PROGRAMMING DENDRITIC CELLS FOR INTERCELLULAR DELIVERY OF T-bet TO ENHANCE FUNCTION OF CYTOTOXIC T-CELLS

by

Pranali Ravikumar

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This thesis was presented

by

Pranali Ravikumar

It was defended on

April 18, 2017

and approved by

Thesis Director:
Robbie B Mailliard, PhD
Assistant Professor
Infectious Diseases and Microbiology
Graduate School of Public Health
University of Pittsburgh

Committee Member:
Walter J. Storkus, PhD
Professor of Dermatology and Immunology
University of Pittsburgh Medical Center

Committee Member:
Velpandi Ayyavoo, PhD
Professor
Infectious Diseases and Microbiology
Graduate School of Public Health
University of Pittsburgh
ABSTRACT

Advances in antiretroviral therapy (ART) have proven successful for controlling HIV-1 in chronically infected individuals. Despite these advancements, curing HIV-1 infection poses a major public health challenge due to the establishment and maintenance of HIV-1 latency in long lasting memory CD4+ T cells during ART. Moreover, the cytotoxic T cells (CTL) needed to effectively target and kill infected cells often become exhausted because of chronic activation. Interestingly, CTL from HIV elite controllers show less evidence exhaustion, which is also associated with higher levels of expression of the Th1-associated transcription factor T-bet. In this study, we hypothesize that type-1 polarized human dendritic cells (DC1) are superior in their capacity to induce and enhance cellular immune responses against virally infected cells partially due to their capacity to express and transfer DC-derived T-bet to effector T cells. Moreover, we propose that overexpression of T-bet in therapeutic DC, through genetic modification, offers another approach to improve DC-induced cellular immunity. Here we show that DC1 indeed uniquely express T bet as a general trait while conventional DC generated in the presence of PGE2 (DC2) are T-bet deficient as determined by western blot and intracellular flow cytometry analysis. We also report that overexpression of T-bet in DC1 (DC1Tbet) through use of an adenoviral vector delivery system enhances their CTL inducing activity. However, DC1Tbet display a reduction in their capacity to produce IL-12p70 upon activation with CD40L. Moreover, using a GFP-based
tracking method, we demonstrate that DC1 have the capacity to directly transfer cytoplasmic content to activated CD8+ T cells in a CD40L dependent manner. These data suggest, both a novel helper function of CD40L expressing CD4+ Th cells, and a mechanism for potential DC to T cell transfer of T-bet. We propose that this immune mechanism of DC1 to CTL intercellular transfer can be exploited to enhance anti-HIV T cell response, or to correct their dysfunction of T cell exhaustion as supported by evidence in DC1-based cancer immunotherapy studies and help develop a better understanding of intercellular communication routes in both health and disease demonstrating the significance of this research in public health.
**TABLE OF CONTENTS**

**PREFACE** .................................................................................................................................. XII

**1.0 INTRODUCTION** .............................................................................................................. 1

**1.1 DENDRITIC CELLS** ........................................................................................................ 2

1.1.1 Dendritic cells: Origin and function............................................................................ 2

1.1.2 Dendritic cell distribution and its role in immunogenicity ..................................... 3

1.1.3 Role of dendritic cells in priming and expansion of T cells ......................... 6

1.1.4 Role of dendritic cells in mediating CD4+ T cell ‘help’ for inducing CTL responses........................................................................................................................ 8

**1.2 IMMUNOTHERAPY OF CHRONIC DISEASES** .......................................................... 10

1.2.1 T cell-based immunotherapies ............................................................................. 10

1.2.2 Dendritic cells as immunotherapeutic tools......................................................... 11

**1.3 HIV IMMUNOTHERAPY** .................................................................................................. 14

1.3.1 “Kick and Kill”- a new approach to the HIV cure and the hurdles ...... 14

1.3.2 Role of DC in promoting the HIV ‘kill’ .............................................................. 16

**1.4 CTL EXHAUSTION/ DYSFUNCTION** ............................................................................. 17

**1.5 TARGETING T-BET** ....................................................................................................... 18

**1.6 DC-BASED INTERCELLULAR TRANSFER OF IMMUNOLOGIC INFORMATION** .......................................................... 20

**2.0 STATEMENT OF THE PROJECT** ............................................................................... 23

**3.0 SPECIFIC AIMS** ........................................................................................................... 24
3.1 AIM 1: TO DETERMINE IF T-BET EXPRESSION IN MATURE DC IS ASSOCIATED WITH POLARIZATION STATUS........................................................ 24
3.2 AIM 2: ASSESS FUNCTIONAL IMPACT OF T-BET OVEREXPRESSION ON DIFFERENTIALLY POLARIZED DC (ADC1 AND DC2)........................................... 24
3.3 AIM 3: DETERMINE IF T-BET CAN BE TRANSFERRED FROM ADC1 TO CD8+ T CELLS (CTL PRECURSOR) ............................................................................. 25
4.0 MATERIALS AND METHODS..................................................................................... 27
  4.1 HUMAN PRIMARY CELL ISOLATION FROM BLOOD ................................... 27
  4.2 RECOMBINANT ADENOVIRUS............................................................................. 27
  4.3 GENERATION OF HUMAN DC FROM MONOCYTES...................................... 28
  4.4 ACTIVATION OF MATURE DC VIA CD40L......................................................... 28
  4.5 IL-12P70 ELISA........................................................................................................... 29
  4.6 GENE EXPRESSION ANALYSIS VIA MICROARRAY ...................................... 29
  4.7 WESTERN BLOT ANALYSIS .................................................................................. 29
  4.8 DC- T CELL CO-CULTURE ..................................................................................... 30
  4.9 ELISPOT ASSAY ........................................................................................................ 30
  4.10 FLOWCYTOMETRY ANALYSIS........................................................................... 31
  4.11 IMAGING STUDIES.............................................................................................. 32
  4.12 TRANSWELL ASSAY ........................................................................................... 32
5.0 RESULTS.......................................................................................................................... 33
  5.1 AIM 1: TO DETERMINE IF T-BET EXPRESSION IN MATURED DC IS ASSOCIATED WITH POLARIZATION STATUS......................................................... 33
    5.1.1 Phenotypic characterization of differentially matured polarized DC ... 33
5.1.2 Morphologic characterization of differentially matured and polarized DC .......................................................... 35
5.1.3 Functional characterization of differentially matured and polarized DC .......................................................... 36
5.1.4 Quantitative analysis of T-bet RNA expression in αDC1 versus DC2 before and after CD40L stimulation .......................................................... 37
5.1.5 Assessment of T-bet expression in differently matured and polarized DC at the protein level .......................................................... 38
5.1.6 Quantitative analysis of T-bet protein expression in αDC1 versus DC2 .......................................................... 40

5.2 AIM 2: ASSESS THE FUNCTIONAL IMPACT OF T-BET OVEREXPRESSION ON DIFFERENTIALLY POLARIZED DC TYPES ............................................................................. 41
5.2.1 Test use of an adenoviral delivery system to determine if DC can be engineered to overexpress T-bet .......................................................... 41
5.2.2 Determine the impact of T-bet overexpression on DC phenotype .......... 43
5.2.3 Determine the impact of T-bet overexpression on DC cytokine production ............................................................................................................. 44
5.2.4 Determine the impact of Tbet overexpression on DC capacity to induce HIV-1 specific CTL responses ............................................................................................................. 45
5.2.5 Assessment of DC, Tbet impact on the long-term function and survival of CTL following challenge with HIV-1 antigen expressing targets .......... 48

5.3 AIM 3: TO DETERMINE THE POTENTIAL FOR ADC1 TRANSFER OF T-BET TO CD8+ T CELLS ............................................................................................................. 49
5.3.1 Proof of principle GFP-based model for intercellular transfer from DC to T cells .......................................................... 51

5.3.2 To determine the mechanism of intercellular GFP transfer from CD40L activated DC.GFP to CD8+ T cells ............................................................... 54

6.0 DISCUSSION........................................................................................................................................... 58

7.0 PUBLIC HEALTH SIGNIFICANCE ........................................................................................................... 64

8.0 FUTURE DIRECTIONS .......................................................................................................................... 65

BIBLIOGRAPHY ........................................................................................................................................... 66
LIST OF FIGURES

Figure 1. Role of immature and mature dendritic cells in generating adaptive immune response. 5

Figure 2. Three signal model of DC induced T cell activation.......................... 7

Figure 3. Environmental signal dependent priming of naïve T cells into different T_H cell types via mature dendritic cells................................................................. 8

Figure 4. Dendritic cell based immunotherapy for cancer....................................... 14

Figure 5. ‘Shock and Kill’ approach to HIV cure.................................................. 15

Figure 6. Role of DC derived T-bet in T_H1 specific priming................................ 19

Figure 7. Mature DC have higher expression of CD86 and CD83.......................... 34

Figure 8. αDC1 show significant morphological changes (reticulation) post treatment with CD40L compared to DC2. ........................................................................ 36

Figure 9. αDC1 produce higher levels of IL-12p70 (+/- CD40L) compared to DC2......... 37

Figure 10. The difference in T-bet expression (+/-) CD40L in αDC1 versus DC2 is insignificant. .................................................................................................................. 38

Figure 11. T-bet is expressed at higher levels in αDC1 compared to DC2, independent of the maturation cocktails used................................................................. 39

Figure 12 Confirmation of higher levels of T-bet protein expression in αDC1 versus DC2 by flow cytometry. ..................................................................................... 40

Figure 13 Ectopic overexpression of T-bet overexpression in DC post infection with recombinant adenoviral vectors............................................................... 42

Figure 14 DC can be engineered to overexpress T-bet using a recombinant adenoviral delivery system. ............................................................................................ 43
Figure 15  T-bet overexpression in differently matured and immature DC does not alter their expression of CD86 and CD83. ................................................................................................................................. 44
Figure 16  T-bet overexpression negatively impacts DC IL-12p70 producing capacity. ............ 45
Figure 17  T-bet overexpression in αDC1 enhances their ability to induce HIV-1 CTL. ............ 47
Figure 18  Higher HIV-1 specific memory CTL responses maintained after challenge with antigenic targets when initially induced with αDC1.Tbet compared to αDC1 .................. 48
Figure 19  T-bet expression in CD8+ T cells is increased when co-cultured with CD40L- activated αDC1 .............................................................. 50
Figure 20  T-bet expression decreases in DC post CD40L stimulation .................................... 51
Figure 21  Transfer of GFP from αDC1 to CD8+ T cells ........................................................... 52
Figure 22  CD4+ T cell ‘help’ promotes transfer of DC derived GFP to CD8+ T cells. ........... 53
Figure 23  CD40L plays a significant role in promoting the transfer of GFP from DC.GFP to CD8+ T cells .................................................. 54
Figure 24  CD40L-mediated GFP transfer from DC1.GFP to CD8+ T cells can occur in a non-contact dependent manner in bystander DC-activated CD8+ T cell recipients. ................. 55
Figure 25  Prior CD8+T cell activation is critical for effective GFP transfer from DC1.GFP..... 57
Figure 26  T-bet localization pattern in various DC populations. (Raw data only) ................. 65
PREFACE

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1.0 INTRODUCTION

Human Immunodeficiency virus (HIV) was identified in the early 1980’s when pandemics were reported in various parts of USA associated with rare types of cancers like Kaposi’s sarcoma and pneumonia predominantly observed in the homosexual male community (1). It was revealed that the HIV originated from chimpanzees because of the virus crossing species barrier and transmission occurred via infected blood of the animal (2, 3). Despite major advancements in HIV research and HIV therapies, HIV is still a tremendous public health concern, with over 36.7 million people being infected across the globe as per WHO reports, 2017. Hence, strong effort continues to exist in the HIV research community aimed towards not only improving our understanding of HIV pathogenesis, but also towards development of more effective methods to limit HIV spread, as well as better strategies to treat or even cure chronic HIV infection. Although the current use of antiretroviral therapy (ART) has significantly reduced the number of AIDS related deaths since its introduction as reported by UNAIDS, ART does not come without side effects (4, 5) and the widespread use of ART has raised concerns for the potential development of HIV drug resistance. Importantly, UNAIDS suggests that although current ART regimens are very effective at controlling HIV, it is ineffective at targeting the long lived cellular reservoir(s) that harbors the latent form of HIV, and therefore, those infected must therefore remain on ART throughout life.

Current HIV Cure research proposes the ‘Shock and Kill’ or ‘Kick and Kill’ approach, which is based on the notion that HIV latency can be reversed under an anti-retroviral therapy to
activate transcriptionally silent HIV proviral DNA for subsequent exposure to immune cells capable and recognizing and killing the infected target. The goal of this strategy would therefore be to eliminate or substantially reduce the size of the HIV reservoir to a point where the infection could be controlled without drug therapy (6). At present, various latency reversing agents (LRA’s) are being explored such as HDAC inhibitors, Disulfiram, BET protein inhibitors, and PKC agonists. However, their efficiency in ex vivo studies have shown to be either limited or toxic, and have yet to be proven effective in humans (7). Moreover, some of these LRA’s have been shown to negatively impact the function of cytotoxic T lymphocytes (CTL) (8), the effector cell type likely to be a critical player in the HIV ‘Kill’. Hence, there is still a critical need to establish safe and effective therapeutic means expose and eliminate the latent HIV cellular reservoir.

1.1 DENDRITIC CELLS

1.1.1 Dendritic cells: Origin and function

The term “Dendritic cell” was coined in 1973 by Ralph Steinman and Zanvil Cohn after observing a unique cell type in murine spleen cells which had dendrite like protrusions and was phagocytic in nature. The discovery of these cells was a huge advancement in immunology research as it helped to unlock the mystery of how antigen specific T cell lymphocytes are initially primed to respond to pathogens (9). Dendritic cells (DC) form an integral part of the immune response, acting as a link between the innate and the adaptive branches of the immune system. Because of the central role played by DC in both initiating and modulating adaptive immune responses, they have become widely recognized as the most potent of the professional antigen presenting cells
(APC). Because of this, they have been explored heavily for their potential as an immunotherapeutic tool for several diseases including cancer and HIV (10,11).

### 1.1.2 Dendritic cell distribution and its role in immunogenicity

DC orchestrate the immune response and act as an important bridge between the innate and the adaptive branches of immunity. In the innate immune setting, immature DC are generally positioned in the peripheral tissues, and are concentrated in areas where they would likely encounter microbial challenges, such as the skin, lung, gut, and mucosal tissues. They are strongly phagocytic, and can efficiently recognize pathogens through expression of various receptors that identify conserved pathogen associated molecular patterns. They can also take up pathogen-derived antigens and tissue associated factors through receptor independent micropinocytosis. At this stage of antigen uptake, the DC can become activated by the pathogens directly, or indirectly by tissue derived factors produced by other cells responding to the pathogen or resulting from tissue damage. During this stage, DC can produce inflammatory factors that can activate other players of the innate immune response including Natural Killer cells, NKT cells. The combination of activation signals the tissue resident DC receive results in their progress towards maturation, in which they undergo phenotypic changes including an increase in surface expression of MHC-class 1 and class II molecules as well as co-stimulatory proteins such as CD80 and CD86. Moreover, they undergo changes in expression of chemokine receptors, such as increasing CCR7 expression, which promotes their migration into draining lymph nodes where they can initiate adaptive immune responses (12).

The antigens taken up by the immature DC are processed and presented in the context of MHCI or MHC II. The MHC I and MHC II peptide loading occurs in distinct ways. In the MHC-
I peptide loading pathway, the peptide loading complex (PLC) ensures accurate peptide loading in MHC I by accommodating 8-9 amino acids in the MHC-1 peptide groove. Dissociation of the PLC allows the MHC I to move to the plasma membrane for antigen presentation to CD8+ T cells, most commonly associated with viral or tumor antigens. The unloaded peptides are then destroyed by the ER associated protein degradation system (13). Unlike ubiquitously expressed MHC I, MHC II are only expressed by professional APC. The peptides (12-25 amino acids) are loaded in by exchanging positions with the CLIP fragment present in the MHC II binding groove and then transported to the plasma membrane to be identified by CD4+ T cells (14,15). The antigenic peptides are not utilized instantly, but instead are retained for few days (16). Upon maturation, DC decrease their antigen uptake capacity while their T cell stimulatory ability is enhanced. Antigenic peptide-loaded MHC- class I and II molecules accumulate on the cell surface along with co-stimulatory molecules including CD86, CD80, and adhesion molecules CD48 and CD58 that are upregulated in the process (17). Once they migrate to the T cell areas of the draining lymph node, these mature antigen-loaded DC can now act as mediators of the pathogenic and tissue associated information gathered from the periphery for subsequent translation into specific adaptive immune responses (Figure 1).
Figure 1. Role of immature and mature dendritic cells in generating adaptive immune response. (A. Karolina Palucka, 2005)
In the absence of inflammation, the DC remain in their immature state and sample some antigens in the environment and migrate to the lymph node in small numbers or induce T cell tolerance. b) In presence of inflammation, the immature DC take up antigens and become mature and migratory in nature. They move to the lymph node in large number to generate adaptive immune responses by priming of CD4+ T helper cells and CD8+ cytotoxic T lymphocytes (CTLs), the activation of B cells and regulating the responses by Tregs.
1.1.3 Role of dendritic cells in priming and expansion of T cells

Once the mature migratory dendritic cells reach the lymphoid tissues they can attract naïve T cells or B cells via release of chemokines to promote potential antigen cognate interactions (18). A cascade of events take place for effective DC-induced T cell activation and differentiation to occur via three critical signals, often referred to as signal 1, 2 and 3. (Figure 2). The antigen specific interaction of DC with T cells occurs via DC MHC- class I and MHC-class II presentation of peptides and their recognition by the T cell receptors of the respective CD8+ and CD4+ T cell (19). This antigen specific presentation is often referred to as ‘Signal 1’. This immunologic synapse also involves adhesion and co-stimulatory molecules. The signals provided by these molecules is referred to as ‘Signal 2’. This determines the magnitude of the response, as well as the extent of proliferation and survival of the DC activated T cells. In absence of ‘Signal 2’, T cells often become anergic leading to tolerance. Examples of such ‘Signal 2’ co-stimulatory molecules presented by DC are CD86 and CD80, which both act as ligands for the CD28 co-stimulatory receptor expressed on T cells (20). The ‘Signal 3’ is conveyed with ‘Signal2’, and is marked by the production of specific DC derived factors that help T cells to differentiate into their functionally polarized effector roles. Examples of such polarized effector CD4+ T cells include Th1, Th2, Th17 and Tregs. The development of polarized T cell driven responses greatly depends on the combination and type of pathogen derived and tissue derived signals received by the DC during their initial activation (21) (Figure 3). The strength of T cell activation depends on amount of peptide -MHC complexes, level of co-stimulatory molecules for amplification, and the duration and stability of this immunologic synapse. Following one cycle of differentiation, the T cells undergo major proliferation in response to IL-2 produce in autocrine and paracrine fashion by activated T cells (22).
Figure 2. Three signal model of DC induced T cell activation. (Martien L. Kapsenberg, 2003)
Signal 1 is defined by the interaction of MHC I or MHC II with specific TCR, determining the specificity of interaction. Signal 2 is the co-stimulatory signal mediated by molecules like CD28, CD80 and CD86 on DC that determine the survival and magnitude of T cell response. Signal 3 is important to determine the polarization status of the T cells based on their cytokine environment and directs the type of response of the T cells.
Figure 3. Environmental signal dependent priming of naïve T cells into different Th cell types via mature dendritic cells.
(Martien L. Kapsenberg, 2003)
Based upon the interaction with specific pathogens the immature DC become mature and can give rise to
different effector TH cell types depending on the DC-tissue polarizing factors produced by tissue resident
cells, NK cell, macrophages, mast cells, fibroblasts and many others. These cells produce different factors
to generate type-1, type 2, Tregs responses based on the way these cells were activated.

1.1.4 Role of dendritic cells in mediating CD4+ T cell ‘help’ for inducing CTL responses

Importantly, DC also play a role as a mediator of CD4+ T cell ‘help’ for induction of robust CTL
responses. It has been reported that CD40, a transmembrane glycoprotein surface receptor of the
TNF-α family plays an important role in the ‘help’. The CD40 signals upregulates MHC’s and
CD80 and CD86 on the DC. The activated CD4+ T cells provide CD40L help to the dendritic cells
to prime cytotoxic T cells with the help of co-stimulatory molecules such as CD28/CD70 (23).
This process occurs via interaction of CD4+ T cells with DC via CD40-CD40L which provides
the “help signal” licensing the DC to promote CTL responses. This signal is preceded by transfer of this “helper signal” by an empowered DC as a message to CD8+ T cell thereby aiding their proliferation and effector function. Moreover, absence of signaling lead to failure of secondary expansion of CTL’s leading to early CTL exhaustion suggesting that the CD40L signaling enhances long term survival of CD8+ T cells upon interaction with targets. CD40L is also important for long term survival of DC (24, 25). However, it is still unclear if these helper signals are provided simultaneously, if it requires just one DC interacting with both CD4+ T cell and CD8+ T cell or if these events vary in space and time. Dendritic cells respond to CD40L depending upon their maturation status and help DC unleash ‘Signal 3” and aid in polarizing naïve T cells. For instance, DC matured in the presence of INF-γ, also known as αDC1 produces enhanced levels of IL-12p70, a vital driving factor of TH1-biased cellular immunity in response to CD40L (26) in contrast to production of TH2 responses when, maturing dendritic cells are exposed to PGE2 (DC-2) and produce IL-12p40 acting as a competitive inhibitor of IL-12p70 (27, 28, 29). Interestingly, Heath et al. it has been shown in a cutaneous HSV infection model that CD4+ T cells were primed earlier by clustering with migratory skin DC and that CD8+ T cell activation occurred later upon interaction with lymph node resident XCR1 (+) DCs. This asynchronous activation of T cell via different DC types suggest a possible transfer of immunologic information and CD4 + T cell ‘help’ between DC subsets (30). Recently, Zaccard et al. showed that immunologic information is transferred from one DC to another via a formation of tunneling nanotubes in response to CD40L unlocking a helper function of CD40L for transfer of information (31) We propose that the finding of Heath et al. and Zaccard at al. could be linked.
1.2 IMMUNOTHERAPY OF CHRONIC DISEASES

1.2.1 T cell-based immunotherapies

Cellular and cytokine-based immunotherapies have been widely explored in chronic diseases such as cancer and HIV (32,33,34,35,36). One of the earliest use of such immunotherapeutic approaches was carried out by Steven Rosenberg’s group at the NIH (37,38). He showed that administration of interleukin-2 (IL-2) to metastatic melanoma cancer patients caused tumor regression via activation of endogenous lymphocytes. He verified that antitumor specific lymphocytes could be expanded ex-vivo from the blood or tumors cancer patients and used for adoptive cell transfer therapies. Such T cell therapy strategies have also been explored in the setting of HIV before any effective ART was available (39,40). However, T-cell based therapies have had several limitations including those related to insufficient TCR avidity for target antigens, the need to overcome T cell tolerance and regulatory mechanisms, as well as lack of proper T cell trafficking, expansion and survival in vivo (41). Only recently have such adoptive T cell therapies shown substantial promise in the setting of cancer, and some these studies include the use of genetically modified cells. However, such T cell therapies require the patients to undergo prior non-myeloablative therapy that results in the destruction of circulating immune cells for effective engraftment and in vivo expansion of the therapeutic cells to occur (42).

In the setting of HIV, therapies designed to target the induction or enhancement of HIV-specific CTL function have also been proposed because effective CTL activity has long been known to be critical for the control of HIV infection (43),(44). It has been observed that the CD8+ T cells of HIV controllers elicit strong CTL responses against HIV to maintain a lower viral load (45,46,47). However, maintaining a low viral load is a challenge for majority of the HIV infected
individual who progress towards AIDS in the absence of ART as their CD8+ T cells do not elicit a broad response during acute stages of infection and may not be able to account for the establishment of CTL epitope escape variants of HIV (48). Moreover, as shown in cancer, chronic immune activation in HIV can give rise to T cell exhaustion, which adds an additional hurdle for controlling HIV. In addition, the fact that HIV mainly infects CD4+ T cells, their ability to support CTL immunity may also be greatly compromised (49). While the concept of targeting immune exhaustion through the therapeutic use of immune checkpoint inhibitors, such as those that act by blocking PD-1/PD-L1 interactions, has been implemented with revolutionary success in cancer immunotherapy (50), such approaches have yet to be exploited successfully to correct or improve T cell responses in HIV. To date, much of the focus of development therapies to target the role of HIV specific T cells has been geared towards modifying CTL function to enhance their ability to recognize and eliminate HIV infected cells. Strategies include polyclonal ex-vivo expansion of existing HIV specific CTL as well as genetic modification to CTL to express artificial T cell receptors to target HIV gag epitopes (35) or chimeric antigen receptors having an antibody-like extracellular region attached with the TCR signaling machinery (51,52). Although such T cell therapies have shown some promise for treatment of HIV, these methods laborious, expensive, and not widely applicable (53).

### 1.2.2 Dendritic cells as immunotherapeutic tools

Recent studies suggest that mounting strong polyfunctional CTL responses that cover a wide breadth of HIV epitopes will offer the best chance for controlling HIV (54). Moreover, inducing de novo CTL responses from the naïve T cell pool capable of targeting the reservoir variant antigens and conserved regions of the virus may be needed to account for any immune escape
already established (55, 56, 57). Utilization of DC as an immunotherapy option to present antigens to T cells offers a means to both activate broad array of pre-existing epitope specific CTL responses while also potentially inducing de novo CTL response (58, 59).

Dendritic cells have been utilized safely in various human immunotherapy trials to treat both cancer as well as HIV. Early in-vivo studies in murine models demonstrated their clinical potential for promoting immune response against pre-established macroscopic tumors, where it was concluded that dendritic cells pulsed with tumor peptide provided constant tumor regression or eliminate the tumor completely in majority of the murine models by priming naïve anti-tumor cytotoxic T lymphocytes (CTL’s) and enhancing the effector function of tumor specific CD8+ CTL’s (60). Once culture methods were established to convert monocytes into DC (61), a path was paved for their use in humans. With time, the major focus of DC use as a therapeutic shifted towards improving their ability to elicit better immune responses. This led to exploring various ways of maturing dendritic cells to enhance their stimulatory capacity, including use of cytokines such as TNF-α, IL-1β, IL-6, and PGE2. While DC derived IL-12p70 was identified as a critical factor for eliciting strong cellular immune responses (26) DC matured using this conventional cytokine cocktail method were found to lose their IL-12p70 producing function. Next, the concept of DC polarization came into light, where it was discovered that mature DC could produce high amounts of IL-12p70 if activated properly during their maturation phase. The alpha type-1 dendritic cells (αDC1), which are matured using a combination of IL-1β, TNF-α, INF-α, Poly I:C and INF-γ, became a particularly attractive alternative because of their characteristic mature status, and enhanced capacity to produce IL-12p70 (62,27). Importantly, they have been shown to be superior inducers of TH1 and CTL responses (62,55, 63), including primary CTL responses (64). Another recently identified novel feature of αDC1 is their unique ability to form functional
tunneling nanotube networks in response to CD40L (31). This immunologic phenomenon termed ‘DC1 reticulation’ enhances the area reach of the DC and facilitates intercellular communication and transfer of cellular material between DC. It remains unclear if this mechanism is utilized during the normal immune response in vivo, but this may partly explain how migratory DC can transfer antigen to resident DC and affect their capacity to drive CTL responses even though they are spatially separated in the lymph node (30).

The therapeutic potential of αDC-1 has been demonstrated in phase I/II clinical trials in cancer (Figure 4) such as melanoma and recurrent malignant glioma. In the glioma trail by Okada, et al, they reported the upregulation of type-1 specific cytokines and chemokine (CXCL10 and INF-α) from peripheral blood samples that were associated with the high immunogenicity and clinical effectiveness of αDC-1-based therapy (65). In melanoma, the αDC1 based vaccine strategy was shown to correct the functional cytokine profile of preexisting CD4+ T cells, and redirect TH-2 biased responses towards a TH-1 function (63). Another study showed that cytotoxic T lymphocyte induction against breast cancer via αDC-1 loaded with allogenic breast cancer cells (66) thereby affirming the performance of αDC-1 to be better over DC-2.
Figure 4. Dendritic cell based immunotherapy for cancer. (Pawel Kalinski et al., 2011)
DC loaded with tumor specific antigen are critical in generating specific effector T cell types. Programming DC with antigens to generate TH1 specific T cell type is desirable and aids in tumor elimination by generating tumor antigen specific CTL and NK cell responses.

1.3 HIV IMMUNOTHERAPY

1.3.1 “Kick and Kill”- a new approach to the HIV cure and the hurdles

The resting memory CD4+ T cells have shown to be the most prominent reservoir of HIV in the form of a ‘provirus’. These cells are a barrier towards curing HIV as they are not affected by ART or immune responses (67, 68). The concept of ‘kick and kill’, also known as “shock and kill” is a proposed approach to cure HIV by exposing and eliminating the latent HIV cellular reservoirs. The main concept of this approach is to reactivate the latent proviral HIV DNA to induce active
viral replication (the ‘kick’), which then would lead to the death of the infected cells by either direct cytopathic impact of the virus replication, or because of immune recognition and elimination of the infected targets (the ‘kill’). Importantly, this reactivation would be done under the cover of ART to keep newly produced virus from infecting other uninfected CD4+ T cells (69). In the pharmaceutical industry, there has been an ongoing quest for the discovery of effective HIV latency reversing agents (LRAs) capable of exposing the reservoir, or ‘kicking’ the virus, without inducing global T cell activation (Figure 5). Although some LRAs have shown limited success in ex vivo studies (7), the most promising candidate, bryostatin, proved toxic at effective doses in cancer therapy studies (70). Moreover, there is intense research focus on devising strategies to prime the immune system to generate an arsenal of immune effectors positioned to attack the infected cells (the ‘kill’) once they are exposed by the an effective LRA. It is believed that HIV-specific CD8+ cytotoxic T cell lymphocytes (CTL) will be the most critical of responder immune cell types needed to effectively target these HIV cellular reservoirs (56). However, major impediments exist for mounting an effective CTL ‘kill’ including the establishment of CTL epitope escape variants within the HIV reservoir (71, 72, 73, 74) as well CTL dysfunction or exhaustion resulting from chronic immune activation associated with HIV infection (75, 76).

Figure 5. ‘Shock and Kill’ approach to HIV cure. (Steven G. Deeks, 2012)
The “Shock and Kill” method involves activating viral reservoir using LRA’s. This is expected to activate the latent virus which can kill the cells by the virus itself or the patient’s immune system. ART can be administered post this to prevent new cells from getting infected by the activated virus.
1.3.2 Role of DC in promoting the HIV ‘kill’

DC have been explored in this scenario of chronic HIV infection for its role in “kill” component of the “kick and kill” approach because of its ability as the most potent of the professional APC with potential for mounting an arsenal of immune effector cells capable of destroying infected cells. DC-centric strategies have already been proven effective as immunotherapeutic tools in the cancer setting (62), and they have also shown promise in the HIV setting (64,77). Advancements in DC based immunotherapies include the implementation of the concept of signal 3, or DC polarization, in the design of clinical trials for the treatment of cancer (63). More recently, the concept of DC polarization as part of the HIV ‘kill’ strategy has been given increased attention, for promoting more effective HIV specific immune responses (31,78).

Recent studies suggest that in the setting of chronic HIV infection the selective priming of highly functional de novo CTL responses from the naïve CD8+ T cell pool will probably be required to effectively target the HIV infected target cells because of the large degree of killing dysfunction associated with memory CD8+ T cells (56), which is likely due to a combination of reasons including epitope alterations as well as immune exhaustion. The need to prime naïve CD8+ T cells also supports the argument for using a DC-based approach to the ‘kill’. Furthermore, the one the most successful HIV immunotherapy clinical trials to date used dendritic cells pulsed with pulsed with inactivated autologous HIV, which resulted in a significant decrease in HIV RNA set point and was associated with increase in anti-HIV CD8+ T cell responses (79,80). However, the dendritic cells used in this study were DC generated using the conventional maturation cocktail containing IL-1β, TNFα, IL-6, and PGE2, which have been shown to have a mature status but are
IL-12p70 deficient (80,64). Although high IL-12p70 producing αDC1 have been widely used with success in the cancer setting (62), they have yet to be adequately explored in the HIV immunotherapy.

1.4 CTL EXHAUSTION/ DYSFUNCTION

CTL exhaustion or dysfunction is commonly addressed as a major barrier in the development of a cure for HIV. In chronic infections, CTL’s can display a loss of effector function and proliferative potential, associated with upregulation of inhibitory or ‘immune checkpoint’ markers including PD-1, Tim-3, LAG-3 and CTLA-4 (81). In HIV infection, it has been established that the extent of CTL exhaustion is directly related to disease progression (82). Various therapies have been centered on blocking these inhibitory pathways to reduce or prevent CTL exhaustion (83, 84). While recent clinical trials targeting immune checkpoints such as the PD-1/PDL-1 pathway have shown remarkable success in the cancer setting, they are not universally effective, and there have also been reports of developing resistance to such therapies, (85). These new findings and drawbacks highlight the need for alternative methods to counteract CTL exhaustion. Interestingly, CTL exhaustion associated with HIV-1 was characterized by increase in expression of check point inhibitors and decreases in type-1 associated polyfunctional responses of CTL along with diminished CTL expression of T-bet. Importantly, HIV non-progressors tend to have more highly functional CTL with maintained T-bet expression compared to HIV progressors suggesting that T-bet may play a critical role in correcting CTL exhaustion or dysfunction (86).
1.5 TARGETING T-BET

T-bet is encoded by the Tbx21 gene and is an important member of the T box family of transcription factors. T-bet is mostly known for its importance as a key regulator of IFN γ production and its association with naïve T helper (T\textsubscript{H}) differentiation into T\textsubscript{H1} effector cells. Therefore, expression of T-bet is considered a common biomarker of T\textsubscript{H1} cells. In addition to T cells, dendritic cells have also been recently shown to express T-bet (87). However, the role of T-bet in dendritic cells is still unclear (88). Interestingly, it has been nicely demonstrated in a murine model of \textit{Listeria} spp. infection that T-bet deficient DC display a greatly diminished capacity to prime T\textsubscript{H1} specific T cells, which resulted in the inability of the mice to clear \textit{Listeria} spp. infection (89,90). This study highlighted a functional role for T-bet expression in DC by directly demonstrating the impact that it had on the DC’s ability to modulate T cell differentiation and function.
T-bet in DC is critical to drive TH1 type of responses as it suppresses TNF production in DC for mucosal homeostasis maintenance. The role of T-bet in DCs has been actively explored in the cancer setting where it has been shown that peptide antigen presenting type-1 polarized DCs can enhance TH1 specific responses (91). Remarkably, these DCs could revive a CD45RO+ subset of defective antigen-experienced CD4+ T cells in melanoma patients by notably promoting an increasing in IL-12Rbeta2, IFNγ and T-bet expression levels, suggesting that dysfunctional or exhausted memory CD4+ cells can be functionally reprogrammed via type-1 DC based therapy (63). Moreover, Storkus group have also shown that DCs engineered to overexpress T-bet using an adenoviral vector model (DC.T-bet) can regulate the tumor microenvironment in favor of durable TH1 responses and central memory T cell responses capable of mediating efficient anti-tumor effects. Hence, it has been established that T-bet ectopically expressed in DC combined with other TH1 type-1
promoting factors, such as IL-12, can contribute towards generating more effective anti-tumor immunity in vivo (92). Interestingly, DC.T-bet was also shown to promote T_{H1} responses via an IL-12p70 independent manner (93). Although, transwell studies indicated that DC and T cell proximity was required for efficiency of such IL-12p70-independent T_{H1} driven responses, the study implicated a novel immunologic mechanism by which DC may transfer T-bet to T cells as a direct means to facilitate T_{H} cell differentiation and functional polarization (93).

### 1.6 DC-BASED INTERCELLULAR TRANSFER OF IMMUNOLOGIC INFORMATION

As discussed earlier, Allan et al. have shown that migratory DCs are required for delivery of antigenic information to draining lymph nodes, and that this information is necessarily transferred to resident DC for induction of protective cellular immunity to HSV via unknown mechanisms (94). Further investigations revealed that the migratory DC interact with a cluster of CD4+ T cells whereas the CD8+ T cells are activated by the resident DCs that presumably receive the peripheral antigenic information from the migratory DC. Interestingly, these events occur in different times and space, thus highlighting the mysterious nature of this information transfer between DCs (30). A recent study by Zaccard et al. identified a novel immunologic process termed as ‘DC reticulation’, in which tunneling nanotube networks are induced exclusively in DCs matured under pro-inflammatory type-1 conditions following their subsequent antigen driven interaction with CD4+ T_{H} cells. It was determined that the induction of DC reticulation was triggered by signaling provided by the T_{H} cell-associated factor CD40L. These CD40L-induced cellular networks were
shown to allow direct intercellular transfer of cytoplasmic and cell surface associated materials between DCs (31).

Interestingly, recent electron microscopy also revealed the potential for this immunologic process of ‘DC reticulation’ to give rise to the release of DC-derived exosomes (unpublished data from Zaccard et al.). However, it is unclear if there are any functional associations with these preliminary in vitro findings. Potentially related to the findings of Zaccard et al. are some additional unpublished data generated from the Storkus group demonstrating that exosomes derived from T-bet overexpressing-DC can themselves indeed contain T-bet, suggesting that they may be capable of being delivered to other cells to have a downstream functional role in the immune response (Storkus et al, unpublished data). These unpublished findings, together with the other published reports give rise to many potentially important questions. One question is whether DC1 are superior at driving TH1 and CTL responses, mostly because of their superior IL-12p70 production capacity, or is that they might also express higher amounts of T-bet? Moreover, could it be possible that one their superior ability to create ‘Intercellular” networks allows DC1 not only to transfer antigenic information, but that they may be able to efficiently transfer transcription factors such as T-bet to T cells, to prime their differentiation towards type-1 effector cells, or to enhance the effector function of these and/or other immune cells?

Based on the presented information and the questions posed, for this thesis proposal, I hypothesized that the clinically applicable αDC1 indeed are superior to other DC types for promoting cellular immunity partly because of their enhanced expression of T-bet, and their ability to transfer DC-derived T-bet to T cells via formation of either tunneling nanotubes or production exosomes. In this study, the goals were to 1) carry out experiments to
determine if high T-bet expression is a general characteristic of DC1 types, including aDC1
2) to test the impact of T-bet expression on DC function using adenoviral expression vectors;
and 3) to determine if DC-derived cellular material, such as T-bet, can be transferred
directly to T cells, including cytotoxic CD8+ T cells, through either nanotube or exosome
delivery. I propose that an increase in knowledge and understanding of such immunologic
transfer mechanism could be important, and potentially targeted as a therapeutic means to
enhance T cell mediated immunity, or to correct dysfunctional T cell responses such as that
related CTL exhaustion typically associated with chronic diseases such as cancer and HIV
infection.
2.0 STATEMENT OF THE PROJECT

The overall goal of this project is to explore the potential of polarized type 1 dendritic cells (αDC1) for therapeutic use in the setting of HIV research. This project deals with the ‘Kill’ aspect of the ‘Kick and Kill’ concept for HIV cure which focuses on enhancing CTL responses against HIV reservoirs to eliminate HIV latency. This ‘Kill’ approach is aimed at programming dendritic cells to drive more T H1 specific responses that would aid in promoting better CTL effector function towards HIV specific targets. Our hypothesis is that T-bet expression in αDC1 plays a critical role in driving effective cellular responses and by using a proper strategy we can correct CTL exhaustion by either inducing new primary CTL responses against HIV, or by restoring the functional capacity of the memory CTL already present maybe through direct intercellular transfer of T-bet to CD8+ T cells via tunneling nanotubes or exosomes. To conclude, this mechanism could be exploited as a potential DC immunotherapeutic strategy to transfer T-bet to exhausted T-cells to restore their function in chronic HIV infection, or to induce HIV latency reversal thereby demonstrating a significant role in public health.
3.0 SPECIFIC AIMS

3.1 AIM 1: TO DETERMINE IF T-BET EXPRESSION IN MATURE DC IS ASSOCIATED WITH POLARIZATION STATUS

This aim focuses on evaluating T-bet expression levels in αDC1 versus DC2 to determine if there is an association between T-bet expression and DC polarization status. We hypothesize that αDC1 express higher T-bet level, which contribute to their superior capacity to induce type-1 immunity based on previous literature (95). We test this hypothesis through the following sub-Aims:

1. Characterize DC polarization status of differentially matured DC by analyzing their morphology, their surface protein expression using flow cytometry, their IL-12p70 producing capability following CD40L stimulation via IL-12p70 ELISA.
2. Assess T-bet gene expression in αDC1 versus DC2 at baseline and after stimulation with CD40L using gene chip analysis.
3. Compare T-bet protein expression levels in αDC1 versus DC2 via western blot and flow cytometry.

3.2 AIM 2: ASSESS FUNCTIONAL IMPACT OF T-BET OVEREXPRESSION ON DIFFERENTIALLY POLARIZED DC (ADC1 AND DC2)

In this Aim, the goals are to engineer mature DC types to overexpress T-bet (αDC1Tbet, DC2Tbet) using an adenoviral vector system, and, to determine the impact of T-bet overexpression on these
DCs. We hypothesize that T-bet overexpression will have no impact on basic phenotypic and IL-12 producing capacity, but will enhance their ability to activate effective HIV-1 specific CTL responses. We will address this hypothesis in the following sub-Aims:

1. Determine the success of the T-bet transduction approach used to establish T-bet overexpression in human polarized DC using western blot and flow cytometry analysis.
2. Determine effect of T-bet overexpression on DC phenotype and functional characteristic including their capacity to produce IL-12p70 and induce HIV-1 specific CTL responses in vitro.

3.3 AIM 3: DETERMINE IF T-BET CAN BE TRANSFERRED FROM ADC1 TO CD8+ T CELLS (CTL PRECURSOR)

Here we aim to determine the potential for DC to delivery of T-bet to CD8+ T cell, and use DC engineered to express GFP to study intercellular transfer of this protein to T cells as a proof of principle approach. We hypothesize that changes in T-bet expression in both αDC1 and CD8+ T cells following their co-culture will support the notion that T-bet is being transferred from αDC1 to the T cells. We also hypothesize that green fluorescent protein (GFP) derived from GFP-transfected αDC1 (αDC1GFP) can transfer to T cells, mediated through CD40L-induced tunneling nanotubes or exosomes. We will test these hypotheses through the following Sub-Aims and approaches:

1. Determine T-bet expression levels in αDC1 and T cells, comparing their respective baseline values following CD40L stimulation (αDC1) and co-culture DC (T cells).
2. Establish a proof of principle transfer study by engineering αDC1 to express GFP (αDC1\(_{\text{GFP}}\)) to track in vitro transfer of GFP to autologous T cells using flow cytometry analysis and confocal microscopy.

3. Study the mechanism of intercellular transfer between αDC1 and T cells
   - Study the role of CD40L.
   - Determine cell contact requirements between αDC1 and T cells for intercellular transfer using a transwell co-culture model.
4.0 MATERIALS AND METHODS

4.1 HUMAN PRIMARY CELL ISOLATION FROM BLOOD

Buffy coats containing whole blood products from healthy and anonymous donors we ordered from the Central Blood Bank of Pittsburgh. In HIV studies, whole blood products were obtained from participants of the multi-center AIDS cohort study (MACS) chronically infected with HIV-1 on ART. The blood samples were processed using the density gradient separation method (59) to obtain specific cells from PBMC. Autologous CD14+monocytes, CD3+ T cells, CD4+ T cells, CD8+ T cells were isolated using immunomagnetic negative selection method using the specific separation kits (EasySep: STEMCELL Technologies Inc., Vancouver, BC, Canada).

4.2 RECOMBINANT ADENOVIRUS

Human T-bet (hT-bet) was PCR cloned from peripheral blood lymphocytes using the following primers: hT-bet: Fwd 5’-GTCGACGACGGCTACGGGAAGGTG-3’, Rev 5’-GGATCCTTAGTCGGTGTCCTCCAACC-3’. The product was then digested with the restriction enzymes SalI and BamHI and the 1.7Kb fragment containing full-length hT-bet was ligated into the adenoviral-Cre-Lox (Ad.lox) vector. The Adv.Tbet was used for overexpression in DC. The mock Adv. (empty) was used as controls and the Adv.EGFP was used for the imaging and transfer studies in DC. All the viruses were provided by the Storkus lab, University of Pittsburgh.
4.3 GENERATION OF HUMAN DC FROM MONOCYTES

Monocytes were cultured for 5 days at 37°C in IMDM (Gibco, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (c IMDM) in the presence of GM-CSF and IL-4 (both 1000 IU/ml; R&D Systems, Minneapolis, MN). On day 5 of the culture, iDC were differentially exposed to maturation factors for 48 h. For mature αDC1, the maturation factors consisting of polyisasinic: polycytidylic acid [poly(I:C)] (20 μg/ml), IFN-α (3,000 units/ml), TNF-α (50 ng/ml), IL-1β (25 ng/ml), and IFN-γ (1,000 IU/ml) was used (62). Alternatively, αDC1 were also generated by maturing with LPS (250 ng/ml) and IFN-γ (1,000 IU/ml). Mature, low IL-12p70 producing DC2 were generated using a modified version of a previously described cocktail consisting of TNF-α (50 ng/ml), IL-1β (25 ng/ml), and PGE2, (10−6 mol/L) and IL-6 (1,000IU/ml) (62). The DC. Tbet, DC.GFP were generated by infecting mature DC on day 6 with T-bet/GFP tagged adenovirus vector at a MOI of 500 respectively in minimum media for 2 hours at 37°C, post which the media containing specific maturation cytokines was replaced.

4.4 ACTIVATION OF MATURE DC VIA CD40L

Differentially matured DC were plated at a cell count of 25,000 DC per well on a 96-well plate and were stimulated for 24 h with either rhCD40L (0.5 μg/ml) (MegaCD40L; Enzo Life Sciences) or CD40L-expressing J558 (J558-CD40L) cells (Dr. P Lane, University of Birmingham, United Kingdom) (27), which were added to DC cultures at a 1:1 ratio.
4.5 IL-12P70 ELISA

Supernatant collected after 24 h from the wells stimulation with either rhCD40L (0.5 µg/ml) or CD40L-expressing J558 (J558-CD40L) cells were tested for the level of IL-12p70 expression via an IL-12p70 ELISA to determine the functionally characterize the DC.

4.6 GENE EXPRESSION ANALYSIS VIA MICROARRAY

DC (+/-) CD40L were evaluated for their T-bet expression at nucleic acid level via gene chip analysis. Representative DC1 and DC2 were generated and Qiagen RNeasy kit (Qiagen, Valencia, CA) used to isolate mRNA followed by a direct hybridization assay using the Illumina Human T-12 Expression Bead Chip Kit (Illumina, San Diego, CA), which contains >47,000 probes corresponding to roughly 35,000 genes (Taylor Poston). Data analysis was conducted via R programming (Dr. Martinson, University of Pittsburgh).

4.7 WESTERN BLOT ANALYSIS

DC (αDC1, DC2, αDC1.empty, DC2. empty, αDC1.Tbet and DC2.Tbet) were characterized for T-bet expression at protein level post harvesting on day 7 of culture via western blot analysis as previously described in (91). Briefly, the DC’s were lysed in 80-100 µl of lysis buffer/RIPA buffer at 4°C for 30 mins. After centrifugation at 13,500 × g for 10 min, the supernatant was mixed 5/1 with SDS-PAGE running buffer, and proteins were separated on 4%-2% SDS-PAGE pre- cast gels
(Lonza). mAbs against T-bet and HRP-conjugated goat anti-rabbit Ab (Santa Cruz Biotechnology, San Diego, CA and Ayyavoo lab) were used to detect the expression of T-bet. The primary antibody was used at a dilution of 1:500 or 1:1000 and the secondary antibody was used at a dilution of 1:3000. Tubulin was used as the loading control. Probed proteins were visualized by a western lightning chemiluminescence detection kit (Western bright ECL, Advansta) and exposed to X-Omat film.

### 4.8 DC- T CELL CO-CULTURE

Differentially matured DC (1.25 ×10⁵ cells/ml) were co-cultured with CD4+ or CD8+ T cells, or both (3.75 ×10⁵) cells/ml in the presence of SEB (1μg/ml) or a pool of HIV-1 Gag (p17 and p65) 18mer peptides (1.25μg/ml). These cultures were grown over a period of 10-14 days prior to testing via flow cytometry/ ELISpot. The co-culture was maintained at 37°C with frequent addition of IL-2 (100 IU/ml) and IL-7 (10ng/ml). The cultures were also challenged with the Gag peptide antigen, including an MHC-class 1 (A2) restricted HIV 9mer TLNAWVKVV along with irradiated T2 cells at day 14, 7 days prior to a secondary IFN-γ ELISpot read-out assay.

### 4.9 ELISPOT ASSAY

ELISpot assay was performed to measure IFN-γ production by memory CTL’s stimulated with αDC1 versus αDC1.Tbet. The assay was performed as previously described in (77) by stimulating αDC1 and αDC1.tbet loaded with S5 peptide pool at a stimulator (DC): responder (T cells) ratio
of 1:10. Briefly, the assay included negative-control wells with T cells or T cells and DC without peptide. ELISpot data were calculated as the means of spots in duplicate wells minus the mean plus 2 standard deviations of spots in duplicate negative controls.

4.10 FLOWCYTOMETRY ANALYSIS

Surface staining and intracellular staining (True nuclear staining kit, BD) was carried out to look at various markers as previously described in (63). The stains used for flow cytometry are as follows: Mouse-anti-human CD83-PE (Beckman Coulter), CD86-PE (Beckman Coulter), OX40L-PE, CD3-FITC, CD4-APC, CD8- Per CPCy-5.5, CD3-PE, INF-γ-PE-Cy7, CD107a-FITC, CD14-Alexa Flour 700 (all from BD Biosciences), T-bet-BV711(Bio legend) and the respective matched isotype controls (BD Biosciences). Prior to analysis of T cell responses generated due to peptide restimulation, the cells for stimulated with anti-CD3/CD28 activating Dynabeads (Gibco, Life Technologies) to mimic interaction with DC and stained for CD107a in one condition to be used as positive and negative controls respectively. Purity was determined by the exclusive expression of either CD4 or CD8 on the CD3+ gated lymphocytes. Analysis was performed using the BD Biosciences LSR Fortessa Cell Analyzer. The data was acquired via FACS Diva software and analyzed using the Flow Jo version 7.6 software.
4.11 IMAGING STUDIES

The imaging studies were done using various imaging techniques. Firstly, bright field microscopy was used to collect morphological images of differentially matured DC. (Leica). The DC-T cell co-culture images for studying GFP transfer from Dc to T cells were obtained via confocal microscopy using Nikon Eclipse Ti and Photometrics Evolve camera system with a Nikon Apo TIRF 60x Oil DIC N2 objective lens with a numerical aperture (NA) of 1.49; and NIS-Elements software was used to collect the images generated. For studying T-bet localization in DC, preliminary studies were conducted via image stream analysis using the Amnis flow cytometer machine and the data was analyzed using the IDEAS software.

4.12 TRANSWELL ASSAY

Transwell assays were performed to study the mechanism of DC-T cell transfer. Briefly, αDC1.Tbet \((5 \times 10^5)\) were plated in the transwell (0.4μm PTFE membrane collagen coated, Costar) along with CD8+ T cells or with control αDC1 in the bottom chamber of a 24-well transwell plate in 400 μl of IMDM ((Gibco, Life Technologies, Grand Island, NY). The CD8+ T cells were also stimulated with anti-CD3/CD28 activating Dynabeads (Gibco, Life Technologies) to mimic interaction with DC in one bottom well of the transwell. All the conditions were stimulated with SEB (1μl/400μl) and 1.25μl of CD40L. The cells at the bottom were harvested 48 h post the experimental setup and were analyzed for GFP positive CD8 T cells. Analysis was performed using the BD Biosciences LSR Fortessa Cell Analyzer. The data was acquired via FACS Diva software and analyzed using the Flow Jo version 7.6 software.
5.0 RESULTS

5.1 AIM 1: TO DETERMINE IF T-BET EXPRESSION IN MATURED DC IS ASSOCIATED WITH POLARIZATION STATUS

5.1.1 Phenotypic characterization of differentially matured polarized DC

Differentially matured human monocyte-derived DC types were generated based on the previously described methods using either a cocktail of factors consisting of poly(I:C), TNF-α, IL-1β, IFN-α, and IFN-γ (62) for αDC1, or IL-1β, TNF-α, IL-6 and PGE2 and for DC2 (27). DC were analyzed phenotypically based on surface expression of protein markers by immunostaining and flow cytometry. The gating strategy for DC is shown in figure 7A. The αDC1 are characterized based on their high expression of the surface markers of CD83, CD86 and low expression of OX40L, while typically DC2 express high levels of all three of these markers, including OX40L (Figure 7B).
Figure 7. Mature DC have higher expression of CD86 and CD83.
A) Flow cytometry gating strategy for analyzing DC population based on light-scatter properties (left) and single cell discrimination (right). B) Surface markers expression analysis of CD86, CD83 and OX40L on differentially mature DC compared to isotype controls.
5.1.2 Morphologic characterization of differentially matured and polarized DC

DC were also characterized based on their changes in morphology in response to the different maturation cocktails, and their subsequent response to the T helper cell associated co-stimulatory molecule CD40L as assessed via standard bright field microscopy. At the end of their initial culture period, it was observed that the αDC1 were semi-adherent, formed some clusters as well as elongated patterns on the tissue culture surface (Figure 8A), while DC2 appeared to form less clusters and were found to be more uniformly rounded (Figure 8B). Upon CD40L stimulation, there were also morphological changes unique to the differentially matured DC types. As previously described (31), the αDC1 showed extensive reticulation by forming ‘tunneling nanotubes’ after stimulation with CD40L (Figure 8C), while no such reticulation was seen in the DC2 conditions (Figure 8D).
Figure 8. αDC1 show significant morphological changes (reticulation) post treatment with CD40L compared to DC2. A) and B) show respective cell morphologies for αDC1 and DC2 prior to CD40L stimulation. Panel C) shows the unique ability of αDC1 to reticulate in response to CD40L compared to DC2 shown in panel D). The tunneling nanotubes are noted by red arrows. (Images were captured at 40X, by standard bright field microscopy, with bottom panel figures being artificially magnified to highlight fine cellular extensions.)

5.1.3 Functional characterization of differentially matured and polarized DC

Differentially matured and polarized DC were also tested by ELISA for their ability to produce IL-12p70 in response to CD40L. As expected, αDC1 produced higher levels of IL-12p70 when compared to DC2 (data not shown). The IL-12p70 ELISA also revealed another characteristic feature of αDC1 compared to DC2, with αDC1 producing significantly higher amounts of IL-
12p70 when treated with CD40L for 24hrs compared to the diminished to DC2+CD40L. (Figure 9).

![Graph showing IL-12p70 levels](image)

**Figure 9.** αDC1 produce higher levels of IL-12p70 (+/- CD40L) compared to DC2. Shows the higher production of IL-12p70 by αDC1 +CD40L over DC2+CD40L. The higher expression of IL-12p70 can be seen in the former compared to the diminished expression in the later. These data were generated for (n=3) and the error bar represents the standard error mean for the 3 donors.

### 5.1.4 Quantitative analysis of T-bet RNA expression in αDC1 versus DC2 before and after CD40L stimulation

To determine relative differences in T-bet expression in αDC1 versus DC2 gene chip analyses of were performed. First, the expression levels of IL-12p70 was assessed to verify the differentially polarized functional status of the DC types being analyzed (Figure 10A). The increased IL-12
gene expression verified that DC1 indeed were being generated, and this was earlier confirmed by measuring IL-12p70 by ELISA (data not shown). With this confirmation, T-bet gene expression levels (+/-) CD40L was assessed. From the analysis, we observed that the differences in the level of T-bet gene expression between αDC1 and DC2 (+/-) CD40L was unremarkable (Figure 10B). This was surprising, and seemed to go against the hypothesis that αDC1 would more highly express T-bet, and warranted further investigation at the protein level.

Figure 10. The difference in T-bet expression (+/-) CD40L in αDC1 versus DC2 is insignificant. 7(A) depicts the functional difference between αDC1 and DC2 characterized via IL-12p70 expression. As seen in 7(B), the levels of T-bet do not vary significantly between αDC1 and DC (+/-) CD40L.

5.1.5 Assessment of T-bet expression in differently matured and polarized DC at the protein level

Next, the level of T-bet protein being produced between αDC1 and DC2 was assessed to determine if the actual translated protein values would correspond to the gene chip analysis findings. The T-bet protein levels of the DC types were first determined via standard western blot analysis. We observed a protein band for T-bet at 62kDa, which was consistent with the expected T-bet protein size previously established in an earlier report (95). Results from the western blots generated
suggested that αDC1s indeed expressed substantially higher T-bet at the protein level when compared to DC2 (Figure 11A). Furthermore, we wanted to determine if this pattern of relative T-bet expression levels was generally consistent with other DC1 and DC2 types, independent of the maturation factors used to achieve their respective polarized status. For this, DC1 were generated by maturing DC in the presence of LPS and INF γ, which is known to yield high IL-12p70 producing DC. Western blot analysis of these DC1 revealed that T-bet expression was indeed clearly expressed, with a band still visible at 62kDa while a band was not visible for the DC2 (Figure 11B). Hence, from these results we conclude that higher expression of T-bet is a general trait of DC1 as compared to DC2. For the rest of the study, the αDC1 maturation method was used to generate DC1.

Figure 11. T-bet is expressed at higher levels in αDC1 compared to DC2, independent of the maturation cocktails used.
(A) Western blot analysis showing higher detectable expression of T-bet at 62kDa in αDC1 compared to DC2. Data is from one experiment representative of 3. (B) confirms the pattern of higher expression of T-bet in αDC1 versus DC2 independent of the maturation cocktails used. Tubulin was used as loading control for western blot analysis (data not shown).
5.1.6 Quantitative analysis of T-bet protein expression in αDC1 versus DC2

On determining the higher T-bet protein expression in αDC1 compared to DC2 and observing it to be a general trait of DC1, we wanted to confirm these data via another protein quantitation method. To analyze T-bet protein expression levels, a flow cytometry approach was used followed by intracellular antibody staining for T-bet using a specific flow cytometry labelled antibody. It was observed that the αDC1 were indeed clearly positive for T-bet expression while there was little evidence of T-bet protein in DC2 when the mean fluorescence intensity of the T-bet antibody stained samples were compared to their respective isotype controls (Figure 12A and B). The data shown in figure 12 is representative of three experiments performed, all of which consistently showed similar results. These flow cytometry results supported the data from western blots analyses suggesting that αDC1 indeed express a substantially higher level of T-bet protein when directly compared to DC2.

![Figure 12](image.png)

**Figure 12** Confirmation of higher levels of T-bet protein expression in αDC1 versus DC2 by flow cytometry.
A) Single parameter histograms showing a clear positive shift in the total population of αDC1 expressing T-bet with respect to the isotype control, and B) showing a very minimal shift in T-bet antibody staining in the DC2 population compared to isotype control staining.
5.2  AIM 2: ASSESS THE FUNCTIONAL IMPACT OF T-BET OVEREXPRESSION ON DIFFERENTIALLY POLARIZED DC TYPES

5.2.1  Test use of an adenoviral delivery system to determine if DC can be engineered to overexpress T-bet

After determining that αDC1 express higher amounts of T-bet at the protein level, we wanted to see if T-bet could be overexpressed in different DC types, including iDC, αDC1 and DC2, using a T-bet gene adenoviral delivery system as previously described (91). The purpose of doing so was to allow for further exploration of the direct impact of T-bet expression on DC phenotype, function, and to establish a method to use as a basis for future intercellular trafficking of DC1-derived T-bet. This T-bet overexpression strategy was preliminarily tested using polarized αDC1. Following maturation, the αDC1 was infected with the adenoviral T-bet vector (Adv-Tbet) for 24hrs. The cells were then lysed and analyzed for T-bet protein expression by western blot analysis. The western blots revealed a very intense band at 62kDa in the transfected DC, which was much more pronounced as compared to the αDC1 and DC2 controls, thus confirming that the protein T-bet was indeed ectopically overexpressed in the Adv-Tbet transfected DC (DC_{Tbet}) (Figure 13).
Next, we wanted to verify the western blot results by assessing T-bet overexpression in DC via intracellular flow cytometry. αDC1 and DC_{Tbet} were stained for T-bet prior to flow cytometry analysis. We observed a larger shift in the peak of T-bet positive cells in case of DC_{Tbet} with respect to the isotype control versus αDC1 and its isotype control (Figure 14A). These results provided a confirmation that T-bet overexpression can be induced via an adenoviral delivery system to establish a DC_{Tbet}. Moreover, we also observed a striking difference between the MFI for the isotype controls for αDC1 and DC_{Tbet} as well as the MFI for the T-bet positive population for αDC1 and DC_{Tbet} (Figure 14B).
5.2.2 Determine the impact of T-bet overexpression on DC phenotype

We further wished to study the impact on DC phenotype resulting from T-bet overexpression. We decided to analyze the impact on DC surface expression of the common maturation-associated co-stimulatory markers CD86 and CD83. DC were also infected with empty vector for use as a negative control. Flow cytometry analysis revealed no substantial differences in expression of CD86 or CD83 specifically resulting from T-bet overexpression. (Figure 15A, B and C). Similar results were seen when testing the impact on OX-40L expression (data not shown).
Figure 15 T-bet overexpression in differently matured and immature DC does not alter their expression of CD86 and CD83. The histograms depict the comparative expression CD86 and CD83 on control and respective Adv-Tbet transfected DC types (αDC1 and DC2). The empty vector infected DC types served as the respective controls.

5.2.3 Determine the impact of T-bet overexpression on DC cytokine production

After analyzing the phenotypic properties, we wanted to see if the overexpression of T-bet alters DC IL-12p70 production capacity. The adenoviral vector system was used overexpress T-bet in αDC1 and DC2, and an adenoviral empty vector served as a transfection control, both at a MOI of 500. Culture supernatant IL-12p70 levels were tested via an IL-12p70 ELISA following DC stimulation with CD40L. Surprisingly, we observed that the T-bet overexpression in DC reduced the IL-12p70 production ability in both αDC1 (Figure 16A) and DC2 (Figure 16B). Importantly, this effect was most pronounced in αDC1. After repeating this experiment in three different donors
and subsequent IL-12p70 ELISAs using donors, we concluded that T-bet overexpression is associated with decreased production of IL-12p70 in DC.

Figure 16 T-bet overexpression negatively impacts DC IL-12p70 producing capacity. T-bet transfected αDC1 and DC2, and their respective DC controls were harvested, re-plated, stimulated for 24h with CD40L. Supernatants were collected and tested for IL12p70 content. (A) IL-12p70 production by αDC1, αDC1 transfected with empty vector, and αDC1 transfected with Adv-Tbet, and B) IL-12p70 production by DC2, DC2 transfected with empty vector, and DC2 transfected with Adv-Tbet. Error bars represent standard error with (n=3).

5.2.4 Determine the impact of Tbet overexpression on DC capacity to induce HIV-1 specific CTL responses

To determine the impact of T-bet overexpression on the CTL inducing capacity of DC, αDC1, αDC1-Tbet, DC2 and DC2-Tbet were used as HIV-1 peptide antigen presenting cells to stimulate autologous responder CD8+ T cells derived from HIV-1 chronically infected MACS participants. On Day 14 of the DC- CD8+ T cell co-culture stimulated with a donor specific conserved HIV peptide pool, we stained the responder cells for markers such as Live and Dead, CD107a, INF γ, T-bet and CD8 to analyze the HIV-1 specific CTL responses via flow cytometry. The cells were gated for the lymphocytes, single cell populations and the live cell populations (Figure17A). We observed that there was a significant difference in CD107a and INF γ responses between αDC1
and αDC1.Tbet. The αDC1.Tbet cells were seen to generate a better CD107a and INF γ suggesting that T-bet overexpression promoted HIV-1 specific memory CTL responses (Figure 17Band 17C) although the CD107a and INF γ responses in DC2 and DC2.Tbet were found to be similar. Furthermore, we gated on the populations that were double positive for both CD107a and INF γ. It was observed that the responses were higher for αDC1.Tbet over αDC1 confirming the ability of T-bet overexpression in DC to enhance superior HIV-1 specific memory CTL responses (Figure 17D and 17E). However, there was no significant change observed between DC2 and DC2.Tbet in inducing HIV-1 specific memory CTL responses. On calculating a percentage increase in HIV-1 specific memory CTL responses between αDC1 versus αDC1.Tbet and DC2 versus DC2.Tbet via quantifying the changes in markers such as CD107a, INF γ and CD107a+ INF γ, we observed that there was a significant increase in case of αDC1.Tbet compared to its control (αDC1). The changes observed between DC2 and DC2.Tbet for increase in CTL responses were found to be very minimal (Figure 17F and 17G). We also looked at percentage of CTL expressing T-bet and observed that T-bet levels were higher in case of αDC1 over DC2 when compared to appropriate controls (Figure 17H and 17I)
Figure 17 T-bet overexpression in αDC1 enhances their ability to induce HIV-1 CTL.

(A) The gating strategy used for data analysis of day 14 DC-primed bulk CD8+ T cells, which represents the gated live, single cell lymphocytes. (B) Flow cytometry analysis show that stimulation with HIV-1 peptide induced expression of CD107a (left panel) and (right panel) INF γ in HIV-1 antigen responsive CTL that were initially primed by HIV-1 peptide antigen presenting aDC1 and aDC1.Tbet. Analysis of the responding CTL producing an enhanced translocation of CD107a and production of INF-γ after initial priming by either αDC1 (C) or αDC1.Tbet (D) antigen presenting cells. Graphs (E) and (F) depict the percent increase in peptide antigen responsive CTL responses resulting from initial CTL priming by αDC1.Tbet compared to control αDC1, and (G) and by DC2.Tbet compared to control DC2 respectively. (G) T-bet expression in HIV-1 specific CTL initially stimulated with either αDC1, αDC1.Tbet or (H) DC2, or DC2.Tbet.
5.2.5 Assessment of DC. $T_{bet}$ impact on the long-term function and survival of CTL following challenge with HIV-1 antigen expressing targets

After determining that $\alpha_{DC1}.T_{bet}$ were superior inducers of HIV-1 specific CTL responses, the impact of their T-bet overexpression on the long-term survival of the CTL they induced was assessed by testing the functional response of the CTL in IFN- $\gamma$ ELISpot at day 22, 7 days after their exposure to HIV-1 peptide antigen pulsed T2 target cells. While there was an overall decrease in the percentage of HIV-antigen responsive CTL surviving at day 22, the cultures that were initiated using the $\alpha_{DC1}.T_{bet}$ cells had a higher percentage of HIV antigen specific IFN-$\gamma$ producing CTL (Fig 18), suggesting a positive effect from the DC T-bet overexpression. However, it is important to stress that this experiment was performed on one donor only, so these interpretations should be viewed with caution.

![Figure 18](image)

**Figure 18** Higher HIV-1 specific memory CTL responses maintained after challenge with antigenic targets when initially induced with $\alpha_{DC1}.T_{bet}$ compared to $\alpha_{DC1}$. INF$\gamma$ ELISpot results of day 22 CTL cultures, 7 days after challenge with Gag peptide antigen and T2 targets. Data represents the results from one donor, with error bars representing the SDEV of the assay triplicates.
5.3  **AIM 3: TO DETERMINE THE POTENTIAL FOR ADC1 TRANSFER OF T-BET TO CD8+ T CELLS**

As previously discussed, the concept of ‘signal 3’ or DC polarization has been actively explored in various cancer immunotherapies. The Storkus group has also shown using a murine sarcoma model that DC transfected to overexpress T-bet skews (DC_{Tbet}) T cell effector function towards type-1 immunity resulting in positive therapeutic impact (91). Moreover, in preliminary studies using a T-bet knockout model, they also have data suggesting that T-bet transfer from DC_{Tbet} to responder T cells can occur through some unknown mechanism (unpublished data, communication from Walter Storkus). In addition, preliminary flow cytometry results from our lab show that when human αDC1 hyper-stimulated with CD40L and co-cultured with T cells, the number of T-bet expressing T-cells generated clearly increases, even in the absence of cognate antigen (Figure 19A, B and C). Interestingly, we show that the T-bet level of expression in αDC1 decreases following exposure to the T helper cell factor CD40L (Figure 20). Importantly, the mechanisms involved in these potentially related findings from our group and the Storkus group remain unclear. We hypothesize that the noted decrease of T-bet expression in αDC1 occurring due to CD40L activation, and the increased T-bet expression in T cells following co-culture with αDC1 are directly related, and represents the direct transfer potential of T-bet from DC to T cells. Therefore, we decided to carry out some exploratory studies to examine the general potential for DC to T cell intercellular transfer of cargo, including T-bet.
Figure 19 T-bet expression in CD8+ T cells is increased when co-cultured with CD40L- activated αDC1.

It has been observed that the T-bet expression in both CD8+ T cells (panel A) and CD4+ T cells (panel B) is almost doubled in T cells co-cultured with αDC1 compared to their endogenous levels in only T cells without DC (analysis courtesy of Tatiana Garcia-Bates). C) is a graphical representation of increase in Tbet levels in T cells due to αDC1 activation relative to their endogenous T-bet levels when cultured with cytokines alone (IL-2 and IL-7).
Figure 20 T-bet expression decreases in DC post CD40L stimulation.
The left panel are the flow cytometry contour plots measuring T-bet expression of the aDC1 that were cultured with and without CD40L stimulation. The right panel bar graphs summarize the flow cytometry data and shows the percent of the DC that were T-bet positive.

Ultimately we wanted to investigate the role of T-bet transfer from DC to T cell. While the planned approach was to use a T-bet expressing vector tagged with a fluorophore to track T-bet transfer from DC to T cell via imaging, due to the unavailability of such a fluorophore-tagged T-bet expression vector, we used a GFP expression system instead to establish a “proof of principle” model for general DC to T cell intercellular transfer of cargo material.

5.3.1 Proof of principle GFP-based model for intercellular transfer from DC to T cells

αDC1 were engineered to express GFP (αDC1.GFP) by transducing them with a replication incompetent GFP-tagged adenoviral vector. The αDC1.GFP were established and these were co-cultured with autologous T cells for 4 days. We were specifically interested in studying the intercellular transfer between DC and CD8+ cytotoxic T cells, the cell type critical for killing HIV-1 infected cells and controlling HIV-1 viral load. On day 4, these cultures were harvested and the CD8+ T cells were analyzed for GFP expression via flow cytometry (Figure 21A). The flow cytometry analysis determined that GFP was indeed transferred from DC to the CD8+ T cell, with 22.8 % of the total CD8+ T cells positive for GFP.
(Figure 21B). These preliminary results showing that DC1-derived GFP could be readily transferred to T cells This was rather surprising and prompted us to verify the results and examine the phenomenon in more detail. We first performed some imaging studies on cultured cells using confocal microscopy to visually confirm the GFP transfer from DC to T cells. Indeed, the GFP signal could be observed within the T cell clusters after co-culture with DC.GFP cells (Figure 21C).

Figure 21 Transfer of GFP from αDC1 to CD8+ T cells.
A) Flow chart of experimental procedure. Purified DC1.GFP were co-cultured with autologous CD3+ T cells in the presence of SEB. On day 4, the T cells were analyzed for the presence of GFP (green). B) Flow cytometry analysis of CD8+ T cells expressing DC1.GFP derived GFP. The percent positive is based on negative control value (not shown) represented by the vertical line. C) Image acquired by confocal microscopy of a cluster of T cells following 4day co-culture with DC1.GFP. The GFP expressing T cells (green) are indicated by the red arrows.

We next decided to repeat the experiment, to see if similar results would be achieved co-culturing the DC1.GFP with again with the purified T cells containing both CD4 and CD8 T cell populations or only the purified CD8+ T cell fraction. When performing the flow cytometry analysis, we expected to see
GFP transfer in both conditions tested, however, efficient GFP transfer to CD8+ T cells was seen only when both the CD4+ and CD8+ T cells were present (Figure 22A), suggesting a novel role of CD4 ‘help’ in DC to T cell intercellular transfer. We wanted to further investigate the role of CD4 ‘help’, and wanted to see if we could substitute the CD4+ T cells with CD40L helper factor as a CD4+ T cell surrogate to generate a similar response. In doing so, we determined by flow cytometry analysis that the CD40L could indeed induced the GFP transfer from the DC1.GFP to T cells, enhancing the transfer greater by greater than 3-fold (Figure 22B). We again verified these findings by, confocal microscopy. Interestingly, the images generated from confocal microscopy supported the data from the flow cytometry shown earlier. GFP transfer from the DC to the CD8+ T cells was observed to be substantially higher when in the presence of CD40L compared to conditions without CD40L. (Figure 23). **Together, these collective findings (imaging and flow cytometry) support our identification of a novel “helper” function of CD40L for facilitating transfer DC cellular cargo to CD8+ T cells.**

*Figure 22 CD4+ T cell ‘help’ promotes transfer of DC derived GFP to CD8+ T cells.*

A) Flow cytometry analysis of CD8+ T cells following their 4day co-culture with DC1.GFP either alone (left panel), in the presence of CD4+ T cells (middle), or in the presence of CD40L (right).  B). Summarized flow cytometry results of percent positive GFP transfer to CD8+ T cells with the various co-culture conditions.
5.3.2 To determine the mechanism of intercellular GFP transfer from CD40L activated DC.GFP to CD8+ T cells

After establishing a “proof of principle” for the potential for intercellular transfer of material from DC1 to CD8+ T cells, we wanted to address the potential mechanism involved. We initially hypothesized that such a transfer could occur either via CD40L-induced ‘tunneling nanotubes’ (31) or ‘exosomes’ (unpublished data Zaccard et, and unpublished data and personal communication from Walter Storkus).

To investigate the mechanism of the observed transfer phenomenon, we used a transwell-based experimental assay system to test if the CD40L-mediated transfer required direct contact between the DC1.GFP and the CD8+T cells, or if this could occur independently of their proximity of DC and T cells (Fig 24A). The cells from all the bottom wells were harvested after 48 hours post experimental setup and were analyzed via flow cytometry to look at GFP positive CD8 T cells. The data from the flow cytometry
analysis showed that while the CD40L-mediated GFP transfer from DC to the CD8+ T cells did not occur when the T cells were separated by a trans-well membrane, transfer occurred when DC had contact with the recipient T cells, with 7.23% of those CD8 T cells having green fluorescence compared to only 0.69% for those T cells having no contact with any DC (Figure 24 B, C). These results verified the possibility of GFP transfer from CD40L activated DC.GFP to CD8+ T cells via extracellular delivery, possibly through extracellular vesicles or exosomes'. However, the results also revealed a new mechanistic parameter for consideration related to the role of the unlabeled control DC in this system, and the role of the activation status of the CD8+ T cell recipients.

Figure 24 CD40L-mediated GFP transfer from DC1.GFP to CD8+ T cells can occur in a non-contact dependent manner in bystander DC-activated CD8+ T cell recipients.

A). Experimental layout of the 48h trans-well co-culture assay. (Left to right) the first and the second wells are the negative and positive control for CD40L-mediated GFP transfer from DC1.GFP to CD8+ T cell respectively. The third well consists of CD8+ T cells + SEB in the bottom well separated from the DC.GFP +SEB+CD40L in the
To study the role of CD8+ T cell activation in the DC1.GFP to T cell transfer, we set up another trans-well experiment with an additional condition where we added anti-CD3/28 T cell activation beads to the bottom well, as a DC mimic to artificially activate the CD8+ T cells (Fig 25A). In doing so, we would be able to determine if indeed there was a specific requirement for there to be DC present in the lower chamber along with the recipient CD8+ T cells (where possibly DC are still needed to directly hand off the extracellular cargo to the T cells), or if there is merely a requirement for the recipient CD8+ T cells to be activated to acquire GFP expression. When the results of the experiment were analyzed by flow cytometry, only minor differences were observed in the percentage of GFP positive CD8+ T cells in the conditions where the control αDC1 were also present in the lower chamber as compared to the CD8+ T cells that were activated by the anti-CD3/28 beads. These results suggest that CD8+ T cell activation plays a critical role in the CD40L-mediated DC1.GFP to CD8+ T cells transfer of cargo such as GFP. (Fig 25 B).
Figure 25 Prior CD8+T cell activation is critical for effective GFP transfer from DC1.GFP. 
A) Experimental layout of trans-well assay to study role of CD8+ T cell activation. From left to right, the first well consists of CD8+ T cells + SEB in the bottom well and DC1.GFP +SEB+CD40L in the upper chamber of the trans-well system; the second well consists of activated CD8+ T cell via DC1+SEB in the lower chamber, and DC1.GFP +SEB+CD40L in the upper trans-well and the third well consists of ant-CD3/28 T cell activation beads and recipient CD8+ T cells on the bottom and DC1.GFP +SEB+CD40L in the upper trans-well. B) represents the flow cytometry data for the above-mentioned conditions.

To conclude, the results from the trans-well assay strongly support mechanism of inter cellular transfer from DC to CD8+ T cell occurs at least in part through extracellular deliver, possibly via exosomes, which optimally requires the activation of the recipient CD8+ T cells.
Despite the success of ART to control HIV-1 in chronically infected individuals, there is still no definite cure to HIV. The establishment and maintenance of HIV latency in memory CD4+ T cells has been a major hurdle towards developing a cure for HIV. Recently, there has been an emphasis on CTL exhaustion or dysfunction due to chronic infection which affects the overall CTL killing potential. Interestingly, previous studies from Hersperger et al. has shown the difference in CTL exhaustion levels between elite controllers and HIV progressors associated with a nuclear transcription factor called T-bet which selectively promote TH1 type of responses (86). Hence, in my project I explore the role of DC derived T-bet in enhancing CTL responses and the potential transfer of T-bet from DC to T cell for reviving CTL dysfunction.

In my first aim, I hypothesized that endogenous T-bet expression in mature DC in DC is associated with their polarization status, and that high IL-12p70 producing type-1 polarized DC (DC1 or αDC1) express higher T-bet levels compared to IL-12p70 deficient DC2 (31). Surprisingly, upon examination of T-bet expression at the nucleic acid level using gene chip analysis, I found no substantial difference between αDC1 and DC2 before or after CD40L stimulation. However, combined results from the western blot analysis and flow cytometry analysis clearly revealed a higher expression of T-bet protein in αDC1 compared to DC2, which contradicted the data from the previous gene chip analysis. It is worth noting that there were several unspecific bands observed on the western blots for both the DC1 (αDC1 as well as DC1 generated with LPS+IFN-γ) and DC2, but only in the DC1 preps could a clear band at the 62kDa be seen. These discordant results seen at nucleic acid and protein levels of T-bet expression could be due to the differences in various post transcriptional or translational modifications between the DC types. This could
result in a higher amount of functional T-bet protein being expressed in αDC1 compared to DC2, despite the RNA message being indistinguishable between them. These modifications could also explain the presence of the unspecific bands in the western blots, which could represent products resulting from different post translational modifications leading to functional or less functional T-bet. Importantly, the higher expression of T-bet protein in αDC1 that was shown both by western blot and flow cytometry analysis strongly supports my hypothesis that endogenous T-bet expression in mature monocyte derived DC is greatly influenced by their polarization status, and that DC1 in general express higher amounts of T-bet than DC2.

Previous studies from the Qu. Y et al. have shown that overexpressing T-bet in CD11c+αDC1 through use of an adenoviral vector delivery system can efficiently reprogram the function of a T cell to promote more TH1 specific responses in a cancer setting (91). For my second aim, I wanted to explore the use of this T-bet overexpressing DC system in my project, to study the function and trafficking of αDC1-derived T-bet. In addition, I wanted to determine the impact of this approach on the phenotype and function of αDC1, including their capacity to induce CTL response in the setting of chronic HIV infection. Although the T-bet overexpression did not alter the phenotypic and morphological characteristics of either αDC1 or DC2, it was surprising that the overexpression had a negative impact on the IL-12p70 production capacity of αDC1, since both IL-12p70 and T-bet are known for promoting IFN γ production and type-1 responses in T cells, and because αDC1 have been thought to be relatively resistant to suppression from exogenous signals (96). While these results were consistent between donors, and consistent with the findings from Lipscomb et al. et.al, who showed that T-bet overexpression decreased IL-12p70 production in immature DC, it is unclear these results are physiologically relevant since this is in fact a very artificial system. Nevertheless, this suppression of IL-12p70 may represent a negative feedback
mechanism triggered by high T-bet accumulation within the DC. However, I speculate that the this finding may be related to the IL-12p70 independent mechanism of transfer that requires close proximity of DC- T cell and therefore maybe pointing towards unique intercellular mechanisms of transfer (exosomes/TNTs).

It is important to note that the alteration in the IL-12p70 production capacity of the αDC1 following adenoviral delivery of the T-bet expression gene did not hinder their ability to effectively induce HIV-1 specific CTL responses. In fact, the magnitude of the CTL responses induced by αDC1.Tbet were higher than that induced by the control αDC1. In addition, the CTL’s induced by the αDC1.Tbet showed enhanced long term survival following challenge with antigen tagged target cells (T2 cells) compared to those induced by αDC1. While, the data suggest that αDC1.Tbet may have a superior ability to drive better HIV-1 specific CTL responses over αDC1, this test was only performed one time with only one HIV positive MACS participant, and therefore must be replicated in multiple donors before claiming that αDC1.Tbet are significantly more effective at inducing HIV-1 specific CTL responses over αDC1. Nevertheless, these data generated are in accordance with the study done by the Storkus group, which suggests the T-bet overexpression in DC can improve T cell responses potentially through a novel IL-12p70 independent mechanism.

For my third aim, I originally set out to test my hypothesis that αDC1 have the capacity to directly transfer T-bet to CD8+ T cells. The rationale for this idea evolved from a combination previous findings and observations. First, through a personal communication the Storkus group I was made aware of their unpublished data showing that a small percentage of T cells from a T-bet knockout mouse expressed T-bet after they were injected with DC overexpressing Tbet. These preliminary results suggest that the T-bet must have been transferred from the DC to the T cells via an unknown mechanism. Another key finding came from an earlier observation made in my
lab where it was found that T-bet levels in CD8+ T cells were upregulated after being co-cultured with αDC1 in the presence of CD40L, even when the antigen was absent. I linked these results with data from one of my experiments, in which I showed that the expression of T-bet protein in αDC1 is downregulated when treated with CD40L, even though there was no difference in T-bet gene expression at the molecular level based on gene chip analysis. I hypothesized that the upregulation of T-bet in CD8+ T cells co-cultured with CD40L-stimulated αDC1 was due to the transfer of T-bet from αDC1 owing to the decrease in αDC1 T-bet levels. Due to the unique capacity of αDC1 to form tunneling nanotubes in response to CD40L (31), and the fact that exosomes might be released as part of this process of DC ‘reticulation’ (unpublished data from Zaccard.et.al), I hypothesized that the transfer of T-bet from αDC1 to CD8+ T cells might occur through one of these routes.

While I was unable to specifically study αDC1 transfer of T-bet due to the limited time and unavailability of a T-bet probe tagged with a fluorophore to monitor transfer via imaging, I did however successfully establish a ‘proof of principle’ transfer study using ADV-GFP to visualize GFP transfer from αDC1 to CD8+ T cells. The striking results of my imaging and flow cytometry studies showing that GFP transfer from αDC1.GFP to CD8+ T cells indeed occurred, and was greatly enhanced in the presence of either CD4+ T cells or recombinant CD40L was fascinating. These data support the notion that the CD4+ T cell factor CD40L plays a critical role facilitating the transfer of cellular cargo from αDC1 to CD8+ T cells, also suggesting the unveiling a novel ‘helper’ function of the CD4+ T cell.

While some of the mechanisms involved in the intercellular transfer remain unclear, trans-well assays revealed that the GFP transfer from the αDC1.GFP to the CD8+ T cells could occur through the trans-well when the cells were separated, but only in cases where the CD8+ T cells
were activated. These observations suggest that transfer is occurring via extracellular vesicles or polarized exosomes, but does not eliminating the potential role that tunneling nanotubes may also play in this transfer phenomenon, since there was some indication of transfer occurring in the absence of CD40L ‘help’ when direct contact was permitted. Importantly, the activation status of CD8+ T cell was found to be an essential component for the transfer to occur. The reason for this activation requirement may be due to the expression of an essential surface receptor that might help capture the extracellular vesicles carrying the cargo, in this case GFP. It is also conceivable that the GFP transfer was not direct protein transfer, but rather transfer of some ADV.GFP that remained and did not fully integrate into the αDC1 genome, and that the CD8+ T cell activation helped to facilitate their trans-infection and transduction. Nevertheless, CD40L-mediated transfer from the αDC1 to the T cells occurred.

These findings from my projects may also link to the studies conducted by Hor JL et al. showing that there was asynchronous T cell activation by distinct DC subsets, and that the CD8+ T cells were activated by LN resident DC and not the migratory DC, yet required the migratory DC to carry the antigenic information and transfer it to LN residing cells via some unknown mechanism to generate the CD8+ T cell responses for clearance of HSV viral infection (30). Interestingly, in that study it was shown that the migratory DC interacted with CD4+ T cells, and that this interaction was required to generate the CD8+ T cell responses distally. It is possible that the transfer of T-bet might also may occur spatially between migratory DC to LN resident DC as proposed in the model by Zaccard et.al via CD40L-induced tunneling nanotubes (31). It might also be possible that DC-derived information can be directly transferred from migratory DC to CD8+ T cells being activated distally by other DC via extracellular vesicles or polarized exosome to drive type-1 specific immune responses. While these scenarios are very speculative, the possibilities are
intriguing, and my findings highlight the fact that there is still so much yet to be fully understood about the human immune system.

Although these findings from my project shed light on the various immunological mechanism of interaction between αDC1 to T cell, it is still early to establish some of the specific mechanism of transfer. Also, it is unclear if there is true biologic significance to these in vitro studies, and further studies would need to be carried out to further elucidate the importance of our findings. To conclude, the various findings from my project provide a different insight into the immunological processes occurring in the human body, which I strongly believe have a biological significance and warrants further investigation.
7.0 PUBLIC HEALTH SIGNIFICANCE

Despite the success of ART in controlling HIV viral load, HIV latency and associated CTL exhaustion or dysfunction is a major hurdle on the road to developing a vaccine or cure for HIV. In my project, I investigated the potential of programming DC to enhance T cell responses in a HIV setting based on work done by various groups such as the Storkus group who have shown significant changes in the tumor microenvironment due to enhance T cell responses against the tumor antigen when stimulated by reprogrammed DC. The strategy of “Kick” and “Kill” for HIV cure is an active area of research and the reprogramming of DC to revive dysfunctional CTL or a better CTL response is a major focus of my project contributing to the “Kill” component of HIV. The results from my project suggesting enhanced HIV-1 specific CTL responses in response to DC. Tbet suggest the potential of DC being used in immunotherapeutic strategies by appropriate reprogramming for treatment of chronic viral infection or development of a therapeutic DC based vaccine. Moreover, I have some interesting findings from my project which sheds light on understanding the interplay of various immunologic factors associated with dendritic cells and T cells. Interestingly, my data proposes the identification of a novel and new helper function of the CD40L to enable transfer of cellular cargo in the form of extracellular vesicles / polarized exosome from DC to CD8+ T cells. This discovery could enable us to understand the complex mechanisms of intercellular transfer in both health and disease. This mechanism could be using in therapeutics targeting chronic infection or to understand the various mechanisms by which a pathogen might facilitate transfer from cell to cell by exploiting this route of transfer.
8.0 FUTURE DIRECTIONS

In the future, we would also like to investigate the following details:

1. The localization of T-bet in dendritic cell in the presence and absence of CD40L stimulation. This will enable us to determine if the T-bet levels are lower in the DC post CD40L stimulation due to higher localization of T-bet in the cytoplasm of the DC. This will also help get a better insight about the transfer mechanism (nanotubes/exosome). Preliminary studies were performed on DC to determine T-bet localization using the AMNIS, Image stream technology. However, the analysis to compare the different localization patterns on a single cell level using the IDEAS software could not be done due to limited time and resources. The preliminary data is shown in the figure below.

![Figure 26 T-bet localization pattern in various DC populations. (Raw data only).](image)

2. Study T-bet transfer from DC to T cell via imaging using a DC infected with recombinant adenovirus encoding a fluorescent T-bet fusion protein

3. Determine if presence of T-bet can be found in DC derived exosomes and/or TNTs.


