Transfer of DC Instruction to T Cells Via Extracellular Vesicles

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B.Tech in Bio-engineering, Shanmugha Arts, Science, Technology and Research Academy,

India, 2018

Submitted to the Graduate Faculty of the

Department of Infectious Diseases and Microbiology

Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Master of Science

University of Pittsburgh

2020

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

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Abstract

An overwhelming push in HIV research has been made towards developing a 'functional cure', with the overarching goal being to eliminate or control the virus without the need for continued antiretroviral drug therapy. Successful immunotherapeutic strategies are now mainstream as anti-cancer treatments have encouraged exploration into the development of novel immunotherapies to treat HIV. Importantly in public health, new insights into the complexities of how the immune system functions in both health and disease continue to provide room for developing novel and improved therapies. Because dendritic cells (DC) play a central role in the crosstalk between the innate and adaptive branches of the immune response, they have been widely considered for their therapeutic potential for both HIV and cancer. Yet in order to capitalize on their strengths in this regard, there is still a need to better understand the basics of how they function and communicate with other immune cells. In this study, we explore the basic role DC-derived extracellular vesicles play in the immune crosstalk between DC and T cells, characterizing mechanisms of their release, their transfer to T cells, their phenotype, and their functional impact on cellular immune responses to viral antigens. It is our position that information gained from this work may contribute to the development of novel and improved therapies to treat chronic diseases such as HIV-1 infection.

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Preface

I would like to thank my advisor, Dr. Robbie B Mailliard from the bottom of my heart for all the support and guidance he provided throughout my journey in IDM. Secondary to being a great mentor, he was also a good friend and a pillar to depend on during times of peril. He taught me the value of patience, hard work and how to enjoy science. I would like to thank my lab members Renee Anderko, Shiv Biradar, Holly Bilben and Tatiana Bates Garcia. Additionally, I would like to thank the members of the Ambrosio Lab for their immense help and guidance. I extend my gratitude to Dr. Ernesto Marques for his valuable advice and suggestions which helped hone my skills. Moreover, I would also like to thank my family Thothathri Visvanathan, Banumathi Thothathri and Visvanathan Thothathri for being such enormous emotional support through my MS program.

1.0 Introduction

Chronic diseases such as cancer and HIV have long been a burden to society, and are observed frequently along with other infectious diseases [1]. Despite advancements in therapeutics, the number of people infected with HIV across the globe is approximately 37.9 million according to WHO reports, and approximately 17 million new cancer diagnoses occur resulting in approximately 9.6 million deaths each year worldwide. The use of various chemotherapeutic approaches and combination anti-retroviral therapy (ART) have played a major role in controlling these diseases [2], but they do not come without major side effects. Moreover, their increased application is also fueled with raised concerns for the potential development of drug resistance [3, 4]. On a positive note, there have been great strides made in terms of not just prevention, but also therapeutics to lessen burden, limit new cases, and to even cure some of those affected by these chronic diseases. Importantly, advances in technologies and our understanding of immunology in general has led to the development of novel and promising immunotherapeutic strategies.

In cancer, the treatments have moved from standard highly toxic and non-specific treatments such as chemo and radiation therapy, to more advanced immunotherapeutic approaches such as developments of tumor vaccines, monoclonal antibodies, immune checkpoint inhibitors, and T cell therapies including the use of genetically engineered chimeric antigen receptor T cells (CAR-T cells) [5, 6]. Likewise, the push in current HIV therapy is to move towards finding either a ‘cure’ or a ‘functional cure’, where the goal is to have people currently living with HIV to acquire the means to clear the infection completely or to control the virus themselves immunologically without the need for continued ART. One concept highly touted is

the “Kick and Kill” approach, whereby latency reversal agents (LRAs) are used to kick-start transcription of integrated inactive/silent HIV DNA for subsequent recognition and elimination by immune effector cells such as HIV specific cytotoxic T cell lymphocytes (CTL) [7]. Currently, various latency reversal targets including HDAC inhibitors, TLR agonists, PKC activators, and cytokines have been tested for therapeutic applications, but have yet to show substantial clinical improvements. Moreover, some of the most effective LRA’s have demonstrated toxicities and/or negative impacts on CTL function [8]. Hence there is still a need to establish novel therapeutics with improved outcomes for treating chronic HIV infection.

A recent report by Kristoff et al brought forward a clever and promising strategy to utilize dendritic cells (DC) as a cellular immunotherapy tool drive both HIV latency reversal as well as the activation of CTL effectors capable of effectively targeting the infected CD4+ T cells exposed [9].

1.1 Dendritic Cells

1.1.1 Origin

The term ‘dendritic cells’ was coined by Dr.Ralph Steinmann and Zanvil Cohn in 1973, after the earlier discovery by pathologist Elie Metchnikoff of cells that had the ability to phagocytose invading pathogens. They were recognized for their integral part in both innate and adaptive immune responses and thus playing an vital role as a natural link between the innate and adaptive immune system [10, 11]. Due to their potent antigen presenting capabilities, DC and their various subsets are collectively referred to as ‘professional’ antigen-presenting cells (APC),

being the primary cell type with the ability to process and cross-present exogenous antigen in the context of both MHC-class I and II molecules to prime naïve T cells. Cross presentation is the ability of an APC to process and present an exogenous antigen via MHC class I to naïve CD8 T-cells mediated via either a cytosolic or vacuolar pathway [12]. Because of this, they have been widely utilized as an immunotherapeutic tool to treat chronic diseases including cancer and HIV [13, 14].

1.1.2 Distribution and Immunological function

Immature dendritic cells (iDC) reside at sites of entry for common infections like gut, skin, mucosal surfaces. iDC have high phagocytic activity but low MHC class I and II presenting capabilities. They efficiently process engulfed microbes and become activated by various microbial derivatives recognized by their highly specialized pattern recognition receptors (PRRs) that identify conserved regions of pathogen associated molecular patterns (PAMPs). During this stage of invasion, in addition to iDC being activated by pathways downstream of PRRs, they also respond to endogenous environmental signals, such as cytokines and inflammatory products from other immune cells responding to the pathogen induced assault. A result of these combined signaling factors ultimately leads to activation and maturation of DC, resulting in decreased phagocytic activity and enhanced phenotypic characteristics such as surface expression of MHC and co-stimulatory molecules such as CD80 and CD86 (Figure 1) and the lymph node homing chemokine receptor CCR7 [15, 16]. Mature DC then migrate to the draining lymph nodes to activate residing naïve and central memory CD8+ and CD4+ T cells by presenting antigen in the context of MHC-class I and MHC-class II molecules respectively (signal 1) along with the co-stimulatory molecules (signal 2) [17-21].

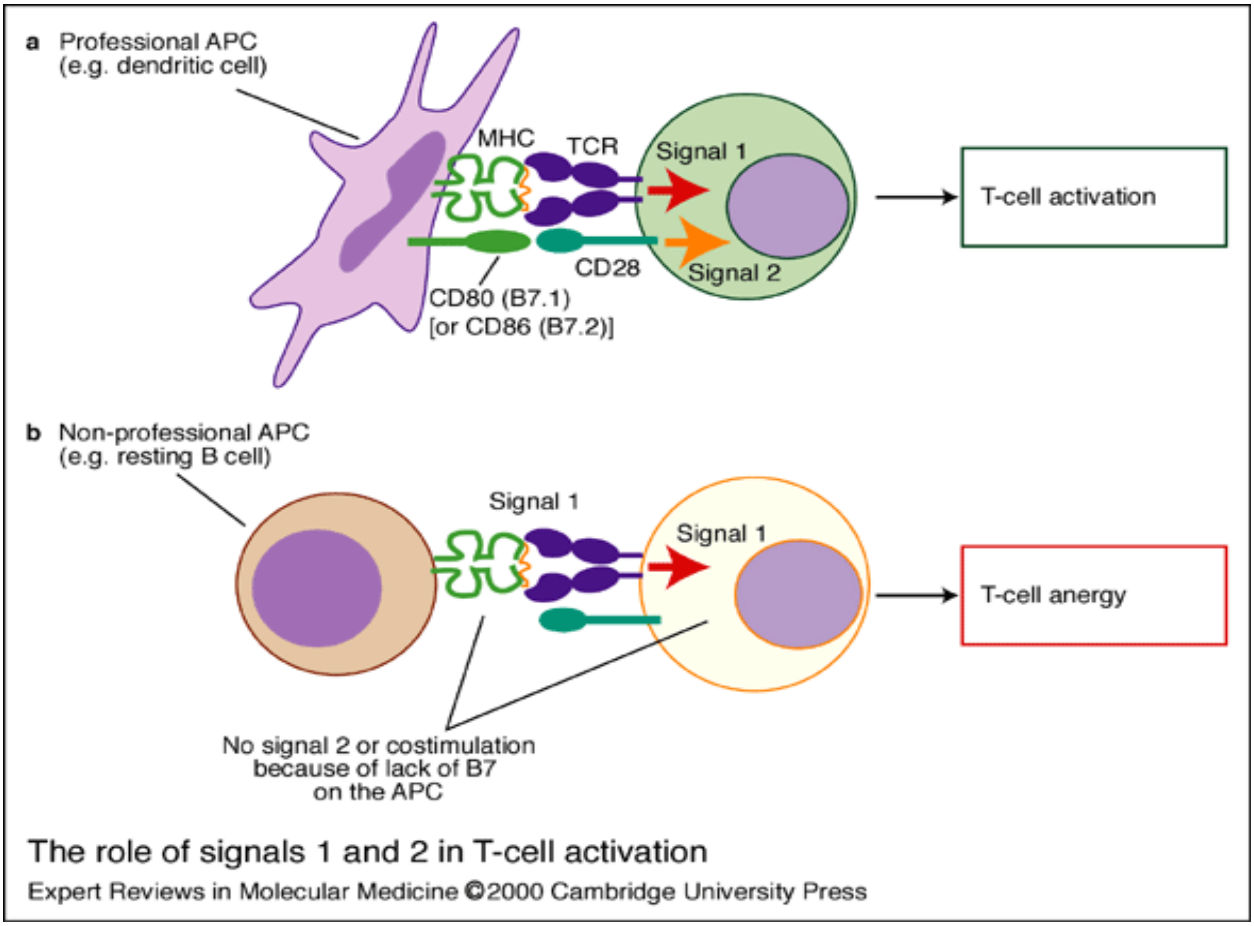

Figure 1. Role of mature DC in activating T-cells
(Horig, H., et al., Expert reviews in molecular medicine, 2000).
(A) In the presence of co-stimulatory molecules CD80 and CD86, DC activate T-cells via interactions with CD28 on T-cells thus activating them in the LN (B) Lack of co-stimulatory molecules, despite presence of signal1 via MHC-TCR interaction leads to anergy of CD4+ T cells [22].

1.1.3 Antigen presentation and priming of T cells

DC process the antigen and present them on MHC (I/II) depending on source of antigen. Exogenous peptides are typically presented via MHC class II, which aids in the activation of CD4+ T cells. Endogenous peptides are typically presented via MHC class I, which helps in triggering CD8+ T cells [23]. This MHC-associated presentation of peptide antigen to activate antigen specific T cell receptors is termed as ‘signal 1’. Simultaneously, the co-stimulatory molecules provided, which is referred to as ‘signal 2’, determines the magnitude and duration of

the T-cell response directed towards the peptide antigen presented by the DC. In the absence of co-stimulatory molecules, the T-cells enter a state of anergy or tolerance [24]. A few examples of this are CD80 (B7.1), CD86 (B7.2) and 4IBBL, which interact with various receptors on T cells. Further, an additional set of factors provided by DC, collectively known as 'signal 3', which include cytokines such as IL-12p70, direct the functional differentiation of T cells to greatly influence the nature and overall character of immune response mediated by the T cells to match the nature of the pathogen and affected tissues (Figure 2). Examples of this includes the differentiation of naïve CD4+ T cells into T helper (Th)1, Th2 and Th17 cells [25]. A few factors that determine the effector response of T cells include combinations of the type of pathogen, tissue derived factors, and type of TLRs activated in the DC [26]. T-cells are then directed by DC to carry out their effector activity in specific tissue sites of infection. This directed homing process is sometimes referred to as DC-mediated 'signal 4'. At this time, it is not entirely clear what combination of factors contribute to signal 4 or what exactly determines the outcome of this process. However, signals provided to T-cells by tissue specific DC greatly influence this process. For example, DC from Peyer's patch have been shown to upregulate $\alpha 4\beta 7$ integrins on CD8+ T cells which aid in homing towards the gut [27]. In a similar manner, DC activated in the skin increased P & E-Selectin on CD8+ T cells [28].

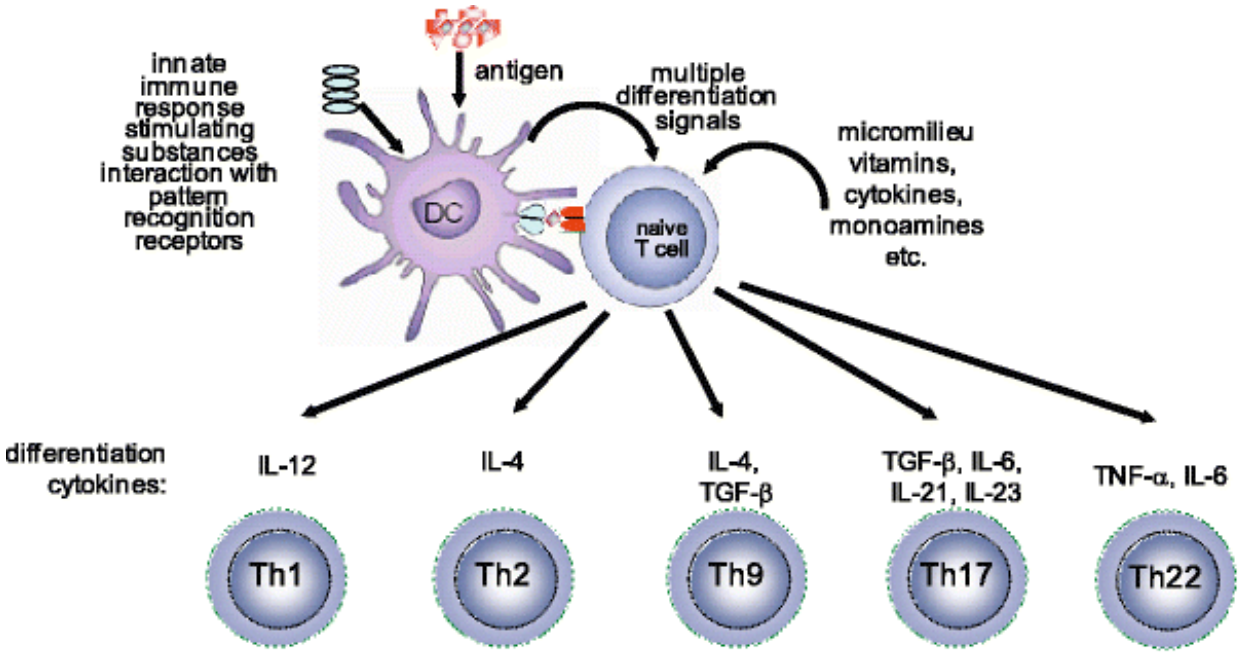

Figure 2. Signal 3 provided by DC skews the immune system
(Marek Jutel et al. Current allergy and asthma report, 2011)
Signal 3 is important to determine the polarization status of T-cells depending on the type of pathogen encountered and cytokines produced in accordance to direct the type of T-cell response [29].

1.1.4 Role of DC in mediating CD4+ T cell 'Help', and the role of CD40L

DC also play a special role in driving the cytotoxic effector functions of CD8+ T cells. Importantly, the capacity of DC to induce effector CTL differentiation and long-term survival is greatly impacted by the nature of their interaction with CD4+ T helper cell. During their antigen specific interaction with DC, CD4+ T cells provide important 'helper' signals to the DC, which hyper-activates mature DC, causing further upregulation in their expression of co-stimulatory molecules and release of cytokines that influences CTL generation. An important factor mediating this enhanced CTL inducing capacity in DC is the CD4 'T-helper' signal CD40L [30-32]. CD40L is a transmembrane glycoprotein that is part of the TNF-superfamily. CD40L expression is induced on the Th cells when they are provided the DC-mediated signals 1 and 2.

CD40L interacts with CD40 expressed on the antigen presenting DC, mediating DC upregulation of surface expression of MHC, costimulatory molecules CD80/CD86, and the production of IL12p70 [33]. This aids in the licensing of DC to promote CTL response, subsequently increasing its proliferative capabilities. Lack of CD40-CD40L signal, reduces the property of DC to induce secondary expansion of CTLs, thus indicating a role of CD40L in the help for long-term CTL survival [30, 31, 34].

Importantly, DC can have very different responses to CD40L depending on the environmental factors they encounter during maturation. DC matured in the presence of PGE-2 and LPS, while highly stimulatory have been shown to have diminished IL-12p70 producing capabilities, but demonstrate enhanced IL-12p40 production [35]. IL-12-p70 is an important regulator of Th-1 mediated responses [36], while IL-12p40 acts as an inhibitor for the biologically active IL-12p70 [37]. The capacity for mature DC to produce IL-12p70 in response to CD40L is influenced heavily by the presence of the cytokine IFN-γ, which can occur either during the environmental instruction or programming phases of DC maturation followed by subsequent interaction with CD40L expression CD4+ Th cells, or when simultaneously present during the moment when mature DC are actively interacting with the CD4+ Th cells in the lymph node. The source of IFN-γ can come from effector cell types including CD8+ T cells, NK cells, or Th1 cells. Importantly, CD40L is most uniquely associated with CD4+ T cells, expressed on the surface following their antigen specific activation [38]. This CD40L- and IFNγ-mediated IL-12p70 together helps to drive primary type-1 responses in responding naïve T-cells [39]. On the other hand, the presence PGE-2, a mediator of chronic inflammation, facilitates DC driven type-2 response [37]. As eluded to earlier, the combination of environmental signals received by the DC during maturation greatly determines the nature of their responsiveness to

subsequent CD40L signaling they may receive during their antigen specific interaction with T cells in the lymph node, and they may be pre-programmed or polarized to bias the ensuing adaptive T cell responses towards a particular immune character (i.e. Th1, Th2, Th9, Th17, Th22, T reg).

More recent studies from our group have shown other interesting effects of CD40L stimulation of mature DC that also impacts the outcome of the adaptive immune response. For example, PD-L1 is expressed moderately on mature DC, but is highly upregulated following their activation with CD40L [40]. PD-L1 is well known as an important immune checkpoint ligand that binds to PD-1 on activated PD-1 expressing CTLs, which results in the suppression of their effector response by inhibiting the action of PI3K [40, 41]. Interestingly, the function of PD-L1 expressed on DC appears to be context dependent, as it supports naïve T cell differentiation into their effector cell phenotype while it inhibits late stage effector T cell function [40]. Another study from our group defined a novel immunologic process referred to as DC 'reticulation', which highlights intriguing differences in the nature of the responsiveness to CD40L expressing Th cells by differentially matured or polarized DC [42]. Type-1 polarized dendritic cells (DC1) in particular respond to CD40L to dramatically undergo this reticulation process, in which they form extensive networks of tunneling nanotubes (TNTs). These TNTs were shown to facilitate intercellular transfer of content, including antigen, between DC [42]. While highly speculative, this may help to explain how migratory DC deliver peripheral antigen to resident DC in the lymph node [43, 44]. Although it is widely accepted that CD40L plays a critical role in DC mediated CTL responses, there are aspects of this interaction that are not clearly elucidated and remain unexplored.

1.2 Role of DC in Immunotherapy

DC have been used as a therapeutic option owing to their ability to elicit strong primary and memory T cell responses. In the setting of cancer, there have been many clinical trials utilizing DC as a cellular vaccine tool [45]. The most common DC type used in early clinical trials have been generated from monocyte precursors that are cultured in the presence of IL-4 and GM-CSF and differentiated into iDC ex-vivo, and then exposed to a cocktail maturation factors including TNFa, IL-1b, IL-6 and PGE2 to generate mature DC [46, 47]. This method yields a DC type that is highly stimulatory, however it was found to be defective in its capacity to produce IL-12p70 in response to CD40L, and has been shown to drive Th2 [47] and T-reg responses preferentially. Other protocols for DC generation have since been created to improve on this point. One clinically applicable DC generation protocol was created to generate a specialized DC1 cellular vaccine tool, referred to as alpha-DC1 (αDC1) [48]. This platform was developed for the specific purpose of generating monocyte derived mature DC that had a superior capacity to produce IL-12p70 and to induce strong CTL responses against cancer antigens, and utilized a combination of type-1 and type-2 interferons and the TLR3 agonist poly-IC to mature the DC. When loaded with tumor antigen, αDC1 were found to drive increased IFN-γ producing CTLs compared to the standard PGE2 matured DC [48]. The increase in IFN-γ is attributed to elevated T-bet expression in the CTLs. During clinical trials, alpha DC1 were shown to re-polarize Th2 skewed CD4+ T cells toward acquiring a Th1 functional status, while simultaneously increasing CTL responses toward particular epitopes in melanoma patients and breast cancer patients [49, 50]. The same pattern was shown in Chronic lymphocytic leukemia (CLL) patient derived αDC1 showing the CTL induction against CLL [51]. One impressive

clinical trial for glioblastoma showed clinical responses of a delay of recurrence of tumor and enhanced longevity with use of αDC1 [52]. The positive responses were associated with the IL-12p70 producing capacity of the αDC1 generated from study participants, and these patients recorded upregulation of mRNA for chemokine CXCL10 and IFN-α, of which CXCL10 was found to home T-cell response towards the brain [52]. Another study showed that delivery of murine DC (DC1-like) engineered to overexpress T-bet promoted superior cytotoxic responses by CTLs, while simultaneously decreasing the level of T-regulatory cells present [53].

In the setting of HIV, there have been a number of clinical trials using autologous DC as a therapeutic vaccine, including the use of antigen in the form of HIV-derived peptides, inactivated HIV, and autologous apoptotic HIV-infected CD4+ T cells [54-56]. Unfortunately, the results of most of these studies have been underwhelming, with limited enhancement of HIV-specific CTL responses, sometimes accompanied with enhancement of T reg activity [57]. But, there have been a few trials in particular that did yield somewhat impressive results though, where treatment with autologous DC loaded with inactivated HIV was found to significantly decrease residual viremia associated with induced CTL responses [58] and increase in the percentage of activated CD4+ T cells [59]. Albeit these studies used MDDCs generated via conventional cocktails involving PGE-2, which produce low levels of IL12-p70 production. More recently, a DC-based clinical trial using DC generated in the presence of IFN-α showed promise, where the authors reported inverse correlation between breadth of IL-13 producing CD4 T-cells and HIV levels and active cytokine production and increased % of polyfunctional CD8+ T cells restricted to specific HIV epitopes post cART interruption 16 weeks after vaccination [60].

A major hurdle in therapy is the latency of HIV in CD4+ T cells. Interestingly, administration of DC pulsed with autologous HIV, meant to enhance CTL responses, instead showed an increase in viremia during ART [61, 62], indicating the ability of DC to 'Kick' HIV from its CD4 reservoir. Related to this notion, recently published data from our lab has displayed the ability of αDC1 (referred to in the paper as monocyte derived DC1 or MDC1) presenting CMV or HIV antigen could specifically reverse latency, and that this effect involved the CD40L/CD40 signaling pathway [9]. This study suggested that a substantial portion of the HIV reservoir may be contained within the CMV antigen specific T cell fraction of the CD4+ T cells. This study also suggested that utilization of heterologous CMV antigen may help to promote CD4+ T cell 'help' to the DC to license CTL inducing capacity while providing specific antigen to drive the LRA effect. Therefore, it is proposed that if programmed properly, DC can be used as an all-in-one cellular therapeutic "Kick and Kill" tool [9] (Fig 3). Again, the bidirectional talk between DC and CD4+ T helper cells and CD40L signaling was found to play a major role in this LR activity. However, the downstream mechanisms involved in the CD40L-mediated LRA effect have yet to be elucidated.

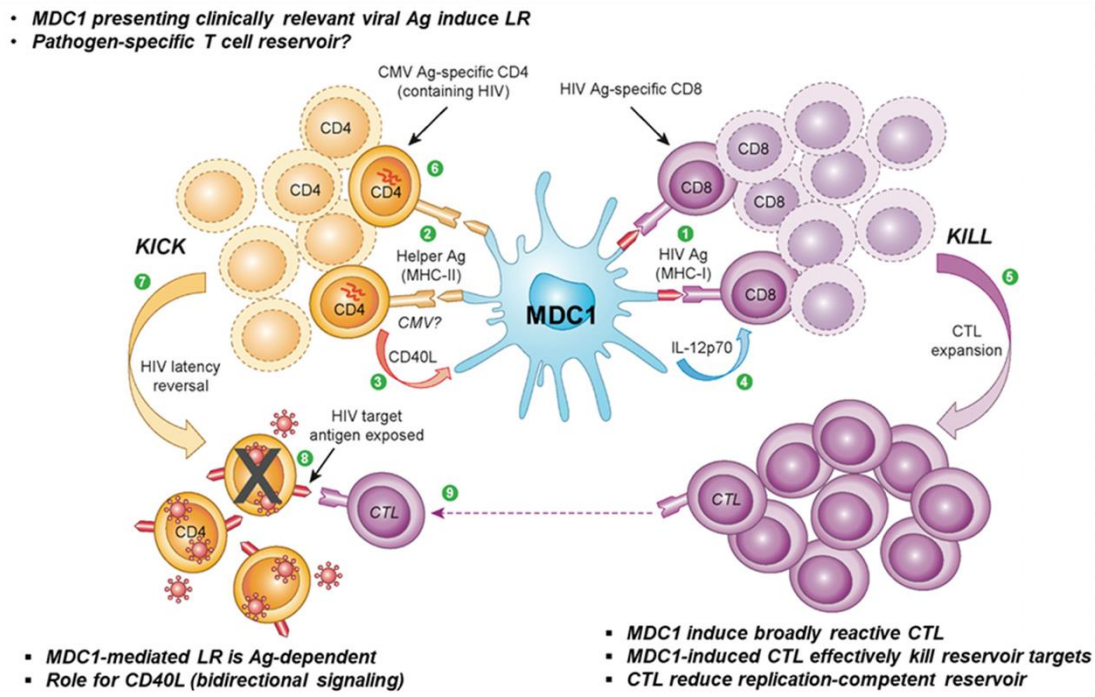

Figure 3. Alpha DC1 driven "Kick and Kill" of HIV
(Jan Kristoff et al. viruses, 2019)
Alpha DC1 induces the antigen-specific CD4+ and CD8+ T cells responses by targeting CMV-specific CD4+ T cells and resulting in latency reversal of HIV and simultaneously signaling via CD40-CD40L interaction to release IL12-p70 to drive the action of cytotoxic CTLs to kill these infected cells [63].

Allan et al. have displayed the ability of migratory DC to transfer antigen to the lymph node and this is needed by the resident DC to induce cellular immunity by interacting with CD8+ T cells, while migratory DC interact with CD4+ T cells [43]. Later, it was shown by these CD8α- migratory DC were more in number in the LN and better at inducing Ifn-gamma production from CD8+ T cells, than CD8α+ resident DC [44]. These suggest an interaction between the various DC subtypes and T-cells, while the nature of the interaction remains to be explored. Recently, Zaccard et al identified a novel process deemed DC reticulation in DC treated with CD4 T helper signal CD40L, which interacts with CD40 on the surface of DC. It was shown that these reticulation aka tunneling nanotubes (TNTs) were shown to enhance transfer of bacteria and other cytoplasmic content between DC [42]. Related findings from the Storkus lab at the

University of Pittsburgh has shown *in-vivo* using murine models, the transfer of T-bet from T-bet overexpressing DC to CD8+ T cells, and their preliminary data suggest a potential role for exosome delivery of this factor [53].

These data, along with previously mentioned interaction between various DC subtypes, gives rise to unknown features of intercellular communication. A driving question is if there is a dependence on CD40L for this communication, and does the polarization status of the DC influence this process, as it does for the TNT-based intercellular transfer [42]. Other unpublished data from our lab indicates the ability for DC to facilitate HIV to *trans*-infection is enhanced DC1 than standard DC types matured in the absence of type-1 polarizing factors, in a manner dependent on subsequent CD40L signaling. The downstream mechanisms involved in this CD40L-mediated *trans*-infection process, whether mediated through TNTs, or extracellular vesicles, or some other processes, have yet to be determined.

1.3 Extracellular Vesicles

1.3.1 Origin

Extracellular vesicles (EVs) were initially discovered in 1946. They were originally postulated to be waste particulate secreted by cells. Later, it was observed in multiple instances that this serves as a means of communication between cells, and that they are produced by most cell types [64-66]. Their content can be highly varied and can change their make up with subtle variations induced in the parent cell of origin. EV recipient cells can be influenced in a wide variety of ways by such EVs depending on cell of origin and context of their release and uptake.

EV-mediated signaling can occur via protein signaling pathways [67]. They have also been noted to play a variety of functions in diseased states such as cancer, dengue, HIV and Tuberculosis.

1.3.2 Classification and Biogenesis

EVs are further classified into 3 subgroups namely (a) Apoptotic bodies (b) Ectosomes and (c) Exosomes. Apoptotic bodies are larger in size, ranging from 800-5000 nm, originating from the blebbing of the plasma membrane of an apoptotic cell. Ectosomes are smaller (50-3000 nm), originating directly from the fusion of the endosome with plasma membrane, while exosomes are the smallest (50-150 nm), arising from invagination of late endosome and being released via exocytosis [68, 69]. The biogenesis of EVs depend on a multitude of factors. Initial research has shown that formation of exosomes is mediated by endosomal sorting complexes required for transport machinery [ESCRT] proteins. This has 4 components ESCRT 0, ESCRT 1, ESCRT 2 and ESCRT 3. ESCRT 1 and 2 exert control over the production of membrane buds, while ESCRT 3 plays a major role in removing the attachments to endosomal complex and aids in generation of intraluminal vesicles (ILV) [70, 71]. There are also ESCRT independent exosome production, via cholesterol/lipid raft and tetraspanin involved pathways [72]. EVs have high cholesterol/ lipid content on their surface, more than that contained on the inner side of the parent cells from which they are derived. Other common molecules observed on the surface of exosomes are CD63, CD81, CD9, MHC molecules, and cell-adhesion molecules [73]. Although, recent data has shown the origin of exosomes from endocytic compartments to sometimes lack these markers, and therefore has led to more refined definitions to the term “exosomes” [74, 75].

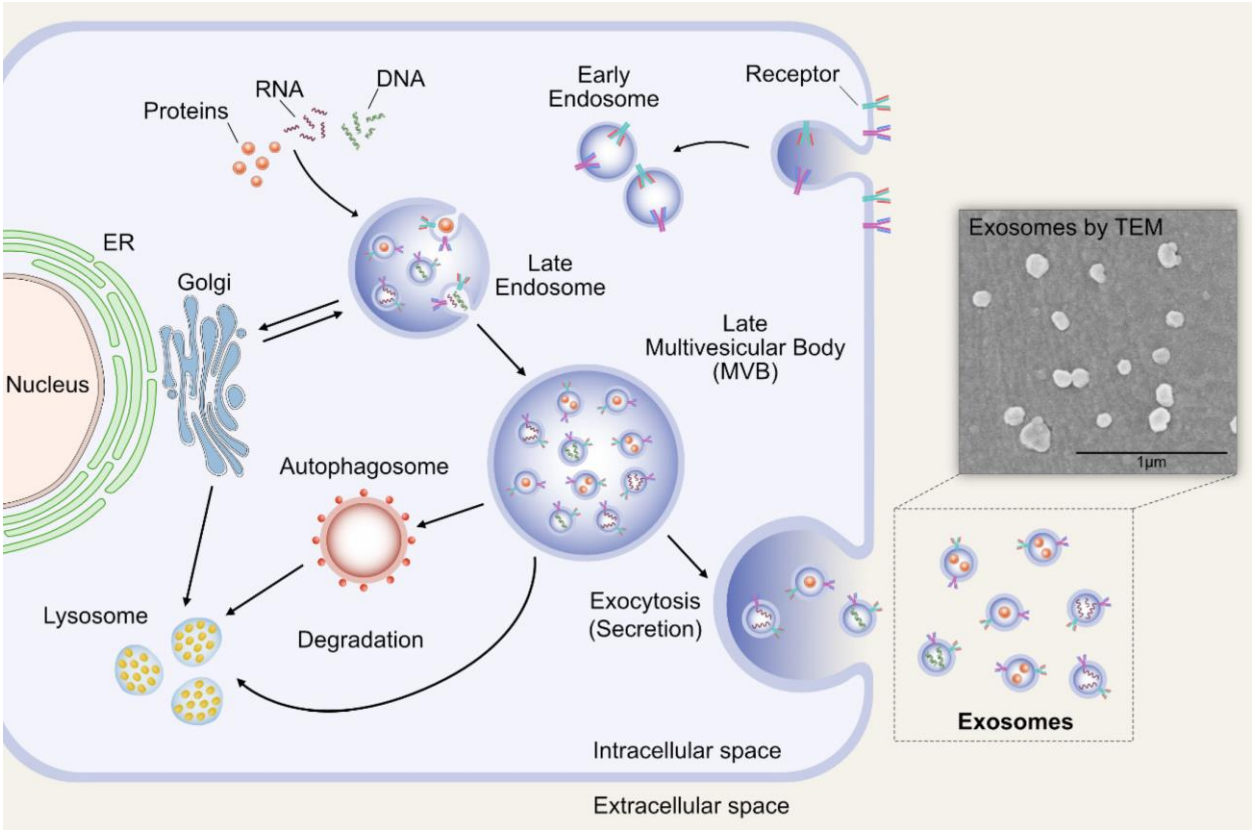

Figure 4. Biogenesis of Extracellular vesicles
(Sangiliyandi Gurunathan et al. cells, 2019)
Late endosomes arising from the golgi invaginate to form Multi-vesicular bodies, which may/may not fuse with the plasma membrane to be released into the extracellular plasma as exosomes [76].

1.3.3 Uptake and influence in immunological function

Despite a lot of research on the intercellular transfer and uptake of EVs, there has been no conclusive element determined to be responsible for cell uptake. Previous research has varied due to many factors including the type of donor and recipient cells being studied, the state of these cells, and the heterogeneity of the EVs themselves based on size, origin and surface markers expression. Early studies involving murine DC indicated heavy dependence on CD81 for uptake into DC [77]. Furthermore, the same group also displayed multiple methods of intake

including micropinocytosis for small EVs, phagocytosis and fusion/hemifusion of membranes [78, 79]. The uptake of EVs can be highly dependent (specific) on the interaction with receptors on the surface of recipient cell or non-specific via phagocytosis. Multiple roles of EVs have been noted, regarding their ability to modulate various immune functions. B-cell derived EVs having enhanced surface expression of MHC II and co-stimulatory molecules, have been demonstrated with the ability to directly activate T cells [80]. EVs also carry lipids, proteins, as well as RNA (mRNA, miRNA, tRNA), all of which can influence various functions of the recipient cells such as cell signaling and blocking of mRNA translation via miRNA delivery to target cells [81]. EVs derived from LPS-activated DC loaded with antigen were shown to enhance CD4+ T-cell proliferation specific to the antigen of interest, showing a novel role of antigen loaded exosomes in priming and polarization of type-1 immune responses, both *in-vitro* and *in-vivo* [82]. Further, a similar interaction was shown to be enhanced when EVs were internalized by iDC [83]. Recently, DC-derived EVs were shown to activate memory CD4+ T cells, but the type and extent of response obtained depended on size of EVs [84]. In other reports, Antigen-pulsed DC exosomes were as efficient as APC in inducing antibody responses in B-cells and CTL responses *in-vivo* [85, 86], although the type of response was dependent on DC-subtype. However, these studies lacked proof of direct interaction of DC-derived exosomes with these immune cells, thus it can be speculated that the effect may have involved the bystander activation of resident DC. Nevertheless, these studies suggest that DC-based EVs can influence the response of B cells and T-cells in an APC-independent manner.

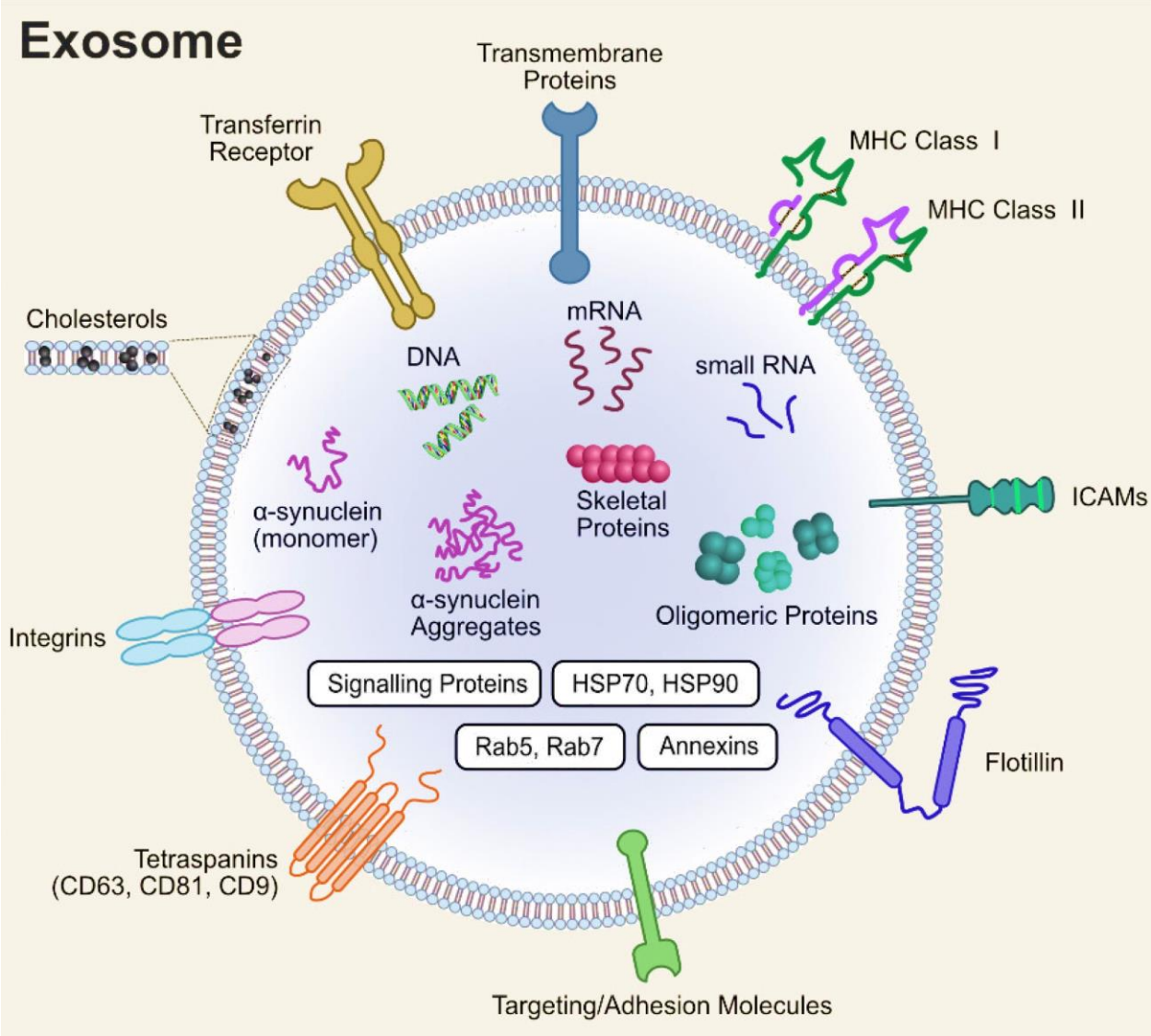

Figure 5. Structure and content of exosomes
(Sangiliyandi Gurunathan et al., cells, 2019)
Exosomes contain various molecules which are parent cell specific, obtained from either the surface (Integrins binding domains, tetraspanins) or from the cytosol including proteins and mRNA. Bio-molecules such as miRNA can be packed which are exosome specific but not found in the parent cell [76].

1.3.4 Role in cancer/cancer therapy

EVs are major role players in the setting of cancer. Since EVs are similar in protein composition as their parent cell, tumor specific EVs have increased levels of tumor antigen on their MHC. Exosomes from cancer environments are being investigated in their role as

functional biomarkers to predict prognosis, via surface markers and miRNA expression levels [87, 88]. Initially, this was thought to play a role in enhancing anti-tumor response. For example, as shown *in-vivo*, tumor-derived EVs from various patients were transferred in-*vitro* to DC, thus enhancing the differentiation and proliferation of antigen specific CD8+ T-cells efficiently and increasing IFN-γ levels [89]. Soon, it was seen that DC-derived EV pulsed with tumor antigens were more potent in inducing CD8 T-cell responses, thus shifting research focus to this area [90]. In 2012, a report published by Stephen et al., displayed the ability of DC-derived exosomes attached to dynabeads to kill tumor cells via TNF-related pathways and increase NK-cell anti-tumor activity [91]. Anti-tumor responses were further enhanced in EVs displaying CD40L obtained from modified tumor cells, inducing increased CD80 and CD86 in DC, along with superior IFN-γ and IL-2 expression from splenocytes [92]. Despite the anti-tumor effects of tumor-derived EVs, it was noted that these EVs also had immune suppression activity and/or aided the spread of cancer in other EV-dependent means. The pro-tumor effect of EVs has been demonstrated in Melanoma based vesicles, carrying FAS-L on their surface, which displayed its ability *in-vitro* to induce apoptosis in Fas sensible T-cells [93]. Between 2008 and 2011, in independent studies, it was reported that cancer-based EVs enhanced angiogenesis and promoted proliferation of endothelial cells via mRNA and other tissue factors packaged in EVs [94, 95]. Further studies revealed the presence of factors on cancer-derived EVs, which activate plasminogen and exacerbate tumor metastasis [96]. All evidence suggests a potential role of EVs in the setting of cancer, thus warranting further studies.

1.3.5 Role in infectious diseases

One of the earliest evidences of EV's role in infection was observed with both BCG and M. *Tuberculosis* infected macrophages, shedding vesicles with various lipid moieties on their surface [97]. In the context of dendritic cells, these cells are the primary source for detection of infection. Intense research on effect of DC-infected EVs has been explored in several diseases such as Hepatitis C virus (HCV), dengue and HIV. All though not directly infecting DC, HCV-infected cells were shown by dreux et al., to have proviral effects by transferring HCV RNA to pDC via exosomes, implicated to reduce IFN-α production during failed cell-cell contact [98]. Infection of DC with various dengue strains revealed differential expression of miRNAs in EVs between high and low virulent strains and associated differences in signaling pathways such as WNT, PI3K/AKT and MAPK [99]. Infection of iDC with HIV-1 particles revealed the association of EVs with HIV, in its role to *trans*-infect CD4+ T cells. Next this was shown to be enhanced with mature DC (mDC), revealing a dose dependent uptake of EVs competing with HIV suggesting the role of a common mechanism of entry into the cells, dependent on lipid raft-like moieties. HIV infection of mDC is considered a factor associated with increased trans-infection while parallel studies indicated a considerable interaction between CD81 and HIV for redistribution to DC-T cell contact zones [100, 101]. In 2001, Juan Martin-serrano et al., have shown the high dependence of HIV on TSG101 for its egress from infected cell [102], which is considered a prominent marker of EVs [74].

1.3.6 Overarching goal of the project

Based on the background and scientific premise presented, I hypothesize that intercellular communication delivered by DC to other cells, including CD8+ T cells, is enhanced as a normal immunologic process during infection. Moreover, I hypothesize that polarized DC1, such as the described clinically relevant αDC1, are superior in this communication process as compared to PGE2-matured DC. This belief is due to not only because of the unique capacity of DC1 to rapidly form TNT networks, but also for their potential to release EVs in response to CD40L. For this thesis, the overarching goals were to 1) Test the ability of differentially polarized DC subtypes to transfer cellular content to CD8+ T cells via EV delivery and 2) Characterize the phenotype of DC-derived extracellular vesicles and their biologic impact on naïve and memory CD8+ antigen specific T cells.

In addressing these specific aims, it could increase the knowledge of basic immunologic functions associated with DC- T-cell communication, which could aid in developing novel therapeutic strategies to enhance cytolytic T-cell function in the setting of chronic disease. Furthermore, information gained may be helpful in explaining how pathogens, such as HIV-1, may utilize or 'hijack' this intercellular communication process to facilitate or enhance the spread of infection.

2.0 Specific Aims

2.1 Aim 1: Determine the Role of CD40L in DC Capacity to Transfer Content to CD8+ T Cells

This aim focuses on developing a model to aid in visualizing the transfer of content from DC to CD8+ T cells and to determine its association with DC polarization status. We hypothesize DC1 will enhance transfer to CD8+ T cells induced by their capacity to form TNTs, DC2 will fail to amplify this transfer. We will test this hypothesis via the following sub-aims:

1. Characterize differentially polarized mature DC by analyzing morphology, surface marker expression, and their IL12-p70 producing ability following CD40L stimulation
2. Generate GFP-expressing human dendritic cells as a model for intercellular transfer
3. Determine impact of polarization status on capacity to transfer
4. Determine the role of TNTs vs extracellular vesicles (EVs) in intercellular transfer

2.2 Aim 2: Assess the Phenotypic and Immunomodulatory Characteristics of EVs Derived from Differentially Polarized DC

For this aim, we study EVs derived from differentially matured monocyte derived DCs, and we assess the impact of CD40L on the production and character of DC derived EVs. We also determine the biologic impact that these EVs have on the activation and survival of CD8+ T

cells. We hypothesize that EVs derived from CD40L treated DC have immunomodulatory properties that will impact the priming and survival of effector CD8+ T cells. This aim will be explored through the following Sub-Aims:

1. Measure quantity and size of EVs produced
2. Determine EV expression of immunomodulatory surface proteins for different DC treatment conditions
3. Characterize protein content of the DC derived EVs
4. Describe the biologic impact of DC derived EVs on induction of primary and memory T cell responses

3.0 Materials and Methods

3.1 Isolation of Primary Cells from Blood

PBMC were obtained from buffy coat or whole blood products from healthy donors obtained from the Central Blood Bank of Pittsburgh. For the HIV related studies, blood was obtained from men who are participants in the Multicenter AIDS cohort study (MACS), Pittsburgh clinical site [103]. The blood samples were isolated by density gradient separation method (Corning Cat# 25–072-CV). These were further separated into monocytes and peripheral blood lymphocytes (PBLs), using immunomagnetic negative selection method (EasySep: STEMCELL Technologies Inc., Vancouver, BC, Canada)

3.2 Generation of Human DC from Monocytes

To generate iDC, monocytes isolated from PBMCs via CD14 magnetic bead positive selection (Miltenyi) were cultured for 5 days in IMDM (Gibco, Life Technologies, Grand Island, NY) containing 10%FBS, or AIM-V (Gibco, Life Technologies, Grand Island, NY) in the presence of GM-CSF and IL-4 (both 1000 IU/ml; R&D Systems, Minneapolis, MN). On day 5, iDC are exposed to a cocktail of maturation factors for 48 h. For generation of αDC1 (DC1), the maturation factors consist of TNF-α (50 ng/mL), IL-1β (25 ng/mL), Ifn-α (3000 units/mL), Ifn-γ (1000 units/mL) and polyisosinic: polycytidilic acid (Poly I:C) (20 ug/mL) [48]. DC2 were generated using a modified cocktail, consisting of IL-6 (1000 units/mL), PGE2 (10^{-6} mol/L), IL-

1β and TNF-α. DC-GFP were generated by infecting iDC at day 4, with an adenoviral-GFP vector [104] (a gift from Dr. Walter Storkus, University of Pittsburgh), at MOI of 25 for 24 hours, followed by the addition of the relevant maturation cocktail used.

3.3 Activation of Mature DC with CD40L

Differentially matured DC were stimulated for 24 hours with rhCD40L (0.5 μg/ml; MegaCD40L, Enzo Life Sciences). Supernatants were collected for IL-12p70 measurements and EV isolation and characterization.

3.4 Extracellular Vesicle Isolation

Supernatant was collected by centrifuging at 2000g for 5 mins from the wells containing mature DC stimulated after 48 h stimulation rhCD40L (0.5 ug/mL). Supernatant were further run through qEV (35-350 nm) columns to obtain purified extracellular vesicles bodies [105].

3.5 Characterization of Extracellular Vesicles

Isolated EVs were viewed and characterized by Nanoparticle Track Analysis (NTA) as previously described [106]. In short, approximately 0.5mL of sample was loaded into sample chamber and 3 videos of 60 seconds were recorded. Using Stokes-Einstein equation,

hydrodynamic scattering and diffusion coefficient were obtained and results are displayed as particle size distribution and concentration by analyses via Nanosight software.

3.6 Flowcytometric Analysis

Cell surface and intracellular staining of CD8+ and CD4+ T cells was carried out as previously described [107]. The stains used are as follows: Mouse Anti-human CD3-BV711 (BD Biosciences), CD4- Percyp-Cy 5.5 (BD Biosciences), CD8- Pe Cy7 (Biolegend), CD27- BV421 (BD Biosciences), CD45RA- BV605 (BD Biosciences), CCR7- FITC (ebioscience), PD1- PE cy7 (BD Biosciences), CD107- FITC (BD Biosciences), INF-γ- Alex 700 (BD Biosciences), CD107- FITC (BD Biosciences), IL2- APC (BD), Mip-1β, TNF-α- PE (BD Biosciences). During experiment and prior to analysis of T-cell responses, stimulation of cells was performed with anti CD3/CD28 dynabeads (Gibco, Life Technologies), to mimic antigen cognate interaction with DC. Data acquisition was performed using the BD Biosciences LSR Fortessa Cell Analyzer, and data was analyzed using the Flow Jo software, version 10.6.1

3.7 SDS-Page Analysis

DC-derived EV content was characterized by SDS-PAGE. Briefly, extracellular vesicles were lysed with 80-100 uL of lysis/RIPA buffer at and at 4°C for 15 seconds. After centrifuging at 14,000 × *g* for 5 minutes, supernatant was further concentrated and centrifuged at 14,000 × *g* for 5 minutes and supernatant was mixed with 5/1 SDS-running buffer and proteins were

separated on 4% SDS-PAGE pre-cast gels. Total protein content was detected with Coomassie blue staining.

3.8 DC T-Cell Coculture

Differentially matured DC were co-cultured with CD4+/CD8+ T cells or both together at a ratio of 1:7 in the presence of SEB (1μg/ml). These cultures were grown together for 5-7 days prior to testing with flowcytometry/ ELISPOT. The co-culture was maintained with the addition of IL2/IL-7. CD8+ T cells were also challenged with extracellular vesicles at 1:20 ratio for 10-12 days prior to flowcytometric testing, to help functionally characterize effect of extracellular vesicles on CD8+ T cells.

3.9 IL12-P70/IFN-γ ELISA

Supernatant was collected after 24 h stimulation of DC with rhCD40L by centrifuging at *2000*× g for 5 minutes and tested for IL-12p70 and IFN-γ expression via an IL-12p70 ELISA to functionally characterize the DC.

3.10 IFN-Gamma ELISpot

ELISpot assay was implemented to determine IFN-γ production in PBMCs of HIV+ donors treated with differentially polarized dendritic cell-derived extracellular vesicles (EV) pre and post CD40L treatment. The assay was performed as previously described in [108] by stimulating PBMCs with CEF-peptide (10 ug/mL) and Gag 9mer, TLNAWVKVV (5 mg/mL). PBMCs were treated with EVs in ratio 1:20. The assay had a positive control of PBMC stimulated with anti-CD3/28 dynabeads and negative controls of PBMCs with no peptide stimulation.

3.11 Recombinant Adenovector

Adenovirus vector was generated using Cre-Lox recombination based on previously described methods [104]. Briefly, the EGFP cDNA was attached to a cytomegalovirus early promoter, while simultaneously E1-E3 substituted by cotransduction of EGFP-N1 into packaging cell line, CRE8 to be further propagated and purified by density gradient centrifugation. All adenoviral vector preparations were handled by Walter storkus's group and provided to our lab as a gift.

3.12 Microscopy

Imaging studies were performed using various imaging techniques. GFP-multivesicular bodies localization in CD8+ T cells were conducted via image stream analysis using Amnis flow cytometer and analyzed using the IDEAS software. Morphological images of differentially matured DC were obtained using Leica bright field microscope.

3.13 In-Well Western Blot

Samples are fixed and incubated for 15-20 mins. Post incubation, they were spun for 30 mins at 4^{o}C at 13,200 rpm., and 0.1% Triton X and 3% BSA were added and the samples were gently resuspended and incubated for 1 hr at room temperature. This was followed by a 30 mins 13,200 rpm centrifugation at 4^{o}C. Supernatants were carefully discarded and blocking buffer was added with appropriate secondary antibody-based serum. Unconjugated primary antibody, either CD81, PD-L1 or CD86 (all purchased from Santa Cruz Biotechnology) was added and incubated overnight at 4C. The next day, samples were spun for 30 mins at 4^{o}C at 13,200 RPM, and pellet resuspended in Triton X. The secondary antibody in (1:500) dilution was added and incubated at room temperate for 1 hr, spun at 20,000 g and analyzed using LICOR Odyssey Imager.

3.14 Trans-Well Assay

GFP. αDC1/DC2 ($3x10^5$) were plated in the upper chamber of a 24 well trans-well system in 400uL (0.4µm PTFE membrane collagen coated, Costar) along with T-cells (+/- DC) in the bottom chamber in a total volume of 1ml IMDM+ 10% FBS (Gibco, Life Technologies, Grand Island, NY). Where mentioned, the cultures also contained SEB (1ng/ml) and CD40L (0.5 µg/ml; MegaCD40L, Enzo Life Sciences). GFP.DC and T-cells were incubated at a ratio of 1:3 as previously described [84]. The cells at the bottom were harvested 48 hrs post incubation and were analyzed for GFP+ content in CD8+ T cells via flowcytometry.

4.0 Results

4.1 Aim 1: To Determine Role of CD40L in DC Capacity to Transfer Content to CD8+ T-Cells

4.1.1 Phenotypic characterization of differentially polarized DC

Differentially polarized DC were generated using previously described methods, with a cocktail of cytokines involving either poly(I:C), TNF-α, IL-1β, IFN-α, and IFN-γ for DC1(αDC1) [48], or IL-1β, TNF-α, IL-6 and PGE2 for DC2 [109]. DC were analyzed for prominent surface protein markers by flow cytometry. The gating strategy is depicted in figure 6A. The alpha DC1 were characterized by their high CD86, moderate CD83 expression and low levels of OX40L (figure 6B), while DC2 express all of these markers at high levels (Figure 6C).

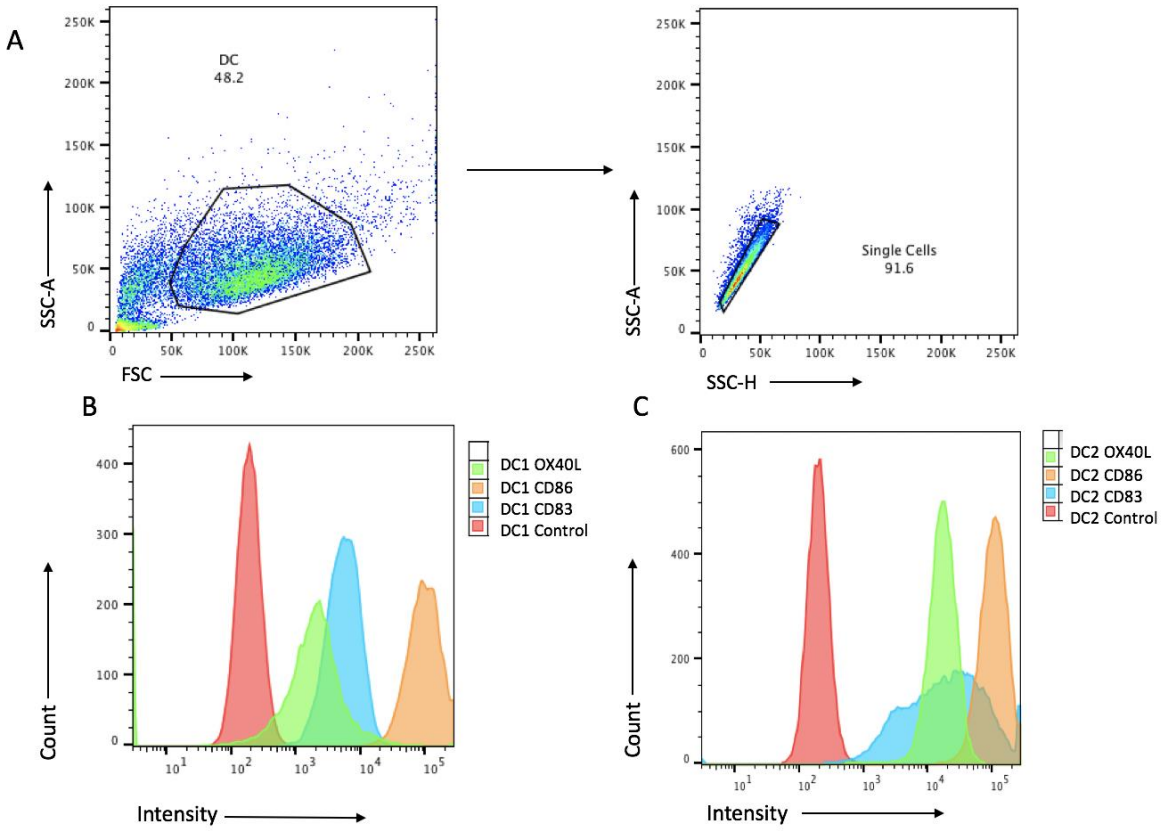

Figure 6. Mature DC have high expression of co-stimulatory molecules
(A) Representative gating strategy applied for data analysis of day 6 DC-culture, depicting the DC gating (Left panel) and single cell DC (Right panel) (B) Surface markers expression analysis of OX-40L, CD86 and CD83 on Alpha DC1 compared to control DC1 unstained. (B) Surface markers expression analysis of OX-40L, CD86 and CD83 on DC1 compared to control DC2 unstained.

4.1.2 Morphologic characterization of differentially polarized DC

Differentially polarized DC were also characterized based on morphological changes in response to cytokines involved in maturation and their ensuing response to CD4+ T-helper signal CD40L assessed by bright field microscopy. On day 6 of culture, DC1 were semi-adherent and formed elongated clusters at certain sites of adherence (Figure 7A), while DC2 were rounded and

formed less number of clusters (Figure 7B). Furthermore, on stimulating with CD40L, as previously shown, DC1 displayed high levels of extensive tunneling nanotube formation (figure 7C), in comparison to DC2 (figure 7D).

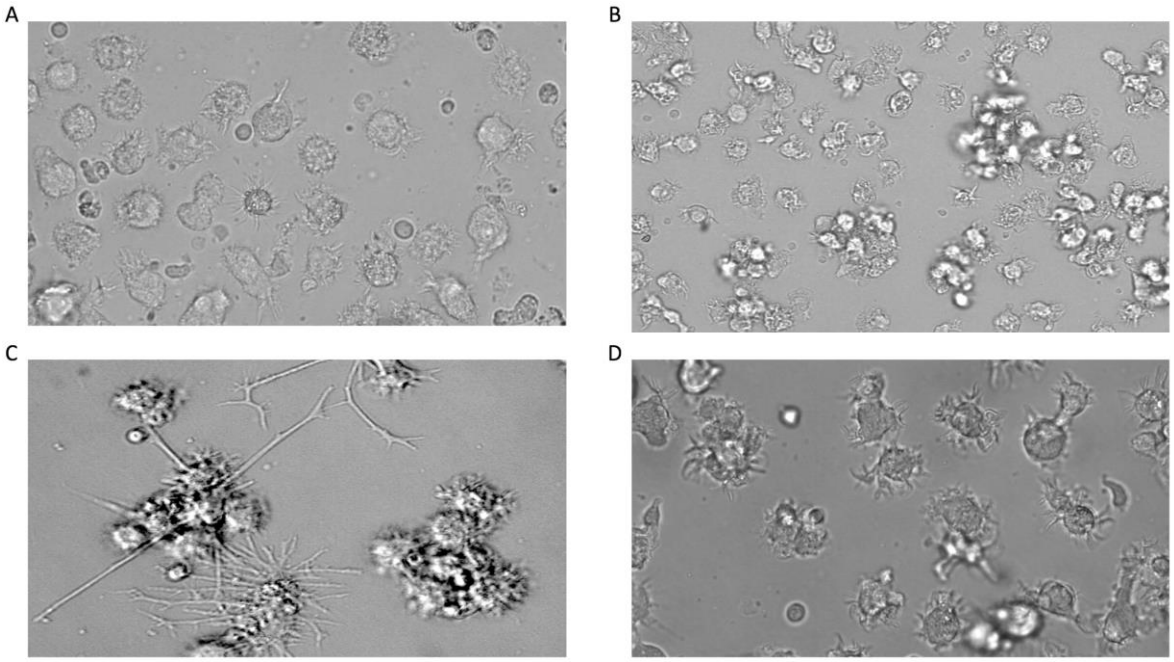

Figure 7. CD4 T-helper signal CD40L induces reticulation via tunneling nanotubes that increases surface area
(A) and (B) show respective morphology of αDC1 and DC2 prior to CD40L stimulation. (C) Shows the unique ability of αDC1 to form tunneling nanotubes (TNTs) in response to CD40L (D) Shows the less efficient ability of DC2 to form TNTs in response to CD40L. The TNTs are denoted by green arrows.

4.1.3 Functional characterization of differentially polarized DC

DC were tested for the ability to produce IL12-p70 in response to CD40L. As expected, DC1 produced higher levels of IL12p70 than DC2 post CD40L exposure (Figure 8A). This was in correlation with previously developed microarray data, which on further analysis revealed an increase in IL12 mRNA in DC1 in response to CD40L (figure 8B), but no relative change in DC2 (data not shown)

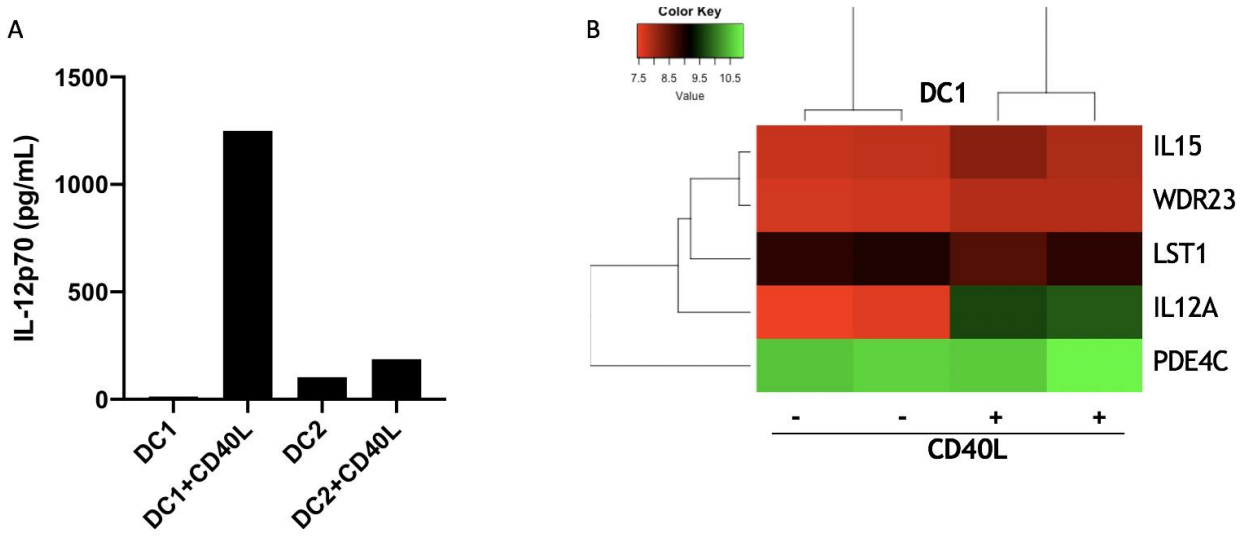

Figure 8. IL12-p70 production of DC1 and DC2
(A) ELISA shows the higher production of IL12-p70 by alpha DC1+CD40L compared to DC2+CD40L, while both have diminished IL12 producing capabilities prior to CD40L exposure. (B) Heatmap from microarray data, showing the increased levels of IL12 mRNA after CD40L stimulation in DC1 from 2 different donors ($p<0.005$).

4.1.4 Generation of GFP expressing differentially polarized dendritic cells as a model to track intercellular transfer

We developed a model to track the transfer of cellular content from DC to other cells, using GFP content as marker to determine transfer. We did this by engineering DC to express GFP, by transducing DC with a replication incompetent GFP-tagged adenoviral vector and adding different maturation factors for 2 days. The DC were inspected for GFP expression via flow cytometry. Initial analysis revealed that both cell types were transduced with GFP, albeit DC1 had lower GFP transduction efficiency than DC2 (Figure 9A, 9B). This led us to hypothesize that the IFN-α used in the maturation cocktail for DC1 might have interfered with adenoviral transduction due to its anti-viral properties. Hence, we adjusted our protocol by transducing the iDC on day 4, exposing them to the adenoviral vector for 24h, and then

subsequently added maturation cocktail on day 5 for a 48 h exposure. This indeed increased the transduction efficiency, resulting in a much higher level of GFP expression in the DC1 (termed αDC1.GFP; Figure 9C).

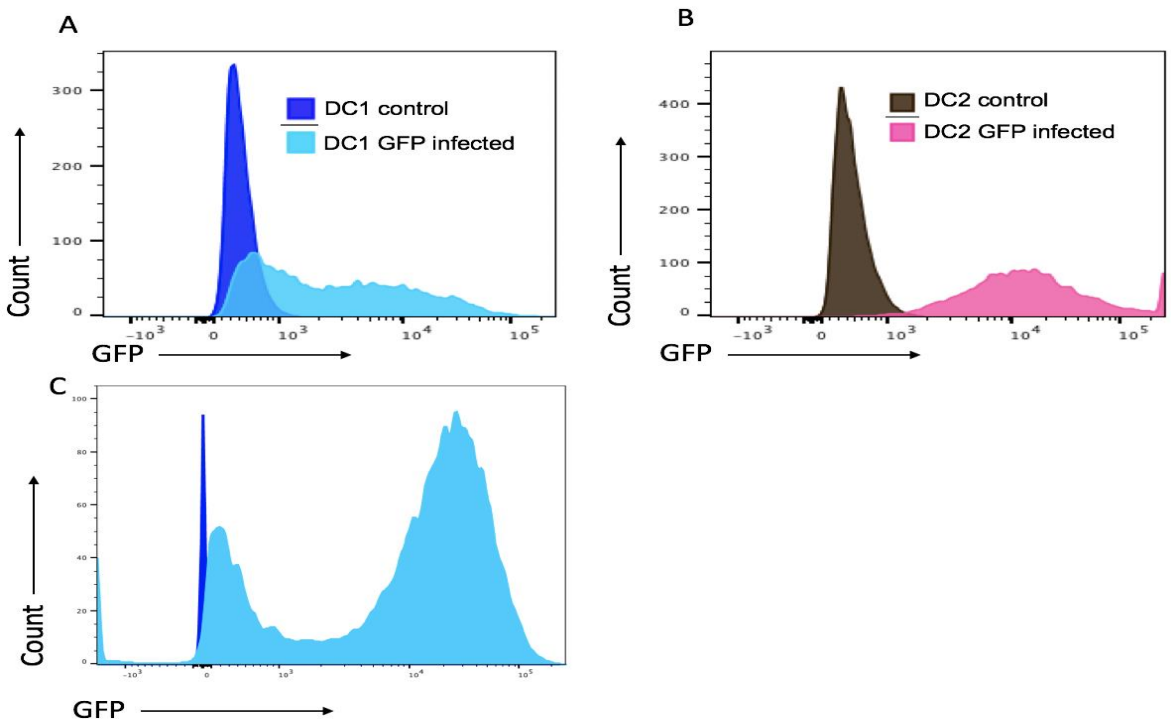

Figure 9. GFP expression in DC1 and DC2 is comparable when transfected in immature DC stage
(A) and (B) show the respective low GFP+ αDC1 and high GFP+ DC2 when transfected after adding maturation cocktail. (C) Shows the high GFP+ αDC1 when transfected before adding maturation cocktail.

4.1.5 Assessing the capacity of DC to transfer cellular content to T-cells : Role of CD4+ T cell 'help'

The αDC1.GFP that were generated as previously mentioned were co-cultured with T-cells in the presence of SEB (used as an antigen surrogate) for 4-6 days. Our specific interest was to determine role of CD4 'help' in transferring content to CD8+ T cells. The conditions for this experiment were

subdivided into culturing αDC1.GFP with purified T cells containing both CD4+ and CD8+ T cell population or with only purified CD8+ T cell fraction. This was followed by flow cytometry analysis of the T cells to determine if GFP was transferred. During the flow cytometry analysis, we observed enhanced transfer of GFP to CD8+ T cells cultured in the presence of CD4+ T cells in comparison to those cultured in the absence of CD4+ T cells. To see if CD40L signaling was playing a role, we used a CD40/CD40L blocking antibody in the assay, which reduced GFP+ CD8 T cells from 25.6% to 9%, (Figure 10A and 10B). To further investigate this role of CD4 'help', we substituted CD4+ T cells with a synthetic recombinant CD40L activating protein. Flow cytometric analysis of these CD40L treated DC revealed an enhanced transfer of GFP to CD8+ T cells from 7.54% to 34% , similar to what was seen in the cultures containing the CD4+ T cells (25.6%). We hypothesized that the transfer of GFP to take place would be at reduced levels in DC2 cultured with CD8+ T cells, due to their inability of DC2 to efficiently express TNTs in the presence of CD40L. Interestingly, we observed that DC2 had similar levels of transfer of GFP to CD8+ T cells as seen in DC1 (Figure 10C).

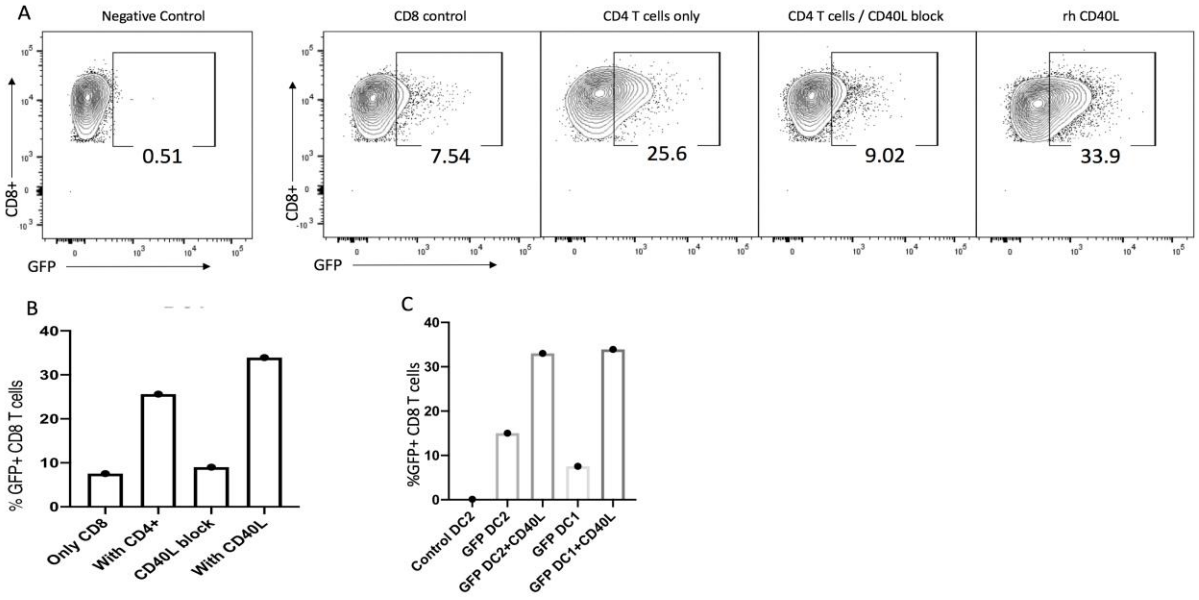

Figure 10. CD4+ T cell 'help' increases efficiency of transfer of DC derived GFP to CD8+ T cells
(A) Flow cytometric analysis plots following 6 days of co-culture of CD8+ T cells and GFP.DC1+SEB (in all conditions from 2nd panel to the left through right). The 1st panel from the left is a negative control with non-GFP.DC1 as negative control. From 2nd panel to the left to right, the respective conditions are only GFP.DC1 to establish baseline transfer of GFP (2nd panel from left), in the presence of CD4+ T cells (middle), in the presence of CD4+ T cells and CD40L blocking mAb (2nd panel from right) and in the presence of rhCD40L (right most panel). (B) Summarized flow cytometry results of % GFP+ CD8 T cells in various conditions. (C) Summarized flow cytometry results of % GFP+ CD8 T cells in DC1 and DC2, enhanced by CD40L

4.1.6 Determination of mechanism for intercellular transfer of GFP from GFP.DC to CD8+ T cells

Having addressed the proof of principle that DC can transfer contents to CD8+ T cells, and that CD40L was playing a role, we wanted to determine if this effect was contact dependent. We hypothesized that the CD40L may be promoting the release of EVs from the DC. To test this idea, we used a trans-well assay system to determine if both DC subtypes invoked similar response to CD40L in-terms of producing GFP-EVs. As a positive control, GFP.DC2 were co-cultured with T-cells in the presence of SEB as described in section 1.1.5 (figure 11A right

panel). The cells from the bottom well were harvested 48 hours post incubation and were analyzed for GFP positive CD8+ T cells via flow cytometry. The data revealed that while GFP.DC1 separated by the membrane did not transfer any GFP to CD8+ T cells (0.41%), activation of CD8+ T cells via DC-based CD80/86 lead to 29.8% of CD8+ T cells and 27.9% of CD8+ T cells to have GFP from GFP.DC1 (figure 11A middle panel) and GFP.DC2 respectively (Data not shown). **Together, these collective findings (flow cytometry of DC1 and DC2) support our identification of a novel "helper" function of CD40L for facilitating transfer DC cellular cargo to CD8+ T cells via EVs.**

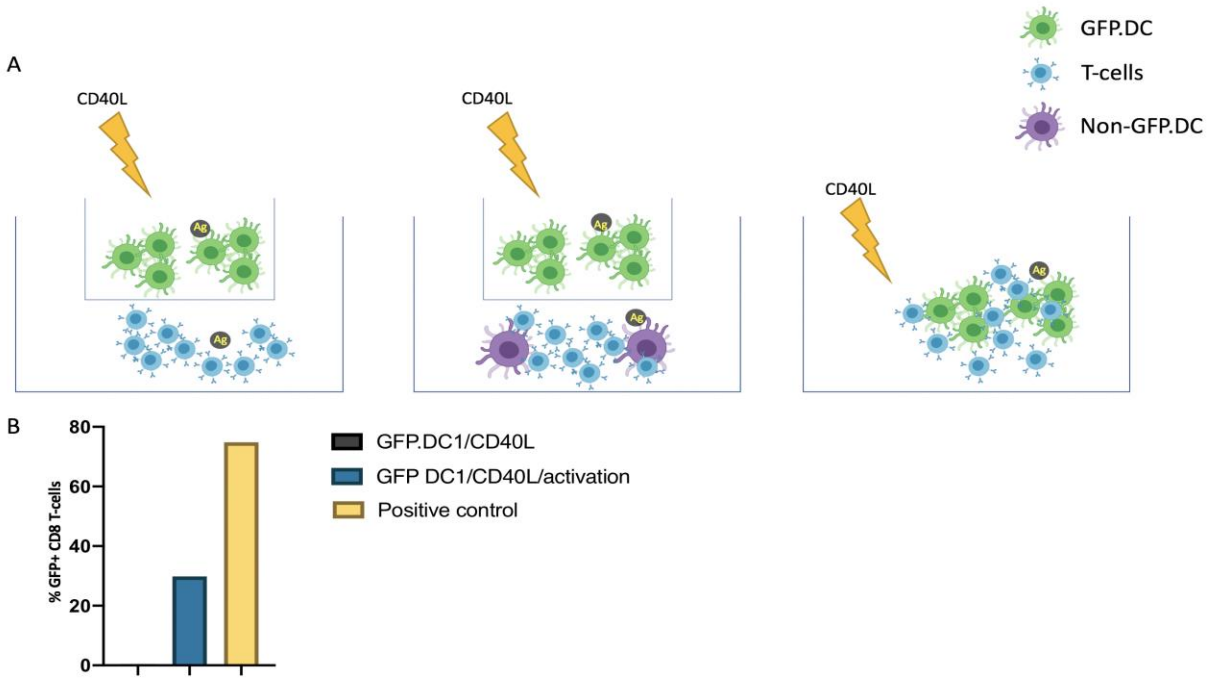

Figure 11. Contact independent intercellular transfer from DC to CD8+ T cells: Requirement for T cell activation
(A) Experimental layout of trans-well assay. From left to right, the first well consists of GFP.DC1stimulated with CD40L in the upper chamber and T-cells with SEB(Ag) in the bottom well and; second well's lower chamber consists of T-cells co-cultured with non.GFP.DC1+SEB(Ag) and GFP.DC1+SEB(Ag)+CD40L in the upper chamber; third well is an acting positive control co-culture of GFP.DC and T-cells with CD40L and SEB(Ag) (B) Data from flow cytometric analysis summarized in the form of bar graphs showing %GFP+ CD8+ T-cells.

Since our experiments suggested that transfer was occurring in a non-contact dependent manner, we set out to investigate the potential role of DC derived EVs in our model. To do this, we first attempted to generate and isolate GFP.EVs derived from the GFP-DC subtypes we engineered through adenoviral transfection as mentioned in section 1.1.4. Using resin-based exclusion columns to avoid apoptotic contaminants, we were able to obtain purified small extracellular EV (sEV) and medium extracellular EV (mEV) having a size range of 35-350 nm (Figure 12A). These EVs were added to cultures of anti-CD3/CD28 bead activated CD8+ T cells and imaged to gain better insights about the mechanism of transfer. Preliminary imaging and analysis for proof of DC-derived GFP.EVs was determined using AMNIS, image stream technology. This revealed an interaction between GFP.EVs and CD8+ T cells (Figure 12B). Furthermore, it could be seen that the number of EVs taken up per-cell varied. However due to time constraints, we were unable to further analyze and determine the spatial specificities of interaction (surface vs internalization) of these EVs.

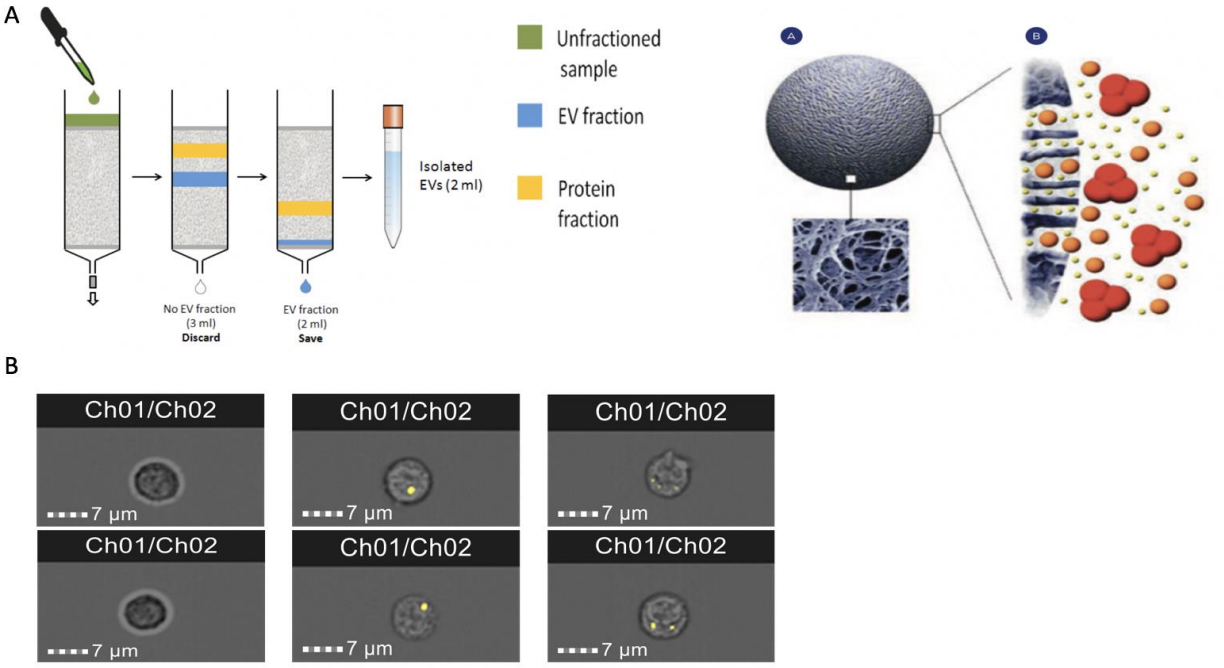

Figure 12. GFP+ extracellular vesicles derived from DC captured by CD8+ T-cells
(A) Graphic image displaying the process of EV isolation using a resin based size exclusion chromatography system isolating EV in 30-300 nm size range. (B) GFP+ EV localization on CD8+ T cells imaged via image-stream flow cytometry (Raw data only).

4.2 Aim 2: Assess the Phenotypic and Immunomodulatory Characteristics Of EVs Derived From Differentially Polarized DC

4.2.1 Characterize EVs by size and surface markers

EVs from differentially polarized DC were isolated and purified from culture supernatant at 48h time point post CD40L exposure to avoid the risk of contamination of EVs with apoptotic bodies. This was to confirm the existence of EVs by testing for predominant surface markers. The EVs showed high levels of tetraspanin (CD81) in the absence of CD40L, while in the case of DC1 post CD40L treatment showed tremendous decrease, while the levels remained the same in

DC2 with or without CD40L (Figure 13A). These EVs also expressed high levels of CD86, a prominent surface marker previously observed on DC-derived EVs [78], with no apparent change in either DC condition post CD40L exposure (Figure 13B).

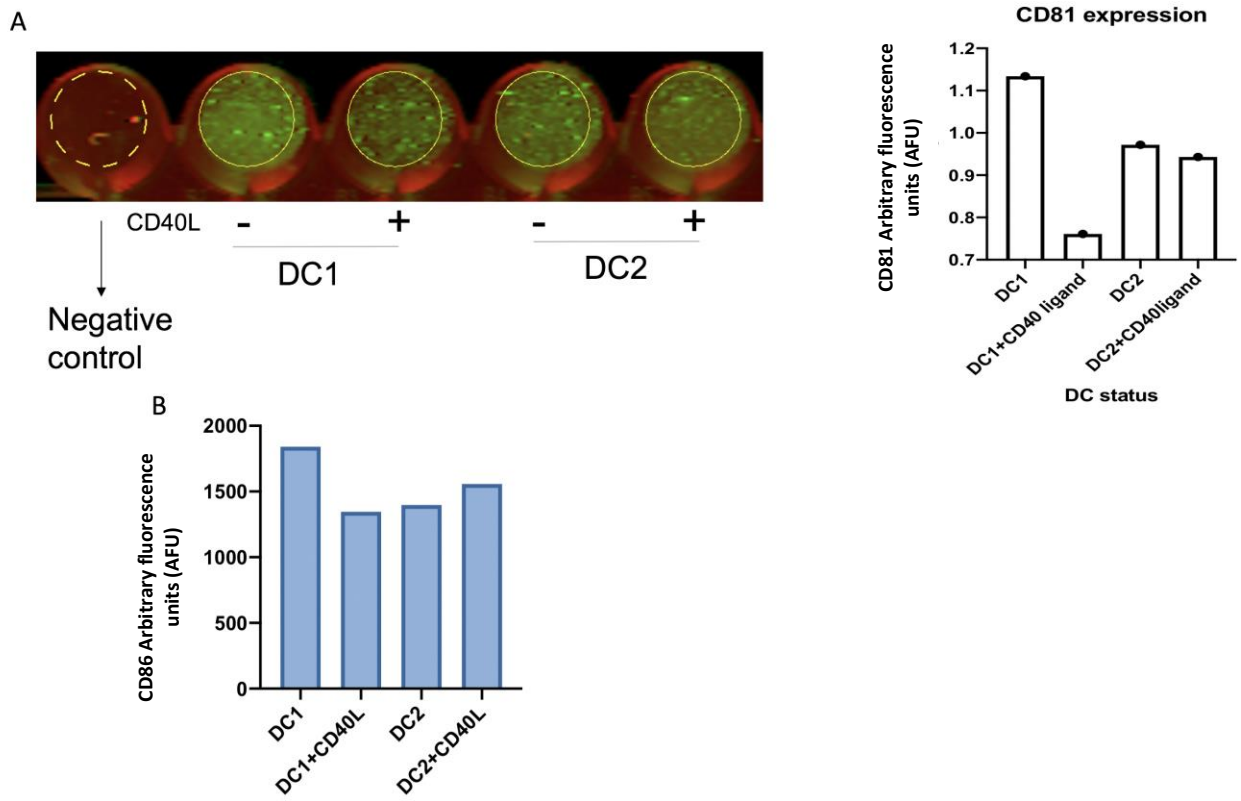

Figure 13. Immunodetection in DC1 and DC2 derived vesicles
(A) In-well western blot image of Immunofluorescent stain for extracelluar vesicle surface marker CD81 (left panel). Alpha DC1+CD40L show reduced expression of CD81, but not DC2 (right panel). (B) DC-derived EVs were assessed for their ability to express Co-stimulatory molecule, CD86 on their surface.

The size of EVs was verified by nanoparticle tracking analysis (NTA), which analyzes Brownian motion of the particles from video recordings, while simultaneously keeping track of individual particle's scattered light. This revealed a significant decrease in size of DC1 based EVs post CD40L (Figure 14B) while significant changes were not observed with DC2 (Data not shown). This was consistent during cultures of DC in serum-free media. The overall size range

of DC-derived EVs ranged from 110 nm to 250 nm. **Thus DC-derived EVs were not similar in size (+/-) CD40L treatment, while they displayed DC-surface markers at similar levels**.

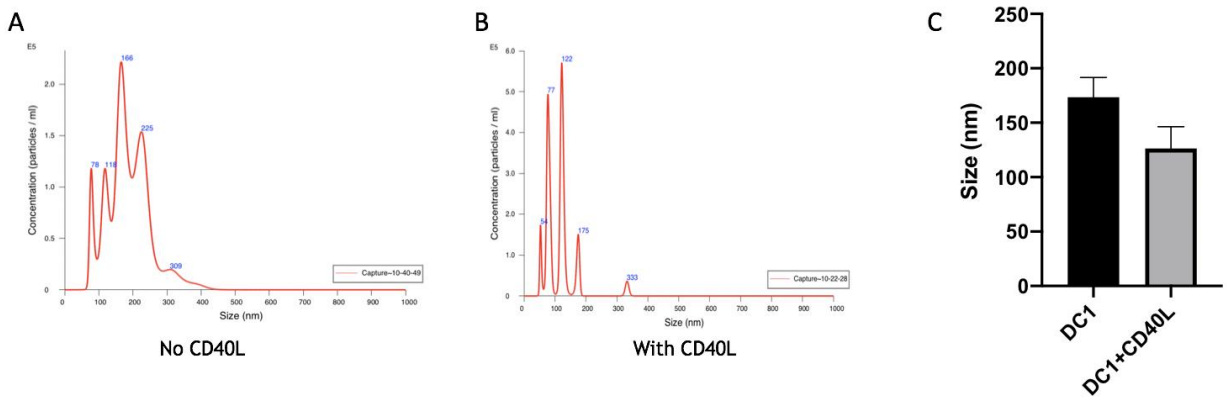

Figure 14. Characterization of Extracellular vesicle size distribution
Alpha DC1-derived EVs were analyzed via nanoparticle tracking analysis (NTA) for relative size distribution with representative graph for DC1.EV (left panel) and DC1+CD40L.EV (middle panel). The summarized graph of mean size from 3 different experiments (n=3) with error bars (right panel).

4.2.2 Characterize impact of CD40L on internal protein content of EVs

Having determined the effect of CD40L on surface markers associated with EVs, we wanted to see if there was differential packaging of proteins in different DC types, including DC1 and DC2 post CD40L stimulation. Following maturation, the DC1 were treated with CD40L for 2 days. The EVs from the supernatant were lysed and subsequently analyzed by SDS-PAGE for total protein expression. The Coomassie blue stain revealed DC1-derived EVs to have 3 distinct protein bands between 35 and 55 kDa and 2 bands between 15 and 25kDa. In CD40L treated DC1-derived EVs, a singular band much stronger in expression was observed between 35 and 55kDa. Additionally, there was secondary band between 55kDa and 70kDa, which is not

observed in control EVs. This confirms that CD40L affects differential packaging of proteins in DC1-derived EVs (figure 15).

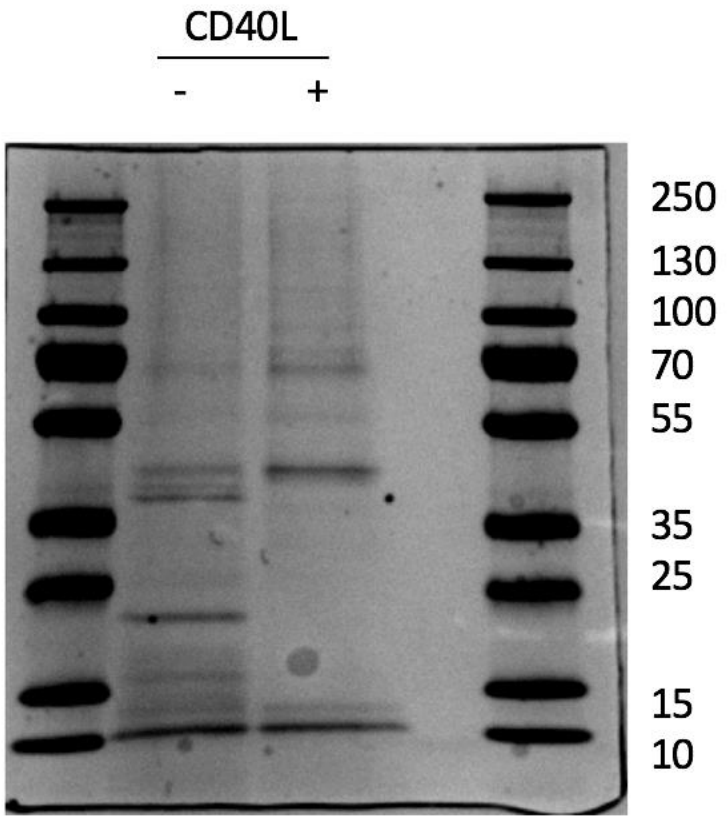

Figure 15. Changes in total-protein of DC1.EV induced by CD40L
Equal concentrations of internal protein from lysed EVs were separated by SDS-PAGE and stained with Coomassie blue for total protein. 2nd lane from the left represents alpha DC1.EV. 3rd lane from the left represents alpha DC1+CD40L.EV. 1st and last lane are ladders.

Next, we wanted to explore the effect of CD40L on DC2-derived EVs protein. Interestingly, the protein expression pattern was comparable in EVs derived from both DC2 and CD40L-treated DC2 (figure 16). This is consistent with the fact that DC2 are less responsive to CD40L signaling with regards to morphological changes and IL12p70 production. Since this does not account for changes in other materials, which can be transferred such as mRNA and miRNA, this warrants further investigation via RNA sequencing.

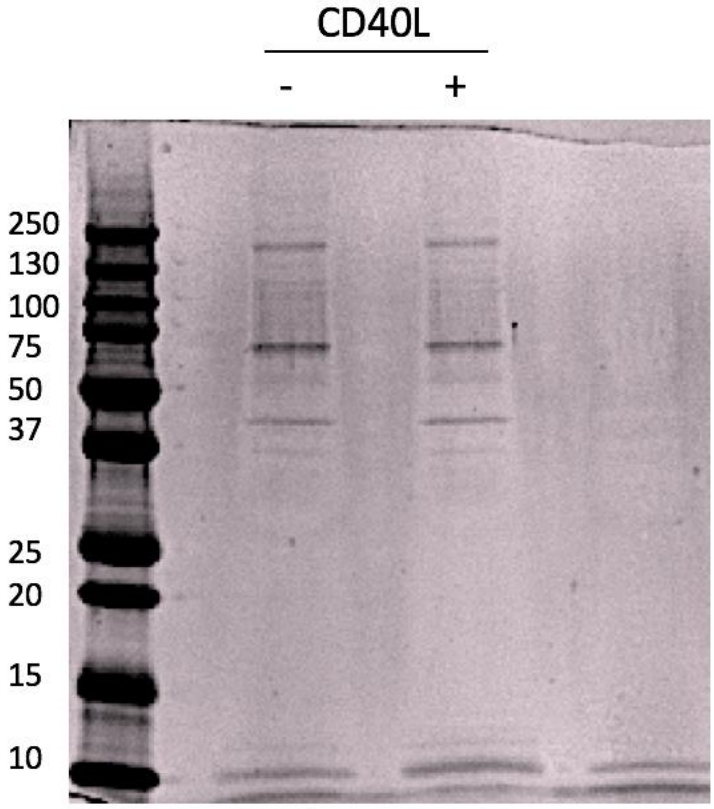

Figure 16. Changes in total-protein of DC2.EV induced by CD40L
Equal concentrations of internal protein from lysed EVs were separated by SDS-PAGE and stained with Coomassie blue for total protein. 3rd lane from the left represents DC2.EV. 5th lane from the left represents DC2+CD40L.EV. 1st lane is the ladder.

4.2.3 Biologic impact of CD40L treated DC-derived EVs on primary T-cell cultures

To study the biologic changes induced by EVs, naïve CD8 T cells were activated with anti-CD3/CD28 dynabeads and treated with DC-derived EVs for 10-12 days. Images of cultures were taken on day 5 post treatment, which showed changes in morphology of the T cell cultures in CD40L treated DC-derived EVs compared to control. EV treated cultures showed enhanced replication compared to control as shown in figure 17A vs C.

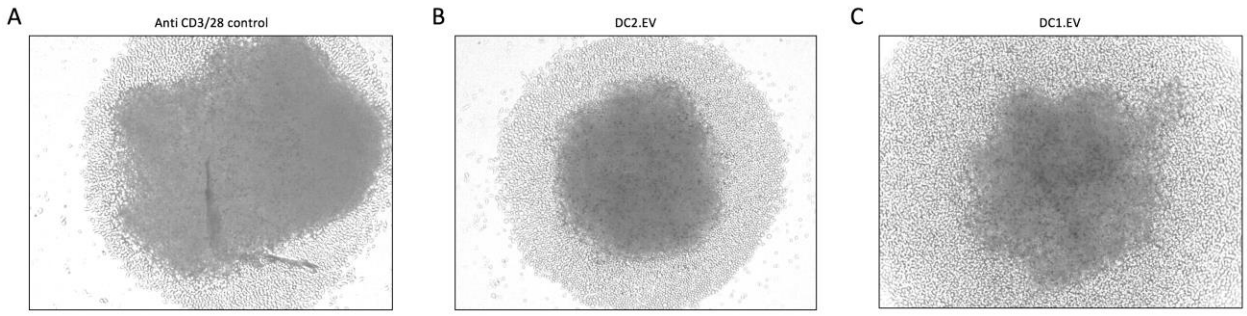

Figure 17. Activated naïve CD8+ T-cells treated with DC-derived EVs
All cultures contained naïve CD8+ T cells stimulated with antiCD3/28 bead (A) Shows the control antiCD3/28 bead-stimulated naïve T-cells (non EV-treated) (B) Shows decrease in T cell expansion (small circumference) in cultures treated with EV derived from CD40L-treated DC2 (C) EVs derived from CD40L-treated DC1 induces higher amount of proliferation of naïve T cells than other conditions. (All images were captured at 20X by standard bright field microscopy).

On day 12 post treatment, the T-cells were stained for markers of effector activity including IL2, CD107a, IFN-γ and Mip-1β to analyze the effect on CD8+ T cells. In the analysis, we gated for lymphocytes, single cell discrimination, and live cells (figure 18A). Furthermore, during analysis Boolean gating was performed on cells for expression of cytokine combinations to obtain data on relative polyfunctionality [107]. Preliminary results indicated a significant change (decrease) in the population of cells producing 0 and 1 cytokine T cell cultures treated with EVs derived from the CD40L-stimulated DC1 compared to control untreated T cells (Ranging between 5-10%), and a significant increase in polyfunctionality especially in % of cells producing 2 and 3 cytokines (%) (figure 18B,C), which is an indicative trait of CD8 T cells having superior overall effector function [110, 111]. However, there was no significant change observed between cultures exposed to the EVs derived from CD40L stimulated DC2 and control conditions in-terms of increase in polyfunctionality. Hence CD40L induced DC-derived EVs treatment appeared to have the capacity to influence the priming and functional properties CD8+ T cells, and this immunomodulatory property is influenced by the mode of DC maturation.

However, these findings are limited by the number of donors tested and should be further explored with other healthy donors before concrete conclusions can be made.

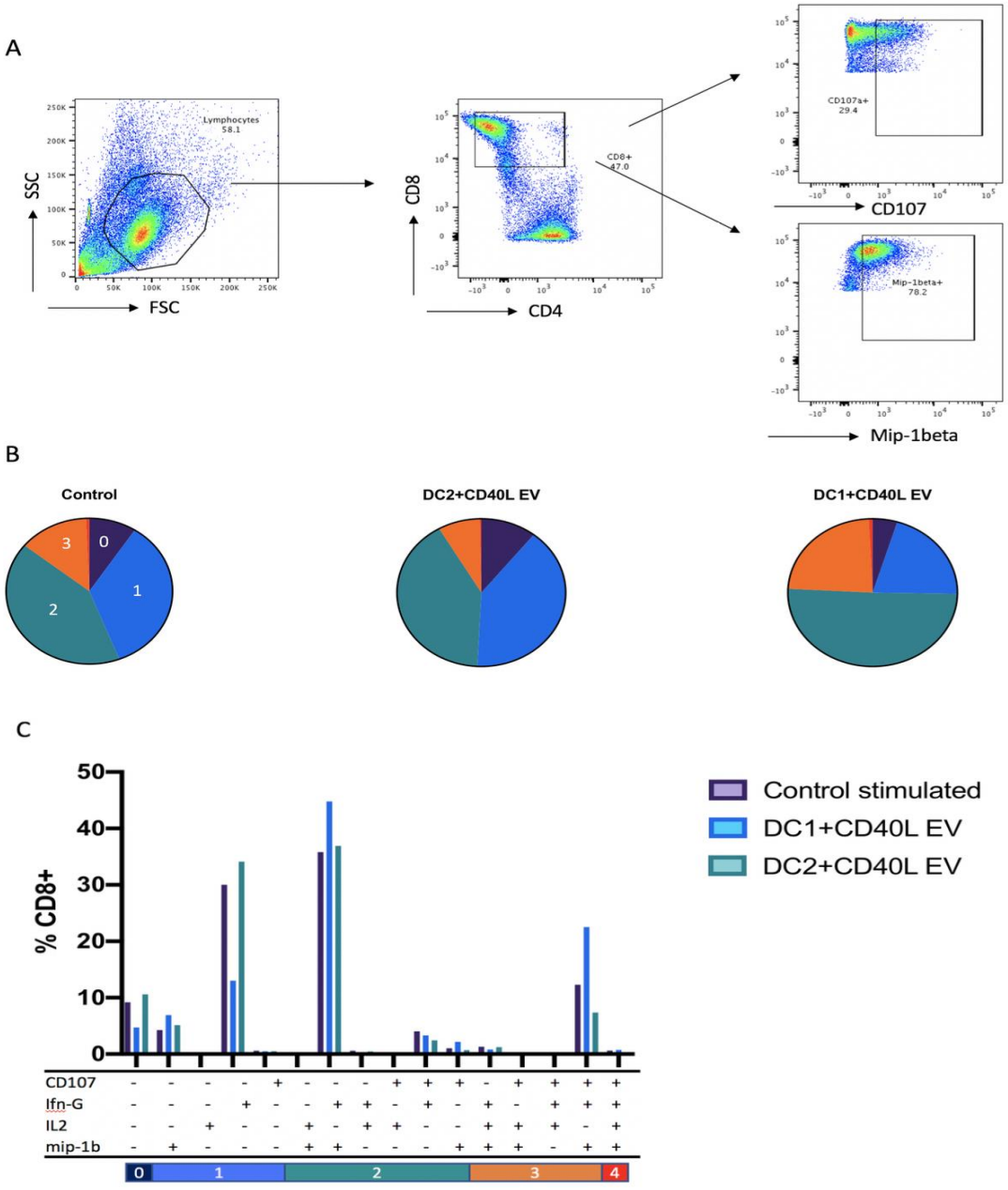

Figure 18. DC1+CD40L-derived EVs displayed enhanced polyfunctionality of naive CD8 T-cells
(A) The gating strategy used for data analysis of day 14 DC. EV treated activated naïve CD8+ T-cells, which represents lymphocytes (left panel), CD4/CD8 stain (middle panel) and Boolean gating of % +CD8 T-cells for each cytokine. (B) Summarized pie charts displaying overall % of CD8+ T cells producing different numbers of cytokines. (C) Bar graph displaying %CD8+ T cells producing different combinationsof cytokines, CD107-a, Interferon-gamma, IL-2 and Mip-1beta.

4.2.4 Assessment of CD40L treated DC-derived EVs impact on the long-term function of memory CD8+ T cells following challenge with HIV-1/CEF antigen expressing targets

Having observed a positive effect of these EVs on priming of naïve CD8+ T-cells, we wanted to study the effect of these EVs on the activation of virus specific memory CTLs CEF and HIV- specific. Using specimens from HIV+ participants from the MACS cohort, we cultured their PBMC in the presence of either CEF (CMV, Epstein-Barr and Influenza) peptide or HIV Gag peptide and cultured them for 10-12 days in the presence or absence of EVs derived from CD40L-treated DC. These cell cultures were supplemented with IL2 and IL7 on day 5 and tested for functional response in IFN-gamma ELISPOT on day 10-12. Overall, compared to the control (Untreated), DC-derived EV treated PBMCs had lower percentage of CEF specific IFN-γ producing CTLs. This effect was exacerbated in CD40L treated DC-derived EV, which held true for the EVs derived from both DC1 and DC2 subtypes (figure 19A). The same effect was observed in HIV-1 antigen specific IFN-γ producing CTLs (figure 19B). These results directly contradict the effect seen in naïve T-cells. We hypothesized a change in impact based on the characteristics of memory vs naïve T-cell and while also taking into consideration previous data published by Garcia-Bates et al, showing the increased PD-L1 expression on CD40L treated DC, and a context dependent and opposing impacts of DC-derived PD-L1 on the function of naïve vs memory CTL [40].

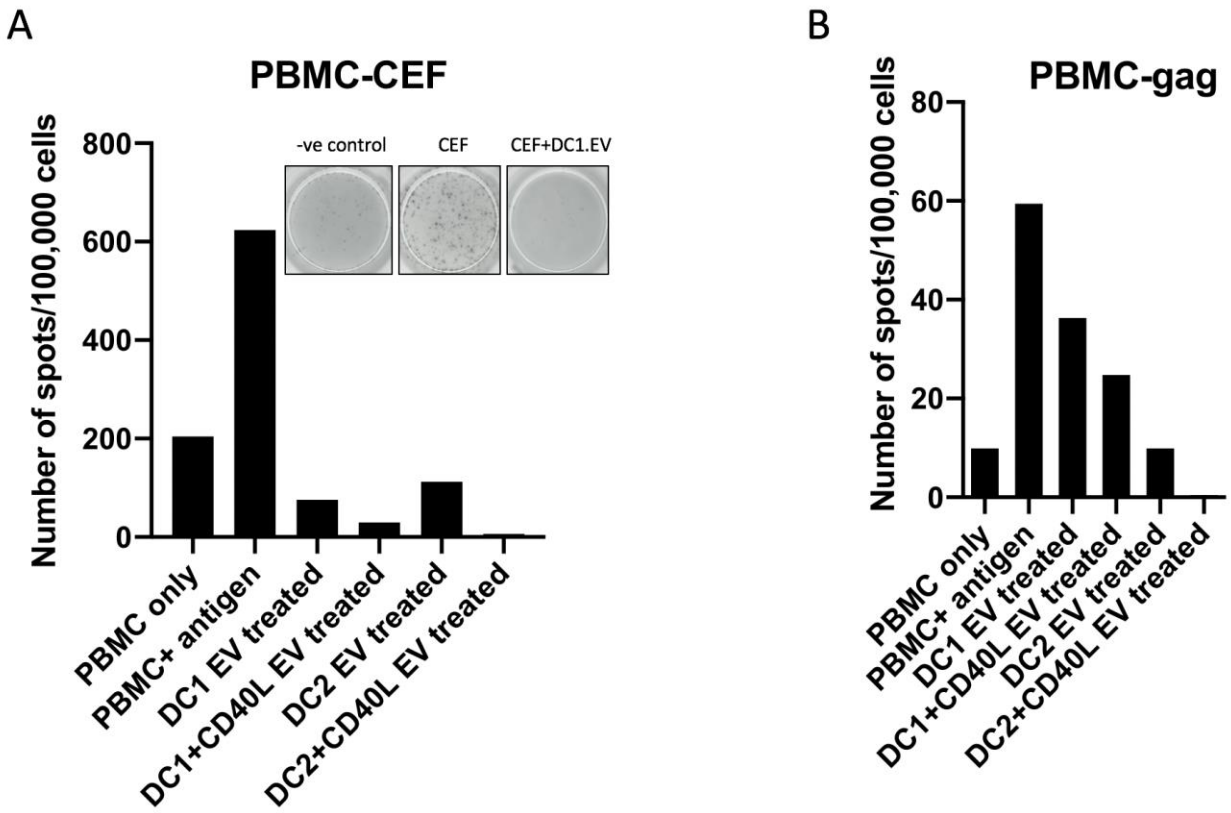

Figure 19. Memory T-cells were negatively regulated by DC derived EVs
(A) IFN-gamma assay results from HIV+ donors of PBMCs stimulated with CMV-EBV-Flu antigen in the presence of various DC-derived EVs. (B) IFN-gamma assay results from HIV+ donors of PBMCs stimulated with HIV-specific antigen in the presence of various DC-derived EVs (Representative of 2 experiments/ n=2).

PD-L1 is an inhibitory ligand found predominantly in cancer patients and long-term HIV progressors [112]. To initially confirm this, we analyzed for PD-L1 expression in both DC-types via flow cytometry (figure 20A), which corroborated with previously seen results. Thus, we performed in-well western blotting on DC-derived EVs for PD-L1 and subsequently found slight increase in PD-L1 expression in CD40L treated DC1.EV, while no apparent changes were observed in DC2 (figure 20B).

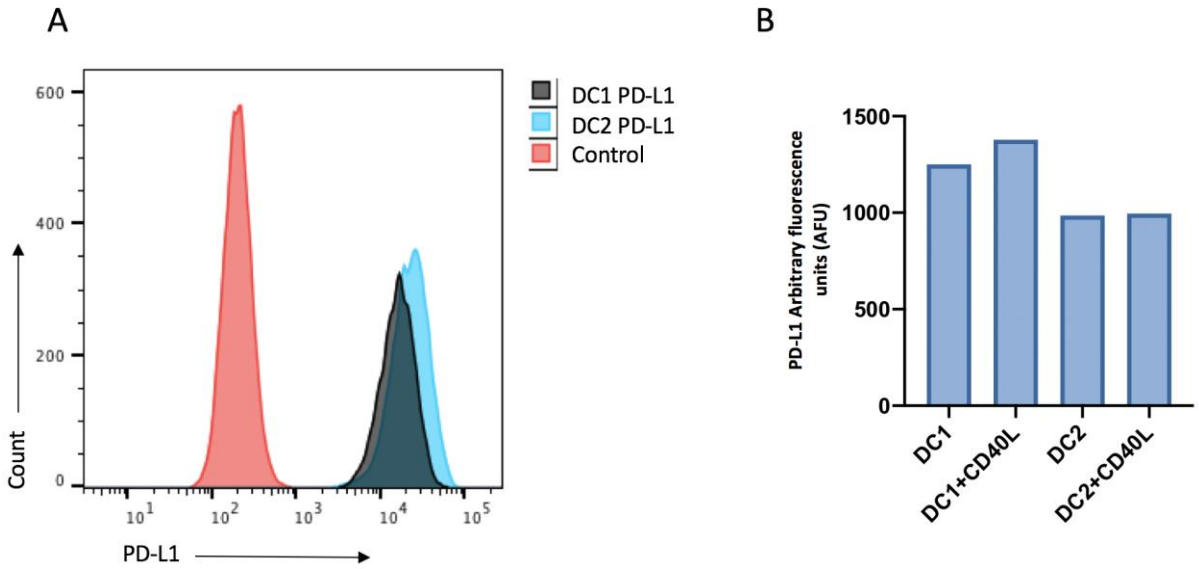

Figure 20. PD-L1 expression is profound on mature DC
(A) Surface marker analysis of PD-L1 on DC1 and DC2 (B) In-well western blot immunofluorescent staining of DC-derived EV for changes in PD-L1 expression induced by CD40L treatment.

5.0 Discussion

The ability of DC to modulate immunologic functions of various adaptive immune cells is a basic area of immunology with enormous potential yet to be completely utilized. Recently, data from the Storkus lab hinted at the ability of DC overexpressing T-bet to transfer content to T-cells in T-bet knocked out murine model [53]. Potentially related to these findings, a study by Zaccard et al., highlighted a pivotal role of CD40L on DC1, facilitating the formation of TNTs in a process referred to as 'reticulation', which aids in inter-DC communication and intercellular transfer of content between DC [42]. While there is emerging evidence of increased CTL killing potential induced by DC1 is related to their superior responsiveness to CD40L on CD4+ T helper cells, however it is unclear if DC reticulation and intercellular transfer of content to CTL directly is playing a role in enhancing CTL priming. Therefore, in this project, we sought to explore the potential for DC and CD8+ T cells to directly exchange of information, with emphasis on T-helper signal CD40L in enhancing such intercellular communication and DC1-mediated CTL responses.

In my first aim, we hypothesized that DC1, because of their unique capacity to reticulate in response to CD40L, would have better potential to communicate with CD8+ T cells as compared to DC2. As a proof of principle, we aimed to visualize the transfer via Adv-GFP transduction of DC. Upon initial examination, we found low baseline levels of GFP transfer was occurring between DC1 and CD8+ T cells and was greatly enhanced in the presence of CD4+ T cells or recombinant CD40L, further corroborated by CD40L blockade (figure 10A). Surprisingly, DC2 displayed similar levels of GFP handover to CD8+ T cells (figure 10C),

revealing a novel function of CD40L in promoting DC transfer of cellular cargo directly to CD8+ T cells, with similar efficiency in both mature DC types tested.

While the exact nuances of the CD40-CD40L signaling pathway responsible for this transfer is yet to be fully explained, we sought to confirm the messenger capability of EVs via trans-well assays and image stream flow cytometry. Preliminary results of DC-generated EVs showed the accumulation of GFP-EVs on CD8+ T cells (figure 12B). While performing trans-well assays, we observed GFP transfer from DC to T-cells only when T-cells were activated. While unclear, this role of activation on T-cells may be due to increased expression of certain surface markers/receptors that may play a role facilitating uptake of EVs via phagocytosis or endocytosis. This does not undermine the potential role of TNTs in this phenomenon, since the efficiency of the transfer in the trans-well assays was lower than when the cells co-culture with direct contact permitted. Our results also indicate the role of CD40L on DC generation and transfer of EVs, potentially with a specialized immunomodulatory function.

For my 2nd aim, we sought out to characterize and explore the functional role of CD40L induced DC-derived EVs. We investigated their ability to modulate the induction of primary and memory CTL responses. We observed that CD40L-treated DC1 generated EVs that were smaller in size than those from CD40L-treated DC2. Subsequently, quantification of the prominent exosome related surface marker CD81, revealed diminished levels in the same sub-type following CD40L stimulation, while the expression of the DC-associated costimulatory molecule CD86 helped distinguish them as indeed originating from the parent DC [78]. Although the implications of this are for the most part still unknown, inhibition of CD81 has been shown to decrease uptake of EVs into other types of target cells [77]. The decrease in size might correlate

with an exosome like feature, which have more potent immunomodulatory capacities than medium or large sized EVs [74, 79].

Although there were phenotypic differences noted, this itself does not indicate differences in content or function. Previously published data from our lab, as also shown in Aim1, has revealed the noticeable reaction of DC1 to CD40L characterized by the high level of production of IL12-p70 and better CTL inducing capacity [48]. We sought to examine the possibility that such CTL responses were partially enhanced as a result of EV delivery, and in particular influenced by modifications in internal content of EVs specifically derived from CD40L-stimulated DC1. From SDS-PAGE, there were several protein bands observed in EVs collected from untreated DC1, whereas in EVs derived from CD40L-treated DC1 did not result in these bands being expressed. Instead a protein band between 55kDa and 70 kDa was upregulated, which could correspond to a higher expression of T-bet or IL12-p70. As expected, the changes in DC2-derived EVs were subtle and showed no major differences resulting from CD40L exposure.

To determine the potential immune impact of these CD40L induced DC-derived EVs, we first specifically tested their impact on the primary activation of naïve T-cells. As a surrogate of antigen specific activation by antigen presenting mature DC, we used anti-CD3/CD28 activation beads as the primary activator of the naïve T cells. Although the results are preliminary, we observed differences in the induction of polyfunctional effector responses when DC derived EVs were present during this primary stimulation, with a significant increase noted in the percentage of CD8+ T cells producing 2 or 3 cytokines in EVs derived from the CD40L stimulated DC1, compared to those derived from DC2-EV. These data are consistent with internal phenotypic and content changes noted with the CD40L stimulated DC1, further suggesting a potential role of CD40L ‘help’ in influencing the quality of primary CTL immunity.

Surprisingly, when we performed similar experiments to induce the activation of memory T-cell responses to HIV and CEF peptides (using PBMC collected from HIV infected MACS participants), the presence of DC-derived EVs resulted in diminished cytolytic responses, suggesting an inhibitory role of DC-derived EVs. Moreover, this highlights that the function of the EVs may be context dependent, greatly influenced by the differentiation stage of the T cells they target. Based on the findings from a previous report from our group describing a similar context dependent effect on T cells resulting from PD-L1 expression on DC, which is enhanced upon CD40L stimulation, we hypothesize that the effect could be due to EV expression of PD-L1 t [40]. Indeed, we did find a high degree of expression of PD-L1 on the DC-derived EVs. This could also imply EV-mediated differential functional impact on different types of T-cells, whereby they function to shut down exhausted memory T-cells and to stimulate priming of naïve T-cells.

As mentioned before, these novel findings may be a novel immunologic mechanism and may help to explain findings from others regarding the mechanisms by which migratory DC mediate cytolytic responses by transferring information to resident DC following their initial interaction with CD4+ T helper cells [44]. CD40L dependent transfer from DC to T cells may also be a mechanism utilized or hijacked by HIV spread infection to other T cells, which theoretically could include CD8+ T cells [113]. While all of these are speculative, to my knowledge this is the first study to dwell into the effect of CD40L on DC-derived EVs and their potential biologic impact on CD8+ T cells, thus describing a novel helper function of CD40L expressing CD4+ T helper cells, and highlighting the fact that there still basic immunological mechanisms that have yet to be fully explored. A better understanding of this immunologic

phenomenon may lead to the development of effective therapeutic strategies to treat chronic diseases such as HIV.

6.0 Public Health Significance

Dendritic cells (DC) are the natural link between the innate and the adaptive immune system. Their central role in the immune response has been the reason that DC have been evaluated and utilized as a tool for the development of vaccines against chronic diseases including cancer and HIV. Despite the success of ART, there still is no cure for chronic HIV infection. Developing an immunotherapeutic method design to effectively control HIV, similar to those few 'elite controllers' who can do this naturally, has been a primary goal in the field of HIV cure research. There are a number of major hurdles to address to achieve this immunologic 'functional cure', which include CTL exhaustion/dysfunction, viral evolution and immune escape, and HIV latency itself. The strategy of "Kick and Kill" for HIV cure is an area of research where DC are actively being explored for their potential in both inducing long-lived polyfunctional CTL that can focus attack on highly conserved regions of HIV, as well as driving HIV latency reversal [9, 114, 115].

Even though DC are amongst the most well studied cells in the immune system, due to high degree of tissue-based differences, and their incredibly wide range of functions, many basic biologic aspects of how they work and communicate with other immune cells still have yet to be fully elucidated. For example, the central dogma in DC biology is that they acquire antigen in the peripheral tissue and migrate to draining lymph nodes to induce CTL responses. More recently, it has been shown that migratory DC are required for delivery of antigen and instruction to lymph node resident DC to induced effective CD8+ T cell responses, and this is done through some unknown mechanism [43]. Moreover, their expression of high levels of PD-L1 have been suggested to play important, albeit opposing roles, on the induction of primary CTL responses vs

the regulation of memory CTL responses, through mechanisms that remain unclear [40]. Importantly, viruses such as HIV have targeted the functional programming DC as a means to escape immune control and to enhance viral dissemination [116, 117]. A clearer picture of their basic function is still critically important for the development of improved immunotherapeutics and for understanding how pathogens may utilize them or modify their behavior to their advantage.

CD40L has long been known to play a major 'helper' role in DC-mediated CTL responses. In recent years, the role of CD40L has been shown to be critical to various and newly discovered aspects of DC functions, including inducing DC1 reticulation, or the immunologic process by which DC form tunneling nanotube networks that support intercellular communication and antigen transfer amongst DC [42]. CD40L has also been shown to mediate opposing immunoregulatory effects on T-cells via upregulation and activation of the PD-L1 signaling pathways [40]. Unpublished data from the Mailliard laboratory has suggested an important role for CD40L in CD4+ T cell : DC interactions leading to enhanced DC-mediated HIV trans-infection. And finally, inhibition of CD40L signaling of DC has been shown to lessen their capacity to drive latency reversal and expression of HIV protein in latently infected CD4+ T cells. In all of these cases, the mechanisms involved in the CD40L mediated effect have yet to be elucidated.

DC-derived extracellular vesicles (EVs) have recently been tied to antigen specific CD4+ and CD8+ T-cell responses and explored as drug delivery systems and vaccination strategies [91, 92]. Until now, DC derived EVs have typically been studied following their collection as a result of their non-specific release in culture supernatants. Our finding that signaling via the CD4+ T helper cell factor CD40L results in the purposeful packaging and release of DC-derived EVs, and

that they serve as a vehicle to transfer cellular content to T cells to influence T cell function suggest the discovery of a novel DC-mediated helper function of CD40L. The link between CD40L-induced DC-derived EVs and T-cells is an unexplored paradigm that may be critical to many immunological functions, situation and outcomes, and thus warrants further investigation.

My thesis research work has significance and potential impact to public health in various aspects. **The first way has to do with improving our understanding of basic immunology**. The immune system is critical to every component of health, including but not limited to wound healing, fighting off microbial invaders, psychology, and digestion. Understanding how EVs derived from such a central player of the immune system are produced and function immunologically could be extremely valuable. One can imagine that such knowledge could then **lead to the bioengineering of EV-based immunotherapeutic strategies** to treat a number of health conditions, including HIV infection, cancer, and autoimmune conditions. Moreover, understanding how to better isolate them and analyze their phenotypic, content, and functional characteristics **could lead to the development of EV-based biomarker assays for the purpose of identifying, assessing, or monitoring certain human health conditions**. And finally, understanding the role of CD40L-induced DC-derived EVs and their mechanisms of formation and action could lead to a **better understanding of how microbes might circumvent, modify, or utilize them for their survival advantage.**

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