOS [99], OX40 [99], and CD27 [100].

1.3.1 General structure of current CARs

Similar to the original CAR design, extracellular domains are developed to recognize a native protein or molecule of choice [88]. Unlike conventional T cells, recognition does not require the TCR and MHC molecules from antigen presenting cells (APCs). Indeed, CARs are designed to recognize the native conformation of the target rather than peptide fragments bound

to MHC receptors. Although other extracellular domains have been used [101, 102], recognition is usually achieved through the expression of a variable fragment of an antibody. These too have been engineered using a single chain polypeptide, rather than co-expression of the variable region of the heavy chain and variable region of the light chain [89]. In the CAR gene, both fragments are linked together with a peptide linker resulting in a single chain variable fragment (scFv) expression cassette [103, 104].

Between the extracellular recognition domain and the transmembrane domain is called the hinge region. The hinge is a peptide sequence that provides a spacer between the ectodomain of the scFv and the transmembrane region, and is a vital part to efficient CAR activity [105, 106]. For example, Guest et al., [105] demonstrated that CAR activation was enhanced or diminished depending on the length of the hinge. They tested four separate CAR T cells and found that all were functionally active against their targets, but two CARs had enhanced activity in the presence of a hinge while the other two CARs did not. CARs that had enhanced activity without a hinge bound the most distal regions of the target protein as determined by scFv binding-site determination [105].

The transmembrane domain is a structural component of the CAR construct. However, a few studies suggest that when 1st generation CARs were used, only the transmembrane domain of CD3ζ provided sufficient signaling to induce activation whereas transmembrane domains from other proteins did not [107]. This was thought to occur because 1) 1st generation CARs only contain CD3ζ signaling motifs without any other co-stimulatory signaling, and 2) the CAR was able to interact with the functional TCR to enhance signaling. Other transmembrane domains typically used are derived from CD4, CD8, and CD28 [89].

1.3.2 Current applications using CAR-T cell therapy

Many researchers have seen the potential of CAR-T cell therapy for treatment of a variety of diseases. Several designs are currently undergoing clinical trials for the treatment of hematologic malignancies [108]. In addition, research is being done to verify proof of concept of CAR-T cells targeting solid tumors [109], autoimmune diseases [110, 111], and infectious disease [112, 113].

The majority of all CAR-T cell therapies have been designed to target and eliminate non-solid tumors. To this end, the first CAR- T cell therapeutic received FDA approval. This therapeutic, Kymriah™ (tisagenlecleucel) is a 2nd generation CAR which recognizes CD19 and contains CD3ζ and 4-1 BB signaling domains [114]. CD19 is a lineage specific marker found on all B cells and thus provides a limited target approach. This anti-CD19 CAR effectively eliminates all $CD19^+$ B cells, both cancerous and noncancerous. Kymriah™ is designed to treat patients, up to 25 years of age, with B-cell precursor acute lymphoblastic leukemia (ALL). ALL represents roughly 25% of all pediatric cancers and is particularly difficult to treat if relapse has occurred, with a 5-year survival rate of less than 10% [115]. When Kymriah™ was used to treat relapsed patients, they were able to induce complete remission in 83% of patients [116].

Like Kymriah™, many CAR-T cell designs have been used to target CD19. Differences in endodomains of the CD19 CAR are the major differences among different CAR designs, although other considerations include dose [117-119], ratio of CD4+ to CD8+ transduced CARs [117, 120], and CD8+ T cell subset used [121], all with very similar efficacies. CD19-CAR-T cells are the most studied and utilized constructs to eliminate non-solid tumors, but other targets exist for CAR-T cell therapy, such as CD20 [122-124] CD22 [125], BCMA [126], CD123 [127], ROR-1[128], and NKG2D [129].

In addition to the application of CAR-T cells for treatment of non-solid tumors, others have attempted to tailor CAR-T cells to eliminate solid tumors [109]. However, the validity of this latter approach has proven less effective [130]. The prevailing challenges to target solid tumors may be due to limited unique tumor associated antigens (TAA) expressed by the tumor [109]; however, without a tumor specific target, off-tumor toxicities occur [131].

CAR-T cells have been tested to treat antibody mediated autoimmune disease [101]. In an attempt to treat pemphigus vulgaris (PV), a disease caused by autoantibodies to the keratinocyte adhesion protein Dsg3, a novel CAR was constructed. It expressed the first four domains of Dsg3, the CD8α transmembrane domain, and the 4-1BB and CD3ζ signaling domains. Using a xenograft model, they showed that this CAR was able to efficiently lyse cells expressing autoantibodies to Dsg3, while maintaining NK cells as well as keratinocytes. CAR-T cells were unresponsive in the presence of the antibody alone, suggesting a requirement for cell-cell contact for a CAR-T cell response.

1.3.3 CAR-T associated toxicity

The greatest concern to CAR-T cell therapy is the associated toxicity. The most prevalent forms of toxicity in patients undergoing CAR-T cell therapy are cytokine release syndrome (CRS) and neurotoxicity [132]. CRS, also known as a cytokine storm, is the result of dangerously elevated levels of cytokines in response to the CAR-T cell treatment [133, 134]. Although the major contributors of CRS are not well defined, it appears to be present in all effective CAR-T cell therapies to date [132]. Some of the elevated cytokines include: IL-6, IL-15, IL-2, IFN-γ, and TNF-α [132, 135]. These cytokine levels, all pro-inflammatory, can result in dangerous symptoms such as high fever, organ failure, and hypotension [132, 135]. The severity of CRS

appears to directly correlate with CAR-T cell expansion in the patient [134]. Neurotoxicity is often manifested in CAR-T cell therapy. This is considered as inflammation in the nervous system. Some of the manifestations of neurotoxicity are cerebral edema, delirium, seizures, and coma [118].

1.3.3.1 Approaches to overcome toxicity - inhibit cytokine function

Attempts to alleviate CRS and neurotoxicity have been reported [136]. Common remedies are to lower cytokine levels and administer immunosuppression reagents such as anti-inflammatory steroids [137]. During a pioneering clinical trial, a pediatric patient experienced CRS [138]. She had a fever of 40.7 °C, and was in the pediatric intensive care unit for nearly two weeks in an induced coma. It was then found that she had ~500-fold increase in serum IL-6 levels. She was then treated with tocilizumab, an anti-IL-6R monoclonal antibody. Within hours, her fever subsided. Since this case, tocilizumab has received FDA approval to treat CRS.

Despite the effective response to tocilizumab and other inhibitors of CRS, there is concern that aggressive treatment of CRS will result in depletion or inefficacy of CAR-T cells [139]. Therefore, caution has been taken to alleviate CRS, unless absolutely necessary. To understand the role of IL-6 in the response to CAR-T cell therapy, Singh *et al.,* [140] showed the origin of IL-6 production in CRS is derived from monocytes. They tested this using a xenograft model lacking monocytes but containing malignant human B cells and CAR-T cells. Interestingly, they were unable to detect IL-6 despite rapid clearance of the B cells. This led to two conclusions: tumor cells or CAR-T cells did not produce the reported IL-6, and the IL-6 had no effect on tumor elimination. They also found that IL-6 had no effect on CAR-T cell transcription or

cytotoxicity. Although they did not address proliferation of CAR-T cells, these results suggest that IL-6 inhibitors regimens should be considered for treatment of CRS.

1.3.3.2 Approaches to overcome toxicity - alternative CAR designs

Other approaches to overcome toxicity are in the design of the CAR-T cell themselves. Transient expression of a CAR through mRNA transfection has been reported [141]. Other strategies have focused on the CAR design such as CAR-T cells with an integrated suicide gene, dual signaling requirements, and universal CARs [142].

1.3.3.2.1 Suicide gene CAR

The suicide gene CARs contain an inducible caspase protein, in addition to a CAR [143]. The caspase requires homodimerization for activation of the caspase system to occur, and thus remains inactive until dimerization is achieved. Linked to the caspase gene is the FK506 binding protein, which is able to bind the chemical inducer of dimerization molecule AP20187 [143] or AP1903 [144]. Upon recognition of the small molecule, two FK506 binding proteins linked to caspase 9, bind the FK506 analog, resulting in dimerization. This dimerization activates the caspase cascade and leads to rapid apoptosis of the CAR T cell. This acts as a safety switch to quickly eliminate the CAR-T cells, preventing fatal CSR or neurotoxicity progression. The major caveat to this treatment is the CAR-T cell therapy is eliminated, and disease progression may continue if not fully eliminated prior to the suicide induction treatment.

1.3.3.2.2 Dual Signaling CAR

Another promising CAR design is one that requires dual signaling for CAR activation. This has taken on many variations [145] including, trans-signaling CARs [146], remote-control CARs [147], and inhibitory CARs [124]. Each utilizes a unique mechanism with the hope of eliminating the dangerous side effects of CRS and neurotoxicity. In each instance, two signals are required to activate the CAR.

In the case of the trans-signaling CAR, this is achieved by placing different signaling molecules on two separate scFv, thereby requiring both molecules to be recognized for CAR activation [146]. This not only increases specificity of the CAR, but also decreases the likelihood of on-target off-tumor effects.

In the remote-control CAR design, a scFv is linked to the co-stimulatory signaling domain followed by an FK binding protein, as in the caspase activating CAR mentioned above [147]. The CD3ζ and other co-stimulatory signaling domains are linked to a separate membrane bound protein and FK binding protein. Upon administration of the small molecule, the two polypeptides are linked together. If the scFv recognizes its target in the presence of the small molecule, all primary and co-stimulatory signaling proteins are activated, and cytolytic activity is induced. If the scFv recognizes the target antigen in the absence of the small molecule, the CAR is only able to signal through the co-stimulatory signal, and no CAR activation occurs. This allows for fine-tuning of CAR activation, simply by dose and timing administration of the small molecule.

Inhibitory CARs are another design proposed by Federov *et al.,* [148]. In an attempt to prevent off-target effects, and excessive activation of the CAR, they utilized a novel mechanism. Their CAR design utilizes two receptors: one for the tumor associated antigen, and the other for a non-tumor associated antigen. The tumor specific CAR contains the activation signaling domains

for cytolytic function. The non-tumor specific CAR binds to a target molecule that is not found on the tumor, but on other cells. The non-tumor CAR is the inhibitory CAR and contains inhibitory signaling domains such as CTLA-4 and PD-1. Upon recognition of the tumor antigen, the CAR becomes activated. However, if the cell recognizes both the tumor-associated antigen, and the non-tumor associated antigen, the CAR is inactive. This prevents lysis of non-tumor cells.

1.3.3.2.3 Universal CAR

Universal CAR-T cells are a novel way of overcoming CSR [149-152]. The universal CAR, similar to the remote-control CAR, utilizes a switch to induce activation. However, the switch does not bind at the level of the signaling domains, but is recognized by the CAR scFv. For example, universal CARs have scFv designed to recognize a FITC or neopeptide molecule. Because FITC is a bioorthogonal molecule, it is not found *in vivo*, eliminating activation in the absence of FITC. FITC conjugated molecules, such as antibodies or vitamins, bind to target cells. After this occurs, the CAR recognizes the FITC conjugated molecule and lyses the labeled cell. However, CAR activation does not occur when the FITC molecule is not bound to a target cell. Therefore, CAR mediated lysis is dependent on the bioavailability, dose administration, and binding affinity of the FITC-conjugated molecule. In order to stop a reaction, excess unconjugated FITC is administered, which will not bind to target cells [150]. This outcompetes the FITC-conjugated molecules, and inhibits CAR signaling.

Toxicity remains a significant barrier that must be overcome for effective CAR-T cell therapy. Improved understanding of CRS and its cause has led to current FDA approved therapies to alleviate the symptoms of CRS. In addition, current research to manipulate the CAR-T cell

design has led to the development of alternative safety mechanisms to prevent inherent complications associated with toxicity.

1.3.4 Failure of CAR-T cells in clinical trials

There are two major causes of failed CAR-T cell therapy: elimination of CAR-T cells, and down regulation of target antigen [124]. In a clinical trial to determine optimal dosing of CAR-T cells, those that received a sub-optimal dose of 2 x 10^5 CAR-T cells/kg body weight did not respond to the treatment [134]. Upon examination, it was found that CAR-T cells did not persist in these patients. In an attempt to circumvent this negative result, a higher dose of 2 x 10^7 CAR-T cells/kg was administered to these patients. All five patients that received the second dose did not manifest any persistence one day later. To explain this, they looked at cytotoxic T cell responses to the CAR-T cell and found that in all the cases (n=5), a cellular immune response had occurred. In 4 of the 5 patients, cytotoxic T cells were detected that recognized the murine sequence of the scFv. They concluded that the patient's own immune system had responded and eliminated the CAR-T cells, because of the foreign nature of the CAR gene. In other trials, they also found relapse in the presence of persistent CAR-T cells [153]; however, the cause of this failure resulted from the downregulation of the target tumor antigen. This decreased antigen expression on the tumor cells allowed efficient escape from the CAR-T cells.

1.3.5 Approaches to overcome CAR-T cell failure

In response to relapse, investigators have suggested several strategies. One important objective of CAR-T cell treatment is to reduce the immunogenicity of the CAR molecule(s). Because the CAR encoded foreign (i.e., xenogeneic) proteins, host cellular response may develop.

In an attempt to overcome this, Sommermyer, et al., developed a fully humanized anti-CD19 scFv [154]. They also tested the peptide linkers between various domains of the CAR for immunogenicity, and altered sequence where necessary. They were able to show improved function of the fully humanized CAR compared to the conventional mouse scFv.

Another obstacle associated with relapse is the down regulation of the target antigen. To overcome this, CARs have been designed to target multiple antigens [124]. One approach to address this issue is to express scFv proteins that recognize two distinct antigens. This dual targeting technique has been achieved using sequential administration of two separate CAR-T cells or by expressing two distinct scFv on a single CAR-T cell (dual CAR).

1.3.6 CAR-T cells for HIV Eradication

The first two CAR-T cell designs to target and eliminate HIV-infected cells were tested in 1994 by Roberts, et al. [102]. They termed their CAR-T cells "universal T cell receptors." The CARs were a first generation design consisting of the intracellular signaling domain of CD3ζ followed by the CD4 transmembrane domain. The extracellular recognition epitope for one CAR consisted of the full-length CD4 receptor. The other CAR design contained an scFv of the anti-gp41 monoclonal antibody 98.6, linked to the remaining heavy chain of the antibody. The inclusion of the constant region of the heavy chain allowed for the formation of dimers, and when assembled in this manner, resembled a membrane bound IgG molecule. CAR receptors were transduced in primary $CD8^+$ T cells. When CAR-T cells were co-cultured with target cells expressing HIV Env, elevated IL-2 secretion was detected. Additionally, CAR-T cells specifically lysed HIV-infected cells in the presence of serum from HIV-infected patients, while uninfected cells were not affected.

The promising preliminary data prompted a clinical trial that tested the efficacy of the CD4 CAR-T cells in HIV-infected patients [155]. The trial also tested whether IL-2 administration influenced the capability of CAR-T cells to decrease viral loads. The administration of the CAR-T cells resulted in an initial decrease in viral load among those not receiving IL-2. Administration of IL-2 caused an initial increase in viral load in the blood, as well as an increase in CD4+ T cells over the period of eight weeks. However, the change in viral load did not persist, and no significant decrease was detected over the course of the eight-week study, despite CAR-T cell persistence. Indeed, CAR-T cells were detected 16 weeks post infusion, accounting for up to 10% of all PBMCs in some patients. In an additional phase II clinical trial, using the same CAR-T cell construct, no change in plasma viral loads was detected over the course of 6 months [156]. In a follow-up study, 98% of samples tested contained CAR-T cells 11 years post infusion [157]. These studies demonstrated the persistence of CAR-T cells despite failed eradication of the virus.

Zhen et al., [158] utilized an identical CAR to that of the Roberts group in a unique way. In their work, they transduced hematopoetic stem/progenitor cells (HSPCs) with the CAR or what they termed the "triple CD4 ζ"- the original CD4 CAR with the addition of short hairpin RNA (shRNA) recognizing CCR5 and the long terminal repeat (LTR) of HIV. Using a humanized mouse model, Zhen found that the transduced HSPCs allowed for CAR expression in T cells as well as NK cells. CAR expression led to clearance of HIV from the blood, although the response varied between mice. The viral load correlated with the expansion of peripheral $CD4^{+}CD8^{+}$ T cells. Mice with prominent viral load suppression had the greatest expansion of CAR T cells, while those with a limited viral suppression had less expansion of CAR-T cells.

Recent CAR-T cell designs have incorporated modern neutralizing antibodies in their extracellular domain [158-163]. The most common neutralizing antibody is VRC01, which blocks

the CD4 binding site of gp120 [164]. The antibody is able to neutralize infection of roughly 90% of known HIV isolates [165]. Liu et al., [161] utilized a scFv derived from VRC01 as a 3rd generation CAR containing CD28, 4-1BB and CD3ζ signaling domains. In their studies, the VRC01 derived CAR was directly compared to a 3rd generation CAR that replaced the scFv molecule with domains 1 and 2 of CD4. The VRC01 CAR demonstrated greater potency than the CD4 CAR, at higher effector to target (E:T) ratios, but insignificant differences at lower E:T ratios. When reactivated latently infected cells from HIV-infected patients undergoing combined anti-retroviral therapy (cART) were used as targets, p24 production decreased in co-culture assays with the VRC01 CAR-T cells. These studies demonstrated the potential combination of a "shock and kill" strategy with CAR-T cells.

The most recent publication of an anti-HIV CAR to date has been the CD4-CAR. Starting from the original CD4-CAR, Leibman et al., [160] systematically optimized the CAR through intracellular domains, transmembrane domains, promoters, and vector choice. By doing so, their CD4 CAR was 50-fold more potent *in vitro* than the original CAR. In an *in vivo* humanized mouse model of HIV infection, their CD4 CAR expanded 389-fold in 22 days, while the original CD4-CAR only expanded 2-fold. In contrast to the work above by Liu et al., [161] the improved CD4-CAR outperformed CARs designed from scFv of neutralizing antibodies.

As apparent in the work done by Leibman, improvement of CAR cytoxocity and potency can be achieved through manipulation of the CAR construct. However, the cytotoxic activity of CAR-T cells requires the inherent capabilities of the CTL as no constructs to date express cytolytic enzymes such as perforin and granzyme. Thus, cytotoxicity is most likely achieved and enhanced by CAR-T cells through similar mechanisms as conventional CTLs, namely: antigen recognition (peptide-MHC of conventional CTLS as compared to native recognition through CAR), signaling

cascades (TCR and co-stimulatory receptors in conventional as compared to signaling molecules fused to antigen recognition in CAR molecules), and the formation of an immunological synapse.

1.4 Immunological Synapse

The immunological synapse (IS) is a direct interface that occurs between T cells and antigen presenting cells (APCs). This structural entity is vital for the communication and response of the immune system to a particular antigen [166]. IS formation results in cytokine release [167], cytotoxic cell-mediated killing [168], and antibody secretion [61]. Because of the vital nature of the IS, it is highly regulated. Multiple receptors play various roles in the formation of the IS including antigen recognition, co-stimulation or inhibition, and adhesion.

1.4.1 IS and antigen presentation

The purpose of the IS is to facilitate an immune response to antigen [169]. Antigen is "processed and presented" to T cells by APCs in a form that optimizes the interactions between these cells. When APCs process antigens, peptides are produced which in turn, become bound to major histocompatibility complexes I (MHC-I) or II (MHC-II) on the surface of the APC. Generally, exogenous antigens are endocytosed and become processed and presented to CD4 T cells on MHC II complexes. In contrast, antigens generated inside the APC are presented to CD8 T cells when associated with MHCI molecules. In addition to these two presentation pathways, some exogenous antigens are "cross-presented" on MHC-I complexes further enabling the interaction of antigen with the type of activated T cell (i.e., CD4 or CD8) that is most efficacious in protecting the host [170].

MHC-I receptors are highly expressed by all nucleated cells. They are composed of two proteins, human leukocyte antigen (HLA), and β-2 microglobulin (β2M). HLAs are divided into several major (A, B, and C) and minor (E, F, and G) classes. Each class has the ability to bind various types of peptides and contributes to antigen presentation. β2M assists in stabilization of the complex between peptide and HLA, but does not directly bind the peptide. This complex of peptide and MHC-I is recognized by T cell receptors (TCRs) and stabilized by the CD8 receptor. Upon recognition by a $CD8^+$ T cell, the APC can be targeted for cell-mediated cytoxicity. Because nearly all cell types express MHC-I, nearly all cells can serve as APCs to CD8+ T cells.

MHC-II molecules are expressed by professional APCs: macrophages, dendritic cells, and B cells, which both express MHC-II and co-stimulatory ligands. The receptors that bind peptides are HLA receptors of several classes, the most common of which are termed DP, DQ, and DR. The MHC-II receptor consists of two polypeptide chains, the α and β, which are always paired together. Peptide is bound in the cleft between the two chains. The main source of peptide comes from exogenous sources, internalized and digested in endosomes and lysosomes. T cells recognize MHC-II receptors through TCRs and an additional receptor called CD4. This recognition results in TCR signaling and is one of the major outcomes from the IS.

1.4.2 T cell co-stimulation

There are several signaling molecules found within the IS. Their main purpose is to provide additional signaling required for a response. As a highly regulated system, if only TCR signaling is present, T cells go into a state of anergy (i.e., specifically non-responsive) without co-stimulation (secondary, antigen-independent signaling required for cell activity) [171]. Thus, the signaling

from co-stimulation provides the necessary requirements for a complete response to antigen recognition.

Well studied signaling molecules come primarily from two families, the Ig superfamily (IgSF) such as CD28 and CD278, the inducible co-stimulator (ICOS), and the tumor necrosis factor receptor superfamily (TNFRSF) including OX40 (CD134) and 4-1BB (CD137) [172]. CD28 on T cells directly interacts with CD80 (B7-1) and CD86 (B7-2) on APCs [173]. Upon interaction with cognate receptors in an IS, signaling cascades instigated by CD28 result in activation of Akt and protein kinase C (PKC) [174]. PKC leads to activation of nuclear factor kappa light chain enhancer of activated B cells (NF-κB). Additionally, CD28 is able to induce activation of nuclear factor of activated T cells (NFAT) [175]. In contrast to CD28, ICOS signaling requires ICOS-ligand (ICOSL) [176]. Signaling resulting from ICOS and CD28 crosslinking is nearly identical. However, ICOS signaling promotes greater Akt signaling [177]. Surprisingly, ICOS does not induce IL-2 secretion, whereas CD28 promotes IL-2 secretion [178].

As mentioned above, members of the TNFR family also contribute to co-stimulation. 4-1BB, like OX40, is an inducible co-receptor. Activation requires interaction with their respective ligands: 4-1BB ligand (4-1BBL) [179, 180], or OX40 ligand (OX40L) on APCs. Signaling involves the TNFR associated factor 2 (TRAF2) [181]. Activation of TFAF2 leads to activation of Inhibitor of kappa B kinase (IKK) and subsequent activation of NF-κB. This results in inhibition of pro-apoptotic signals, and promotion of IL-2 production.

1.4.3 Negative regulation in the synapse

In addition to co-stimulation, there are receptors within the IS that assist in negative regulation. These receptors are termed repressors and are induced upon TCR activation.

Moreover, the inhibitory receptors can be detected at high levels within hours of TCR signaling [182]. Repressors are responsible for downregulating signaling through various mechanisms. First, upon activation, they recruit tyrosine phosphatases to remove phosphates from activated motifs on CD3, thereby inhibiting signaling through the TCR [183]. In the case of cytotoxic T-lymphocyte associated protein 4 (CTLA-4), it competes with CD28 for interaction with CD80 and CD86 [184, 185]. Because CTLA-4 has a higher affinity for these receptors than does CD28, it sequesters these co-stimulation ligands and prevents CD28 signaling. In addition to the intrinsic signaling, CTLA-4 may also inhibit co-stimulation through an extrinsic mechanism [186]. As CD80 and CD86 bind to CTLA-4, they are internalized within the T cell, where CD80 and CD86 are targeted for destruction, whereas CTLA-4 is recycled to the membrane. This recycling quickly removes costimulatory ligands from the APC.

Another regulator is programmed cell death 1 (PD-1). This protein does not compete with CD28 for CD80 and CD86, but rather binds to PD-1 ligand 1 and 2 (PD-L1 and PD-L2, respectively) found on APCs [187, 188]. PD-1 also contains domains that recruit tyrosine phosphatases, which inhibit TCR signaling [189]. However, an alternative mechanism has also been proposed for PD-1. In a mouse model for type I diabetes, as well as hypersensitivity, it was shown that PD-1 ligation prevents stable interactions between T cells and APCs [190, 191]. However, in a viral infection model, PD-1 was responsible for T cell exhaustion through stabilization of the IS [192]. The viral infection model looked at T cells in the spleen, which may explain potential differences, since TFH cells are unique in lymphoid tissue, and express high levels of PD-1 [61].

1.4.4 Adhesion molecules

The Springer lab in the early 80s attempted to define the receptors required for cytotoxic T lymphocyte (CTL) activity [193]. They assessed CTL activity in the presence of individual monoclonal antibodies known to bind CTLs and found that one antibody efficiently inhibited cytolytic activity. In an elegant study, it was determined that the newly identified receptor was not the TCR, but an accessory receptor they termed lymphocyte function-associated antigen (LFA-1). Through a similar antibody screening process, other LFA receptors were found and characterized [194].

Later, LFA-1 was discovered to assist in cellular adhesion [195]. Using an antibody screening approach, the Springer lab identified the ligand for LFA-1 and called it intercellular adhesion molecule (ICAM-1) [196]. Both LFA-1 and ICAM-1 were found to be important for IS formation and signal strength [197]. However, why adhesion was required for TCR signaling was not apparent until 15 years after LFA-1 was discovered. Bachmann, et al., [198] showed that LFA-1 lowered the threshold for the amount of antigen required to activate T cells by nearly 100-fold by stabilizing interactions between T cells and APCs.

In addition to the LFA and ICAM families of adhesion molecules, other adhesion molecule pairs assist in the IS including CD2 and CD58 [199]. The extracellular domains of CD2 and CD58 are similar in length to the TCR and peptide-MHC molecules, suggesting that they may interact in close proximity [200]. Indeed, it was shown that the interaction between CD2 and CD58 assisted in T cell activation in response to APCs, suggesting a possible role for signaling in addition to adhesion [198]. Further studies demonstrated that CD2 had similar signaling cascades as TCR signaling, and enhanced TCR signaling [201]. In addition to this, CD2 signaling was shown to be the major co-stimulator signal involved in CD8+CD28- T cells [202].

1.4.5 Supramolecular activation clusters

Understanding why and how all these receptors were involved in antigen recognition required the ability to visualize the IS. Two methods were employed to achieve this: immunofluorescence of key receptors during an IS between T cells and APCs [203], and purified receptors reconstituted on lipid bilayers supported on slides [166]. Based on this work, well-orchestrated organization of the IS came into view. The model proposed by these seminal experiments showed that the IS was organized in a "bull's eye" structure with the TCR, co-stimulatory, and inhibitory interactions at the center, and adhesion molecules surrounding the central structure. These structural organizations were termed supramolecular activation clusters (SMACs) [203]. The formation of the SMAC is largely dependent on actin remodeling [204].

Based on the original definition, SMACs were arranged in two regions: the central SMAC (cSMAC) and the peripheral SMAC (pSMAC) [203]. This has been expanded to include a distal SMAC (dSMAC) [205]. It was later shown that the dSMAC contains clusters of TCR that are activated and migrate towards the cSMAC [206]. Further work showed that the TCRs in the cSMAC are generally devoid of signaling, while co-stimulation continues through CD28 [207].

Within the cSMAC, cytolytic granules are released [168, 208]. The pSMAC forms a protective ring around the cytotolytic granules that allows focused secretion towards the target cell. The pSMAC was also shown to increase efficiency of target cell lysis by three-fold [209]. Interestingly, the cSMAC also plays a role in secretion of TCRs [48]. This was found using the lipid bilayer method to detect SMACs. Choudhuri et al., found that when cells were removed from the bilayers, TCRs remained bound to MHC molecules within the bilayer. Through their work, the endosomal sorting complexes required for transport (ESCRT) pathway was shown to be

necessary for vesicle secretion. The secreted vesicles are internalized by target cells [169, 210]. Choudhuri et al., [48] also showed that HIV utilized the ESCRT pathway for cell to cell transmission and this observation may help explain the role of the IS in the context of the virological synapse described in the HIV section.

Chapter 2 Capacity of HIV Targeting CAR T cells to Eliminate FDCs Bearing HIV Immune Complexes

2.1 Abstract

A functional cure that no longer mandates continued antiretroviral therapy is a major goal in current HIV research. Recent *in vitro* studies testing HIV-targeting chimeric antigen receptor (CAR) T cells suggest the potential to achieve this goal. However, all studies to date have overlooked important *in vivo* reservoirs of HIV, including follicular dendritic cells (FDCs) that trap infectious HIV immune complexes (HIV-IC) on their surfaces but are not themselves infected. The goal of this study was to determine the potential of CD4- and CD4-MBL CAR-T cells to eliminate cells bearing HIV-immune complexes (HIV-IC), and to determine the requirements for this elimination (i.e., cell killing). In this study, we tested a second-generation CAR-T in which native viral gp120 recognition was achieved through domains 1 and 2 of CD4 linked to the mannose binding lectin (MBL). We determined the capacity of the CD4-MBL-CAR-T cell to eliminate envelope (Env) expressing cells, HIV-infected cells, and FDC bearing HIV-IC through a novel CFSE release assay. We also determined the functional activity of CD4-MBL-CAR-T cells through detection of IFN-γ production and CD107a membrane expression. Our data indicated that CD4-MBL-CAR-T cells displayed potent lytic and functional responses to Env expressing cells and HIV-infected cells. However, CD4-MBL-CAR-T cells were ineffective at targeting FDCs bearing HIV-IC. Additionally, CD4-MBL-CAR-T cells were unresponsive to cell-free HIV or concentrated, immobilized HIV-IC in cell-free experiments. These data suggested that other adhesion molecules were necessary for the activation of our CAR T cells. Indeed, blocking ICAM-1 via antibody potently inhibited the capacity of the CD4-MBL-CAR-T cell to respond to Env-expressing, and HIV-infected cells. Our results suggested that CD4-MBL-CAR-

T cells were unresponsive to HIV-IC due to a lack of stabilization between the CAR T cell and Env proteins. Our data indicated that all reservoirs of HIV, including the viral reservoir on FDCs, should be considered when testing a functional cure strategy.

2.2 Introduction

One of the major challenges to a functional cure for HIV is the ability of the virus to reside in sanctuary sites termed reservoirs [80]. These are protected environments that prevent HIV eradication and sustain disease progression. The most studied reservoir of HIV is the latently infected $CD4^+$ T cell, an infected T cell that becomes quiescent shortly after infection, as reviewed in [211]. Upon reactivation, the cell becomes a site for active viral replication, production, and spreading infection. Though often overlooked, other viral reservoirs also exist and contribute to HIV pathogenesis [212]. Additional cellular reservoirs consist of macrophages and follicular dendritic cells (FDCs).

The focus of this study is the FDC reservoir [60, 71, 75, 78, 81, 213, 214]. FDCs reside in the germinal centers (GC) of lymph nodes. They are not infected by HIV, but trap the virus in the form of an immune complex (IC) on their surface [60, 72, 77, 215]. Despite being in the form of an immune complex, even with neutralizing antibodies, the virus remains infectious [60]. In addition, the virus found on FDCs is genetically diverse and some of the variants are only found on FDC networks [71]. Not only do FDCs establish a unique, persistent viral reservoir, they contribute to disease progression by increasing co-receptor expression on permissive cells [81], preventing T cell migration via CXCL12 from the FDC viral reservoir [82], and enhancing HIV replication [83]. Moreover, HIV specific cytotoxic T lymphocytes (CTLs) have restricted access to GCs [76] further limiting the eradication of this viral repository. The importance of the FDC-HIV reservoir in viral pathogenesis is supported by the observation that T follicular helper (Tfh) cells, the T cells that reside adjacent to FDCs, have the highest infection rates and produce more virus than other T cell subsets [216, 217]. Thus, the FDC reservoir poses an important and unique challenge for eradication strategies.

Recent advances in immunotherapies have led to innovative strategies to target and eliminate HIV and its reservoirs [218-221]. Because virus trapped on FDCs remains in its native configuration (i.e., intact virus particles) any immunotherapy designed to eliminate the FDC reservoir must target this form of the virus. The work conducted herein utilized a novel second generation CAR designed to recognize native gp120 on the surface of the virion or infected cells themselves. Developed by the Berger lab at the National Institute of Allergy and Infectious Disease (NIAID), the CAR, designated as CD4-MBL-CAR (**Figure 1**), contains the extracellular domains 1 and 2 of CD4 linked to human mannose binding lectin (MBL), the CD28 transmembrane and signaling domains, and the CD3ζ intracellular signaling domain. With domains 1 and 2 of CD4, the CAR is able to recognize infectious HIV variants, thereby minimizing viral escape that remains possible with specific scFv. The MBL protein further enhances the binding to HIV as it efficiently recognizes the heavily glycosylated gp120 [222]. Containing both the CD4 and MBL domains, the CAR is able to bind gp120 through two distinct epitopes. *In vitro* studies have shown that two-epitope recognition has prevented infection of the $CD8^+$ CAR-T cells [162], while other CAR designs utilizing the CD4 domain have been permissive to infection [160]. The presence of both the CD3ζ and CD28 signaling domains provides the CAR-T cell with primary and secondary signaling requirements for efficient activation, thereby bypassing conventional T cell receptor signaling.

Lysis of target cells is achieved in conventional CTLs through structural organization of antigen recognition receptors, signaling molecules, and adhesion receptors called an immune synapse [203]. A vital aspect of the immune synapse is the formation of an adhesive ring surrounding antigen recognition receptors, primarily through lymphocyte function-associated antigen (LFA-1) and intercellular adhesion molecule (ICAM-1). Indeed, not only does adhesion

lower the threshold for antigen recognition [198], it also provides a protective ring that allows directional release of cytotoxic granules and lysis of target cells bearing the antigen [223]. CAR cytotoxicity is suggested to use a similar mechanism [110].

Our work demonstrates the efficacy of the CD4-MBL-CAR-T cell design to lyse HIV-infected cells and identifies limitations in the system. The data suggest the CD4-MBL-CAR in its present form does not lyse uninfected cells bearing HIV-IC, such as FDCs. The findings also indicate that the CD4-MBL-CAR does not respond to free HIV or concentrated, bead-immobilized virus. Lastly we identified intercellular adhesion molecule (ICAM-1) as necessary for CD4-MBL-CAR-T cell activation.

2.3 Materials and Methods

2.3.1 Virus propagation

HIV-1_{IIIB} stock was prepared by infecting the human T cell line H9. After a 2-hour incubation with the virus at 37°C, the cells were washed and cultured in 20% complete medium consisting of RPMI 1640 supplemented with 20% FBS, 1 X Glutamax, 1 mM HEPES, 50 μg/ml Gentamicin (Lonza). The cells were cultured for 9 days and supernatant was collected by centrifugation at 300 x g for 10 min. The supernatant was then filtered through a 0.45 μm syringe filter and stored in 1.8 ml aliquots in liquid nitrogen. The medium was replaced and the cells were cultured for 3 days (day 12) at which time the supernatant was collected again and stored as described above. Viral stocks were quantified via quantitative-polymerase chain reaction (Q-PCR) for quantification of reverse transcriptase message.

2.3.2 HIV infection

At least 50 μl of HIV_{IIIB} viral stock was used to infect target cells. For infection, target cells were washed in PBS and resuspended in pre-warmed HIV viral stock. Cells were incubated in 12 x 75 mm FACS tubes at 37 °C for 2 hours. After 2 hours, the cells were washed at least twice in PBS, and resuspended in the appropriate complete medium.

For infection using the spinfection method, cells were treated as above with the following exceptions. Instead of placing the cells in the incubator for 2 hours, the cells were pelleted with the virus at 1200 x g for 2 hours at room temperature. After the centrifugation step, the cells were either washed and used directly in downstream experiments, or incubated for another 2 hours as outlined above.

2.3.3 HIV immune complex formation with anti-gp120 monoclonal antibody

50 µl of viral stocks of $HIV_{IIIB,}$ containing approximately 5 x 10^7 virions, were thawed and added to 1.4 – 2.1 ng of purified non-neutralizing anti-gp120 (Chessie 13). This corresponded to 1:1 – 1:1.5 ratio of gp120 molecules to anti-gp120 recognition sites assuming 72 spikes per virion. The immune complex was incubated at 4 °C for 1 hour, then added to 2 x 10^4 cells. The immune complex preparation was scaled as necessary. After incubation, the immune complexes were added to the target cells and incubated for 45 minutes at room temperature. At this time, the cells were washed in PBS at least 2 times, and then used for further experiments.

2.3.4 Monoclonal antibody purification from hybridoma culture

Chessie 13-39.1 and anti-HIV-1 gp120 Hybridoma (902) (AIDS Reagent Program) were cultured in 10% complete medium consisting of RPMI 1640 supplemented with 10% FBS, 1X Glutamax (ThermoFisher Scientific), 1mM HEPES, 1X non-essential amino acids (Hyclone), and 50 µg/ml Gentamicin (Lonza). The 0.22 µm filtered FBS used for culture was previously depleted of IgG molecules through addition to a 1 ml Hi-Trap protein G column (GE Healthcare) at a flow rate of 1 ml/min. Flow-through was collected and used as the medium supplement. For 902 recovery post-thaw, the cells were first grown in culture medium outlined above with the addition of 4% human cord blood plasma. This was done for two passages after which the cells were maintained in 10% complete medium for the remainder of use. Once cells, either Chessie 13 or 902, were recovered from thawing, the supernatant was collected at each passage and stored at 4°C. When final passage was performed, cells were grown in 2-4 T175 flasks (Corning) at 2-5 x 105 cells/ml in ~100 ml 10% culture medium for 2-3 weeks. At this time, most of the cells were

dead as verified by Trypan Blue staining. Cells were pelleted and supernatant was collected and stored at 4°C until purification.

For purification of antibody, supernatant was first filtered through a 0.22 μm cellulose acetate membrane after which the sample was run on a 1 ml HiTrap protein G column (GE Healthcare) at a flow rate of 1 ml/min. Once all supernatant passed through, the column was washed with 10 ml PBS, then antibody was eluted with 0.1 M glycine, pH 2.7. 1.7 ml Eppendorf tubes containing 60 μl 1 M Tris-HCl, pH 9.5 were used to collect a total volume of 1ml of eluent. Fractions were assayed via SDS-PAGE and BCA assays for protein purity and concentration, respectively.

2.3.5 Isolation of follicular dendritic cells from tonsils

FDCs were isolated by placing tonsils in a 10 cm plate with 5 ml of Tonsil Buffer (RPMI 1640, 50 μg/ml Gentamicin, 10 mM HEPES). Using sterile scalpels, the tonsils were cut into small 2 mm pieces and the entire solution was placed in a 50 ml conical tube. To it was added 1 Wünsch unit of Liberase TM (Roche) and 175 IU of DNase I (Sigma). This was placed in a 37°C water bath for 45-60 minutes with occasional mixing. Then tissue free cells were set aside and the remaining tissue was suspended in 5ml of Tonsil Buffer containing 1 Wünsch unit of Liberase TM and 175 IU of DNase I. This was incubated for another 45-60 minutes at 37°C. After digestion, the tissue was triturated gently with a 25 ml serological pipet until it passed freely through the tip. This was then repeated with a 10 ml serological pipet. The tissue-free cells were passed through a 100 μm nylon filter into 10% complete medium and centrifuged for 6 minutes at 350 x g. The cell pellet was suspended in complete medium and layered on top of a 25-43% discontinuous Percoll gradient. The gradients were spun at 2000 x g for 30 minutes at room temperature. Cells

were collected at the interface representing the low-density cell fraction up to 1.06 g/ml. The cells were then washed in complete medium to remove Percoll solution and resuspended in 1-3 mls of complete medium.

To label FDCs, the cells were treated with either normal goat serum (Jackson Immunoresearch) when using HJ2 as a primary antibody, or 20 μg unlabeled mouse IgM (Southern Biotech) when using biotin-CNA.42 as the primary antibody. After 10 minutes, either 250 μl of dialyzed HJ2 hybridoma supernatant, or 6-8 μg of purified biotin-CNA.42 (eBiosciences) was added and the cell were incubated at 4 °C for at least 2 hours to overnight. The cells were then washed and suspended in 100-500 μl FACS buffer. For HJ2 FACS sorting, the normal goat serum was added to 5% v/v. Then PE-goat anti-mouse IgM was added at a ratio of 1:100 (Jackson Immunoresearch) and incubated for 1 hour. When using CNA.42, the cells were suspended in complete medium and APC-Streptavidin was added at a dilution of 1:100 (Jackson Immunoresearch) and incubated for 45-60 minutes. The cells were then washed, filtered to a 30 μm filter tube (Corning) and 7-AAD was added to discriminate between live and dead cells. The cells were then run on the BD FACSAriaFusion and FDCs were isolated gating the population of 7-AAD$^-$ HJ2 or CNA.42$^+$. FDCs usually ranged between 0.5-2.5 % of the total population. For FDC$^-$ populations used in the following studies, the FDC$^-$ cells were 7-AAD$^-$, HJ2 or CNA.42$^-$. After sorting, the cells were washed, irradiated at 1200 RADs, and then used for further experiments.

When using MACS technology, the FDCs were prepared as above up to the point of adding the secondary antibody. At this point, the cells were washed, blocked, and magnetic labeled secondary antibodies/streptavidin was added according to the manufacturer's protocol (Miltenyi, StemCell Technologies, and ThermoFisher).

2.3.6 Chimeric antigen receptor T cell production

Fresh heparinized blood was diluted with an equal volume of cold PBS and layered on Ficoll Paque (GE Healthcare) at a ratio of 1 part Ficoll 2 parts diluted blood. This was spun at 350 x g for 25 minutes without the brake. The buffy coat was removed and washed in 35 ml of PBS and pelleted at 300 x g for 6 minutes. For platelet removal, the cell pellet was again suspended in 35ml of PBS and centrifuged at 100 x g for 10 min. At this point, the cells were counted using a haemocytometer, and cultured at $2x10^6$ cells/ml in CAR T cell medium consisting of AIM-V medium (ThermoFisher Scientific) containing 5% heat-inactivated human AB serum (Valley Biomedical) and 300 IU/ml IL-2 (AIDS Reagent Program) in round bottom 96 well plates with the addition of 50 ng/ml soluble anti-CD3 (LEAF OKT3, BioLegend). This was cultured for 2 days at 37°C, 5% CO_2 after which the cells were washed in CAR T cell medium without IL-2, then suspended in CAR T cell medium at a concentration of $5x10^5$ cells/ml. PBMCs were added to wells containing CAR retrovirus pre-bound to the plates.

To prepare the retrovirus plates, Retronectin (Clontech) was added to 6-well non-tissue culture treated plates at a concentration of 15 μg per well in 1.5 ml of PBS. The plates were sealed with Parafilm and stored at 4°C overnight. The next day, the Retronectin solution was removed and 3 ml of 2% bovine serum albumin (BSA, Sigma) in PBS was added to the wells. This was incubated at room temperature for 30 min after which the BSA solution was removed and the wells were washed with PBS. 1 ml aliquots of CAR retrovirus were thawed rapidly in a 37°C water bath and diluted with an equal volume of RPMI 1640. The PBS was removed from the well, and the diluted retrovirus was added to the plates. The plates were sealed with Parafilm and spun in a pre-warmed centrifuge at 2,000 x g for 2 hrs at 32°C noting the orientation of the plates. After

centrifugation, the retrovirus solution was removed and 1.5 ml of activated PBMCs ($0.75x10^6$ cells) was added to each well containing pre-bound retrovirus. The plates were spun at 1,000 x g for 10 min at 32°C after which the plates were placed in an incubator and cultured at 37°C, 5% CO_2 overnight. The next day, the process was repeated with new plates containing fresh pre-bound retrovirus. The cells were then cultured for 3 days in CAR T cell medium. After this, cells were cultured in CAR T cell medium with 30 IU/ml IL-2 and maintained at 0.5-1 x 10^6 cells/ml every 3 days.

2.3.7 Carboxyfluorescein succinimidyl ester (CFSE) release assay

Target cells used in cytotoxicity assays were labeled with CFSE (ThermoFisher) by first washing the cells with PBS twice. Cells were resuspended at a final concentration of 1 x 10^6 cells/ml in PBS and 1 µl of CFSE was added representing a final concentration of 5 µM. CFSE was incubated with the cells for 15 minutes at 37 °C. CFSE quenching was accomplished by added an equal volume of complete medium and incubating for 5-10 minutes at room temperature. This was washed twice and the cells were resuspended in CFSE buffer (PBS supplemented with 10 % FBS, 1 X Glutamax, 1 mM HEPES, 1 X Non-essential Amino Acids, 1 X Sodium Pyruvate). The target cells were typically used at 2 x 10^4 cells/ well. To prepare the cells for plating, the cells were resuspended in CFSE buffer at 2 x 10^5 cells/ml and 100 µl of cells was added to the well of a 96 well round-bottom plate. For spontaneous release of CFSE from target cells, target cells were placed in wells without effectors and the final volume was kept constant at 200 µl. For 100% lysed controls, wells were prepared as described for the spontaneous release samples with the addition of 1 µl of Digitonin (20 mg/ml stock solution).

Effector cells were added at the ratios indicated in each experiment. The effector: target ratio usually ranged from 0.1:1 to 10:1. After addition of effectors, final volume of the well was always set at 200 µl. The plates were centrifuged at 400 x g for 2 minutes and placed in a 37 °C incubator for 4 hours.

100 µl of the supernatant from wells was then placed into a black 96 well plate and read on a Synergy HT plate reader using the fluorescent settings with the filters 485/20 for excitation, and 528/20 for emission. Sensitivity was adjusted as necessary. Specific lysis was quantified with the following equation: (sample – spontaneous release) / (100 % lysed – spontaneous release) x 100.

2.3.8 ^{51}Cr release assay

Target cells were washed in PBS and resuspended in 200 µCi $Na_2{}^{51}CrO_4$ supplemented with FBS to a final concentration of at least 10 % and incubated at 37 °C for 1 hour. The sample was then washed twice in PBS and once in 10 % complete medium. The pellet was resuspended in complete medium at a concentration to allow 100 µl to be added to a 96 well round-bottom plate. Effector cells were diluted in complete medium to allow 100 µl to be added for each well at the appropriate ratio. Spontaneous Release and 100 % lysed samples were prepared as described above. The plate was centrifuged at 400 x g for 2 minutes and placed in a 37 °C incubator for 4 hours. 100 µl from each sample was removed carefully to avoid the disturbing the cells, and placed in a 12 x 75 tube. The tubes were placed in a γ counter

2.3.9 Protein extraction

Cells were pelleted to remove culture medium and washed at least twice in PBS. The cell pellet was resuspended in whole cell lysis buffer consisting of 10 mM Tris-HCl pH 7.4, 0.4 %

sodium dodecyl sulfate, 10 % glycerol, 1 X Protease and Phosphatase Inhibitors (ThermoFisher Scientific), and 250 U Pierce Universal Nuclease (ThermoFisher Scientific). The sample was placed at room temperature for 10 minutes to alleviate viscosity through nuclease activity. The lysate was then passed through various gauged needles until it was able to pass through a 28-gauge needle. The lysate was then spun at 14,000 x g for 10 minutes to remove cell debris. The supernatant containing whole cell proteins was preserved at -20 or -80 °C while the pellet was discarded.

2.3.10 Western blot

Samples containing 20-100 µg protein were diluted in 4 X loading buffer (50 mM Tris-HCl pH 6.8, 4 % SDS, 4 % v/v β-mercaptoethanol, 40 % Glycerol, Bromophenol Blue) heated to 95 °C for 10 minutes. The samples were then loaded on an SDS-PAGE consisting of a 10 % resolving gel and a 4 % stacking gel prepared in either a Tris-glycine solution, or a Tris-tricine solution. The samples were run at 12 mA through the stacking gel, then increased to 24 mA for the resolving gel. Once the bromophenol blue reached the end of the resolving gel, the gel was removed and pre-equilibrated in transfer buffer consisting of 25 mM Tris-HCl, 192 mM Glycine, 20% Methanol. Protein was transferred to the nitrocellulose membrane using a Mini Trans-Blot Cell (BioRad) in cold transfer buffer at 20 V for 10 minutes followed by 100 V for 60 minutes.

After transfer, the membrane containing protein was dried overnight at 4 °C. The next day the membrane was blocked with 5 % milk in TBS buffer (25 mM Tris-HCl pH 8.0, 150 mM NaCl). The blot was washed once in TBS-T (TBS buffer with the addition of 0.1 % Tween 20). After blocking, primary antibody was added according to the manufacturer's recommendation in TBS-T and incubated for at least one hour at room temperature or overnight at 4 °C. The blot was

washed in TBS-T twice, and secondary, IRDye800CW-donkey anti-mouse or donkey anti-rabbit (Li-COR) at a concentration of 1:10,000. This was incubated for 1 hour at room temperature protected from light. After washing the membrane in TBS-T twice, the blot was imaged on an Odyssey Li-COR scanner on both the 700 and 800 nm channel to detect the pre-stained molecular weight ladder and target protein, respectively.

2.3.11 FACS analysis

Cells for staining were prepared by suspending in 100 µl PBS containing 10 µg Chrompure IgG (Jackson ImmunoResearch) to block irrelevant IgG binding domains. After 10 minutes, primary antibody was added and incubated at 4 °C for 1 hour. Samples were then washed in 3 ml PBS, and resuspended in 300 µl PBS if ready for FACS analysis. If a secondary antibody was necessary, cells were suspended in 100 µl of PBS with the addition of fluorochrome labeled secondary antibody. After 45 minutes, the samples were washed in PBS, and resuspended in 300 µl for FACS analysis. At least 10,000 events were recorded per sample when possible.

2.3.12 Syncytia Formation Assay

TF228 cells stably expressing gp160 or the parental cell line BJAB were co-cultured with the CD4 expressing T cell line SupT1 at a ratio of 1:1 in 24 well plates in complete medium. After 16 hours, wells were imaged for the presence of syncytia using an AMG Evos Imager at 10 X magnification.

2.3.13 CAR activation assay

Equal concentrations of target and effector cells were added to each sample in FACS tubes in a total volume of 200 µl of assay media (10% HI Human serum, 1X Glutamax, 1X NEAA, 10mM HEPES, in PBS). 100 µl of assay media, supplemented with 3X Monensin, 3X Brefeldin A, and 2 µl of APC-α-CD107a was added. Cells were incubated for 6 hours at 37°C after which the cells were washed, and treated with 100 µl PBS containing 2µl APCFire-750-α-CD4, 4 µl FITC-α-CD8, and 10 µg Chrompure Mouse IgG. Samples were incubated 20 minutes at RT. The samples were washed and resuspended in 200 µl Cytofix/Cytoperm. After 20 minutes at RT, the samples were washed in 1X perm/wash buffer. The cells were resuspended in 100 µl perm/wash buffer with 2 µl PE-anti-IFN-γ. After 20 minutes at RT, the samples were washed in perm/wash buffer and resuspended in 100 µl PBS. To this, 100 µl of 4% paraformaldehyde was added, and the samples were placed at 4°C overnight. The next day, the cells were washed and analyzed. Single cells gated on $CD4^+CD8^+$ were analyzed for CD107a and IFN-γ expression. Plots are representative of two experiments, one in triplicate.

2.3.14 Mixed lymphocyte reaction

PBMCs were isolated as described in *2.3.6* from two donors. PBMCs from one donor were γ-irradiated at 1200 RAD and labeled as activators. Non-irradiated PBMCs were labeled as responders. Equal concentrations of activators and responders were plated in 10% RPMI complete medium, and cultured for 6 days. After 6 days, PBMCs were isolated from the donor who supplied activator cells. 2×10^4 activator cells, termed targets, were placed into each well. Responders, termed effectors, were plated at ratios of 3:1, 10:1, 30:1, and 100:1. After 4 hours, 50 µl of

CytoTox-Glo assay reagent was added to the wells and incubated for 15 minutes. After incubation, the samples were analyzed on a BioTek Synery HT plate reader using luminescence settings.

2.4 Results

2.4.1 Expression of CAR molecules on transduced PBMCs

We first focused our work on creating three CAR T cells: CD4-MBL-CAR, CD4-CAR and a control labeled 139 CAR (**Figure 1**). While the first two CARs recognize HIV, the control CAR targets a mutated form of the epidermal growth factor receptor (EGFR). We obtained peripheral blood from HIV-uninfected donors and used retroviral transduction to create PBMCs expressing each CAR. To determine whether transduction of the CD4 and CD4-MBL receptors were expressed on the transduced cells we examined CD4 expression on the transduced cells in comparison with cells transduced with the 139 CAR as a control (**Figure 2**). We observed that PBMCs transduced with either the CD4- or CD4-MBL- CAR constructs showed CD4 expression in $CD8^+$ T cells as well as in $CD4^+$ T cells, while the 139-CAR showed no increased CD4 expression in either subset of PBMC. When we examined the mean fluorescence intensity (MFI) of CD4 expression in the transduced cells, we found that CD4 MFI increased about 1.5 fold (compared to the control 139 vector) in $CD4^+$ T cells regardless of whether the CD4- or CD4-MBL- CARs were transduced. Additionally, we found that while the starting ratios of CD8:CD4 cells varied by donor, the transduction efficiency and CAR expression levels were similar irrespective of the donor.

2.4.2 Evaluation of BJAB and TF228 cells as control cells

To begin to test the function of the transduced CARs, we used cell lines that expressed gp160/120 at the cell surface. The use of cell lines rather than primary cells allowed us to propagate sufficient cells for all of our testing and to use cells that stably expressed the HIV Env. TF228 and BJAB cells are B cell lines: TF228 expresses gp160 while the parental line, BJAB,

does not. To confirm that TF228 cells expressed gp160/120, we performed immunoblotting on cell lysates obtained from both BJAB and TF228 cells (**Figure 3**). As expected we observed robust expression of gp160 on the TF228 cells but were unable to detect gp160 expression on BJAB cells. We reasoned that if TF228 gp160 were fully functional, we would be able to observe syncytium induction when these cells were co-cultured with SupT1 cells that are known to express human CD4 and the HIV co-receptor, CXCR4 (**Figure 4**). Syncytia could not be detected on BJAB cells but multiple syncytia were evident in the co-cultures bearing TF228 cells.

We also examined CD32 expression on both TF228 and BJAB cells to determine if the cells might be suitable for initial testing of the ability of the CAR-T cells to recognize virus on the surface cells such as would occur on FDCs. Because our previous studies of FDCs and HIV had pointed to the use of FcγRIIB (CD32) as a principal receptor used by FDCs to trap and retain HIV immune complexes [71, 77], we reasoned that TF228 and BJAB cells could provide a test system to examine HIV-ICs, provided these cell lines expressed CD32 as reported. We found that both BJAB and TF228 cells expressed CD32 (**Figure 5**).

2.4.3 Evaluation of cytotoxicity assays

Because FDCs are rare cells and there are no cell lines that fully recapitulate these cells, we next sought to examine cytotoxicity assays that could detect cell death in samples containing ~2 x 10^4 target cells or less. Because ^{51}chromium release is considered the "gold standard" for detection of cytotoxicity, we performed a labeling efficiency experiment using ^{51}Cr and different concentrations of FDCs (**Figure 6**). We found that at the maximum concentration of FDCs (6 x 10^5), we detected fewer than 10,000 counts per minute (CPM) and using doses of 100,000 cells yielded only 1,200 CPM. Importantly, at the desired concentration of 10,000 FDCs, only 130

CPM were observed with background readings of 16 CPM. Thus, 1 x 10^4 cells would only provide a maximum readout of 115 CPM above background and was insufficient for reliable detection of cell lysis. Because of this result, we sought alternative methods to detect cell lysis.

We next examined the commercially available assay CytoTox-Glo, to detect cell lysis. Control experiments suggested by the manufacturer demonstrated proof of concept (**Figures 7** and **8**); however, when we analyzed a mixed lymphocyte reaction, background levels from wells containing only effector cells alone was similar to wells containing the allogeneic target cells (**Figure 9**).

Because both ^{51}Cr-release assays and commercially available kits did not provide the sensitivity and low backgrounds needed, we developed a novel assay we termed the "carboxyfluorescein succinimidyl ester (CFSE) release assay". In theory, this assay works identically to the ^{51}Cr release assay, except it uses a fluorescent label, CFSE, instead of a radioactive one. Importantly, in contrast to ^{51}Cr-based assays, labeling with CFSE was consistent. Using different ratios of lysed and live PBMCs, CFSE release was linear over a broad viability range (**Figure 10**). In addition, lysis was readily detectable in wells containing 1 x 10^4 target cells as evidenced by a 34,000 RFU difference above spontaneous release (**Figure 11**). We then tested CFSE labeling of 2 x 10^4 FDCs and detected a 5.8-fold difference in relative fluorescent units (RFU) between lysed and spontaneous release samples (**Figure 12**). Thus, this assay allowed us the range necessary for sensitive and repeatable detection of lysis, even when used with FDCs.

2.4.4 HIV targeting CAR T cells efficiently lyse gp160-expressing cells

Because TF228 cells bear surface gp160 and were available in numbers sufficient for ^{51}Cr-release, we compared our CFSE assay with the standard ^{51}Cr-release to determine the

comparability of the two methods (**Figure 13**). Using BJAB and TF228 cells as targets, we measured lysis of target cells after a four-hour co-culture with our three types of CAR-T cells (**Figure 13**). The CD4-CAR-T and CD4-MBL-CAR T cells lysed TF228.1.6 cells but spared BJAB cells. In contrast, 139-CAR T cells were unable to induce killing of TF228 or BJAB cells. This experiment demonstrated that our CFSE-release assay was comparable to the ^{51}Cr-release assay. We also found that the CD4- and CD4-MBL-CAR-T cells demonstrated potent cytolytic activity even at an effector:target ratio of 2.5:1.

We next determined the activation state of the CAR-T cells in the presence of TF228 or BJAB cells (**Figure 14**). The CAR activation assay indicated that CD4- or CD4-MBL-CAR-T cells were activated as determined by the detection of IFN-γ and CD107a, in the presence of Env expressing TF228, while no response occurred alone, or in co-culture with BJAB cells. Additionally, the 139-CAR control was unresponsive to both BJAB and TF228. These data suggest the specificity of the CD4-based CAR-T cells, their lytic potential to Env-expressing cells, and confirm the utility of the CFSE release assay.

2.4.5 FDCs bind infectious HIV-IC

Now that we had reliable assays to detect CAR-T cell activation and sensitive lysis of target cells, as well as appropriate controls for each, we sought to optimize HIV-IC binding to FDCs. Because of the difficulty of obtaining tonsils from HIV-infected patients, we optimized the formation of HIV-IC using a monoclonal anti-gp120 antibody, Chessie13. To determine the ratio of antibody to gp160 molecules on the virion, we quantified the viral RNA in our HIV_{IIIB} stock. Using Q-PCR to detect RT RNA, and ACH2 cells for a standard curve, we calculated 1.06 x 10^6 virions/µl. Assuming a maximum of 72 spikes per virion [224], we estimated a maximum of 216

antigenic sites per virus particle. Because IgG molecules contain two antigen binding sites, there would be 108 antibody molecules/virion. This estimated that 1.4 ng of Chessie13 was needed for each 50 µl of our viral stock. To confirm this experimentally, ICs were formed using a range of 14 pg to 140 ng of antibody with 50 µl of viral stock and this was added to 2 x 10^4 FDCs. After a 1-hour incubation, samples were washed twice in PBS and prepared for Q-PCR as described in materials and methods. Quantification of viral RT RNA was performed using Q-PCR and the ACH-2 cell line standard that bears a single HIV provirus per cell. Binding of HIV-IC to FDCs was optimal with 1.4 ng of antibody (**Figure 15)**.

We then sought to confirm that virus trapped on FDCs was infectious as would occur in an *in vivo* setting. We formed HIV-IC using two separate antibody concentrations of 2 ng and 2 µg and bound them to irradiated FDCs. FDCs were irradiated so that HIV replication would not occur in any contaminating tonsillar T cells. As a control, we used $CNA4.2^-CD45^+$ irradiated tonsillar cells from the same tissue used to isolate our FDCs. After binding HIV-IC to the FDCs and the control cells, we monitored the amount of HIV released into the cultures (**Figure 16)**. The data indicated that HIV production peaked in co-cultures with FDCs when 2 ng of Chessie13 was used to form the HIV-IC. Although FDCs were able to bind infectious HIV in the absence of antibody, virus production was less than samples utilizing HIV-IC formed with 2 ng of Chessie13 antibody. All other experiments containing HIV-IC were formed using 2 ng of Chessie13 antibody and 50 µl of HIV_{IIIB} viral stock. These experiments confirmed that FDC-trapped HIV-IC was infectious.

2.4.6 CD4-MBL-CAR-T cells do not respond to HIV-IC

We next determined whether CD4-MBL-CAR T cells could lyse FDCs bearing HIV-IC. FDCs were incubated in the presence of HIV, HIV-IC or an irrelevant immune complex

consisting of OVA-anti-OVA. Cell lysis was only detected when TF228 cells were incubated with CD4-MBL-CAR T cells (**Figure 17a**). Surprisingly, CD4-MBL-CAR T cells did not kill FDCs bearing HIV or HIV-IC, although HIV-IC was bound to FDCs (**Figure 17b**). To determine if the lack of CD4-MBL-CAR-T cell killing of FDCs was a unique feature of FDC-associated virus, we repeated this assay using BJAB or TF228 cells in the presence of free HIV or HIV-IC. Because BJAB and TF228 cells express CD32 (**Figure 5**), we reasoned they should trap HIV-IC, just as FDCs, allowing us to distinguish whether HIV-ICs could not be recognized or whether only FDC-HIV-IC evaded CAR-mediated killing **(Figure 18)**. BJAB cells, in the presence of HIV or HIV-IC, were not lysed by CD4-MBL-CAR-T cells, while TF228 were efficiently killed irrespective of the presence of HIV-IC.

Because we found that CD4-MBL-CAR-T cells did not lyse cells bearing HIV-IC, we reasoned that the antibody used to form the IC might block CAR recognition of the virus. To determine whether Chessie13 inhibited CD4-MBL-CAR-T cell killing, we tested TF228 cells in the presence or absence of excess Chessie13 (**Figure 19**). TF228 killing was not inhibited in the presence of excess Chessie13 antibody.

Because FDCs are not permissive to HIV infection [225] and would therefore not express gp160, we sought to determine whether HIV or HIV-ICs themselves were sufficient to induce CD4-MBL-CAR-T cell activation. To test this, HIV or HIV-ICs representing ~5 x 10^7 virions were added to CD4-MBL-CAR-T cells in the presence or absence of BJABs or TF228 cells. The data indicated that CD4-MBL-CAR-T cells were not activated in the presence of 5 x 10^7 virions or HIV-IC (**Figure 20**). CAR activation only occurred in the presence of membrane expressed gp160 on TF228 cells. The possibility also existed that even if CAR-T cells could recognize FDC-virus, they could not kill FDCs because of inhibitory mechanisms present with this cell type. Our

group has not been successful in either transducing or transfecting primary human FDCs. Because we were unable to cause FDCs to express gp160, we postulated that we might use HIV or HIV-ICs bound to beads to determine whether CAR T cells could be activated directly. We formed HIV-IC on 4.5 μm Sheep-anti-Rat Dynabeads, which efficiently bound HIV in the presence of Chessie13 (**Figure 21**). However, no CAR activation occurred **(Figure 22).** We also bound anti-CD4 to the same beads to test whether targeting the CAR directly would lead to CAR activation (**Figure 22**). Although HIV-IC or free virus was unable to induce CAR activation, immobilized anti-CD4 coated beads induced both CD107a and IFN-γ detection in $CD4^{+}CD8^{+}$ CD4-MBL-CAR-T cells (**Figure 22**). Remarkably, soluble anti-CD4 antibody also induced IFN-γ but not CD107a production.

2.4.7 ICAM-1 is required for CD4-MBL-CAR-T cell activation

Because concentrated, immobilized HIV was unable to induce CAR activation, we reasoned that additional interactions at the cell interface were required for CD4-MBL-CAR-T cell activation and killing of target cells. Because immune cells including FDCs form immunological synapses that stabilize cell:cell interactions, we reasoned that adhesion molecules might positively affect CAR activation. FDCs bear high levels of Intracellular adhesion molecule 1 (ICAM-1)[226]. We therefore tested whether ICAM-1 was necessary for CD4-MBL-CAR-T cell activation and killing. Culturing TF228 cells with CAR T cells in the presence or absence of an ICAM-1 specific blocking antibody revealed that blocking ICAM-1 had a pronounced inhibitory effect on the lytic ability of the CD4-MBL-CAR-T cells targeting TF228 cells (**Figure 23**). We next tested infected cells (H9) to determine the importance of ICAM-1 (**Figure 24**). Again, the data clearly indicated that CAR activation was inhibited in the presence of blocking ICAM-1

specific antibody. Lastly, we used primary CD4 T cells to assess the contribution of ICAM-1 on CAR T cell activation. HIV-infected $CD4^+$ T cells (obtained from the same donor as were the CD4-MBL-CAR T cells to avoid allogeneic reactivity) were used as targets with the CD4-MBL CARs (**Figure 25**). Blocking ICAM-1 specific antibody again blocked CD4-MBL-CAR activation. Uninfected autologous $CD4^+$ T cell targets were unable to induce CD4-MBL-CAR-T cell activation.

Collectively these data indicate that CD4-MBL-CAR-T cells are unable to recognize free or immobilized HIV or HIV-IC, despite profound cytolytic activity against Env expressing cells. Our data suggest that CD4-MBL-CAR-T cell activation and induced lysis of HIV-infected cells are dependent on adhesion molecules such as ICAM-1 for activity (**Figure 26**). Because of this, FDCs bearing HIV-IC are inadequate targets for CD4-MBL-CAR-T cell killing.

2.5 Discussion

A major obstacle to HIV eradication is the persistence of infectious virus in sanctuary sites termed reservoirs [80]. The FDC represents a large, secondary lymphoid tissue (e.g., lymph nodes, spleen) reservoir of infectious virus trapped and retained on the surface of the extensive FDC-network [75]. Even though the FDCs trap infectious HIV, they do not become infected by HIV [215]. In fact, native HIV is "stored" on FDCs by specific antibodies, including neutralizing antibodies, and/or complement proteins (i.e., immune complexes, IC) by virtue of their interaction with FDC- Fcγ and complement receptors [60, 72, 77-79, 215]. We reason that HIV in this format, would "display" the virus in a manner that would be significantly different than infected cells where newly formed virus buds from the cell membrane and where viral peptide is present on MHC molecules. This distinctive "display" of HIV on FDCs suggests the need of a unique targeting molecule or cell capable of recognizing native, surface-bound HIV.

One way of targeting FDC-trapped HIV could rely on the use of a CAR specially designed to recognize native HIV and/or HIV-IC. The CAR-T cell used in our studies is a second-generation CAR, designed to recognize gp120 through the extracellular domains of CD4 (D1 and D2) linked to an MBL protein. By nature of this unique design, unprocessed virus on FDCs (or HIV-IC on the surface of other sensitive cells) is recognized through CD4 and/or MBL on the CAR. As a second-generation CAR, it also contains the intracellular CD3ζ and CD28 signaling proteins necessary for potent signaling, activation, and cytolysis of target cells. Our studies indicate that while the CAR T cells can recognize and kill cells that express HIV gp160, FDCs and other cells that bear surface HIV and HIV-IC are not capable of being lysed by the CAR, nor capable of inducing CAR activation (e.g., generation of IFNγ or CD107a). This dissertation presents these data and postulates potential reasons for this failure.

Essential to interrogating CAR lytic potential is a sensitive assay to detect lysis of FDC and other target cells that may be present in small numbers (i.e. $\leq 1 \times 10^4$). Using standard ^{51}Cr labeling techniques, we determined that this technique would not sufficiently label FDCs to repeatedly detect their lysis. In fact, we found that we only were able to label FDCs to a level of 130 CPM per 1×10^4 cells. Moreover, the Cr-labeling was quite variable and seemed to depend on the activation state of the cells being labeled. When we used commercially available labeling reagents (i.e., CytoTox-Glo) the assay displayed high backgrounds with our cells and reagents. After the failure of our initial attempts using more conventional cytotoxic assays, we focused on determining whether CFSE, which labels internal proteins of cells, could be adapted to our use in cell lysis detection. We discovered that the use of CFSE in release assays was highly dependable, sensitive and readily adaptable to our systems. Utilizing our novel CFSE release assay, we were unable to detect any CD4-MBL-CAR-T cell mediated lysis of FDCs bearing infectious HIV-IC. This result was in marked contrast to CAR-mediated killing of HIV-infected T cells and the Env-expressing cell line TF228, both of which were efficiently lysed.

FDCs are post-mitotic [227], reside in a highly inflammatory environment [61], and bear complement-coated immune complexes that typically induce cell lysis [73]. Because of these observations, we rationalized that FDCs might be resistant to CAR lytic mechanisms. In addition, we reasoned that BJABs bearing HIV-ICs should readily be lysed by CAR-T cells as evidenced by the efficient lysis of the BJAB derived, Env-expressing TF228 cells. However, CD4-MBL-CAR-T cells did not lyse BJAB cells bearing HIV-IC. We interpreted the failed lysis of FDCs and BJABs bearing HIV-IC as potentially resulting from the failure of the chimeric antigen receptor to recognize HIV-ICs.

This hypothesis could be clearly detected by looking at the CAR-T cells to determine if activation was occurring. Alternatively, if activation occurred, this could suggest that inhibitory mechanisms might be at play, thereby prohibiting CAR-mediated lysis. We assessed CAR activation by detecting generation of IFNγ and CD107a (LAMP1). Our results indicated that the CD4-MBL-CAR-T cells were inactive in the presence of high concentrations of soluble HIV or HIV-IC. Even when the virus was concentrated and immobilized onto beads, CD4-MBL-CAR-T cells were unresponsive. Surprisingly, this was in direct contrast to both soluble and immobilized CD4 antibody, which did cause CAR activation.

The CD4 antibody clone used in our experiments was SK3, also known as Leu3a, and was calculated in one report to bind to CD4 with a K_d of 1.8×10^{-10} [220]. This antibody clone was also shown to prevent gp120 binding to CD4 [228]. These results suggested that the affinity of SK3 for CD4 is greater than that of gp120, although the affinity of gp120 for the CD4-MBL CAR has not been determined. We postulate that the higher affinity of SK3, and the inherent bivalent nature of antibodies, resulted in CAR signaling and activation. In favor of this, soluble anti-CD4 antibody was able to efficiently induce IFN-γ production. However, when immobilized, the antibody not only induced IFN-γ production, but also induced CD107a detection. We reasoned that the high affinity of soluble anti-CD4 antibody was sufficient to induce IFN-γ production. Once immobilized, however, the antibody-bead complex mimicked a target cell, and allowed the polarity required for targeted release of cytotoxic granules by the CD4-MBL-CAR-T cell [229]. The high affinity and bivalent nature of SK3 overcame the need for stabilization of the interaction with the CD4-MBL-CAR-T cell.

Unlike the anti-CD4 antibody used in our experiments, our data suggested that gp120 recognition and binding required stabilization to induce CD4-MBL-CAR-T cell activation. Our

results indicated that blocking ICAM-1 inhibited CD4-MBL-CAR-T cell lysis of HIV-infected primary CD4+ T cells, while the isotype control antibody had no effect. Indeed, blocking ICAM-1 inhibited not only cytotoxicity but also activation of CD4-MBL-CAR-T cells, regardless of whether the target was an HIV-infected primary $CD4^+$ T cell, an HIV-infected H9 neoplastic T cell, or a TF228 env-expressing B cell.

Several studies indicate the importance of ICAM-1 in HIV pathogenesis and infection. For example, ICAM-1 is important for the virological synapse [230], cell:cell viral transmission [231], syncytia formation [232], and is incorporated into native virions [233]. HIV associated ICAM-1 correlates with viral infectivity and is suggested to enhance cell surface binding [234]. However, ICAM-1 associated HIV, if present in our studies, was insufficient to induce CD4-MBL-CAR-T cell activation.

Although HIV-associated ICAM-1 did not appear to influence CAR recognition, we theorize that ICAM-1 plays an important role in the stabilization of the gp120:CD4-MBL interaction through the formation of a CTL-like immune synapse to induce CAR-T cell activation and targeted lysis. Others recently proposed that formation of an immune synapse was important for CAR mediated activation [101].

The immune synapse is a highly structured arrangement of recognition, signaling, and adhesion receptors at the interface between a T cell and an antigen-presenting cell [203]. For example, adhesion molecules are spatially separated from T cell receptor and peptide:MHC receptors due to differences in the length of extracellular domains [235]. Recent reports conclude that manipulating the distance between the target cell and the CAR alters cytolytic activity of CAR-T cells, even when the same target receptor is used for recognition [151]. In some cases, target receptor recognition proximal to the target membrane enhances CAR-T activity, while other

target receptors enhance cytotoxicity when CAR-T cells interact distally. Although not considered in their work, one possible explanation is that CAR-T cell activity is enhanced when proper spacing allows for the formation of an immune synapse.

Orchestrated CTL-like immune synapse formation seems highly unlikely for an FDC bearing HIV-ICs. In the case of HIV-IC bound to FDCs, HIV is held intact on the surface of the cell, rather than budding from the plasma membrane of an infected cell. Thus, the intact virion, roughly 120 to 140 nm in diameter, provides spatial separation between the target cell and the CAR-T cell. Although FDCs and BJABs express ICAM-1 [226, 236-238], we speculate that gp120 on the virion and ICAM-1 receptors on FDCs are incapable of an arrangement that resembles a CTL-like immune synapse. Without the capability to form a CTL-like immune synapse with HIV-IC bearing FDCs, CD4-MBL-CAR-T cells remain inactive.

Ironically, FDCs bind native antigen in IC form in a manner essential for B cell recognition and response. Thus, the IC must be arranged in a fashion that allows for detection and immune synapse formation with a cognate GC B cell. However, unlike typical immune synapses, the immune synapse formed between FDCs and GC B cells is a uniquely structured organization of antigen clusters found in the periphery of the interface rather than in the centrally located region common found in other immune synapses [239]. This immune synapse structural arrangement was exclusively found in GC B cells, but absent in $CD4^+$ T cells, CTLs, naïve B cells, and mantle zone B cells. These data may further explain why CAR-T cells did not recognize HIV-ICs on FDCs.

The importance of the viral reservoir on FDCs is well documented. The capacity to eliminate this reservoir is critical to a functional cure for HIV eradication. We recognize that this work was performed *in vitro* with FDCs isolated from uninfected tonsils. Although our pre-formed

HIV-ICs bound to FDCs and permitted infection of H9 cells, FDCs bearing HIV-ICs were unable to induce CAR activation. Other factors found in the highly activated environment of the GC, where FDCs reside, may influence the activation state of FDCs and their capabilities to trap HIV-ICs or express adhesion molecules. Additional factors in the GC may also influence CD4-MBL-CAR-T cell activity as well. Because of this unique environment, it is currently unknown whether FDCs can be lysed by cytolytic mechanisms. Future work to determine the *in vivo* efficacy of CD4-MBL-CAR-T cells targeting the HIV reservoir on FDCs will be highly informative.

To our knowledge, this is the first report to not only demonstrate the efficacy of CAR-T cells to target the viral reservoir on FDCs, but also describe the requirement of adhesion molecules for CAR-T cell activation. Our work herein demonstrates that CD4-MBL-CAR-T cells are ineffective at eliminating FDCs bearing HIV-IC *in vitro*. In support of this, our results indicate that CD4-MBL-CAR-T cells are unable to recognize free virus or immobilized HIV-IC. To explain this, we demonstrate that ICAM-1 is required for efficient lysis of HIV-infected $CD4^+$ T cells. We conclude that adhesion molecules are required for CD4-MBL-CAR-T cell mediated recognition and lysis of cells bearing native HIV antigen. We recommend future HIV cure strategies consider all reservoirs for HIV, including that found on FDCs, and that future CAR designs consider the role of adhesion molecules and/or CAR affinity when determining cytolytic activity.

2.6 Figures

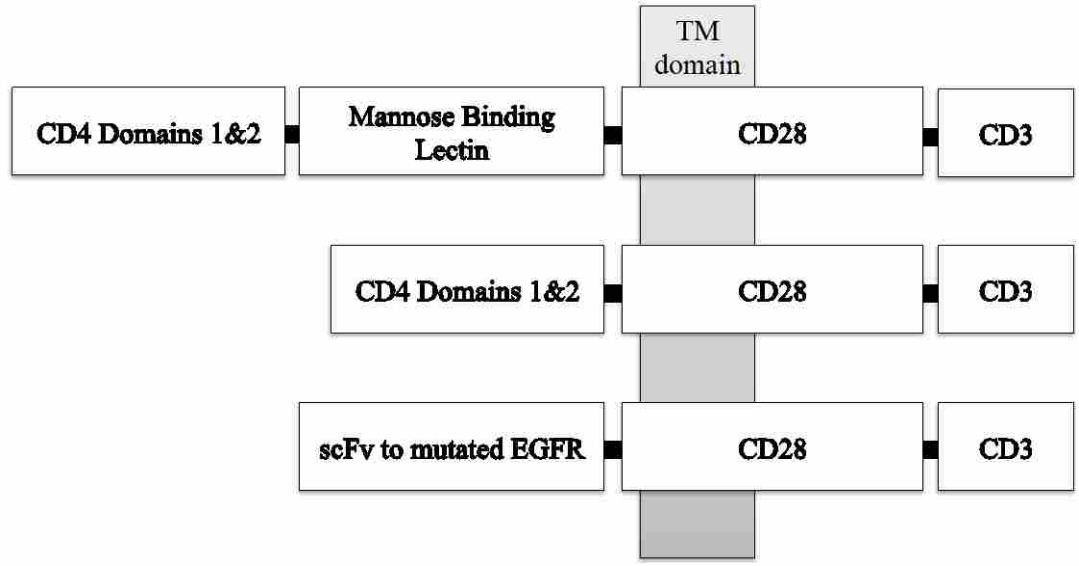


Figure 1 CAR constructs utilized in this study. Each construct contains identical transmembrane and signaling domains. The CD4-MBL construct varies from the CD4 construct by the presence of the mannose binding lectin. As a control, the 139 CAR expresses an scFv to a mutated EGFR. The Berger lab at the National Institutes of Health designed and provided the constructs used in this study.

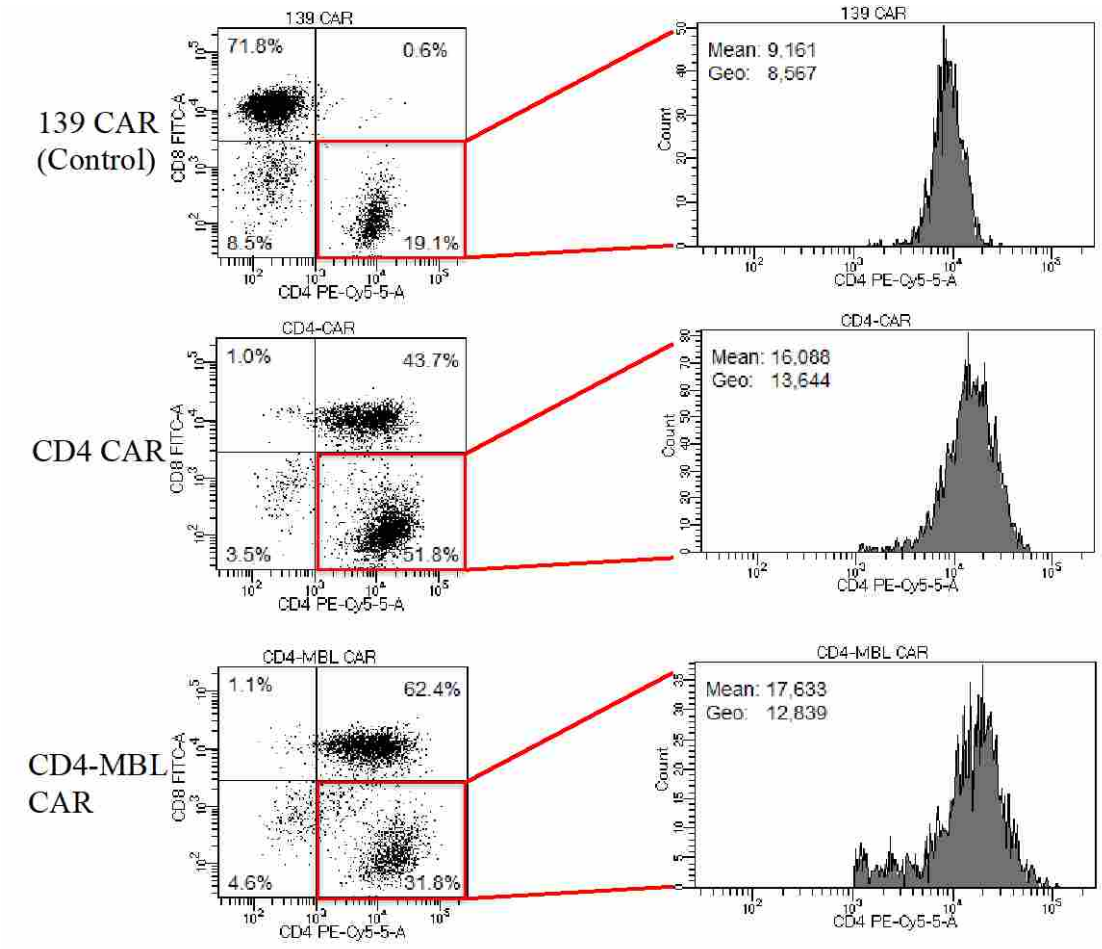


Figure 2 CD4 and CD8 expression on transduced cells. One week after retroviral transduction of activated PBMCs with the indicated constructs, the cells were analyzed by flow cytometry for CD4 and CD8 expression. Cells were incubated with Chrompure mouse IgG in PBS to inhibit Fcγ interactions, and labeled with PE-Cy5 mouse anti-human CD4 (clone RPA-T4) and FITC-mouse anti-human CD8 (clone B9.11). After 1 hour, the cells were washed and 10,000 events examined. Bivariate plots on the left show percentages of each phenotype present in each quadrant with the upper right-hand quadrant demonstrating the CD4 and CD8 double positive cells. Histograms on the right show mean fluorescence intensity (Mean) and geometric mean fluorescence intensity (Geo) of CD4 single positive cells from the lower right quadrant as outlined in red. Although donors varied in CD8:CD4 ratios, similar results were found for mean CAR expression values. These data are representative of five independent experiments using three different donors.

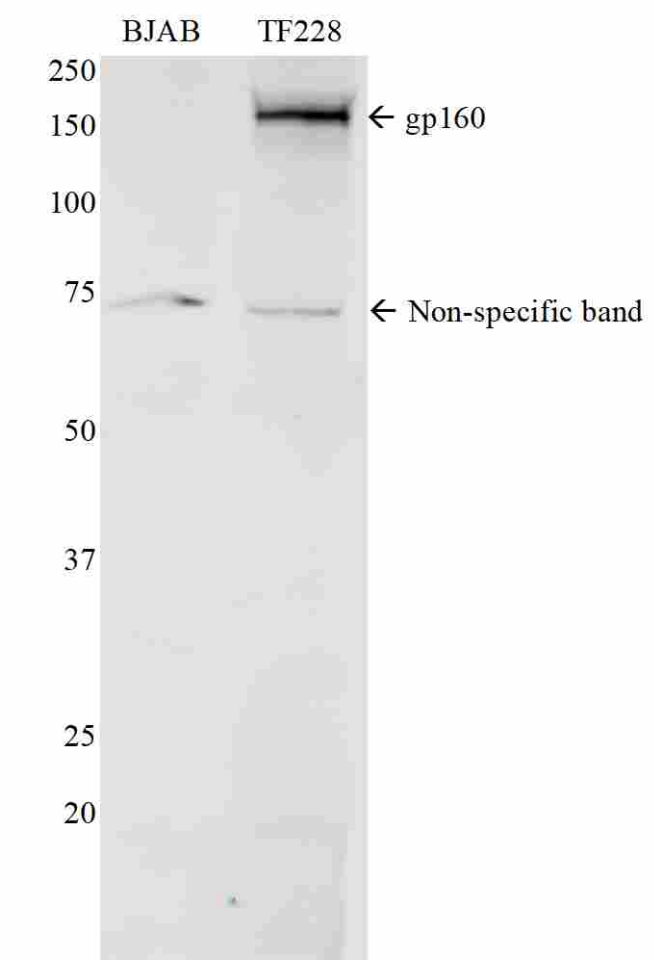


Figure 3 TF228 stably express gp160. 1 x 10^7 BJAB and TF228 cells were lysed as described in *Materials and Methods*. Equal protein concentrations were loaded on an SDS-PAGE and then transferred to a nitrocellulose membrane. After blocking in milk, gp160 was detected using mouse anti-gp120 antibody (Chessie 13). Primary antibody was detected using IRDye 800-donkey anti-mouse, and imaged on a Li-COR Odyssey scanner. The arrows demonstrate gp160 and non-specific bands. Molecular weights are labeled on the left.

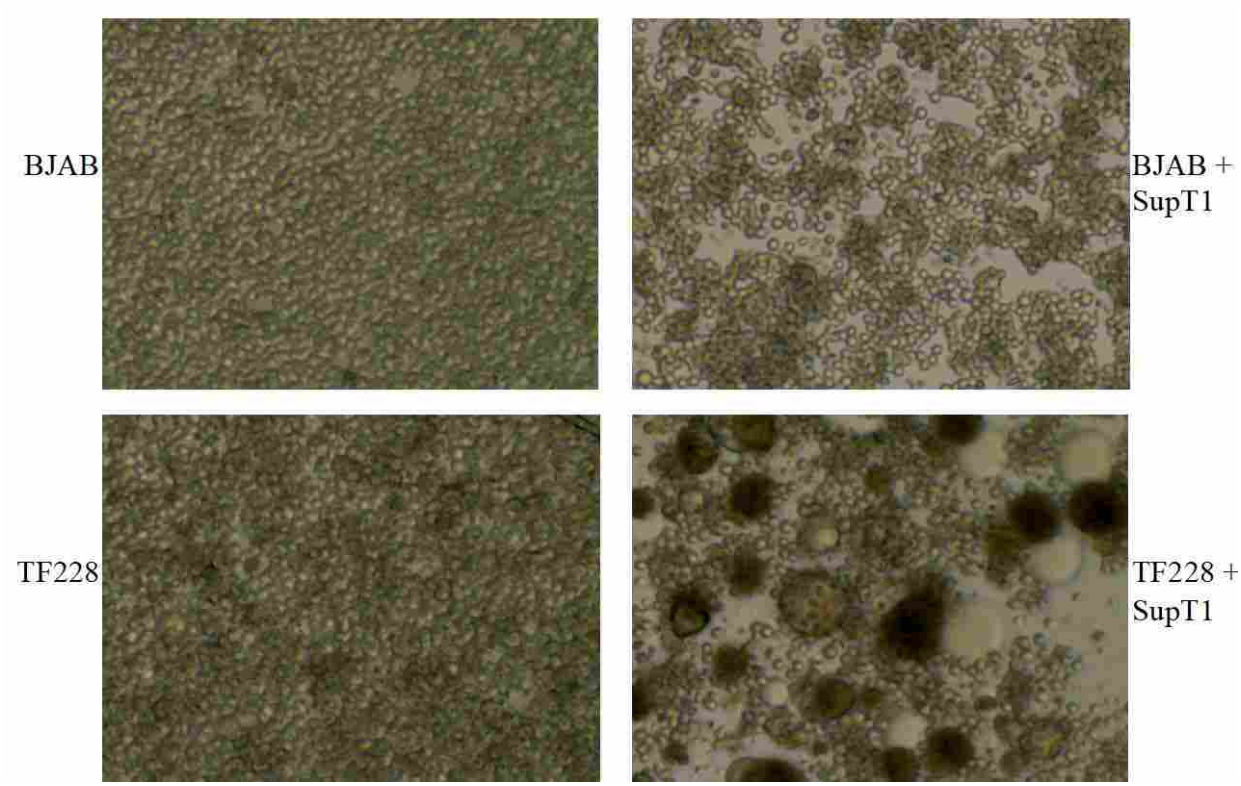


Figure 4 gp160 surface expression on TF228 cells induces syncytia with CD4 expressing SupT1 cells. 2 x 10^5 total cells were added to individual wells in a 24-well plate. When SupT1 cells were added, 1 x 10^5 of each cell type was added. After 16 hours, wells were imaged using an AMG Evos Imager at 10X magnification. Images represent one of three experiments.

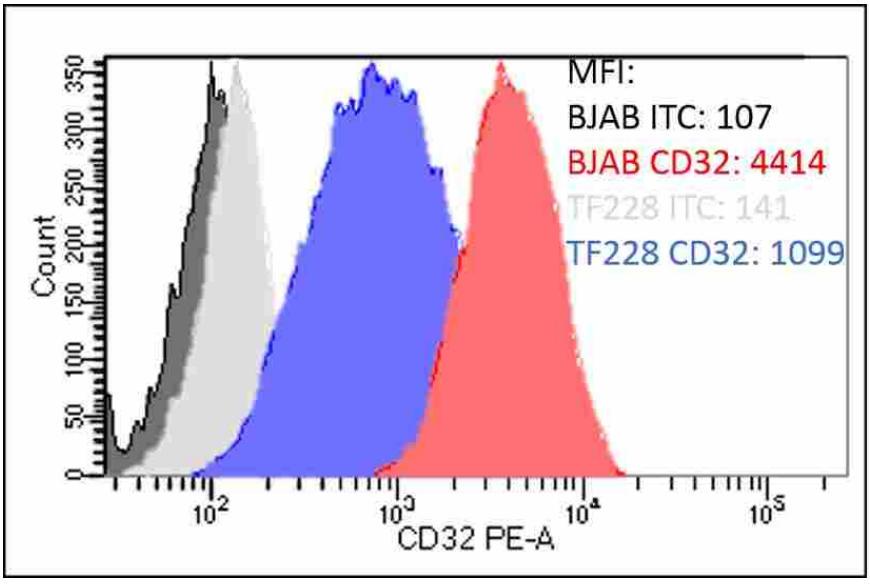


Figure 5 BJAB cells express CD32. 1 x 10^6 BJAB cells were suspended in PBS and blocked with 5 µg of Chrompure Mouse IgG (Jackson ImmunoResearch). Cells were labeled with either PE-mouse anti-human-CD32, or isotype control. After 45 minutes on ice, the cells were washed and resuspended in PBS. 2 µl 7-AAD was added to exclude dead cells from analysis. The samples were then run on a BD FACSfusion, and 30,000 events were recorded.

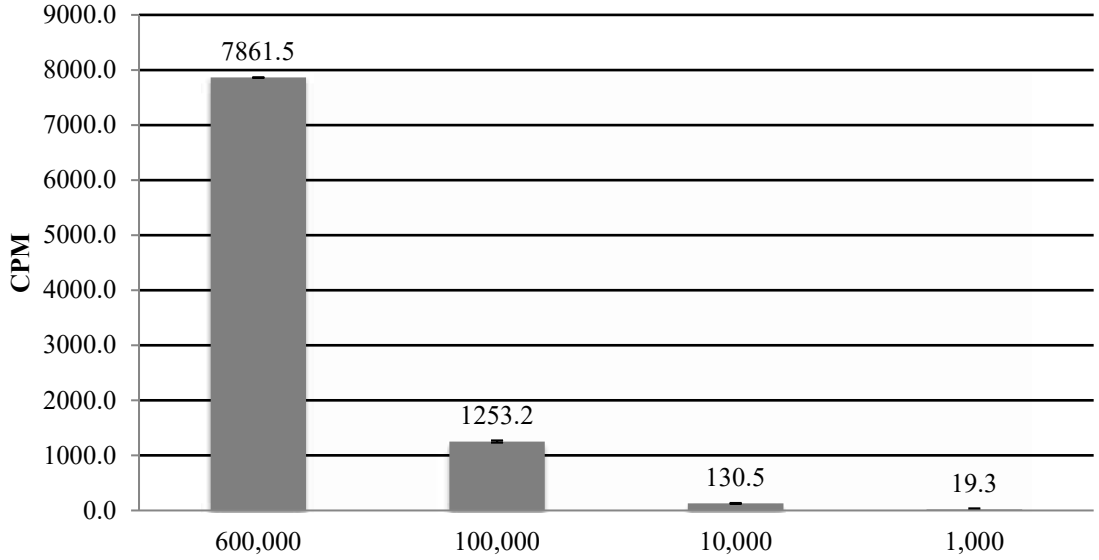


Figure 6 ^{51}CR-labeling of FDCs. FDCs were prepared by magnetic cell sorting and the cells labeled with ^{51}Cr as described in *Materials and Methods*. After 1 hour, the cells were washed, suspended in 100 µl complete medium, and counted in a Packard Cobra II gamma counter. Triplicates were examined for each cell density. The data presented represent the mean and the error bars represent standard error of the mean (SEM). These data are representative of two independent experiments.

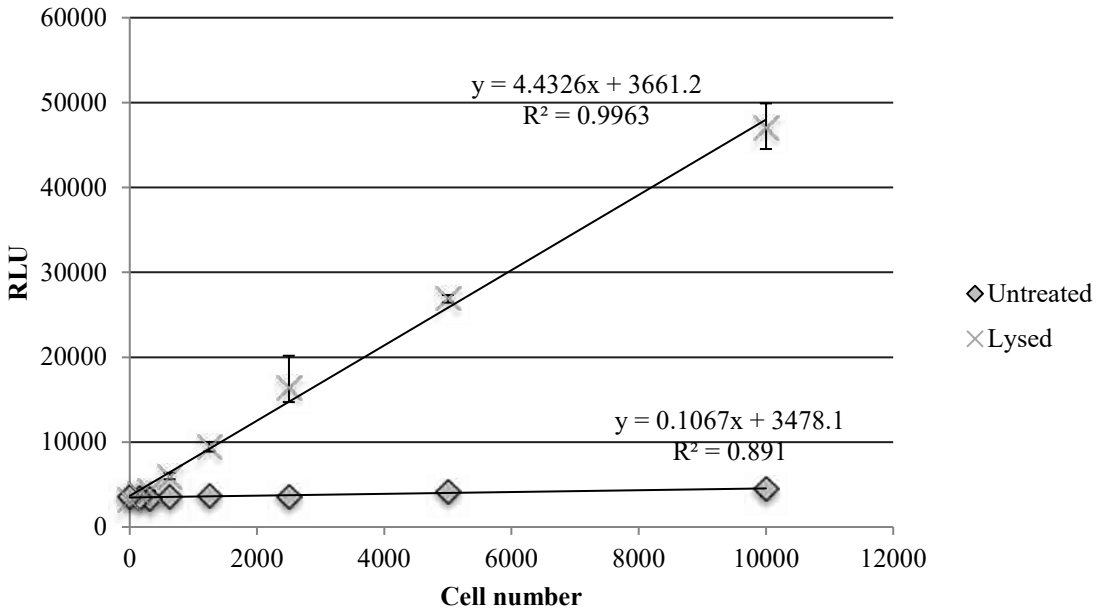


Figure 7 CytoTox-Glo detects lysis in low cell quantities. Using freshly isolated PBMCs, cells were plated in triplicate at various quantities up to 1 x 10^4 cells. Lysed samples were treated with digitonin and assay buffer was added per manufacturer's instructions. Plates were read using on a BioTek Synergy HT plate reader with luminescence settings. Graph points represent the mean of each data set with error bars representing SEM. Data are representative of two independent experiments.

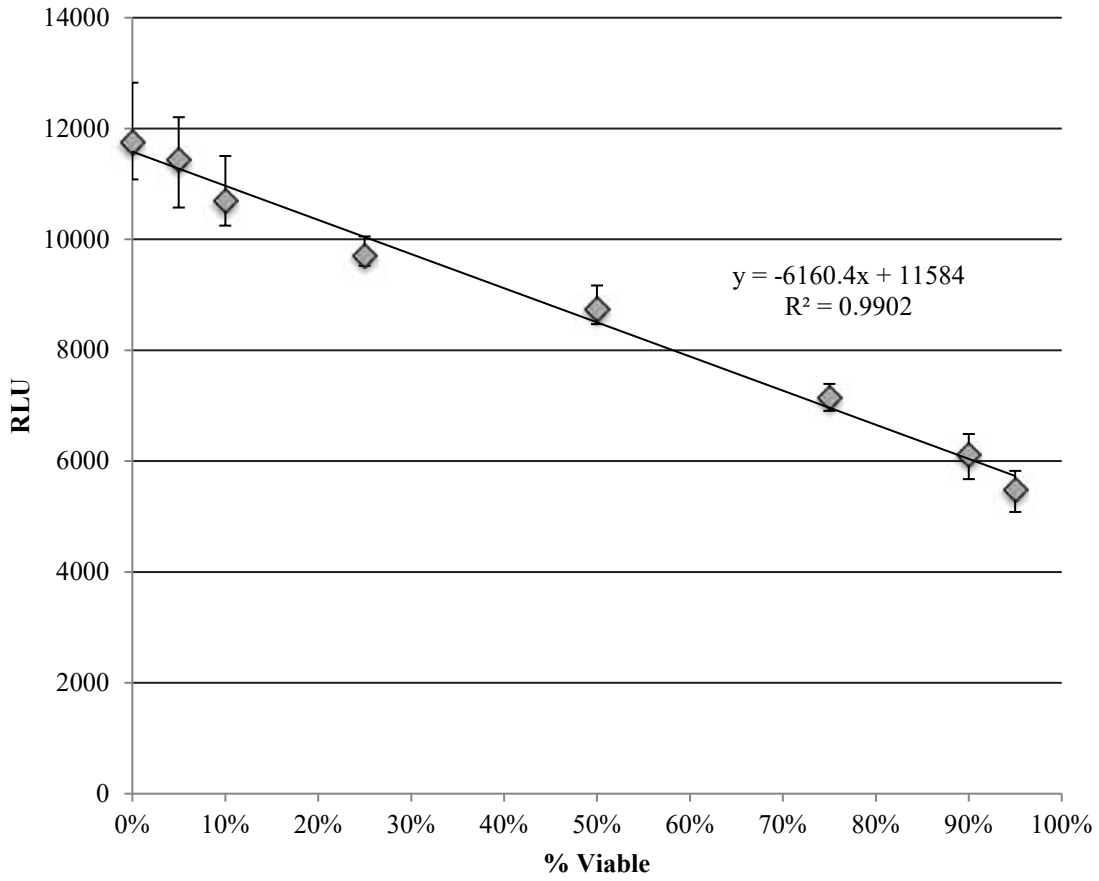


Figure 8 CytoTox-Glo detection is linear over a broad range of viability. Freshly isolated PBMCs were separated into two samples. One sample was prepared in assay buffer, while the other sample was lysed in a minimal volume of distilled water. Once lysis was complete, as detected by trypan blue staining, 1.5 M NaCl was added to bring the final concentration to 0.15 M NaCl. This was then diluted in assay buffer and added to live PBMCs at appropriate concentrations to achieve correct viability percentage. 1 x 10^4 total PBMCs were added to wells in triplicate and samples were processed according to manufacturer's instructions. Samples were analyzed on BioTek Synergy HT plate reader in under luminescent settings. Data points represent mean relative luminescent units (RLU) and error bars, the SEM. Graph is representative of two independent experiments.

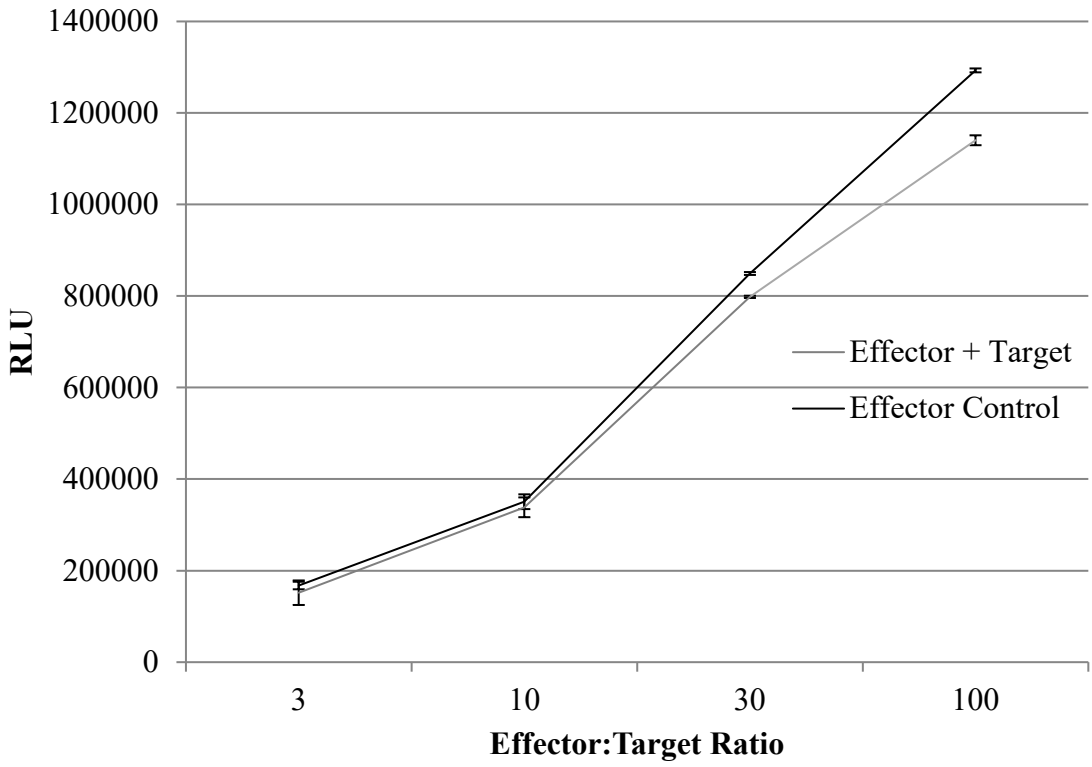


Figure 9 CytoTox-Glo displays high background in cytotoxicity assays. Using conventional allogeneic CTL assays as described in Materials and Methods, the efficacy of CytoTox-Glo for cytotoxicity assays was demonstrated using various effector:target ratios. Cell lysis was undetectable due to the high background associated with the effector cells. Samples were processed per manufacturer's instructions and RLU was detected using a BioTek Synergy HT plate reader. Data points plot the mean and error bars demonstrate SEM. Data are representative of three independent experiments

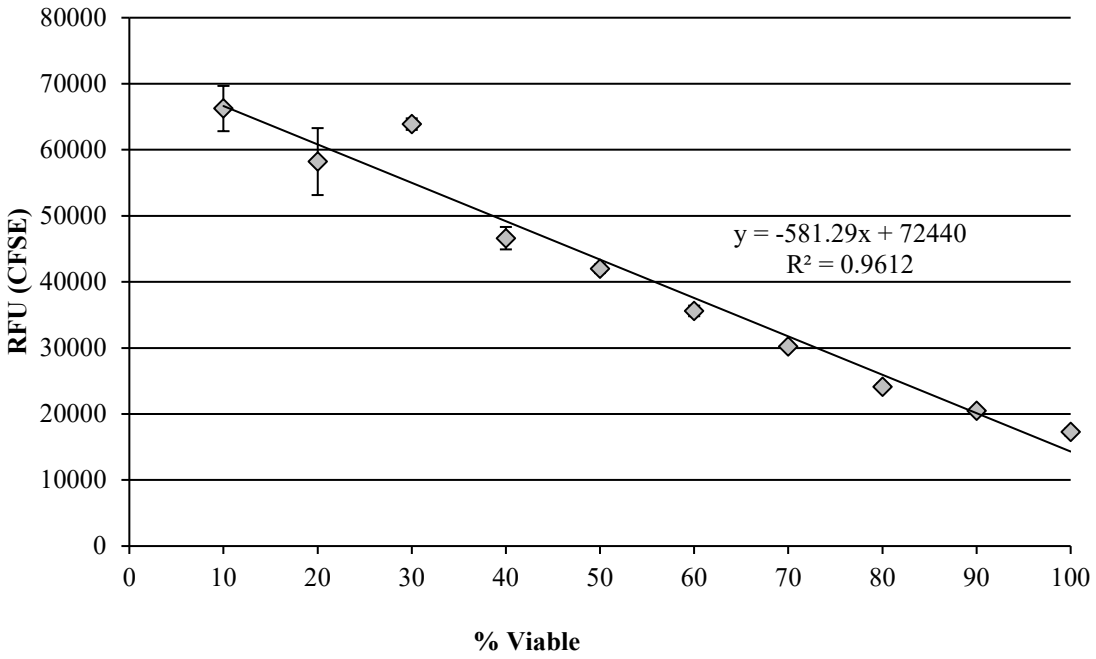
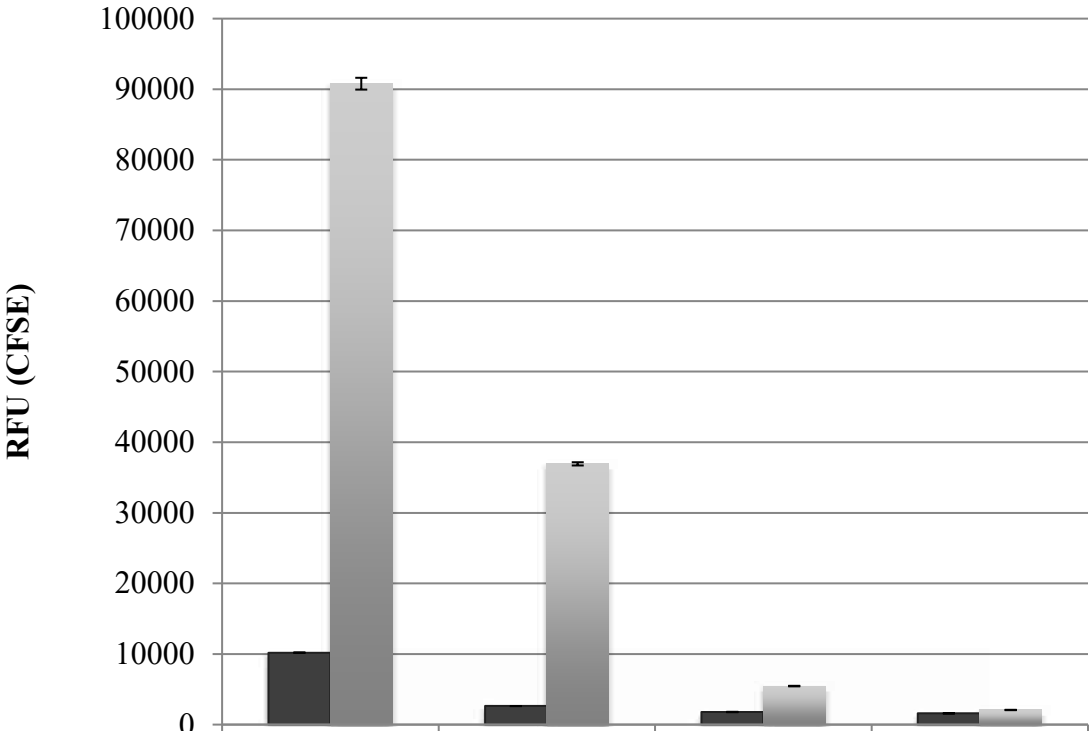


Figure 10 CFSE release detection is linear over a wide range of viability. PBMCs were labeled with CFSE as described in *Materials and Methods.* After quenching, the samples were equally divided into two. To one, the cells were resuspended in CFSE buffer. To the other, the cells were resuspended in a minimal volume of distilled water. After lysis, as determine by trypan blue staining, 1.5 M NaCl was added at 10 % of total volume to obtain a 0.15 M NaCl solution. This was then diluted in CFSE buffer to obtain the final volume as the first sample. Samples of varying viable percentages were obtained by combining the viable sample with the lysed sample at different ratios. Triplicates were placed in a 96 well plate. Samples were centrifuged and supernatants were collected for measurement using the Synergy HT plate reader (BioTek) using fluorescence settings with the 485/20 excitation filter and the 529/20 emission filter. The data represent the mean and SEM of samples run in triplicate.



	10^5 Cells	10^4 Cells	10^3 Cells	10^2 Cells
TF228	10189	2624	1785	1587
Lysed TF228	90784	36934	5454	2070

Figure 11 Effect of cell count on CFSE Release Sensitivity. TF228 cells were labeled with CFSE in bulk as described in *Materials and Methods*. Cells were then plated at the appropriate concentrations in triplicates in a round bottom 96-well plate. The final volume was adjusted to 200 µl. For lysed TF228, 1 µl of 20mg/ml digitonin was added. After four hours, the cells were pelleted and 100 µl supernatant was placed in a black 96 well plate. The samples were read using the Synergy HT plate reader. Bars and values indicate mean relative fluorescence intensity. Error bars represent standard error of the mean.

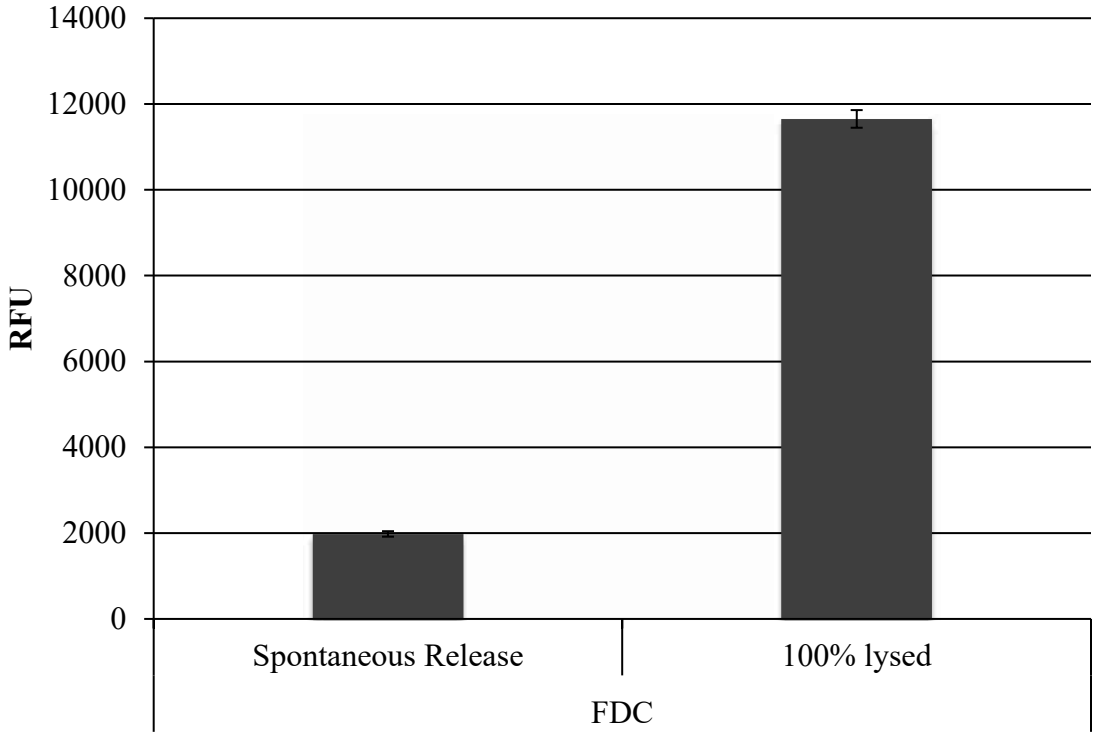


Figure 12 CFSE release from labeled FDCs. 8 x 10^4 FACS isolated FDCs were labeled with CFSE and plated in a round bottom 96 well plate at 2 x 10^4 cells/well in 200 µl CFSE assay buffer as described in materials and methods. 1 µl Digitonin (20 mg/ml) was added to 100% lysed wells and the plate was incubated at 37°C for 4 hours at which point the plate was spun, and 100 µl of supernatant was placed in a black 96-well flat bottom plate and analyzed using a BioTek Synergy HT plate reader. The graph shows the mean and SEM from one experiment of duplicate wells. The graph represents one of three independent experiments.

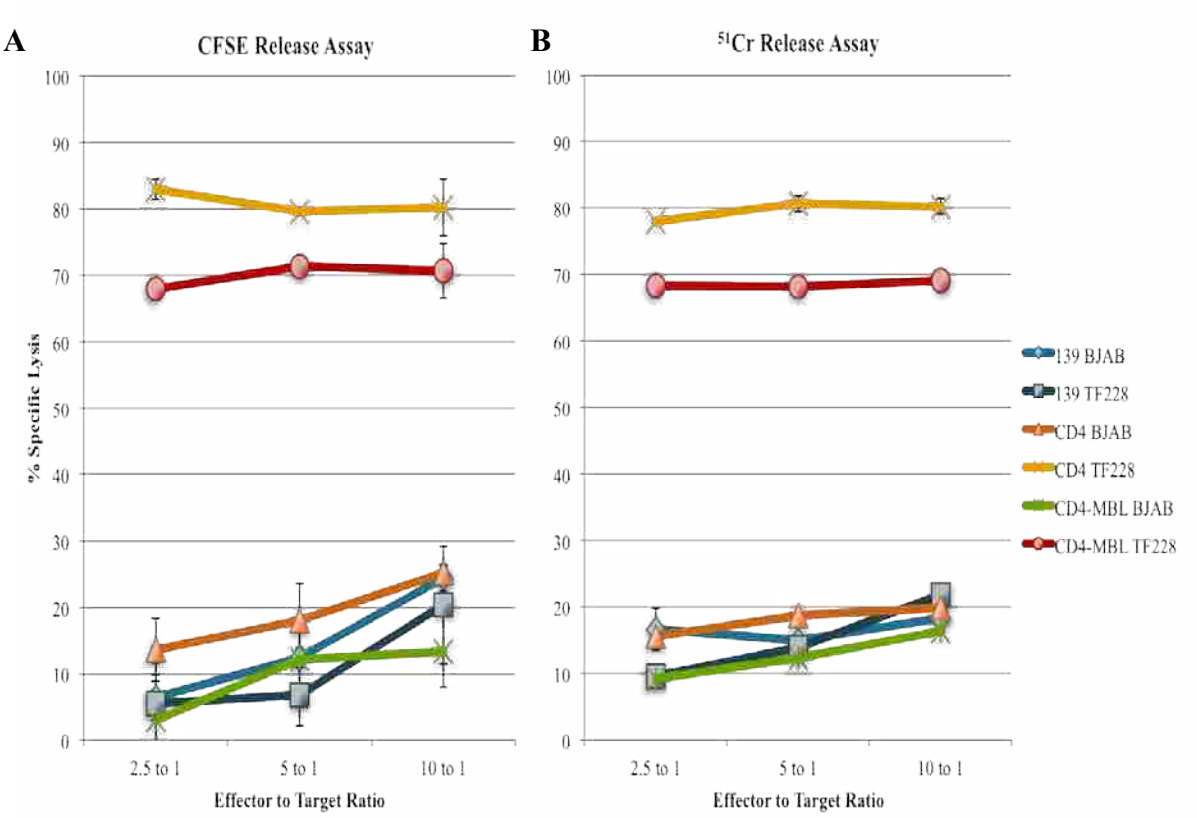


Figure 13 CFSE Release Assay is comparable to ^{51}Cr Release Assay. BJAB and TF228 cells were labeled with ^{51}Cr or CFSE and experimental procedures were performed as outlined in *Materials and Methods*. 3 x 10^4 target cells were added to a round-bottom 96 well plate in triplicate. The appropriate amount of effector cells was added to the wells. Final volume was 200 µl. After 4 hours, 100 µl supernatant was collected and analyzed. Specific lysis was calculated for the CFSE Release Assay (A) and ^{51}Cr release assay (B) as described in the methods section.

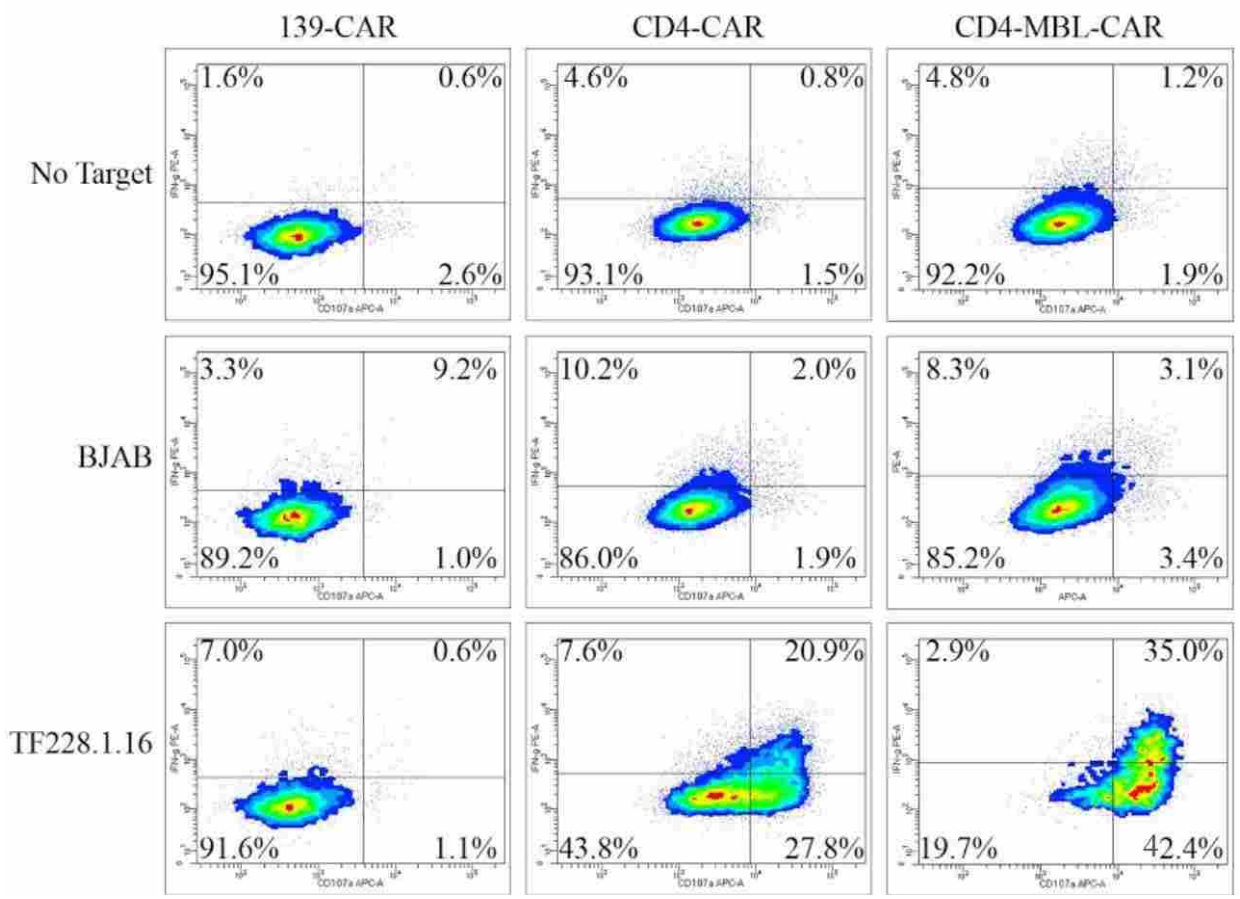


Figure 14 CD4- and CD4-MBL-CAR-T cells are activated in the presence of Env-expressing TF228 cells. Equal numbers of 139-, CD4-, and CD4-MBL-CAR-T cells were cultured alone or with BJAB (Env-) or TF228 (Env+) cells. The CAR activation assay was performed as described in Materials and Methods. Plots represent $CD8^+$ CAR T cells gated as follows: lymphocyte→ single cell→ $CD8^+$ or $CD8^+$ $CD4^+$ for CD4-expressing CAR-T cells. Plots represent activation as determined by an increase in CD107a and IFN-γ. Plots are representative of one of four experiments.

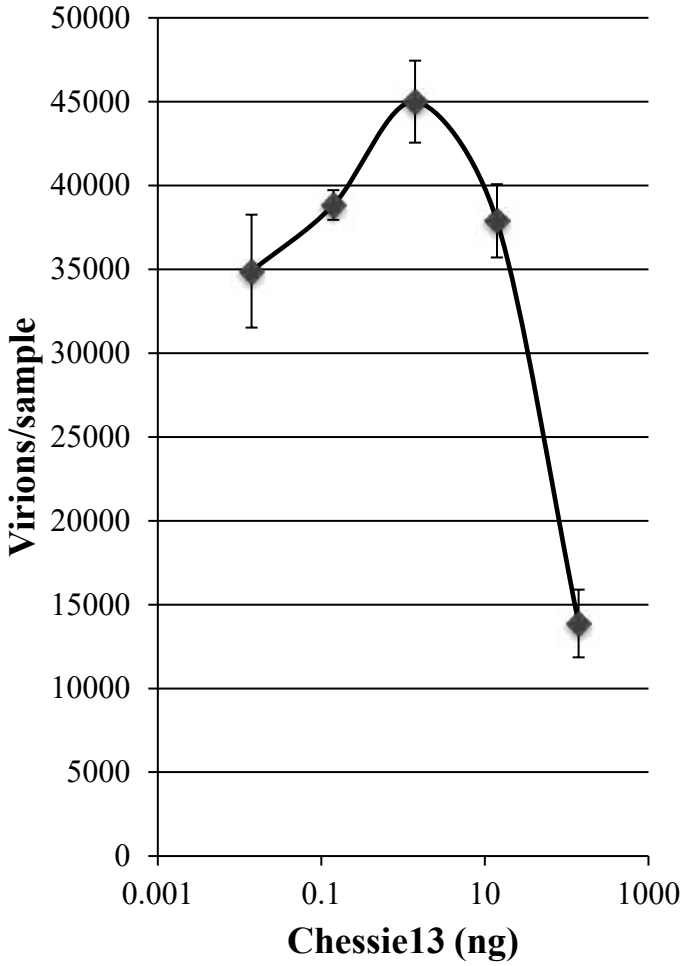


Figure 15 Efficient HIV-IC binding to FDCs is dependent on optimal α-gp120 concentration. HIV-ICs were formed when 5 x 10^7 virions were incubated with a range of α-gp120 (Chessie13) from 14 pg to 140 ng. After incubation, HIV-ICs were then added to 2 x 10^4 FDCs and incubated for 1 hour at 37°C. The cells were washed to remove unbound virions. Samples were lysed and viral RNA was isolated (QIAGEN), and reverse transcribed using Superscript IV (ThermoFisher). Q-PCR was performed using a primer and probe set targeting the reverse transcriptase gene in HIV. Values were compared to a standard curve of ACH-2 cell DNA. Graph is representative of one of three experiments.

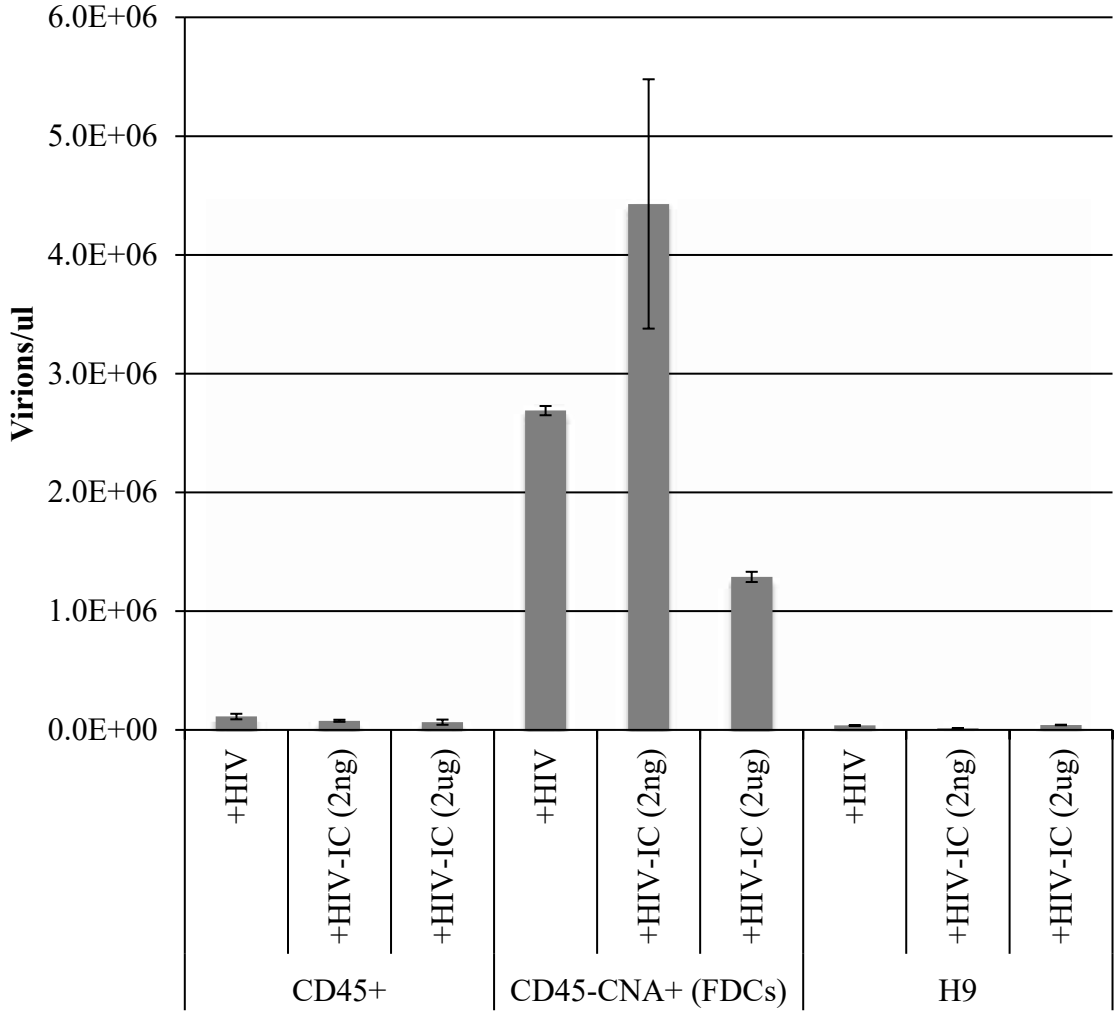


Figure 16 FDCs trap infectious HIV-IC. FDCs were defined as $CD45^{-}CNA^{+}$ while tonsilar cells were sorted from $CD45^{+}$ cells. Cells were irradiated at 1200 RAD. 3 x 10^4 FDCs were placed in 12x75 Falcon Tubes and either preformed HIV-IC or HIV stock was added. HIV-ICs were prepared by combining 50 µl of HIV stock (equivalent to 5 x 10^7 virions) and 2 ng or 2 µg of anti-gp120 (Chessie13). After 45 minutes at 4°C, the HIV or HIV-ICs were added to appropriate tubes. Samples labeled H9 contained equal volumes of HIV or HIV-ICs without any cells for the loading period. Samples were incubated at 37°C to load HIV or HIV-ICs onto cells. Then, the samples were washed twice with 4 ml PBS. Tubes were resuspended with 10% CM and 3 x 10^5 H9 cells were added for a total volume of 1 ml. Samples were incubated at 37°C for 3 days. Then, the cells were pelleted at 300 x g for 5 min, and 140 µl of supernatant was removed and subject to vRNA isolation. Elution volume was 40 µl. 11 µl of vRNA were used for cDNA synthesis in a total volume of 20 µl. 2 µl of cDNA were used for QPCR in triplicate. Error bars are standard error of the mean. Graph represents one of two experiments in triplicate.

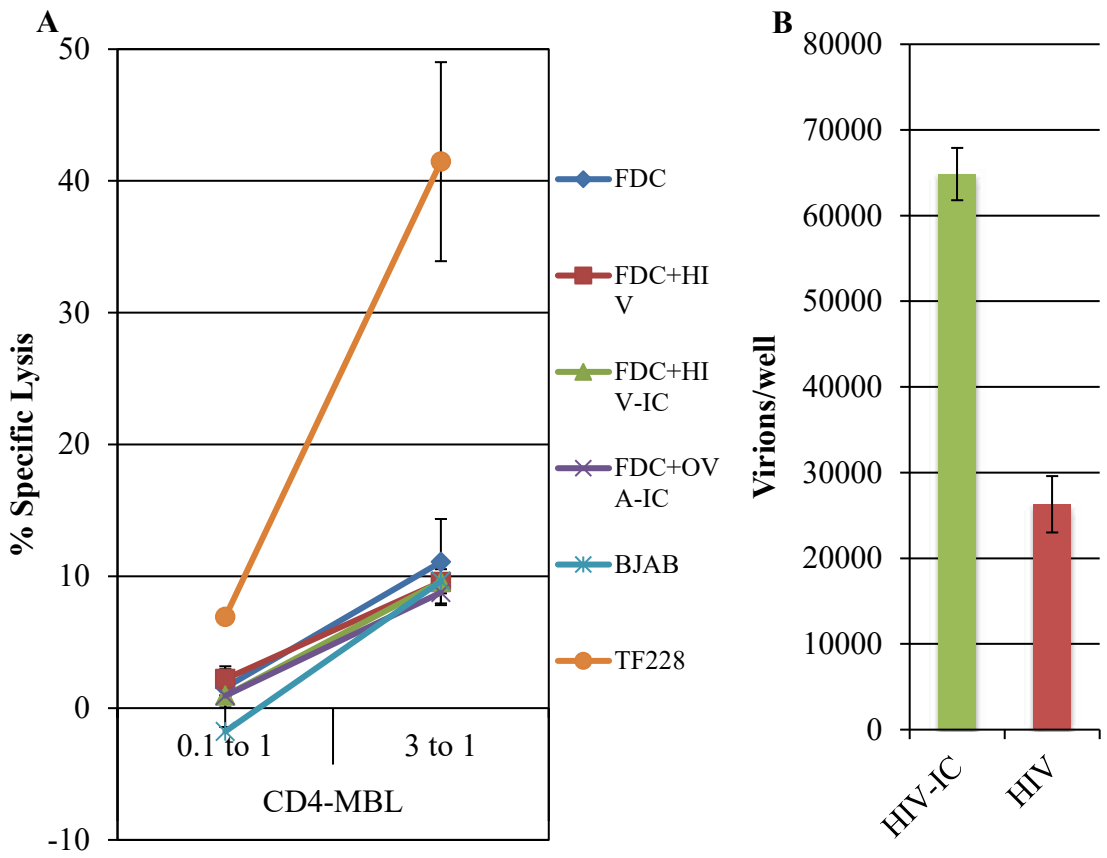


Figure 17 CD4-MBL-CAR-T cells are unable to kill FDCs in the presence of HIV or HIV-IC. **A)** FDC, BJAB, and TF228 cells were labeled with CFSE as described in the methods. HIV-IC were formed by incubation of 5 x 10^7 virions and 2 ng anti-gp120 (Chessie 13) for 45 minutes at 4 °C. OVA-IC was formed by incubation of 7.4 ng OVA with 25.2 ng anti-OVA for 45 minutes at 4°C. 2 x 10^4 FDCs/well were incubated with HIV stock (5 x 10^7 virions), HIV-IC, or OVA-IC for 1 hour at 37°C. The cells were washed 2X in PBS, then resuspended in CFSE assay buffer and plated in a round-bottom 96 well plate. Either 2 x 10^3 or 6 x 10^4 CD4-MBL CAR-T cells were added where appropriate and incubated at 37°C for 4 hours. After 4 hours 100 µl of assay buffer from each well was added to a black 96-well plate and analyzed on a fluorescent plate reader (Synergy H4) using fluorescent setting with an excitation of 488 nm and emission of 529 nm. Specific lysis was calculated as described in Materials and Methods. Graph represents one of 3 independent experiments performed in triplicate. **B)** HIV quantification from samples containing HIV or HIV-IC from **A**. After CFSE assay, cells were washed stored at -80 °C. Viral RNA was isolated from samples using a Viral RNA isolation kit (QIAGEN). Reverse transcription was performed with Superscript IV and random hexamers. Q-PCR was performed with HIV RT specific primers and probes. Quantitation was compared to a standard curve prepared with ACH-2 cells.

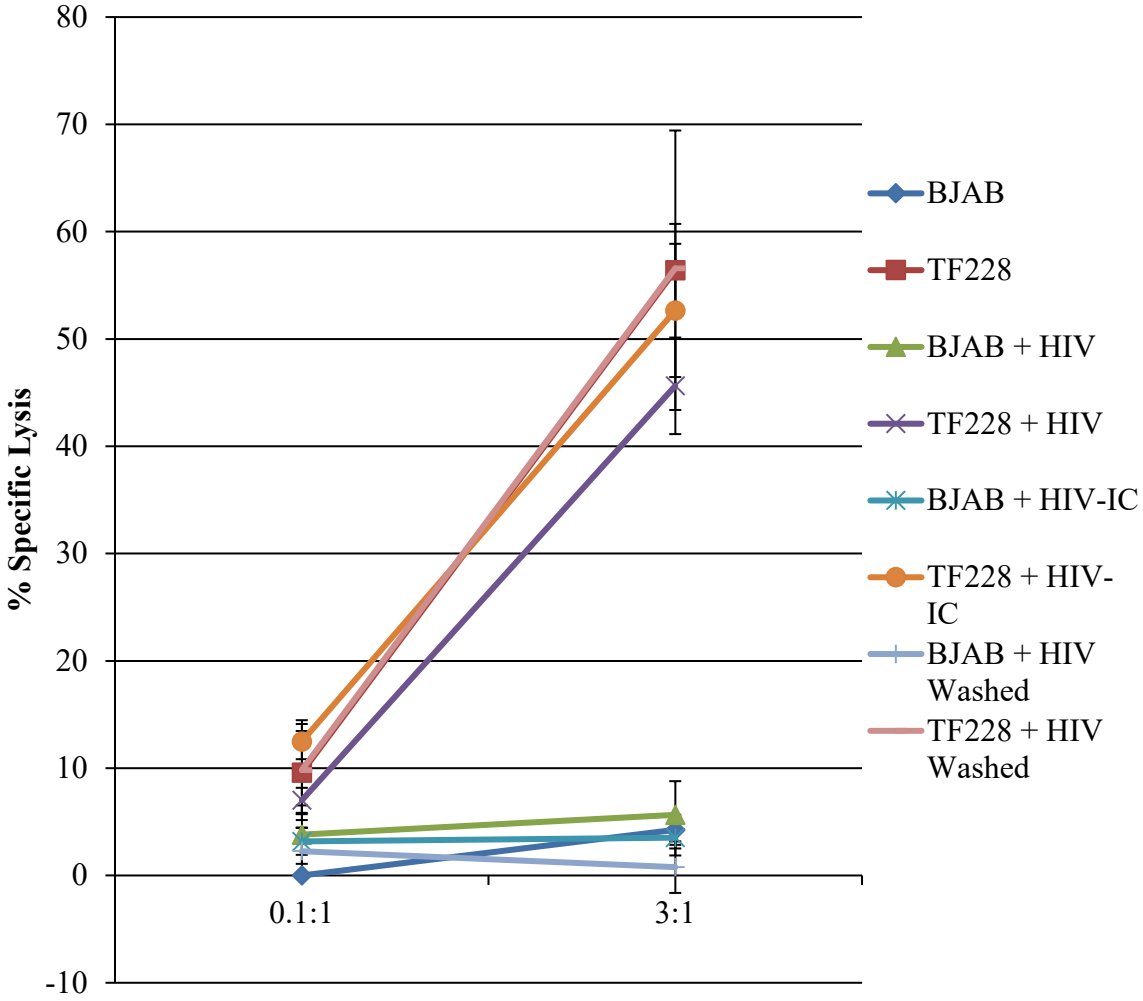


Figure 18 HIV or HIV-immune complexes do not induce CAR-T mediated killing. TF228 and BJAB target cells were labeled with CFSE as described in methods section. After labeling, target cells were pre-incubated with HIV or HIV-IC. For the control, an equal volume of complete media was added. After 1 hour, the cells were washed 2x and resuspended in CFSE Assay buffer. CAR-T cells were added at the appropriate ratio and incubated for 4 hours. After 4 hours, 100 µl of each well was placed in a black 96 well plate and fluorescence was read using 488 nm excitation and 529 nm emission. Specific lysis was calculated as outlined in methods section. Graph represents one of two experiments performed in triplicate.

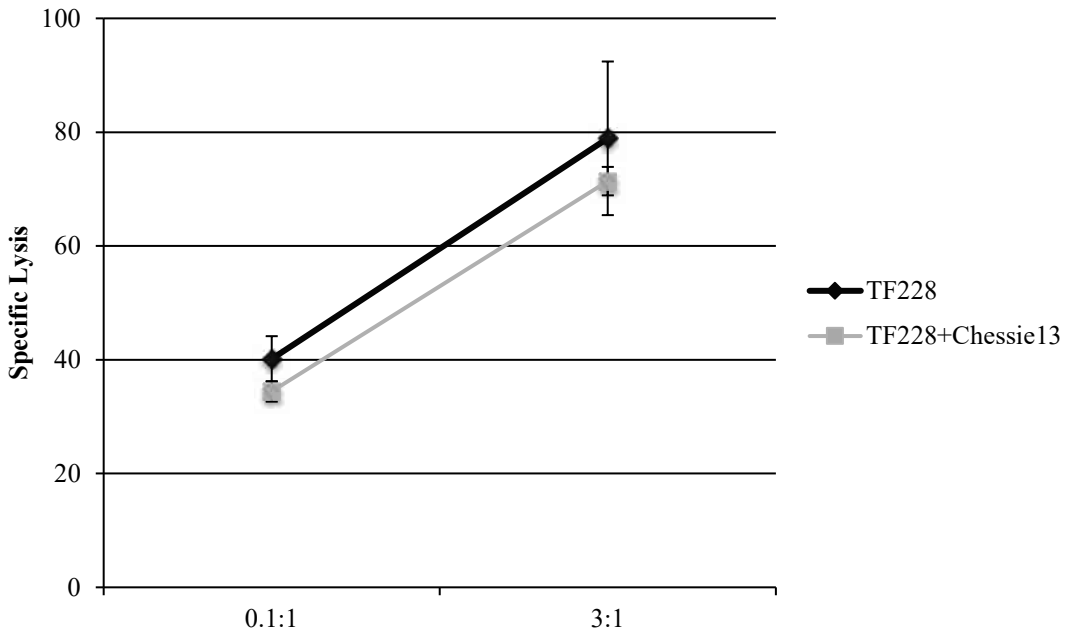


Figure 19 Presence of α-gp120 does not influence CD4-MBL-CAR-T killing of TF228 cells. CFSE-labeled TF228 cells were incubated with 30 μg of α-gp120, Chessie 13, or without antibody. After 30 minutes, 3 x 10^4 TF228 cells were plated in triplicate for each E:T ratio, and CD4-MBL-CAR-T cells were added at the appropriate ratio. After 4 hours, specific lysis was quantified using a BioTek fluorescent plate reader.

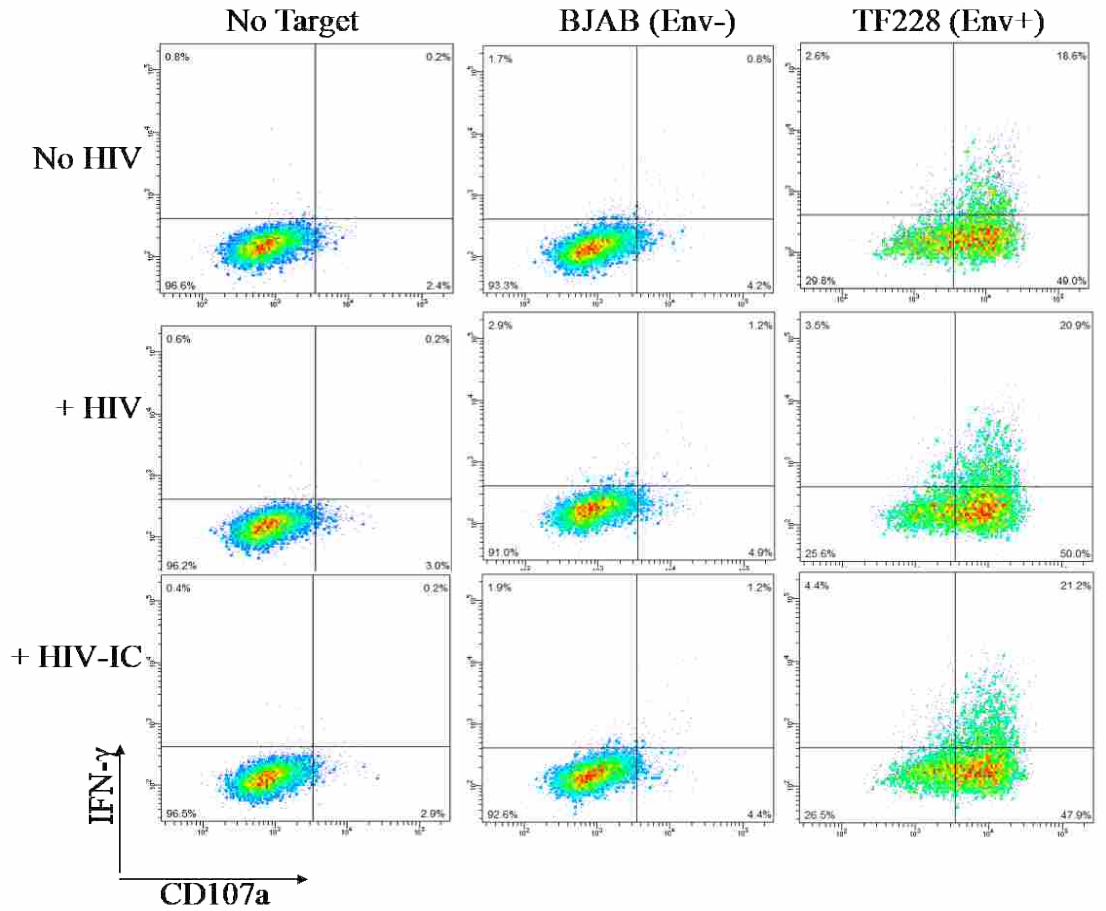


Figure 20 The presence of HIV or HIV-IC is insufficient to activate CD4-MBL CAR-T cells. HIV-IC were prepared by incubation of $5x10^7$ virions with 2 ng non-neutralizing α-gp120 Chessie 13 at 4°C. HIV or HIV-IC was added to 1 x 10^5 BJAB or TF228 cells and incubated for 45 minutes at 37°C. $1x10^5$ CD4-MBL CAR-T cells were added to each sample in 100 µl volume. 100 µl of assay media was added (10% HI Human serum, 1X Glutamax, 1X NEAA, 10mM HEPES, in PBS) supplemented with 3X Monensin, 3X Brefeldin A, and 2ul of APC-α-CD107a. Cells were incubated for 6 hours at 37°C after which the cells were washed, and treated with 100 µl PBS containing 2µl APCFire-750-α-CD4, 8 µl FITC-α-CD8, 10 µg Chrompure Mouse IgG. Samples were incubated 20 minutes at RT. The samples were washed and resuspended in 200 µl Cytofix/Cytoperm. After 20 minutes at RT, the samples were washed in 1X perm/wash buffer. The cells were resuspended in 100 µl perm/wash buffer with 2 µl PE-anti-IFN-γ. After 20 minutes at RT, the samples were washed in perm/wash buffer and resuspended in 100 µl PBS. To this, 100 µl of 4% paraformaldehyde was added, and the samples were placed at 4°C overnight. The next day, the cells were washed and analyzed. Single cells gated on $CD4^+CD8^+$ were analyzed for CD107a and IFN-γ expression. Plots are representative of two experiments, one in triplicate.

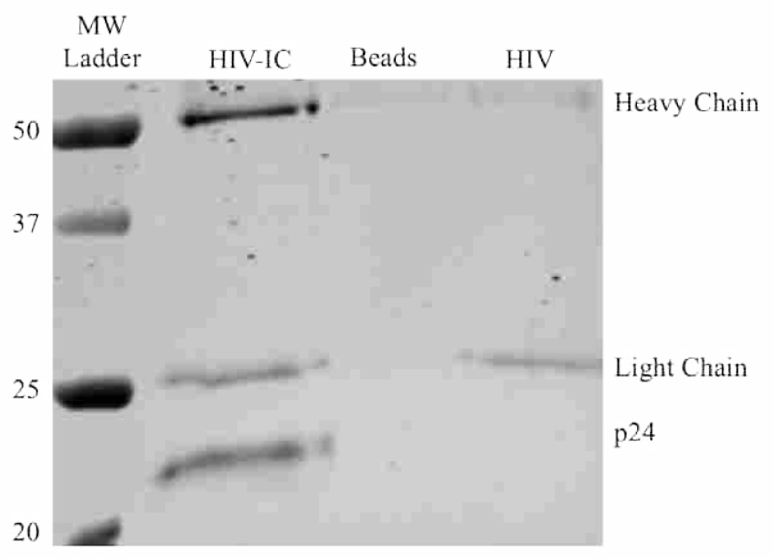


Figure 21 Sheep α-Rat Dynabeads efficiently bind HIV-IC. 25 µl of washed sheep α-rat Dynabeads (ThermoFisher) were incubated with α-gp120 antibody, Chessie 13, (HIV-IC) or PBS for 30 minutes at 4°C. The beads were then washed in PBS twice, and HIV or CAR Activation Assay buffer was added. Beads were incubated at 37°C for 1 hour, then washed 3 times in PBS. 40 µl of 4X loading buffer was added to the beads and heated at 98°C for 15 minutes. Samples were run on 12 % SDS-PAGE and transferred to a nitrocellulose membrane. p24 was detected with in house prepared mouse α-p24 antibody and IRDye800 CW donkey α-mouse (Li-Cor) and imaged on a Li-Cor Odyssey Scanner.

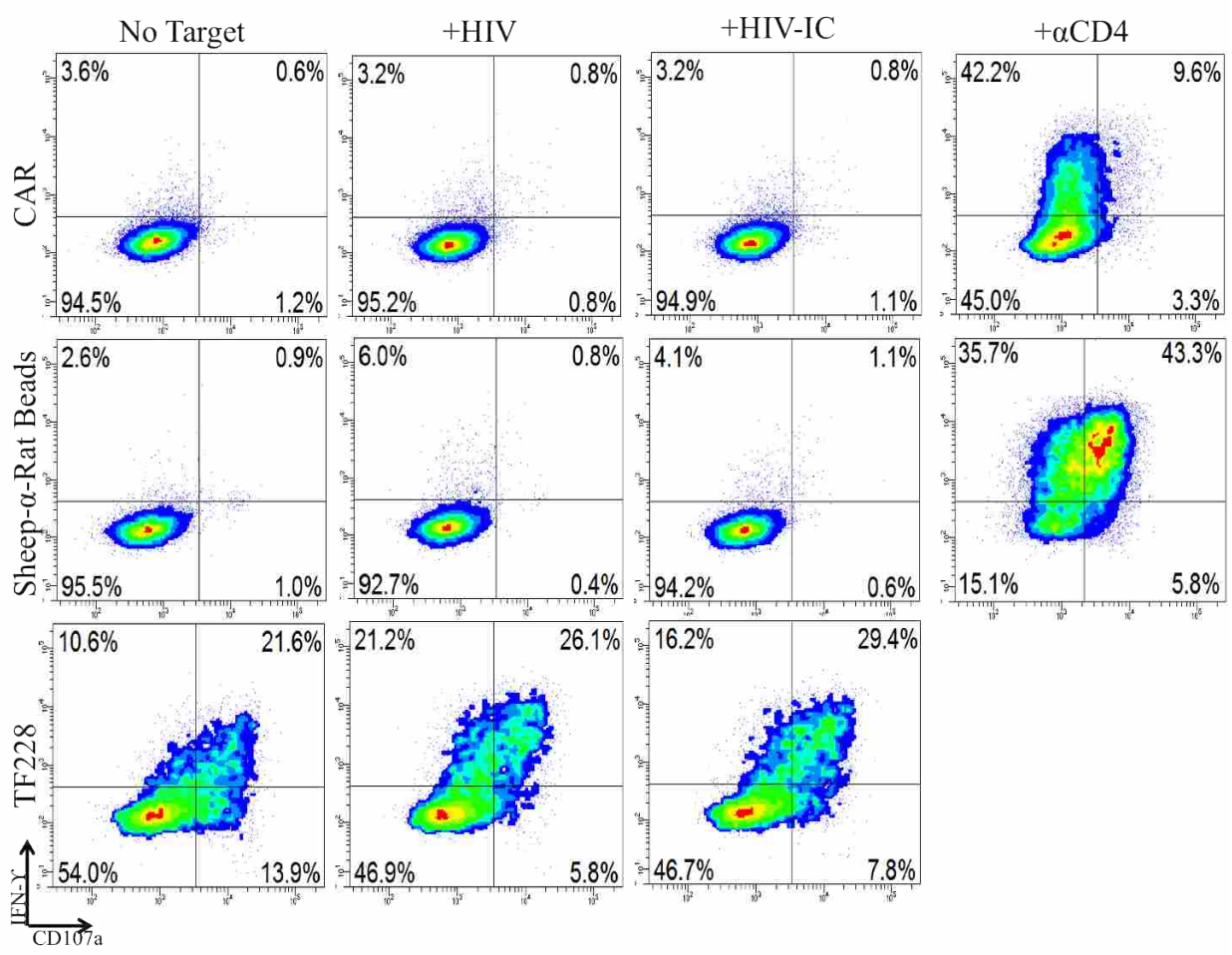


Figure 22 CD4-MBL-CAR-T cells are not activated in the presence of immobilized HIV-IC. HIV was added to control beads or α-gp120 coated beads for 45 minutes at 4 °C. Beads were then washed in PBS and 2 x 10^5 CD4-MBL-CAR-T cells were added. In separate tubes, α-CD4 (clone SK3) was added to CD4-MBL-CAR-T cells or pre-bound to Dynabeads. TF228 cells acted as a positive control. Plots represent $CD8^+CD4^+$ effector cells.

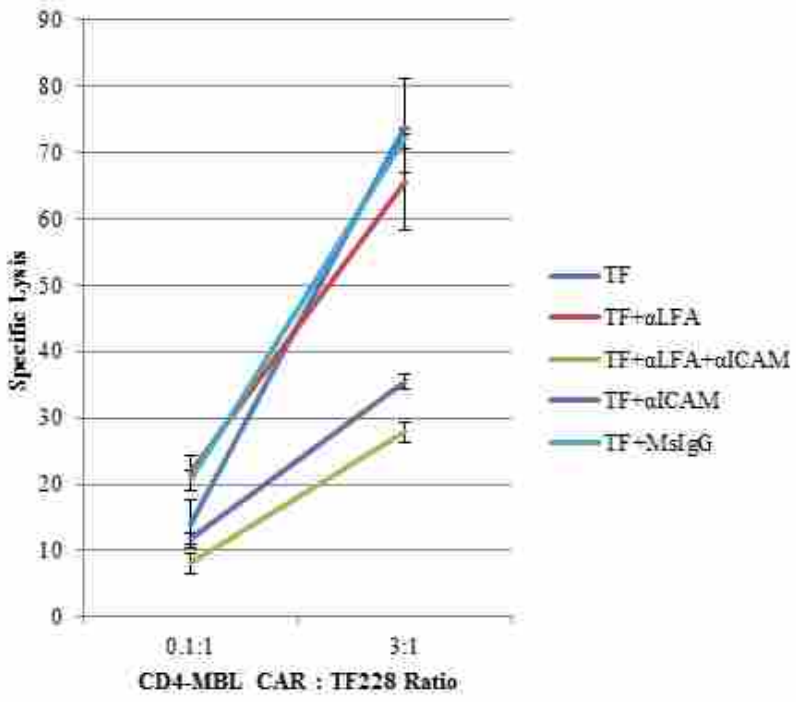


Figure 23 Blocking ICAM-1 inhibits CD4-MBL-CAR-T cell lysis of TF228 cells. CFSE release assay was performed using 3 x 10^4 TF228 cells as targets. ICAM-1 and LFA-1 were blocked with neutralizing antibodies. Mouse IgG was used as a control. Corresponding CD4-MBL-CAR-T cells were incubated with antibodies prior to addition to the targets. After 4 hours, supernatant was analyzed on a BioTek Synergy HT plate reader. Data points and error bars represent the mean and SEM of triplicate wells, respectively. Graph represents one of three independent experiments.

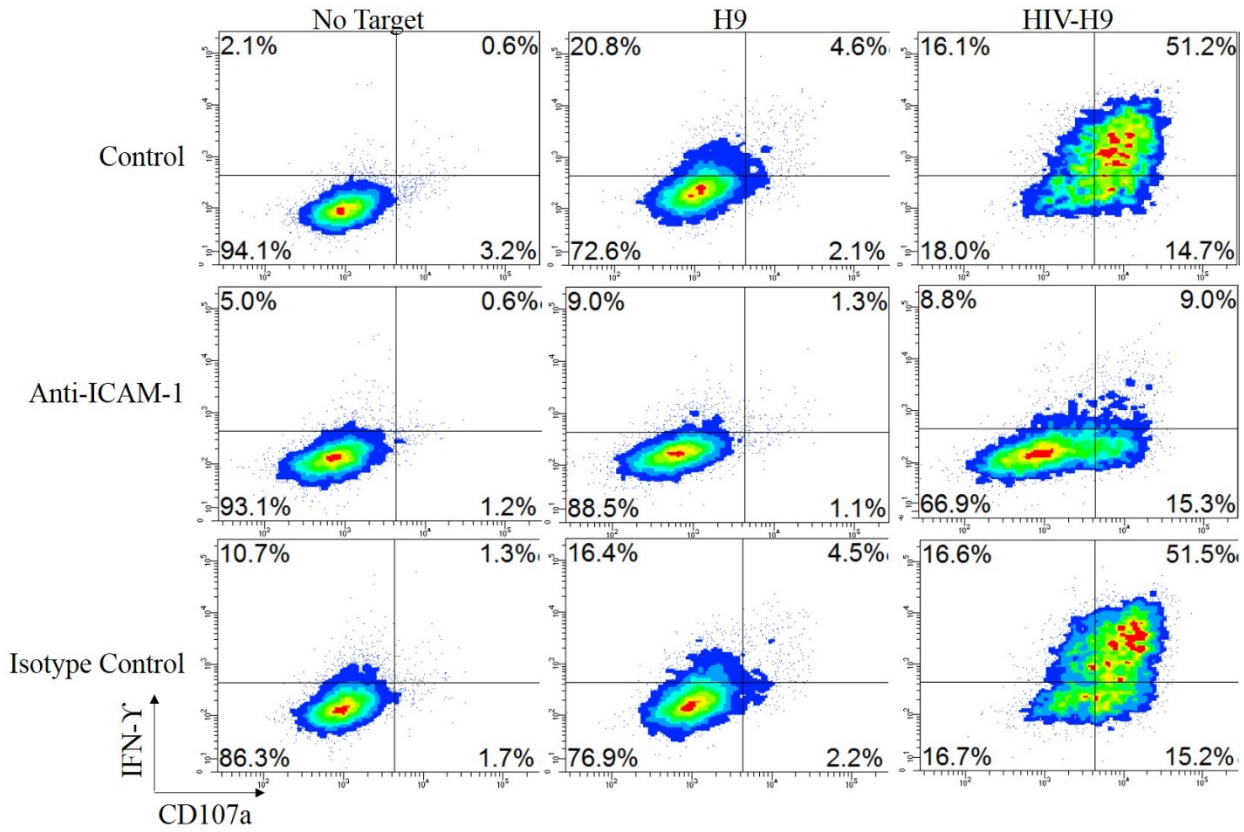


Figure 24 Blocking ICAM-1 inhibits CAR mediated activation in presence of HIV-infected H9 cells. H9 cells were either cultured alone or infected with HIV_{IIIB} and grown for 3 days. Cells were then washed and co-cultured with CAR-T cells for 6 hours in the presence of Brefeldin A, Monensin, and APC-anti-CD107a. The samples were then stained for CD4 and CD8, fixed, permeabilized and stained for IFN-Ɣ. Plots represent $CD4^{+}CD8^{+}$ CAR T cells.

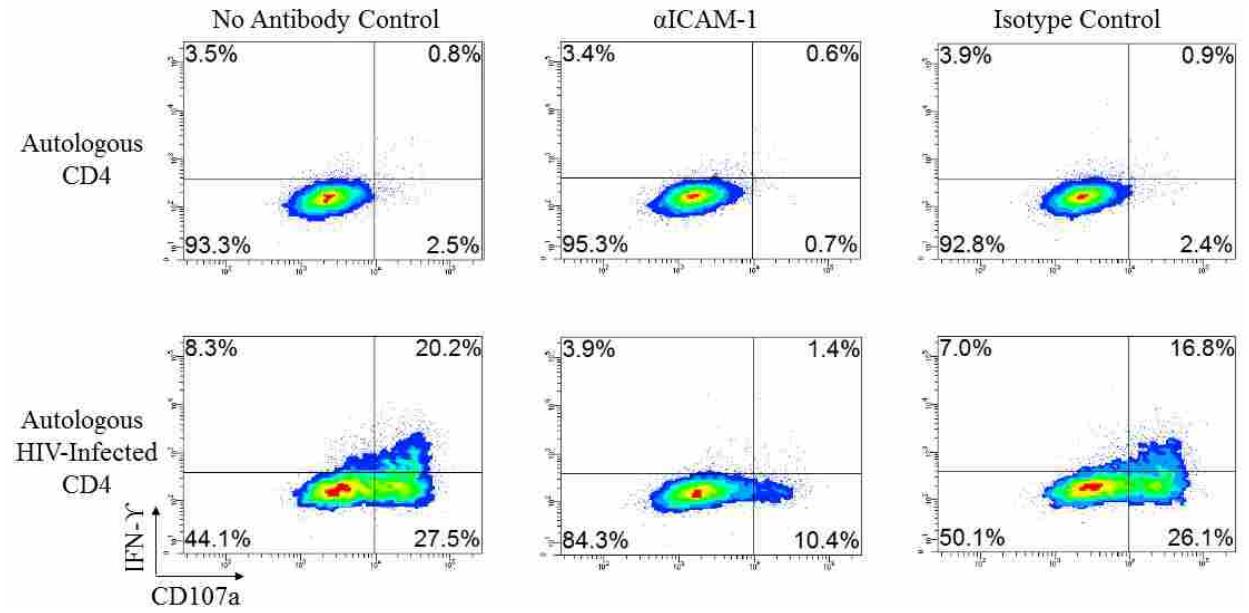


Figure 25 Blocking ICAM-1 inhibits CD4-MBL-CAR-T cell activation in presence of autologous HIV-infected CD4+ T cells. Negatively selected, MACS purified primary $CD4^+$ T cells were activated 3 days with anti-CD3/CD28 beads after which the beads were removed and cells were cultured in 30 IU/ml IL-2 for 24 hours. Subsequently, half of the T cells were infected with HIV_{IIIB} for 2 hours, or left uninfected. Both infected and uninfected $CD4^+$ T cells were cultured separately in 20% FBS RPMI + 30 IU/ml IL-2 for 3 days. Both infected and uninfected $CD4^+$ T cells were washed twice in PBS and 3 x 10^5 cells were incubated with 1 µg of anti-ICAM-1 (clone W-CAM-1) or MsIgG for 30 minutes in 100 µl assay buffer. Similarly, 6 x 10^5 CD4-MBL-CAR-T cells, day 24 post transduction, were incubated with 2 µg of antibody in 200 µl assay buffer. After 30 minutes, 100 µl of CD4-MBL-CAR-T cells +/- antibody were added to appropriate tubes of $CD4^+$ T cells. CAR activation assay was performed as described. Plots are gated to represent $CD8^+CD4^+$ CD4-MBL-CAR T cells.

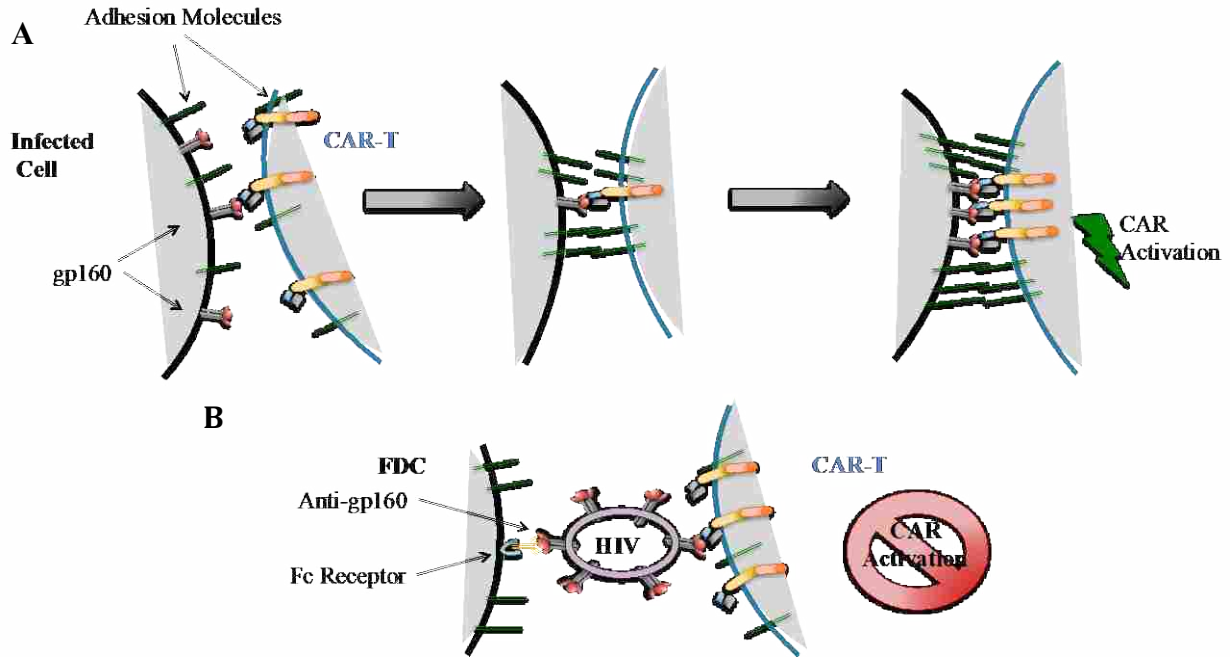


Figure 26 Proposed roles of adhesion molecules in CD4-MBL-CAR-T cell activity. **A** HIV-infected cells express both gp160 and adhesion molecules on the cell surface. As a CD4-MBL-CAR-T cell comes in contact with an HIV-infected cell, interactions form between the CAR-gp120 and adhesion molecules on the surface between the cells. The interaction between the CAR and the gp120 is stabilized through adhesion molecules. Clustering through arrangement of receptors induces the formation of an immunological synapse and allows for CAR activation and subsequent secretion of cytolytic granules. **B** Cells bearing HIV-IC are unable to induce CAR activation. FDCs trap HIV-IC through Fc receptor binding. CD4-MBL-CAR-T cells may recognize gp120 on trapped HIV-IC. However, because of the spatial separation, the adhesion molecules on FDCs are insufficient to stabilize the interaction between the CAR and gp120, and assist in the organization of an immunological synapse required for cytolytic granule release.

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