IL-7 Responses In Th17 Cells Are Dysregulated During HIV Infection

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ABSTRACT

In the gut-associated lymphoid tissues, Th17 cells mediate mucosal homeostasis and inflammation. During HIV infection, Th17 cells become depleted and functionally impaired, which is implicated in the pathogenesis of chronic inflammation in patients treated with highly active antiretroviral therapy. IL-7 is a cytokine that mediates homeostatic responses in T lymphocytes, such as proliferation and survival, which are dysregulated during HIV infection. Whether similar dysregulation occurs in Th17 cells has yet to be reported. IL-7 receptor α (CD127) expression and IL-7 responses were therefore measured in blood-derived Th17 cells from uninfected individuals and effectively treated, HIV-infected individuals by flow cytometry. Th17 cells from uninfected individuals expressed CD127 and, in response to IL-7, exhibited phosphorylation of STAT5, upregulation of Bcl-2, and proliferation. During HIV infection, expression of CD127 and pSTAT5 in Th17 cells was comparable to that observed in cells from uninfected individuals. Interestingly, expression of Bcl-2 was upregulated while proliferation was dramatically impaired. These findings may provide further insight into the mechanisms by which Th17 cells fail to become restored during HIV infection.

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LIST OF ABBREVIATIONS

AIDS: Acquired immunodeficiency syndrome
APC: Allophycocyanin (fluorochrome conjugated to antibodies)
APC: Antigen-presenting cell
ATP: Adenosine triphosphate
CD127: IL-7 receptor α
CD132: common γ
Bcl: B-cell lymphoma
CFSE: Carboxyfluorescein succinimidyl ester
DMSO: Dimethyl sulfoxide
FACS: Fluorescence-activated cell sorting
FBS: Fetal bovine serum
FMO: Fluorescence minus one
FS: Forward scatter
GALT: Gut-associated lymphoid tissue
HAART: Highly active antiretroviral therapy
HBSS: Hank's Balanced Salt Solution
HIV: Human immunodeficiency virus
IFN: Interferon
IL: Interleukin
IQR: Interquartile range
LPS: Lipopolysaccharide
MFI: Mean fluorescence intensity
MIP: Macrophage inflammatory protein
PBS: Phosphate-buffered saline
PdBU: Phorbol 12,13-dibutyrate
PE: Phycoerythrin
PECy or PC: Phycoerythrin cyanin
PFA: Paraformaldehyde
PHA: Phyothaemagglutinin
PI3K: Phosphoinositide 3-kinase
PMA: Phorbol 12-myristate 13-acetate
RORC: Retinoic acid-related orphan receptor C
RORα: Retinoic acid-related orphan receptor α
RORγt: Retinoic acid-related orphan receptor γt
Rpm: Revolutions per minute
RPMI: Roswell Park Memorial Institute
SEM: Standard error of the mean
SFB: Segmented filamentous bacteria
SIV: Simian immunodeficiency virus
SOCS: Suppressor of cytokine signalling
SS: Side scatter
STAT: Signal transducer and activation of transcription
TAC: Tetrameric antibody complex
TCR: T cell receptor
Th cell: Helper T cell

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1.0 INTRODUCTION

1.1 The innate and adaptive immune system: Innate and adaptive immunity are the two main branches that constitute the immune system, which protects the host from infectious disease and repairs damaged tissue [1]. First, innate lymphoid cells (ILCs), which constitute the innate immune system, trigger several inflammatory responses upon pathogen recognition [2]. Foreign antigens are recognized by patter-recognition receptors (PRRs), which are expressed on the surface of ILCs [3]. ILCs have been divided into different groups based on function. Cytotoxic ILCs, such as natural killer cells, provide defense against intracellular pathogens, or against host cells that have been altered, such as malignant cells [4]. The remaining ILCs, of which are non-cytotoxic, mediate immune responses against various infectious agents through cytokine secretion, and repair damaged tissue [5]. In addition, granulocytes, such as neutrophils, kill pathogens by oxidative stress [6]. Finally, antigen-presenting cells (APCs), including dendritic cells and macrophages, engulf pathogens through a process known as phagocytosis, and function as a liaison between the innate and adaptive branches [7].

The second main branch, known as the adaptive immune system, becomes activated in the lymph nodes [8]. The main role of the adaptive immune system is to establish "immunological memory," in which stronger responses are elicited against the same antigen following first-time exposure, resulting in rapid clearance [9]. Adaptive immune cells are therefore able to recognize subtle differences between antigens [10], unlike innate cells, which only recognize whether antigens are foreign, and thus elicit responses of the same intensity against the same antigen regardless of prior exposure [11]. It has recently been suggested, however, that innate immune cells are also capable of retaining immunological memory [12].

The adaptive immune system is composed of B cells and T cells. B cells function as APCs and regulate humoral immunity through antibody-mediated responses [13, 7]. T cells, on the other hand, are further divided into two major subgroups. Cellular immunity is regulated by CD8+ T cells, which eliminate infected and altered cells through immune-mediated death [14, 15]. The other subgroup consists of CD4+ T cells, known as helper T (Th) cells, which regulate a plethora of effector functions carried out by both innate and adaptive immune cells [16].

1.2 CD4+ T cells: development and function: T cell precursors originate in the bone marrow, then enter the thymus to undergo maturation into either CD4+ or CD8+ T cells [17]. Once early development is complete, naïve CD4+ T cells, which have yet to be exposed to antigens, enter circulation and migrate to the secondary lymphoid organs [18]. Naïve CD4+ T cells then undergo clonal expansion into short-lived CD4+ effector T cells following antigen-mediated activation. This process involves interactions between the T cell receptor (TCR) and segments of antigens, which are bound to the surface of APCs via the major histocompatibility complex II (MHC II) [19, 20]. Antigen-induced activation is further enhanced by co-stimulatory signals mediated through the surface protein CD28, expressed by T cells [21]. CD4+ effector T cells induce several immune responses through secretion of pro-inflammatory cytokines. Once the infection is cleared, the CD4+ effector T cells undergo immune-mediated apoptosis, which marks the contraction phase of the inflammatory response [22]. A small percentage of CD4+ effector T cells remain viable and transition into CD4+ memory T cells, which are resting cells that retain immunological memory and provide life-long protection to the host [23]. These memory cells consist of CD4+ effector-memory T cell and CD4+ central-memory T cell intermediates, which provide immunosurveillance in various tissues and lymph nodes, respectively [24].

Once activated, a small percentage of CD4+ effector and memory T cells become polarized into one of two classical Th cell lineages, represented by Th1 or Th2 cells. Lineage polarization is dependent on antigen-mediated TCR stimulation as well as the cytokine milieu secreted by surrounding immune cells, such as dendritic cells [25, 24]. These different lineages are reactive to specific antigens and thus exert unique effector functions. Moreover, each lineage is identified based on the differential production of cytokines, and expression of transcription factors and surface markers, which will be discussed below.

The differentiation of Th1 cells is dependent on IL-12, which is secreted by dendritic cells and macrophages [26, 27]. Th1 cells initiate cytotoxic responses against intracellular pathogens, including *Listeria monocytogenes* [27], *Toxoplasma gondii* [28], and hepatitis B through secretion of IFNγ [29]. Th1 cells are also identified based on expression the Th1-specific transcription factor T-bet [30] and surface marker CXCR3 [31], a chemokine receptor also expressed by CD8+ T and natural killer cells [32, 33]. In turn, Th2 cells activate macrophages to defend the host from extracellular parasites, such as helminths and nematodes [34], and are identified by expression of the chemokine receptor CCR4 [35] and Th2-specific transcription factor GATA3 [30]. Th2 polarization is dependent primarily on IL-4 [36], which is produced by various immune cells, including eosinophils and T cells [37, 38]. The main effector cytokines secreted by activated Th2 cells are IL-4, IL-5 and IL-13 [39].

More recently, two additional Th cell lineages have been established, which are represented by the immunosuppressive regulatory T cells (T_{regs}) and pro-inflammatory Th17 cells. The co-existence of these two lineages is essential for proper health and immune function, and some cytokines that foster polarization of one lineage will antagonize

polarization of the other lineage [40, 41, 42]. Induced by TGFβ [41], T_{regs} promote tolerance to self antigens and prevent allergenic and autoimmune responses [43]. T_{regs} are identified by expression of the T_{reg}-specific transcription factor Forkhead box P3 (FoxP3) and the IL-2 receptor α component CD25 [44]. Finally, Th17 cells are defined by expression of their main effector cytokine, IL-17A, and the Th17-specific transcription factor retinoic acid-related orphan receptor γt (RORγt) [45]. Th17 cells are also largely implicated in the pathogenesis of autoimmune diseases [46].

1.3 The roles of Th17 cells: Th17 cells mainly reside in the gut-associated lymphoid tissue (GALT), but are also located in the bloodstream, tonsils, bronchioles and cervical tissues [47, 48, 49]. Through secretion of several effector cytokines, including IL-17A and IL-22, Th17 cells regulate mucosal homeostasis and immune responses in the GALT [50]. When cultured in the presence of pathogens including *Staphylococcus aureus*, *Candida albicans* and *Staphylococcus pneumonia*, Th17 cells undergo variable degrees of proliferation [51], indicating that these cells exhibit different degrees of antigen specificity. Moreover, culturing CD4+ T cells with *Staphylococcus aureus, Streptococcal kinase*, tetanus toxoid, or *Candida albicans* induces IL-17A production [48].

IL-17A, the main Th17 effector cytokine, initiates several homeostatic and pro-inflammatory responses [50]. IL-17A maintains the structural integrity of the intestinal epithelium [52], and is essential for defense against various pathogens, such as *S. typhimurium*, *Pseudonomas aeruginosa* and *C. albicans* [53, 54, 55]. IL-17A induces production of antimicrobial peptides and chemokines in epithelial cells [56, 57], which promote neutrophil chemotaxis [58]. In rhesus macaques, IL-17A prevents intestinal *S. typhimurium* infection from becoming systemic [59]. Moreover, IL-17A is involved in the onset and severity of autoimmunity [60].

1.4 Th17 cell development and the intestinal microenvironment: Th17 cell differentiation occurs as a result of multiple processes, which have mostly been characterized in mice. *In vitro*, IL-6, TGFβ and IL-21 promote Th17 cell development by inducing IL-17A gene expression in naïve CD4+ T cells [42, 61]. Together, in murine CD4+ T cells, IL-6 and TGFβ trigger expression of RORα and RORγt, transcription factors that upregulate IL-17A gene expression [62]. TGFβ promotes Th17 cell polarization by suppressing that of Th1 cells [63]. Interestingly, in small concentrations, TGFβ favors Th17 polarization, while in larger concentrations, it promotes T_{reg} polarization by inducing gene expression of FoxP3 and simultaneously antagonizing that of IL-17A [41]. On the contrary, IL-6 promotes RORγt expression, thereby favoring Th17 cell differentiation [41]. IL-6 also induces phosphorylation of signal transducer and activator of transcription 3 (STAT3), a transcription factor that enhances RORγt expression [64]. Furthermore, IL-6 induces IL-21 production in the polarizing CD4+ T cells, which further enhances Th17 development through phosphorylation of STAT3 and activation of RORγt, while simultaneously increasing its own production through mechanisms of positive feedback [42]. In addition to IL-6, IL-21 and TGFβ, IL-1β also promotes Th17 cell development. Specifically, in mice, IL-1β has been shown to downregulate suppressor of cytokine signaling 3 (SOCS3), which inactivates STAT3, to promote Th17 cell differentiation and simultaneously prevent T_{reg} differentiation [65].

Finally, IL-23, which is produced by dendritic cells and macrophages [66, 67], also regulates Th17 cell polarization and effector function by inducing IL-17A expression [68]. In murine CD4+ T cells, IL-23 induces phosphorylation of STAT3 and simultaneously inhibits Th1 and T_{reg} polarization [69]. IL-23 also promotes expression of RORγt and multiple Th17 effector cytokines, such as IL-17A, IL-17F and IL-22 [70, 69]. IL-23 is also

implicated in Th17-mediated autoimmunity, as gut-derived CD4+ T cells from patients with colitis express elevated levels of IL-23R, IL-17A and RORC, the gene encoding RORγt [71].

The symbiotic interplay between intestinal immune cells and the microbiota is influential in regulating mucosal homeostasis and immune responses. In the intestine, the microbiota promotes immunoglobin secretion and prevents pathogen growth on the epithelium [72]. In particular, Th17 differentiation is dependent on the microenvironment in the intestines. In mice, adenosine triphosphate (ATP) generated by the micro flora in the small intestine induces IL-17A production in CD4+ T cells, thereby favoring Th17 cell polarization [73]. Studies have shown that segmented filamentous bacteria, presented by dendritic cells and macrophages, play a pivotal role in Th17 polarization [74, 75]. Furthermore, in mice, gut-residing microbes were shown to regulate IL-23 production in both dendritic cells and macrophages [76]. In summary, Th17 cell development is regulated by a discrete cytokine milieu, which is influenced by the intestinal microenvironment.

1.5 Th17 cell phenotype and plasticity: Th17 cells are CD4+ T cells defined primarily by their production of IL-17A [77], although evidence suggests that this definition is not entirely accurate (to be described below). As Th17 cells are functionally volatile, they are difficult to study as a single population [78]. Moreover, no single surface marker has been established to be expressed exclusively by Th17 cells and, consequently, a definitive phenotype remains to be defined. Despite these limitations, others have studied unique combinations of surface markers on different Th lineages and established that CD4+ memory T cells bearing the CCR6+ CCR4+CXCR3- phenotype exhibit characteristics of Th17 cells, as exemplified by expression of IL-17A and RORγt [79, 80, 81].

CCR6 is a chemokine receptor that promotes the chemotaxis of Th17 cells to the GALT upon interaction with its ligand, macrophage inflammatory protein-3α (MIP-3α) [82, 83]. CCR6 is a well-established surface marker for Th17 cells [51, 80], mainly because IL-17A expression [84, 85] and RORC expression [86] are primarily evident in CCR6+ cells but rare among CCR6- cells. Moreover, CCR6+ cells play an active role in autoimmunity and hypersensitivity [87, 88]. IL-17A and CCR6 expression do not exclusively identify Th17 cells, however, as different Th subsets occur within CCR6+ cells [78].

First, Th1/Th17 cells, which are related to, but different from, conventional Th17 cells, demonstrate Th17 characteristics through expression of CCR6, IL-23R, RORγt and IL-17A. Unlike Th17 cells, however, Th1/Th17 cells simultaneously demonstrate Th1 characteristics through expression of IFNγ, T-bet and CXCR3 [51]. Accordingly, Th1/Th17 cells could be distinguished from Th17 cells based on expression of CXCR3 [51]. Furthermore, Th1/Th17 cells exhibit antigen specificity different than that of Th17 cells, as they become activated by *Chlamydia muridarum* [89], and *Mycobacterium tuberculosis* antigen [80], whereas Th17 cells become activated by different antigens, such as tetanus toxoid [48]. In addition, while both Th17 and Th1/Th17 cells are responsive to *C. albicans* and *S. aureus*, Th17 cells exhibit a higher degree of reactivity, defined by DNA synthesis, in response to the two pathogens [51]. Furthermore, a small frequency of Th17 cells (approximately 10%) was found to be responsive to *Escherichia coli* stimulation, which is lower than what was observed for Th1/Th17 cells (roughly 40%) [51].

Next, CCR4 identifies Th2 cells [35], but also identifies Th17 cells when expressed in combination with CCR6 [80, 81]. One study has shown that Th17 cells can be further characterized by expression of CCR4 and CCR6 in the absence of CXCR3 and CCR10 [51].

CCR10 is a marker that identifies Th22 cells, which represent a newly-discovered lineage involved in epidermal protection [90]. In addition, Th2/Th17 cells, which express both IL-4 and IL-17A, as well as GATA3 and RORγt [91], can potentially be identified by co-expression of CCR6 and CCR4 [92]. Th2/Th17 cells mediate bronchial inflammation, and are implicated in the pathogenesis of asthma [91].

As mentioned, Th17 cells exhibit functional fluidity and polarize into other Th subsets [78], such as Th1 cells [93]. One study has shown that Th17 cells can be stimulated to express IFNγ in the absence of T-bet [51]. To further support the notion of Th17 cell plasticity, CD4+CCR6+ memory T cells expressing CCR4 or CXCR3 were found to express RORC mRNA in combination with T-bet mRNA or GATA3 mRNA, respectively. In addition to IL-17A, IFNγ and IL-4 expression was also evident in the aforementioned cells [80, 81]. In addition, IL-17A, RORγt CCR6 and CCR4 expression has also been reported in T_{regs}, suggesting that these immunosuppressive cells can also acquire a pro-inflammatory role [94].

Evidence indicates that cytokines and different antigens induce Th17 cell plasticity. For example, one study demonstrated that Th17 cells undergo polarization into Th1/Th17 cells in response to Th1-polarizing cytokines [95]. In agreement with this finding, Duhen et al. reported that culturing Th17 cells in the presence of IL-1β and IL-12 promotes their polarization into Th1/Th17 cells [51]. Furthermore, another study has shown that *in vitro*-generated Th17 cells primed with *C. albicans* take on a Th1/Th17 functional role by co-expressing IL-17A and IFNγ, whereas those primed with *S. aureus* take on a Th17 functional role by expressing IL-17A in the absence of IFNγ [96].

In summary, IL-17 expression is only evident in CCR6-expressing CD4+ T cells [84, 85]. Furthermore, on CD4+ memory T cells, the Th17 cell phenotype is characterized as being CCR6+CCR4+CXCR3- [79, 80, 81]. In this study, Th17 cells will be identified as $4_3 6$ cells (CD4+CXCR3-CCR6+ memory T cells), and those that produce IL-17A will be defined as functionally active Th17 cells.

1.6 Consequences of HIV infection: HIV infection results in a continuous decline in CD4+ T cell populations and immune system dysfunction, leading to overall impairment of the immune system and a compromised ability of the host to clear infections [97]. Lower CD4 counts and higher plasma viremia are standard markers of disease progression and are thus associated with a greater possibility of developing acquired immunodeficiency syndrome (AIDS) in a shorter period of time [98]. Patients who have progressed to the AIDS stage of infection typically present with extremely low CD4 counts (<200) and develop rare, opportunistic infections that cannot be efficiently cleared [99]. Furthermore, HIV establishes a life-long latent infection in CD4+ cells, defined as an inactive state of infection in which the viral DNA is integrated into the host genome [100].

While there is currently no cure, the progression of HIV infection to AIDS has been dramatically delayed over the past few years by highly active anti-retroviral therapy (HAART), which suppresses life cycle progression of the virus and increases circulating CD4+ T cells in most patients [101]. Despite the major advances with HAART, some patients are unable to exhibit increased peripheral CD4+ T cells despite successful suppression of plasma viral loads [102]. Moreover, in the GALT, CD4+ T cell restoration is often delayed and incomplete, even with long-term therapy [103, 104].

1.7 Non-AIDS complications and implications of Th17 cells: Despite the advances of HAART in preventing AIDS-associated illnesses, a high mortality rate remains evident among most individuals living with HIV due to other, non-AIDS-related complications, such as cardiovascular and metabolic disorders [105]. Such complications are thought to develop as a result of chronic inflammation, an additional indicator of disease progression characterized by upregulated markers of immune cell activation as well as pro-inflammatory cytokines [105, 106]. Furthermore, patients often present with circulating microbial components, including lipopolysaccharide (LPS) and LPS-binding protein (LBP) [107], which are implicated in causing systemic inflammation [108].

While the causes of inflammation and circulating microbial products are poorly understood, evidence suggests that abnormalities in the GALT act as a driving factor. In the context of both Simian Immunodeficiency Virus (SIV) and HIV infections, studies suggest that damage to the intestinal mucosa allows for the translocation of bacterial components into circulation [108, 109, 110], which is associated with altered GALT immunity [111]. Furthermore, alterations in the composition of intestinal microbial populations in patients, such as increased frequencies of Proteobacteria and reduced frequencies of Bacteroides, are correlated with increased markers of microbial translocation and indicators of immune activation [112].

In addition to the above complications, Th17 cells become functionally impaired and rapidly diminished from the GALT following HIV infection, which is associated with microbial translocation and chronic inflammation [113, 48, 114]. Furthermore, Th17 cells have also been reported to be depleted from the bloodstream [113, 115] and cervical tissue [49] during HIV infection. Importantly, maintaining functional Th17 cells and overall GALT immunity is implicated in controlling chronic inflammation during HIV infection

[113, 114, 116, 117]. While others have presented evidence suggesting that Th17 cells are more liable to HIV infection than other Th cells [81, 118, 119], the mechanisms by which Th17 cells become depleted are poorly understood. Th17 dysfunction may occur as a result of impaired IL-23 signalling, as previous members in our lab have shown that patient-derived Th17 cells exhibit reduced STAT3 gene expression and phosphorylation in response to IL-23 (Fernandes et al., manuscript in preparation), while others have shown that Th17 polarization in patient-derived naïve CD4+ T cells is impaired [120]. Alternatively, some evidence indicates that Th17 cells may acquire immunosuppressive roles during HIV infection [113], and even become polarized into T_{regs} [121].

Generally, a longer duration of HAART leads to more promising outcomes, such as a higher degree of Th17 cell recovery and considerable suppression of associated microbial translocation and inflammation [113]. In particular, such outcomes are observed when HAART is commenced during the earliest stages of infection, before HIV-specific antibodies can be measured [114]. As it may be difficult and sometimes impractical to detect HIV infection at such an early stage, alternative therapeutic approaches resulting in complete Th17 cell reconstitution would be essential for treated patients who are unable to fully restore Th17 cells [114].

1.8 Mechanisms underlying Th17 cell apoptosis during HIV infection: While the events resulting in CD4+ T cell depletion are under continued investigation, studies have concluded cell death to be a major cause, resulting from several mechanisms, including those directly mediated by the virus or inflammation [122, 123]. These phenomena are less characterized in Th17 cells, although one study has documented increased levels of pro-apoptotic and pro-pyroptotic mediators in Th17 cells following *in vitro* HIV infection [119]. This finding

suggests that Th17 cells also undergo apoptosis and pyroptosis during infection, although the causative mechanisms are unknown.

As a result, many studies have been focusing on the potential effects of HIV infection on mechanisms of homeostasis and survival in T cells. In particular, IL-7 is well known to regulate T cell homeostasis [124], and has frequently been studied in the context of HIV infection [125]. Responses to IL-7 become dysregulated in both CD8+ and CD4+ T cells during infection, which in turn negatively affects the mechanisms that regulate cell growth and survival [126, 127]. These mechanisms have been scarcely reported in Th17 cells.

1.9 IL-7 biology and homeostatic responses: IL-7, which is generated by stromal cells and intestinal epithelial cells [128, 129], regulates T cell growth and survival [124], and even promotes lymph tissue development in the GALT [129]. IL-7 also regulates Th17 cell polarization, although whether it favors or disfavors Th17 polarization remains debatable. One study has shown that IL-7 further upregulates IL-17A expression in murine CD4+ T cells [130], while another study has found that IL-7 reduces RORγt and IL-17A expression in murine CD4+ T cells, and instead promotes Th1 cell polarization [131]. Furthermore, IL-7 enhances IL-17A production in human Th17 cells [86]. In summary, the roles of IL-7 in Th17 cell homeostasis require further characterization.

IL-7 mediates its effects through interaction with the IL-7 receptor, consisting of the unique α chain CD127 and common γc chain CD132 [124]. Virtually all CD4+ memory T cells express CD127 [132, 133]. Furthermore, CD127 expression has even been reported on murine and human Th17 cells [130, 131, 51, 134]. In lymphopoenic conditions, IL-7 levels become upregulated to promote T cell expansion, which is observed in parallel with suppressed expression of CD127 [135]. Following receptor binding, IL-7 has been shown to

downregulate CD127 through mechanisms of homeostasis [136, 137]. Upon T cell reconstitution, IL-7 levels and CD127 expression revert back to normal [135,136].

Upon interaction with CD127, IL-7 elicits its homeostatic effects by inducing phosphorylation of JAK1 & 3/STAT5 [138]. Phosphorylated STAT5 (pSTAT5) is mainly involved T cell survival [139], but has also been shown to enhance proliferation [140]. IL-7 induces pSTAT5 expression in murine Th17 cells [130], although whether the same phenomenon occurs in human Th17 cells is unknown. IL-7 also induces phosphorylation of phosphoinositide 3-kinase (PI3K) and Akt [138], which promotes T cell metabolism and expansion [141, 142]. In murine CD4+ T cells, TCR-mediated PI3K/Akt signalling promotes IL-17A and IL-23 receptor gene expression [143], although the effects of IL-7 in this matter have not been investigated. Furthermore, another study has demonstrated that IL-7 enhances IL-17A expression in Th17 cells through PI3K/Akt signalling [86].

In addition, B cell lymphoma 2 (Bcl-2) is a protein that prevents mitochondria-induced apoptosis [144]. Following IL-7 stimulation, Bcl-2 becomes further upregulated, which is dependent on JAK1 & 3/STAT5, but not PI3K/Akt [132]. Others have demonstrated, however, that this particular response is, in fact, dependent on PI3K/Akt [145]. While IL-7 has been shown to upregulated Bcl-2 in murine Th17 cells [130], this particular response has yet to be characterized in human Th17 cells.

1.1.0 HIV infection and IL-7 responses: As spontaneous apoptosis plays an active role in reducing CD4+ T cell counts during HIV infection [122, 123], many have studied CD127 expression and IL-7 responses in T cells in this regard. It is well known that CD127 becomes downregulated on both CD8+ and CD4+ T cells, while CD132 and circulating levels of IL-7 become upregulated [146, 147, 148]. The degree of CD127 downregulation is associated with inflammation and reduced CD4+ T cell counts, and therefore indicates the

degree of disease progression [147]. Furthermore, HIV tat protein has been shown to directly downregulate CD127 on CD8+ and CD4+ T cells, suggesting a direct role for viral components in altering receptor expression [149, 133]. The effects of HAART on CD127 are somewhat promising, as some studies report complete or near complete restoration on CD4+ memory T cells [127], while others report complete recovery on CD8+ memory T cells but partial recovery on central memory CD4+ T cells [150]. In Th17 cells, the effects of HIV infection on CD127 expression are unknown.

During HIV infection, IL-7 effector functions also become dysregulated in T cells, which is associated with decreased CD4+ T cell counts [151]. Furthermore, evidence suggests that these perturbations in IL-7 responses occur independently of CD127 expression [152, 153]. In blood-derived CD127+ CD8+ T cells from treated patients, IL-7-induced proliferation and phosphorylation of STAT5 become reduced [126]. STAT5 phosphorylation and Bcl-2 expression in CD4+ memory T cells are also altered in response to IL-7, and become completely and partially normalized by HAART, respectively [127]. Furthermore, IL-7-mediated proliferation of CD4+ effector memory T cells derived from HIV-infected individuals becomes suppressed [154]. These findings collectively demonstrate that dysfunctional IL-7 signalling occurs in CD8+ and CD4+ T cells during HIV infection. Whether HIV infection compromises IL-7 responses in Th17 cells has not yet been studied.

1.1.1 Hypothesis: HIV infection dysregulates IL-7 responses in Th17 cells.

1.1.2 Rationale: Characterizing CD127 expression and IL-7 signalling mechanisms in Th17 cells will improve our understanding of the basic homeostatic responses that regulate survival and proliferation in this lineage. Determining whether such phenomena become altered in HIV-infected individuals receiving HAART may delineate the mechanisms accountable for preventing the complete reconstitution of Th17 cells, and may therefore provide alternative targets for therapeutic intervention. As indicated elsewhere [113, 114], maintaining functional Th17 cells during HIV infection may substantially control the degree of microbial translocation and chronic inflammation, which could ultimately reduce the mortality rate associated with non-AIDS complications.

1.1.3 Statement of Objectives:

i) To characterize CD127 expression on Th17 cells:

Specific aim i) a: To measure CD127 expression on all Th17 cells, defined as $4_3$6 cells

Specific aim i) b: To measure CD127 expression on active, IL-17A-producing Th17 cells

ii) To evaluate IL-7 signalling in Th17 cells:

Specific aim ii) a: To measure the effects of IL-7 on expression of pSTAT5 in Th17 cells

Specific aim ii) b: To investigate the effects of IL-7 on Bcl-2 expression in Th17 cells

Specific aim ii) c: To determine the role of IL-7 in Th17 cell proliferation

iii) To examine the effects of HIV infection on IL-7 responses in Th17 cells from patients receiving HAART

Specific aim iii) a: To measure CD127 expression on Th17 cells from patients

Specific aim iii) b: To determine the ability of IL-7 to induce pSTAT5, Bcl-2, and proliferation in Th17 cells derived from patients

2.0 MATERIALS AND METHODS:

2.1 Study participants: Uninfected individuals (N=23) and HIV-infected individuals receiving HAART (presenting with total CD4 counts > 250 cells/µL of blood, and viral loads <50 copies/mL for ≥ six months) (N=27) were recruited at the Ottawa General Hospital and Faculty of Medicine at the University of Ottawa, Ottawa, Ontario, Canada. The study was approved by the Ottawa Health Science Network Research Ethics Board. Clinical parameters of patients are summarized in Table 1.

2.2 PBMC isolation and cell culture: Heparin (100 international units (i.u.)/mL) (LEO Pharma, Thornhill, ON) was added to the syringes at 1 mL/60 mL of blood. Blood was collected from consenting participants, then poured over Lymphoprep™ (STEMCELL Technologies, Vancouver, BC) and centrifuged at 1600 rpm (revolutions per minute) for 30 minutes (brakes off). Buffy coat (containing PBMCs) was collected and topped to 50 mL with 1x Hank's Balanced Salt Solution (HBSS):phosphate buffered saline (PBS) (Gibco® Life Technologies, Waltham, MA), then centrifuged at 1200 rpm for 20 minutes. In some cases, cells were washed again with 50mL 1x HBSS:PBS at 1600 rpm for 10 minutes. PBMCs were then resuspended in 10mL 1x HBSS:PBS and centrifuged at 1600 rpm for 10 minutes, then stored or used immediately to sort bulk CD4+ T cells or Th17 cells. All cells were stored at 37˚C and 5% CO_2 conditions in 1x Roswell Park Memorial Institute (RPMI) supplemented with 10% fetal bovine serum (FBS), 10% L-glutamine, and 5% penicillin/streptomycin (Gibco® Life Technologies, Waltham, MA). Furthermore, all stimulation assays were carried out one day following isolation, unless otherwise specified.

2.3 CD4+ T cell isolation: Bulk CD4+ T cells were purified from PBMCs by negative selection using the EasySep™ Human CD4+ T cell Enrichment Kit (STEMCELL Technologies, Vancouver, BC). The manufacturer's protocol [155] had been slightly

modified to increase CD4+ T cell purity, represented as the percentage of cells expressing CD4 (Fig. 1B). PBMCs were resuspended in CD4+ T cell separation buffer (2% FBS and 2mM EDTA in PBS) at 50 million cells/mL. The enrichment cocktail (containing antibodies targeting CD8, CD14, CD16, CD19, CD20, CD36, CD56, CD66b, CD123, TCRγ/δ, glycophorin A, and dextran) was added to the cells at 50μL/mL for 10 minutes at room temperature. D magnetic particles (TRIS buffer containing dextran-coated, magnetic iron particles) were added to the cells at 100μL/mL for 5 minutes at room temperature. Cells were topped with CD4+ T cell separation buffer to a total volume of 2.5mL (25 – 100 million starting PBMCs), 5mL (100-200 million starting PBMCs) or 10mL (200-400 million starting PBMCs). Cells were incubated in the magnet for 5 minutes at room temperature. The supernatant (containing CD4+ T cells) was collected and incubated in the magnet for an additional 5 minutes. The CD4+ T cells were centrifuged at 1600rpm for 10 minutes, then resuspended in 1x RPMI at a concentration of 1 or 2 million cells/mL. Cells were either immediately assessed for expression of surface markers or stored overnight for use in future experiments.

2.4 Th17 cell enrichment: CD4+CXCR3-CCR6+ memory T cells (Th17 cells) were enriched from PBMCs using EasySep^{TM} Th17 Human Enrichment Kit (STEMCELL Technologies, Vancouver, BC). The manufacturer's protocol involves two steps, with the first being negative selection of CD4+CXCR3- memory T cells, and the second being positive selection of CCR6+ cells [156]. The manufacturer's protocol was slightly modified to increase Th17 cell purity.

To purify CD4+CXCR3- memory T cells, PBMCs were resuspended in the recommended buffer (referred to as Th17 enrichment buffer, which is composed of 2% FBS and 1mM EDTA in PBS, free of Ca^{+2} and Mg^{+2}) at 50 million cells/mL. The CD4+CXCR3-

memory T Cell Pre-Enrichment Cocktail (TACs targeting CD8, CD14, CD16, CD19, CD20, CD36, CD56, CD66b, CD123, TCRγ/δ, glycophorin A, CD45RA^{high}, CXCR3 and dextran) was added to the cells at 50 µL/mL for 10 minutes at room temperature. Magnetic D2 particles (Tris buffer containing dextran coated, magnetic iron particles) were then added to the cells at 100 µL/mL for 5 minutes at room temperature. Cells were topped with Th17 enrichment buffer to a total volume of 2.5 mL (25-100 million starting PBMCs), 5 mL (100-200 million starting PBMCs), or 10 mL (200-400 million starting PBMCs), then placed in the magnet for 10 minutes at room temperature. The supernatant, containing the CD4+CXCR3- memory T cells, was collected then placed in the magnet for an additional 5 minutes. The CD4+CXCR3- memory T cells were centrifuged at 1100rpm for 10 minutes then used for the positive selection of CCR6+ cells.

To sort CCR6+ cells, the CD4+CXCR3- memory T cells were resuspended in Th17 enrichment buffer (250 µL for 25-125 million starting PBMCs, 1 mL for 125-200 million starting PBMCs, or 2 mL for 200-400 million starting PBMCs). The CCR6 Positive Selection Cocktail (TACs targeting CCR6 and dextran) was added to the cells at 50 µL/mL for 15 minutes at room temperature. Next, nanoparticles (Tris buffer containing dextran-coated, magnetic iron nanoparticles) were added to the cells at 100 µL/mL for 10 minutes at room temperature. Cells were topped with Th17 enrichment buffer to a total volume of 2.5-5 mL (25-100 million starting PBMCs), 5 mL (100-200 million starting PBMCs), or 8-10 mL (200-400 million starting PBMCs). Cells were then placed in the magnet for 5 minutes at room temperature. The supernatant containing the CCR6- cells was discarded, and the remaining CCR6+ cells were resuspended in Th17 cell enrichment buffer (same volumes as described) and incubated in the magnet for a total of three times. The sorted Th17 cells were

then washed, resuspended in 1x RPMI, and either assessed for expression of surface markers or stored overnight for use in experiments.

The CCR6 positive selection protocol was modified separately for patient-derived cells. CD4+CXCR3- memory T cells, which were always sorted from <100 million PBMCs, were resuspended in 250 µL Th17 enrichment buffer. The CCR6 Positive Selection Cocktail and nanoparticles were added at the same concentrations as described above. Cells were topped to 2.5 mL using Th17 enrichment buffer, then incubated in the magnet for a total of two times. Cells were stored as described.

2.5 Measuring expression of surface markers: To analyze expression of surface markers and thus characterize phenotypes of given cells, PBMCs, CD4+ T or Th17 cells were resuspended in 1% bovine serum albumin (BSA):PBS at 1 million cells/mL. A total of 50,000 cells per condition were stained in 50 µL 1% BSA:PBS for 30 minutes at 4˚C using anti-CD4-PC7 (clone A161A1, 2.5 µL), anti-CXCR3-Alexa Fluor 488 (clone G025H7, 2.5 µL of 1:10 dilution in 1% BSA:PBS), anti-CCR6-APC (clone G034E3, 5 µL), anti-CCR4-PE (clone L291H4, 2.5 µL), anti-CD161-PC7 (clone HP-3G10, 2.5µL), anti-CXCR4-PE (clone 12G5, 2.5 µL), anti-CCR5-PE (clone J418F1, 2.5 µL), anti-CD25-FITC (clone BC96, 2.5 µL) (BioLegend, San Diego, CA), or anti-CD127-PE (clone R34.34, 5 µL) (Beckman Coulter) as indicated in the Results/Figures section. Cells were washed once (centrifuged at 1600 rpm for 5 minutes unless otherwise indicated) with 1% BSA:PBS and resuspended in 400 µL PBS. Patient-derived cells were fixed using 1% paraformaldehyde (PFA) prior to flow cytometric analysis.

2.6 Gating strategies: All events were analyzed within the live cell gate. To set markers for multi-colored stains, fluorescence minus one (FMO) controls were used, which represent cells that have been stained with all antibodies used in one experiment except for the one

targeting the protein of interest. FMO controls were established using PBMCs or bulk CD4+ T cells, depending on availability, as sorted Th17 cells were limited in number. Unstained cells, or cells that had been stained with an isotype control, as indicated in each figure, were used to set auto-fluorescence for single-color stains. Depending on availability, these controls were established using Th17 cells, CCR6- cells, bulk CD4+ T cells, or PBMCs.

To measure Th17 frequencies within the cell population of interest, gating strategies described by the manufacturer were used (STEMCELL Technologies, Vancouver, BC) [156]. First, using the one-dimensional histogram, PBMCs were gated on total CD4+ lymphocytes. This gate was then applied to the two-dimensional scatter plot representing CXCR3 and CCR6 expression in order to determine the frequencies of CD4+ T cells expressing these two markers, in PBMCs and within gated, total CD4+ lymphocytes. The same gating strategy was applied to sorted Th17 cells to determine CD4+CXCR3-CCR6+ frequencies within the gated CD4+ lymphocytes.

To measure CD127 expression on Th17 cells, a Th17+ gate was created using the CXCR3-CCR6+ quadrant from the two-dimensional scatter plot, which had already been gated on CD4+ lymphocytes. On sorted Th17 cells, CD127 expression was represented as the percentage of gated Th17+ cells. On bulk CD4+ T cells, CD127 expression was represented as the percentage of gated CD4+ lymphocytes.

2.7 Measuring expression of intracellular IL-17A: The IL-17A staining protocol has been adapted from the manufacturer's (BD Biosciences, San Jose, CA) [157]. To quantify expression of intracellular cytokines, Th17 cells were stimulated at 1 million cells/mL in 1x RPMI for 6 hours (at 37˚C and 5% CO_2) using 50 ng/mL PMA and 1000 ng/mL ionomycin (R&D Systems, Minneapolis, MN). Golgi transport inhibitor Brefeldin A was added for the entire duration at 1 μL/1 mL of media, in accordance with the manufacturer's instructions

(BD Biosciences, San Jose, CA). Cells were washed once with 1% BSA:PBS and fixed for 10 minutes at 37˚C in 4% PFA (50 µL per 100,000 cells) (BDH GPR^{TM}, NASDAQ: VWR, Radnor, PA). Cells were washed once with 1% BSA:PBS and permeabilized for 30 minutes at room temperature using 0.5% saponin:PBS (150 µL/100,000 cells) (Sigma Aldrich, Oakville, ON). Cells were washed once with 0.5% saponin:PBS and resuspended in 0.5% saponin:PBS (60 µL per 100,000 cells). Cells were stained for 30 minutes at room temperature using anti-IL-17A-PC7 (clone eBio64DEC17) or isotype-PC7 (mouse IgG1κ, clone P3.6.2.8.1) (both 5 µL) (eBioscience, San Diego, CA). Cells were washed once with 0.5% saponin:PBS and resuspended in 400 µL PBS. To measure CD127 expression on Th17 cells that actively produce IL-17A, cells were stimulated as described, washed once with 1%BSA:PBS, and stained in 100 µL 1% BSA:PBS for 30 minutes at 4˚C using anti-CD127-PE (10 µL). Cells were washed once with 1% BSA:PBS, then subjected to the intracellular IL-17A staining protocol as described.

2.8 Measuring phosphorylation of STAT5: This staining protocol was modified from the one kindly provided by Dr. Angela Crawley and a Master's thesis (personal communication) at the Ottawa General Hospital/University of Ottawa [158]. To induce phosphorylation of STAT5, Th17 cells were stimulated at 1 million cells/mL 1x RPMI for 15 minutes (at 37˚C and 5% CO_2) using 0.01, 0.1 or 1 ng/mL IL-7 (R&D Systems, Minneapolis, MN), either one or two days following cell separation. Cells were washed once with PBS and fixed for 10 minutes at 37˚C using 4% PFA (50 µL per 100,000 cells). Cells were washed once with PBS and permeabilized for 10 minutes on ice using cold, 100% methanol (100 µL per 100,000 cells) (Fisher Scientific, Waltham, MA). Cells were washed once with PBS, resuspended in 100 µL 1% BSA:PBS, then stained for 30 minutes at room temperature using anti-pSTAT5-Alexa Fluor 488 (pY694, clone 47/Stat5(pY694) (RUO), 5 µL) (BD Phosflow, BD

Biosciences, San Jose, CA). Cells were washed once with 1% BSA:PBS and resuspended in 400 μL PBS prior to analysis.

2.9 Measuring expression of Bcl-2: This protocol was adapted from one kindly provided by Dr. Angela Crawley and a Master's thesis at the Ottawa General Hospital/University of Ottawa [158] (personal communication). To upregulate Bcl-2, Th17 cells were stimulated at 1 million cells/mL in 1x RPMI for 48 and 72 hours (at 37˚C and 5% CO_2) using 0.1, 1 or 5 ng/mL IL-7. Cells were washed once with PBS and fixed for 20 minutes at room temperature using 4% PFA (100 μL per 100,000 cells). Cells were washed once with PBS and permeabilized for 20 minutes at room temperature using 0.5% saponin:PBS (100 μL per 100,000 cells) in the presence of anti-Bcl-2-FITC (clone Bcl-2/100) or the isotype-FITC (mouse BALB/c IgG1κ, clone MOPC-21) (both 5 μL) (BD Pharmingen™, BD Biosciences, San Jose, CA). Cells were washed once with PBS and resuspended in 400μL PBS.

2.1.0 Measuring Th17 cell proliferation: To measure cell proliferation, Th17 cells were subjected to a carboxyfluorescein succinimidyl ester (CFSE) dilution assay, which was modified from the manufacturer's protocol (Molecular Probes - Cell Trace, Life Technologies, Waltham, MA) [159]. Cells were resuspended in PBS at 20 million cells/mL and stained for 8 minutes at 37˚C and 5% CO_2 using 2.5 μM CFSE (lyophilized powder was prepared in dimethyl sulfoxide (DMSO) at 5 mM then added to PBS at 1:1000, which was then added to the cells at equal volume) (Molecular Probes - Cell Trace, Life Technologies). Cold FBS was added at equal volume and cells were incubated for 10 minutes on ice. Cells were topped to 50 mL total volume with PBS and centrifuged at 1400 rpm for 10 minutes. Cells were resuspended at 1 million cells/mL in 1x RPMI and stimulated at 500,000 cells/mL for 5 days (at 37˚C and 5% CO_2) using 0.25 μg phytohemagglutinin (PHA) (R&D Systems, Minneapolis, MN) alone, or combined with 0.1, 1 or 10 ng/mL IL-7. This stimulation

protocol has been adapted from another study showing that IL-7-mediated proliferation is enhanced in the presence of PHA [126]. Following stimulation, cells were then diluted in 400 μL PBS then analyzed. Patient-derived cells were fixed in 2% PFA and topped to a total volume of 500 uL using PBS prior to flow cytometric analysis.

2.1.1 Data and Statistical analysis: All results were acquired using Beckman Coulter 500 flow cytometer (Beckman Coulter, Pasadena, CA), then analyzed using FCS Express 4 Flow Research Edition (De Novo Software™, Los Angeles, CA). Bar and line graphs were constructed using GraphPad Prism 5 (GraphPad Software, San Diego, CA). Results are expressed as mean values ± standard error of the mean (SEM), or median values with interquartile range (IQR) as indicated in each figure.

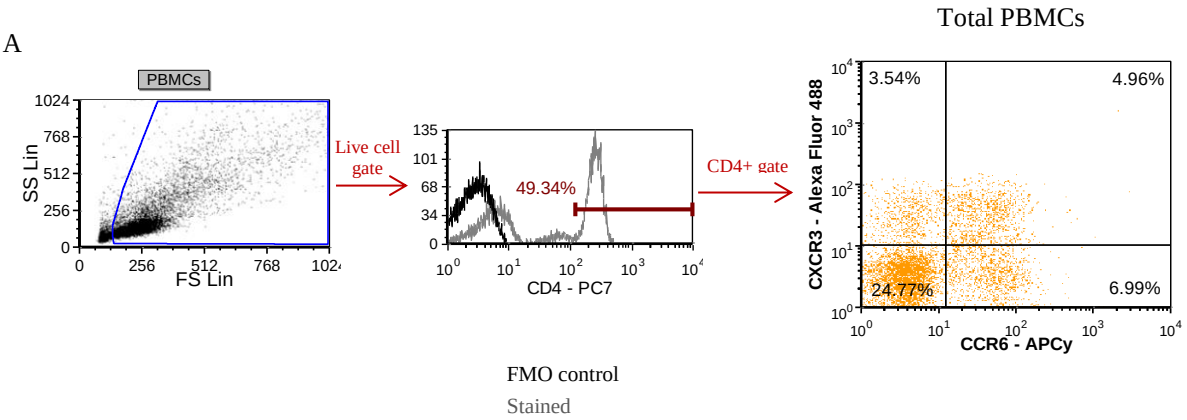
Statistical tests were also calculated using GraphPad Prism 5 based on GraphPad Statistics Guide (GraphPad Software, San Diego, CA) [160], and are indicated in each figure. Student's Unpaired and Paired T Tests (parametric) were used to calculate statistical significance between two sets of data, either from matched subjects or between two different groups of subjects, respectively. One-way ANOVA was used to determine whether IL-7 exerted a significant effect in a dose-dependent manner, while Dunnett's Post Hoc Test was used to determine which dose of IL-7 elicited a statistically significant effect. In each case, the distribution of the data was assumed to be normal. Finally, Mann Whitney Test (non-parametric) was used to determine whether two sets of data obtained from two different groups of study subjects reached statistical significance. In this case, the distribution of the data was not assumed to be normal.

3.0 RESULTS:

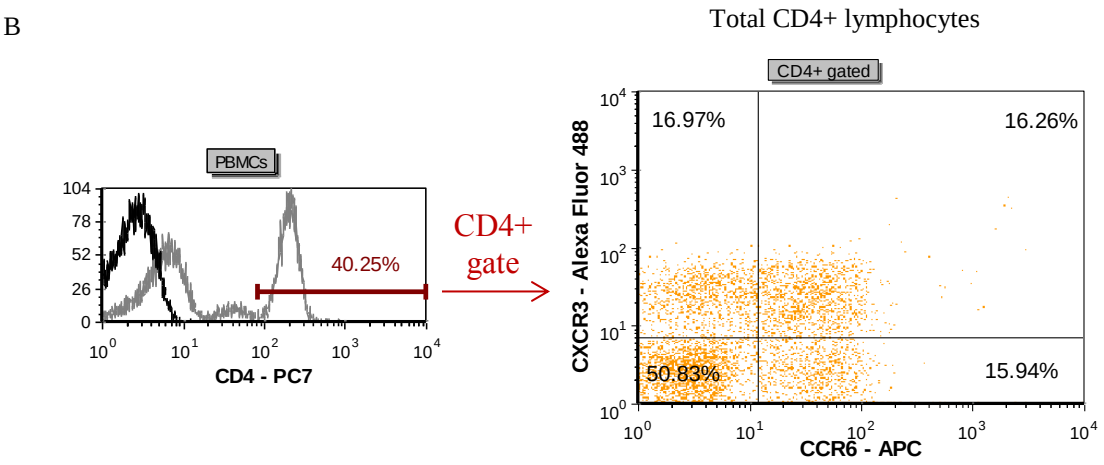
3.1 Characterizing the phenotype of Th17 cells. Th17 cell (4_36 cell) frequencies were evaluated in cells from uninfected individuals prior to and following magnetic separation by measuring expression of CD4, CXCR3 and CCR6. First of all, approximately 7% of PBMCs represented Th17 cells (Fig. 1A). Next, when analyzing Th17 cell frequencies within total CD4+ lymphocytes, roughly 15% expressed the Th17 phenotype (Fig. 1B), which is similar to previous findings [51, 81]. Following Th17 cell isolation, approximately 80% of the sorted cells represented Th17 cells (Fig. 1C), with values ranging between roughly 60-92% among the different donors. Despite this variation, CD4 expression was evident on roughly 95% of the cells, while CXCR3 expression was evident on approximately 1% of the cells (Fig. 1C). Overall, the majority of sorted cells demonstrate the Th17 cell phenotype.

Next, additional surface markers were measured on Th17 cells to further characterize their phenotype. First, CCR4 identifies Th17 cells when co-expressed with CCR6 [79 80], and was therefore measured on sorted cells. Next, CD161 is another characteristic marker of Th17 cells [79, 161], and was also evaluated on sorted cells. Approximately 65% Th17 cells expressed CCR4, while roughly 30% expressed CD161 (Fig. 1D). Furthermore, as $CD25^{hi}$ cells identify T_{regs} [162], expression of CD25 was measured to exclude the possibility of T_{regs} being present within the sorted population. Notably, CD25 was expressed by approximately 10% Th17 cells (Fig. 1D). Next, CXCR4 and CCR5 were also measured on the sorted cells, as their expression has been previously established on Th17 cells [81]. Nearly 90% Th17 cells expressed CXCR4, while roughly 20% Th17 cells expressed CCR5 (Fig. 1D).

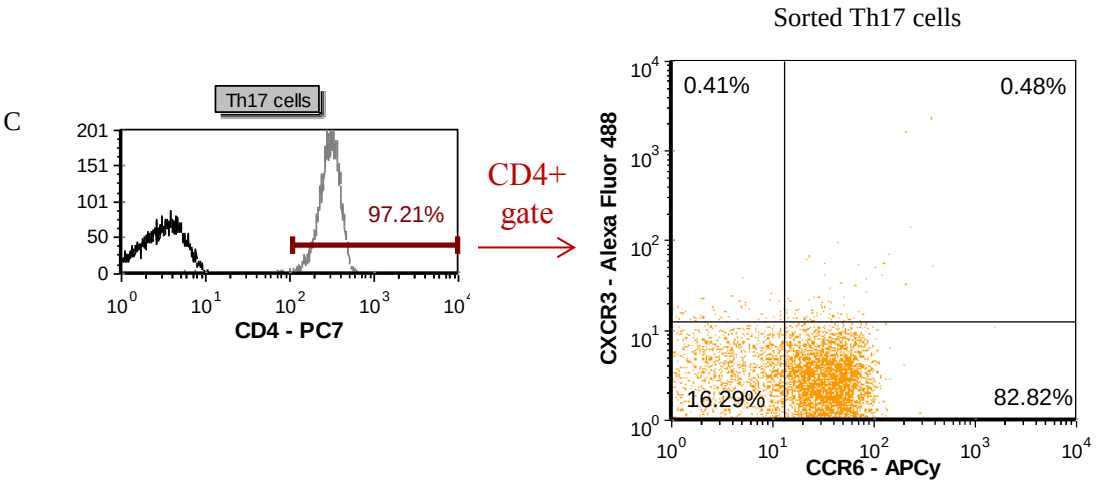
A


Total PBMCs
PBMCs
SS Lin
FS Lin
Live cell gate
49.34%
CD4 - PC7
CD4+ gate
CXCR3 - Alexa Fluor 488
CCR6 - APCy
3.54%
4.96%
24.77%
6.99%
FMO control
Stained

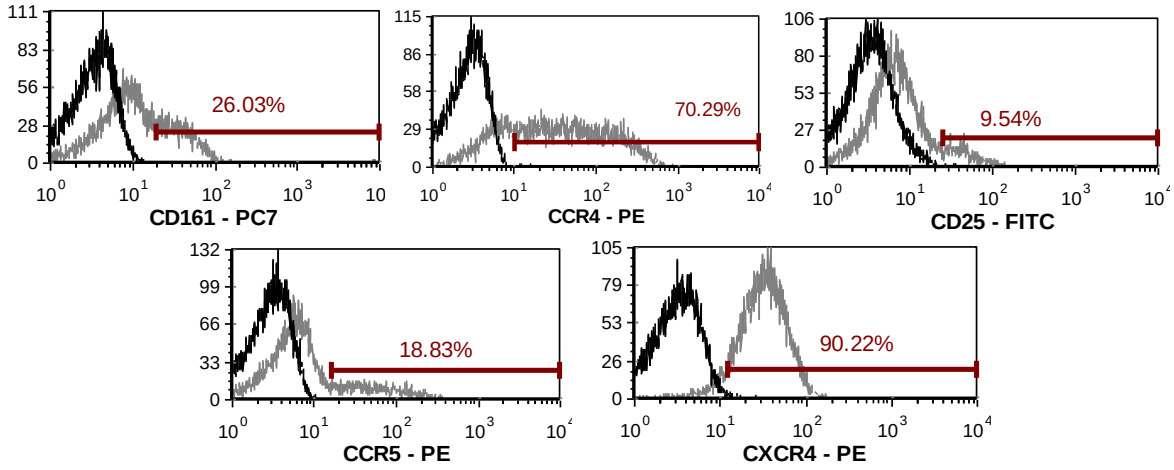
B


Total CD4+ lymphocytes
PBMCs
CD4 - PC7
40.25%
CD4+ gate
CD4+ gated
CXCR3 - Alexa Fluor 488
CCR6 - APC
16.97%
16.26%
50.83%
15.94%

C


Sorted Th17 cells
Th17 cells
CD4 - PC7
97.21%
CD4+ gate
CXCR3 - Alexa Fluor 488
CCR6 - APCy
0.41%
0.48%
16.29%
82.82%

D


26.03%
CD161 - PC7
70.29%
CCR4 - PE
9.54%
CD25 - FITC
18.83%
CCR5 - PE
90.22%
CXCR4 - PE

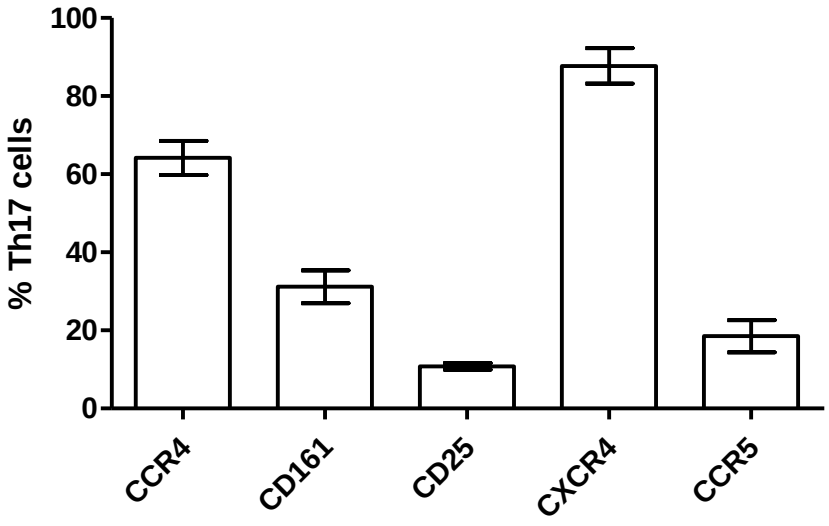
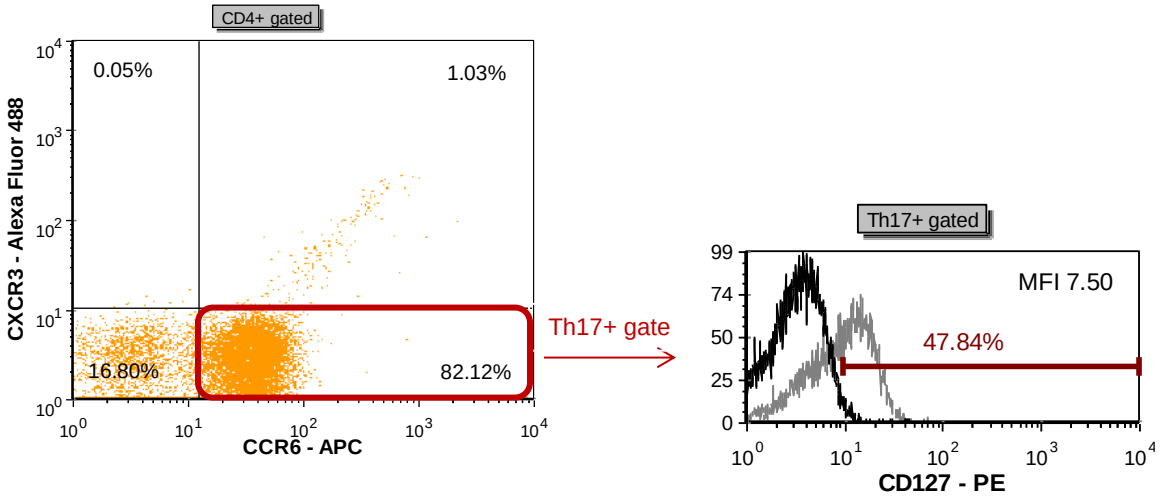

% Th17 cells
CCR4
CD161
CD25
CXCR4
CCR5

Figure 1: Sorted Th17 cells express a variety of Th17 lineage-associated surface markers. A) PBMCs are demonstrated in the FS Lin/SS Lin scatter plot, which represents size and granularity, respectively. Total CD4+ lymphocytes are represented in the histogram, and all CD4+ lymphocytes expressing CXCR3 and CCR6 are represented in the orange scatter plot. The frequency of Th17 cells ($4_3$6 cells) within PBMCs is represented in the lower right-hand corner of the yellow scatter plot, in which CD4+ cells are CXCR3-CCR6+, representative of one donor (mean=5% $\pm$0.35%, N=17). **B)** PBMCs were gated on CD4+ lymphocytes then evaluated for the Th17 ($4_3$6) phenotype, representative of one donor. The mean frequency of Th17 cells within total CD4+ lymphocytes is 14% $\pm$1.17 (N=17). **C)** The Th17 cell frequency following magnetic separation is represented in the histogram and orange scatter plot (mean=78% $\pm$1.77%, N=17). **D)** Expression of CCR4 (N=4), CD161 (N=6), CD25 (N=5), CXCR4 (N=5) or CCR5 (N=5) on sorted Th17 cells is demonstrated in the histograms, representative of one donor, and summarized in the bar graph. The mean frequency was 64% $\pm$4.38% for CCR4+ cells, 31% $\pm$4.23% for CD161+ cells, 11% $\pm$0.85% for CD25+ cells, 88% $\pm$4.55% for CXCR4+ cells, and 19% $\pm$4.16% for CCR5+ cells. Error bars represent SEM.

In conclusion, based on phenotypic analysis, these results confirm that 4_36 cells sorted from PBMCs accurately represent Th17 cells.

3.2 Th17 cells express CD127. Total CCR6+CD4+ memory T cells [118] and murine Th17 cells [130, 131] express high levels of CD127. Furthermore, others have indicated that Th17 cells occur amongst human CD127+ CD4+ memory T cells, respectively [51, 134]. CD127 expression has not been directly quantified on sorted, human Th17 cells, however. Therefore, to characterize CD127 expression on Th17 cells, expression of CD4, CXCR3, CCR6 and CD127 was measured on the sorted cells using flow cytometry. Notably, surface CD127 was expressed by 45% $\pm$4.32% Th17 cells (Fig. 2A), varying from roughly 10-75% between donors. Next, to compare CD127 expression on Th17 cells to that of CD4+ T cells, CD127 was evaluated bulk CD4+ T cells by measuring expression of both CD4 and CD127. Accordingly, CD127 expression was comparable to that of bulk CD4+ T cells, as 58% $\pm$5.64% cells expressed CD127 (Fig. 2B). Moreover, the mean fluorescence intensity (MFI) values for CD127 were approximately 7 in both groups of cells (Fig. 2B).

A


CD4+ gated
0.05%
1.03%
16.80%
82.12%
CXCR3 - Alexa Fluor 488
CCR6 - APC
Th17+ gate
Th17+ gated
MFI 7.50
47.84%
CD127 - PE

B

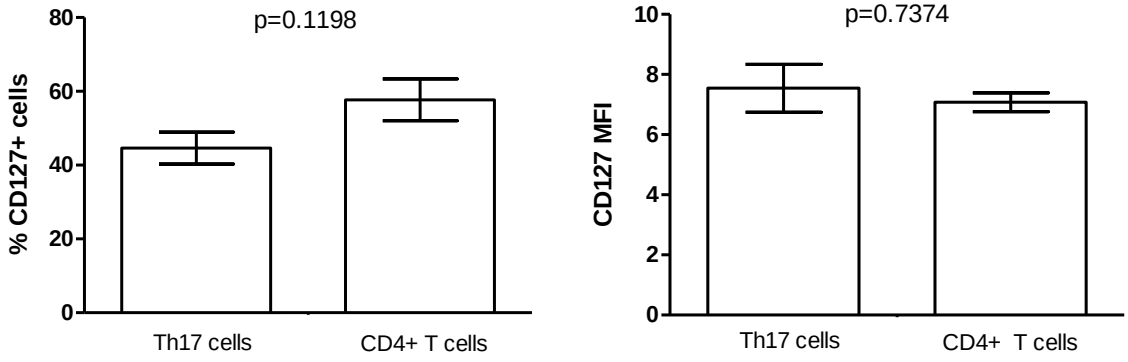
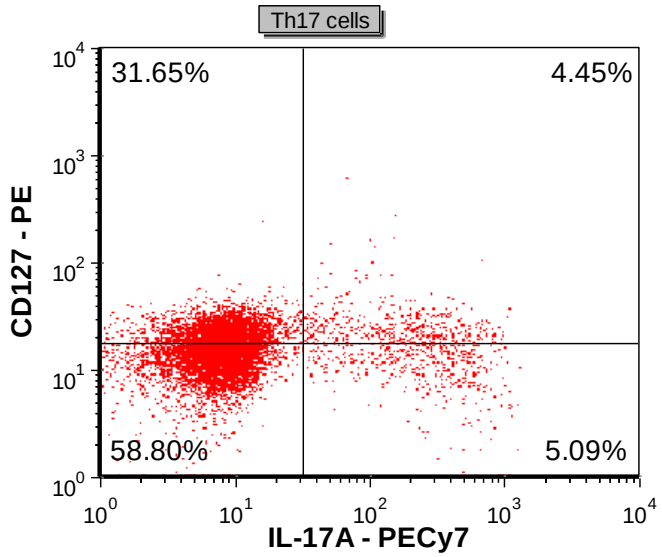

p=0.1198
% CD127+ cells
Th17 cells
CD4+ T cells
p=0.7374
CD127 MFI
Th17 cells
CD4+ T cells

Figure 2: Th17 cells express CD127. A) CD127 expression on Th17 cells is identified in the histogram, representative of one donor. **B)** Comparative frequency of CD127+ Th17 cells (mean=45% $\pm$4.32%, N=17) and CD127+ CD4+ T cells (mean=58% $\pm$5.64%, N=6) (left) and CD127 MFI of Th17 cells (mean=7.54 $\pm$0.79, N=17) and bulk CD4+ T cells (mean=7.08 $\pm$0.31, N=6) (right) are summarized in the bar graphs. p values were calculated using Student's Unpaired T Test. Error bars represent SEM.

Next, CD127 expression was measured on functionally active, IL-17A-producing Th17 cells. Similar to what others have done [51, 54], cells were first stimulated for six hours with 50 ng/mL PMA and 1000 ng/mL ionomycin to induce IL-17A production. Brefeldin A was added for the entire six hours (1 µL/1 million cells) to contain IL-17A inside the cells [163], in accordance with the manufacturer's instructions (BD Biosciences). Once the cells were stimulated, they were stained for surface CD127 then intracellular IL-17A. CD127 expression was compared between total, unstimulated Th17 cells (Th17 unstim.), total, PMA/ionomycin-stimulated Th17 cells (Th17 STIM.), IL-17A+ cells and IL-17A- cells. Notably, 60% $\pm$6.82% unstimulated Th17 cells expressed CD127, while 32% $\pm$4.41% of total, stimulated Th17 cells expressed CD127 (Fig. 3B). Furthermore, 45% $\pm$4.73% IL-17A+ cells and 31% $\pm$4.42% IL-17A- cells expressed CD127 (Fig. 3B). Overall, CD127 expression became downregulated upon stimulation with PMA/ionomycin ($p<0.01$ by Student's Paired T Test). Interestingly, amongst the three groups of stimulated Th17 cells, the highest proportion of CD127+ cells was evident amongst the IL-17A+ cells ($p<0.05$ by Student's Paired T Test).

In conclusion, Th17 cells express CD127, both in resting and in activated states.

A


Th17 cells
31.65%
4.45%
58.80%
5.09%
CD127 - PE
IL-17A - PECy7

B

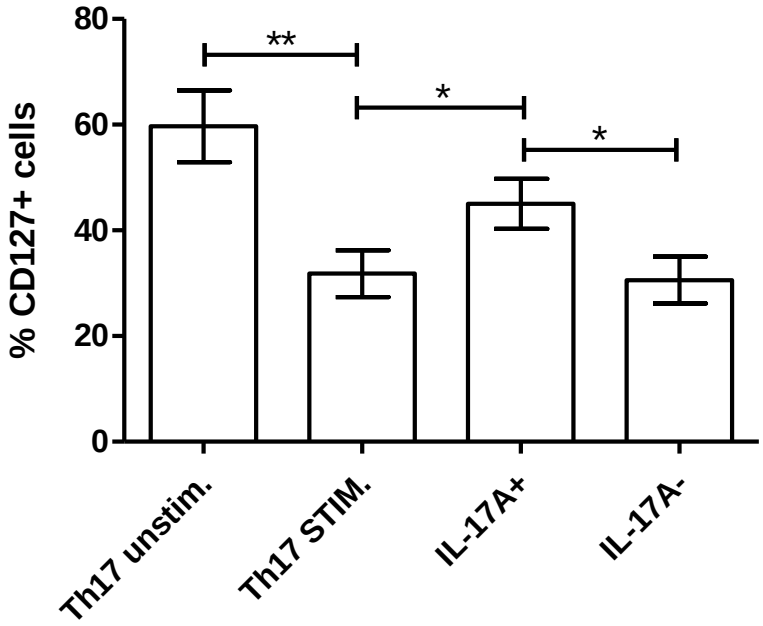
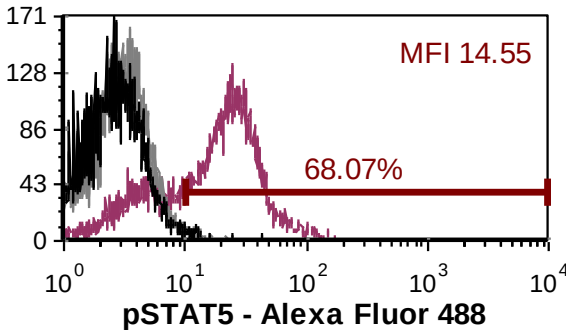

% CD127+ cells
**
*
*
Th17 unstim.
Th17 STIM.
IL-17A+
IL-17A-

Figure 3: IL-17A-producing Th17 cells express CD127. A) The frequency of active, IL-17A+ Th17 cells expressing CD127 is represented in the red scatter plot, representative of one donor. **B)** Frequencies of CD127+ cells between total, unstimulated Th17 cells (Th17 unstim.), total, PMA/ionomycin-stimulated Th17 cells (Th17 STIM.), IL-17A+ Th17 cells (IL-17A+) and IL-17A- Th17 cells (IL-17A-) (N=4) are summarized in the bar graph. The mean CD127+ cell frequency was 60% $\pm$6.82% in unstimulated Th17 cells, 32% $\pm$4.41% in stimulated Th17 cells, 45% $\pm$4.73% in IL-17A+ cells, and 31% $\pm$4.42% in IL-17A- cells. **$p<0.01$ and * $p<0.05$ by Student's Paired T Test. Error bars represent SEM.

3.3 Th17 cells are responsive to IL-7. Responses to IL-7, indicated by phosphorylation of STAT5, expression of Bcl-2, and cell proliferation, have been previously characterized in CD4+ T cells [132, 154]. Furthermore, such responses have been reported in murine Th17 cells [130], although not in human Th17 cells. Therefore, Th17 cells from healthy individuals were assessed for their ability to respond to IL-7 by measuring phosphorylation of STAT5, expression of Bcl-2, and proliferation.

Since the Tyr694 site on STAT5 becomes phosphorylated upon stimulation with IL-7 [132, 138], an antibody targeting this specific epitope was used to detect IL-7-inducible pSTAT5 in Th17 cells. First of all, very few (less than 5%) Th17 cells expressed basal levels of pSTAT5. Following stimulation with a low dose of IL-7 (0.01 ng/mL), roughly 30% Th17 cells expressed pSTAT5. Next, the frequency of pSTAT5+ cells doubled to 60% in response to a higher dose of IL-7 (0.1 ng/mL), and plateaued at 60% in response to the highest dose of IL-7 (1 ng/mL) ($p<0.001$ by one-way ANOVA and $p<0.05$ by Dunnett's Post Hoc Test) (Fig. 4). Overall, IL-7 induces a dose-dependent increase in STAT5 phosphorylation in Th17 cells.



IL-7 unstained
Unstimulated pSTAT5
IL-7-stimulated pSTAT5

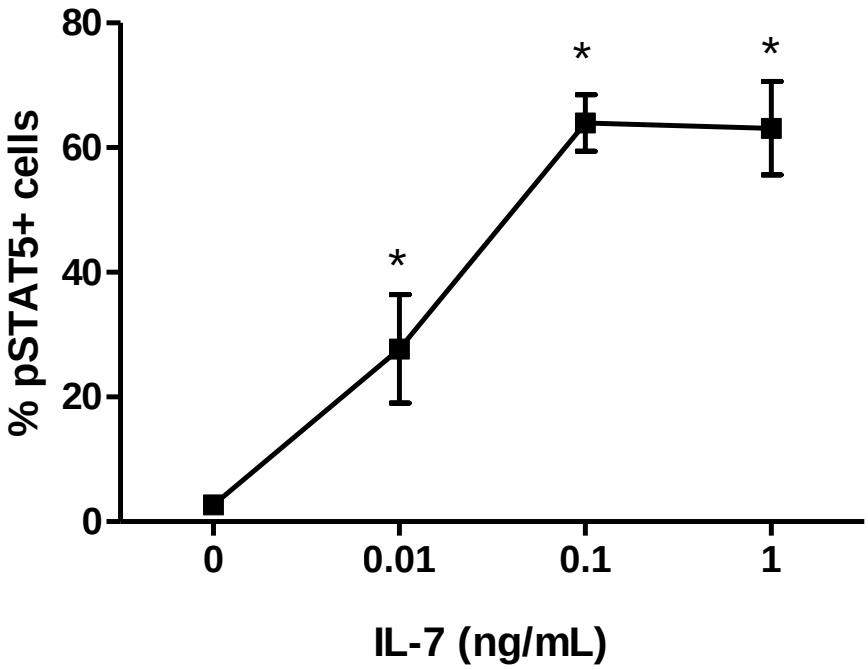
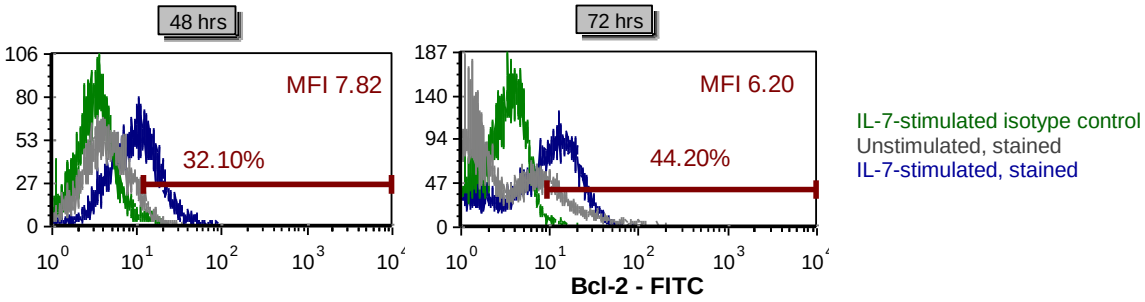


Figure 4: IL-7 induces phosphorylation of STAT5 in Th17 cells. STAT5 phosphorylation in Th17 cells following stimulation with IL-7 (0.1 ng/mL) is demonstrated in the histogram, representative of one donor. The dose response to IL-7 is summarized in the line graph. The mean frequency of pSTAT5+ Th17 cells was 3% $\pm$0.93% in unstimulated cells, 28% $\pm$8.69% in cells stimulated with IL-7 (0.01 ng/mL), 64% $\pm$4.52% in cells stimulated with IL-7 (0.1 ng/mL), and 63% $\pm$7.52% in cells stimulated with IL-7 (1 ng/mL). * $p<0.001$ by one-way ANOVA and $p<0.05$ by Dunnett's Post Hoc Test. Error bars represent SEM.

CD4+ memory T cells express basal levels of Bcl-2, which become increased in response to IL-7 [132, 133]. Following analysis of basal Bcl-2 after 48 and 72 hours, expression was evident in roughly 10% and 15% Th17 cells, respectively. Next, stimulation with IL-7 (5 ng/mL) for 48 and 72 hours increased the mean frequency of Bcl-2+ Th17 cells to 35% $\pm$5.73% and 41% $\pm$6.55%, respectively ($p<0.01$ and $p<0.05$, respectively, by Student's Paired T Test) (Fig. 5). In summary, a small percentage of Th17 cells exhibit Bcl-2 expression at the basal level, which is further upregulated in response to IL-7.


48 hrs
72 hrs
MFI 7.82
32.10%
MFI 6.20
44.20%
106
80
53
27
0
187
140
94
47
0
Bcl-2 - FITC
IL-7-stimulated isotype control
Unstimulated, stained
IL-7-stimulated, stained

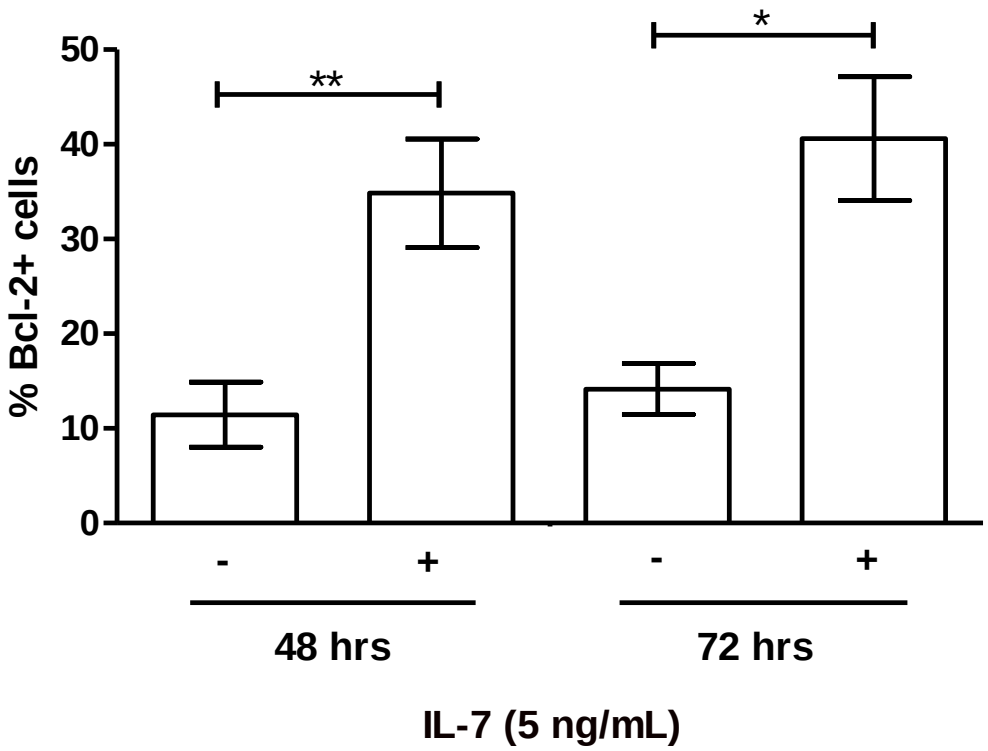
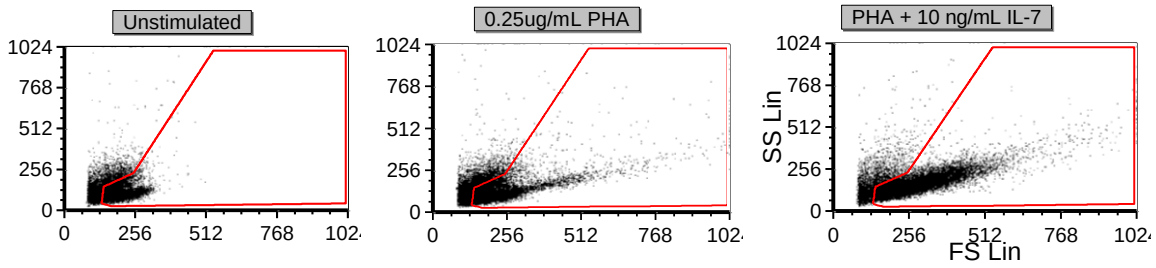

**
*
50
40
30
20
10
0
% Bcl-2+ cells
-
+
-
+
48 hrs
72 hrs
IL-7 (5 ng/mL)

Figure 5: IL-7 upregulates Bcl-2 in Th17 cells. Bcl-2 expression in Th17 cells following stimulation with IL-7 (5 ng/mL) after 48 (left) and 72 hours (right) is represented in the histograms, representative of one donor. The frequencies of Bcl-2+ Th17 cells at the basal level and in response to IL-7 (5 ng/mL) for 48 hours (N=6) and 72 hours (N=8) are summarized in the bar graph. The mean frequency of unstimulated Bcl-2+ cells was 12% $\pm$3.43% after 48 hours and 14% $\pm$2.69% after 72 hours, while the mean frequency of IL-7-stimulated Bcl-2+ cells was 35% $\pm$5.73% after 48 hours and 41% $\pm$6.55% after 72 hours. ** $p<0.01$ and * $p<0.05$ by Student's Paired T Test. Error bars represent SEM.

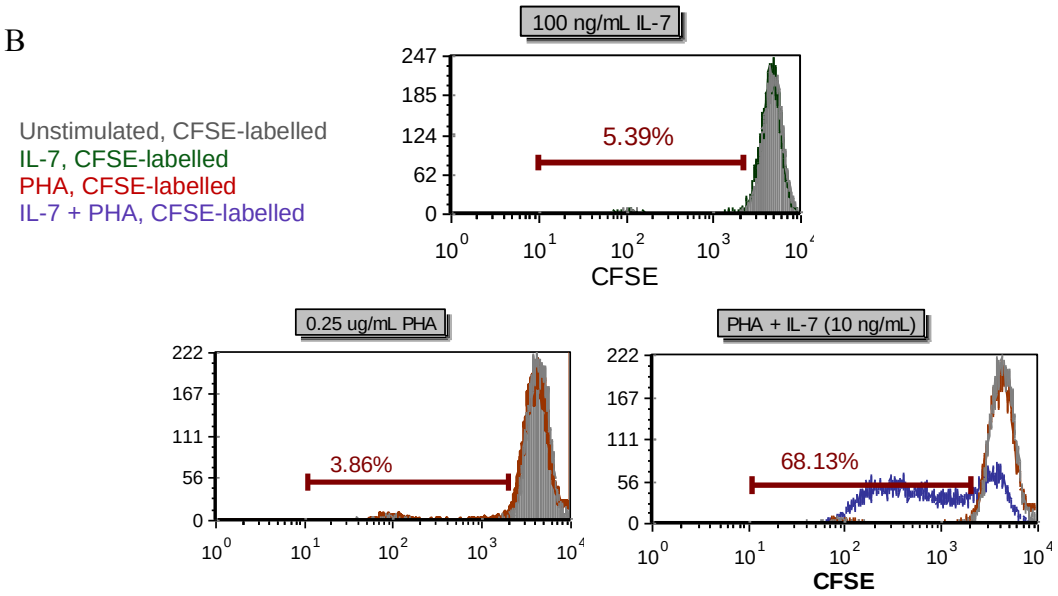
CFSE is widely used to measure T cell proliferation by flow cytometry [164] and was thus used to measure Th17 cell proliferation. IL-7-mediated T cell proliferation is significantly enhanced in combination with sub-optimal TCR signalling [165]. PHA is a lectin that induces cell division by upregulating CD25 [166, 167], and thus enhances the proliferative effects of IL-7 [168]. Th17 cells were therefore stimulated with a low dose of PHA (0.25 μg/mL) in combination with increasing doses of IL-7 (0.1, 1 or 10 ng/mL). While this small dose of PHA did not induce proliferation on its own, it permitted proliferation when used in combination with IL-7. In this regard, the proliferative response elicited by IL-7 would be better represented. The ability of IL-7 to induce Th17 cell proliferation was distinguished from that of PHA by setting the marker on cells that had been stimulated with PHA alone, similar to a strategy reported elsewhere [126].

When cultured in either media or PHA alone, fewer than 5% Th17 cells proliferated, as represented by $CFSE^{lo}$ cells (Fig. 6). Addition of 1 and 10 ng/mL IL-7 to PHA induced proliferation in approximately 30% and 60% Th17 cells, respectively ($p<0.001$ by one-way ANOVA and $p<0.05$ by Dunnett's Post Hoc Test) (Fig. 6). As some studies have reported that, in the absence of co-stimulation through the TCR, higher doses of IL-7 (10-100 ng/mL) induce T cell proliferation [169], Th17 cells were also stimulated with 100 ng/mL IL-7, in the absence of PHA, for five days. On its own, IL-7 did not induce a proliferative response in the Th17 cells (Fig. 6B). In summary, IL-7, when used in combination with a sub-optimal dose of PHA, induces Th17 cell proliferation in a dose-dependent manner.

A


Unstimulated
0.25ug/mL PHA
PHA + 10 ng/mL IL-7
SS Lin
FS Lin

B


Unstimulated, CFSE-labelled
IL-7, CFSE-labelled
PHA, CFSE-labelled
IL-7 + PHA, CFSE-labelled
100 ng/mL IL-7
5.39%
0.25 ug/mL PHA
3.86%
PHA + IL-7 (10 ng/mL)
68.13%
CFSE

C

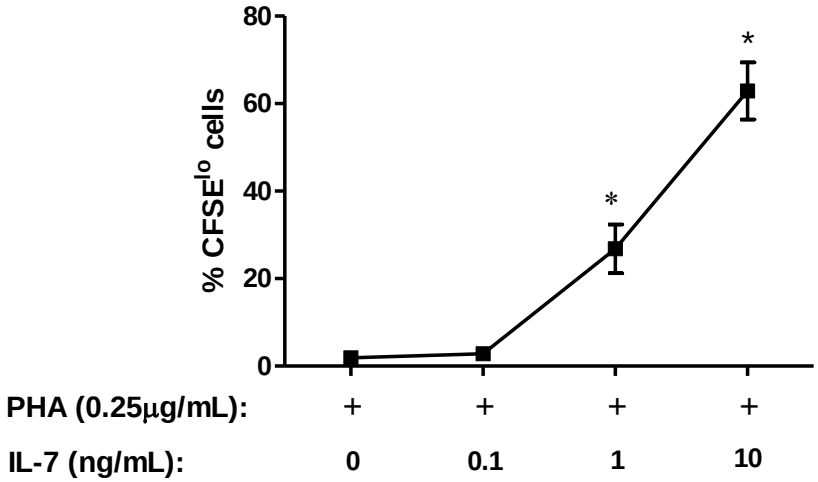

% CFSE^lo cells
PHA (0.25µg/mL):
IL-7 (ng/mL):
0
0.1
1
10

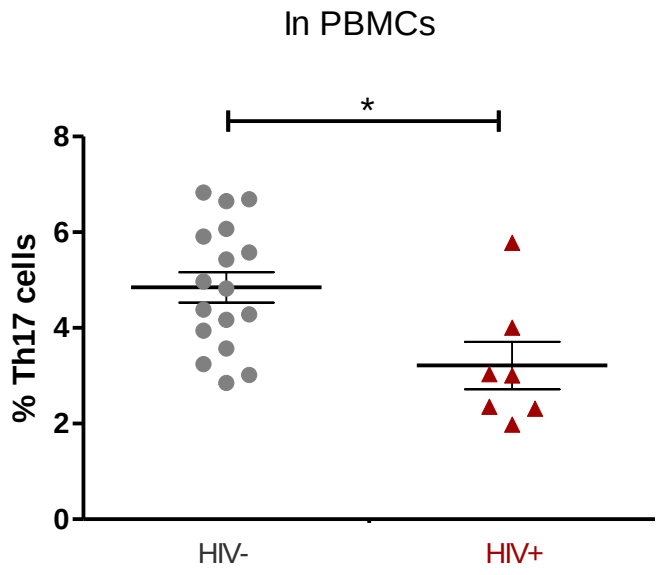
Figure 6: IL-7 induces Th17 cell proliferation. A) Th17 cell granularity (SS Lin) and size (FS Lin) following 5 days of cell culture either in media alone, PHA (0.25 μg/mL) alone, or IL-7 (10 ng/mL) + PHA (0.25 μg/mL), are represented in the scatter plots, representative of one donor. **B)** Proliferating Th17 cells ($CFSE^{lo}$ Th17 cells) following stimulation with IL-7 (100 ng/mL) (top), PHA (0.25 μg/mL) (bottom left), or PHA (0.25 μg/mL) and IL-7 (10 ng/mL) (bottom right) are demonstrated in the histograms, representative of one donor. **C)** The degrees of Th17 cell proliferation, represented by the percentages of $CFSE^{lo}$ cells, in response to 0.25 μg/mL PHA, either on its own (mean=1.8% $\pm$0.48) or in combination with IL-7 (0.1 ng/mL) (mean=3% $\pm$0.42%), IL-7 (1 ng/mL) (mean=27% $\pm$5.57%), or IL-7 (10 ng/mL) (mean=63% $\pm$6.52%) (N=6), are summarized in the bar graph. * $p<0.001$ by one-way ANOVA and $p<0.05$ by Dunnett's Post Hoc Test. Error bars represent SEM.

3.4 Th17 cell frequencies are reduced in PBMCs but maintained in total CD4+ lymphocytes HIV-infected, HAART-treated subjects. Demographics and clinical parameters of HIV-infected, HAART-treated subjects are outlined in Table 1. First, Th17 cell frequencies were measured in PBMCs from uninfected individuals and HIV-infected subjects. Notably, 3% $\pm$0.5% PBMCs in HIV-infected subjects were Th17 cells (Fig. 7A), which is approximately 40% lower than those observed in uninfected individuals ($p<0.05$ by Student's Unpaired T Test). These results are similar to previous reports obtained elsewhere [81]. Next, the frequencies of Th17 cells within total CD4+ lymphocytes were also compared between uninfected individuals and HIV-infected subjects. Notably, roughly 17% $\pm$2.3% CD4+ lymphocytes were Th17 cells in HIV-infected subjects (Fig. 7B), which is similar to those present in uninfected individuals. Overall, while Th17 cell frequencies are reduced in PBMCs from HIV-infected, HAART-treated subjects, their frequencies within total CD4+ lymphocytes are similar to those observed in uninfected individuals.

Sex	Age	CD4 Count (cells/mm^3)	CD4 %	Nadir CD4 Count (cells/mm^3)	Viral Load (RNA/mL)	Duration of Undetectable Viremia (Years)
Male	54	616	37.8	278	<40	5.5
Male	55	282	10	4	<50	4
Male	47	728	31.4	344	<40	3
Male	49	298	21.7	297	<40	1
Male	64	657	40.7	N/A	<40	N/A
Female	49	786	30.5	486	<40	17
Male	51	324	22	211	<50	1
Male	61	395	41	111	<50	4
Male	63	488	32.2	99	<40	17
Male	45	1093	37.4	311	<40	5
Male	27	946	43	321	<50	1
Female	51	1290	52	308	<40	17
Male	45	490	25	301	<50	1
Male	53	603	32.9	250	<40	5
Female	52	616	41	117	<50	1
Male	44	475	36.4	68	<50	2
Male	50	443	22.6	N/A	<40	N/A
Male	69	315	27	198	<40	20
Female	60	274	12	222	<40	0.5
Female	72	493	50.7	462	<40	2
Male	53	335	27	223	<50	1
Male	45	569	23.9	442	<50	4
Male	33	697	32	225	<50	0.5
Male	25	719	32	N/A	<50	2
Male	67	745	39.5	N/A	<50	>7
Male	51	328	40.3	128	<40	11
Male	32	801	47.8	322	<40	1

Table 1: Demographics and clinical parameters of HIV-infected, HAART-treated subjects. Demographics, as characterized by sex and age as of year 2015, are listed in the above table. Furthermore, clinical parameters, including total CD4 counts (cells/mm^3 of blood) and frequencies, nadir CD4 counts (cells/mm^3 of blood), viral loads (material RNA/mL of blood), and the duration for which plasma viremia has been undetectable (represented in years), are also presented.

A


In PBMCs
*
% Th17 cells
8
6
4
2
0
HIV-
HIV+

B

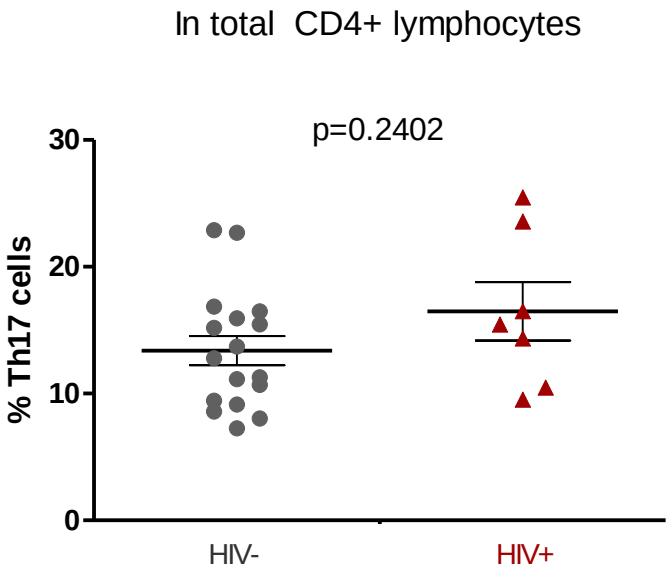
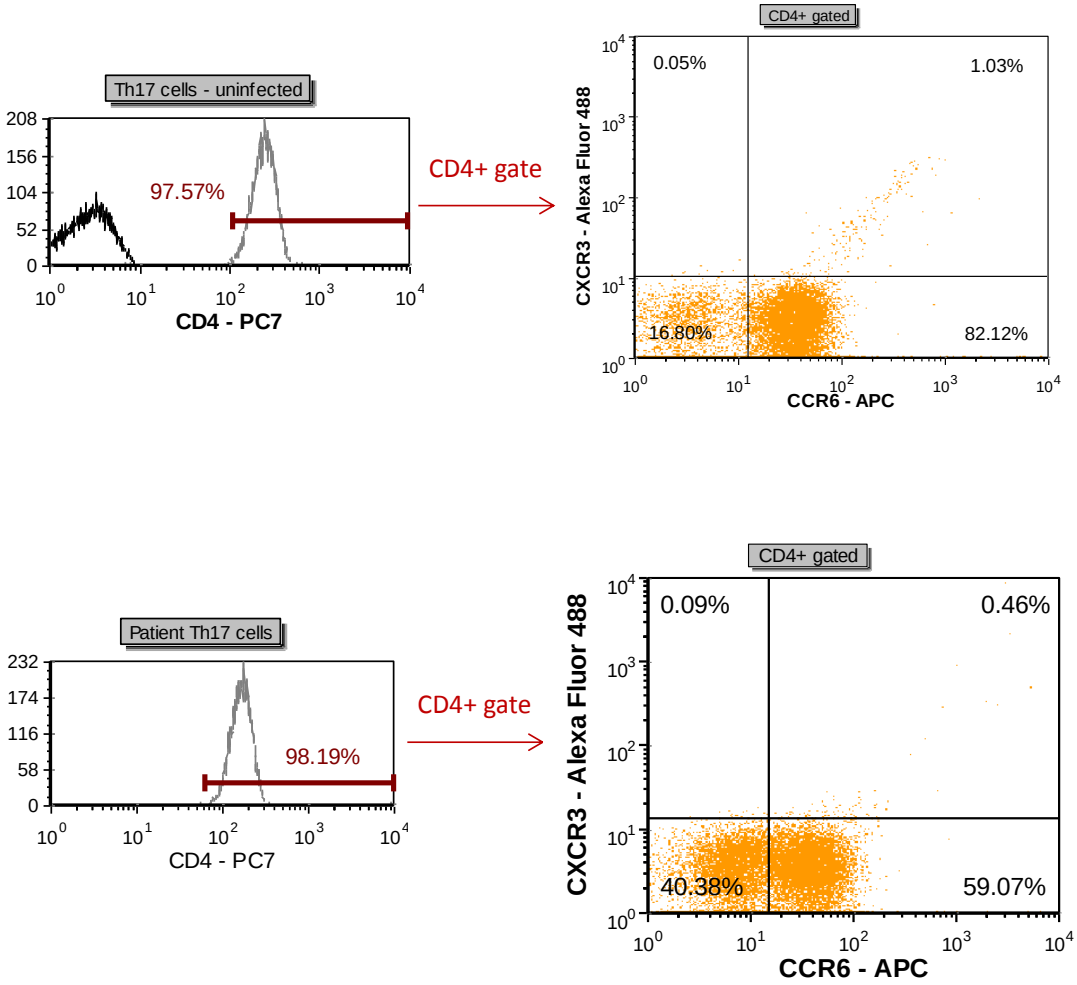

In total CD4+ lymphocytes
p=0.2402
% Th17 cells
30
20
10
0
HIV-
HIV+

Figure 7: Total Th17 cell frequencies are reduced in PBMCs but maintained in total CD4+ lymphocytes in HIV-infected, HAART-treated subjects. A) Th17 frequencies within PBMCs in uninfected individuals (grey dots) (mean=5% $\pm$0.34%, N=16) and HIV-infected subjects (red triangles) (mean=3% $\pm$0.5%, N=7), (* $p<0.05$ by Student's Unpaired T Test) and **B)** Th17 frequencies within total CD4+ lymphocytes in uninfected individuals (mean=14% $\pm$1.17%, N=16) and HIV-infected subjects (mean=17% $\pm$2.3%, N=7) are summarized in the bar graphs ($p=0.2402$ by Student's Unpaired T Test). Error bars represent SEM.

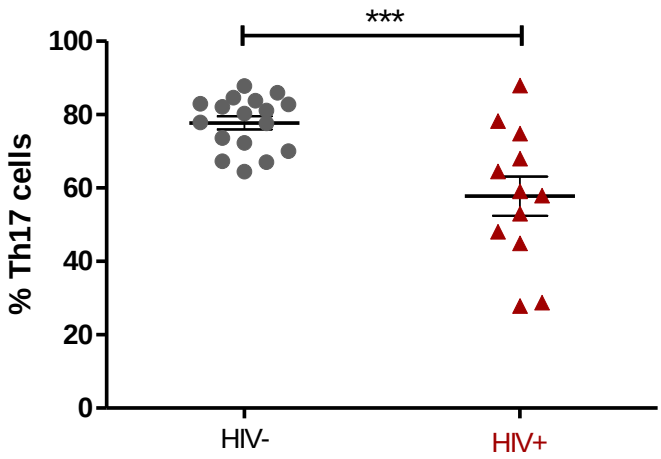
3.5 Sorted cells are primarily representative of Th17 cells in both HIV-infected, HAART-treated individuals and uninfected individuals. Th17 cell purity, defined as the frequency of Th17 cells (CD4+CXCR3-CCR6+ cells) within the sorted population of cells, was measured in cells from HIV-infected individuals. The average Th17 cell purity of cells from HIV-infected individuals was approximately 60%, which is lower than the approximate 80% observed in uninfected individuals ($p<0.001$ by Student's Unpaired T Test) (Fig. 8A & 8B). Next, the mean frequency of CD4+ and CXCR3+ cells was comparable between sorted cells from both HIV-infected and uninfected individuals (Fig. 8C), indicating that the first step of the Th17 isolation protocol (negative selection of CXCR3-depleted CD4+ memory T cells) was effective. Furthermore, the frequency of Th17 cells occurring amongst total CD4+ lymphocytes (prior to separation) (T) and sorted cells (post separation) (S) was compared in both groups of subjects. The mean frequency of Th17 cells within total CD4+ lymphocytes was approximately 15% in both groups, and increased to approximately 60% in HIV-infected individuals ($p<0.001$ by Student's Paired T Test) and 80% in uninfected individuals ($p<0.001$ by Student's Paired T Test) (Fig. 8D), indicating that Th17 cells were significantly enriched following cell separation.

Overall, these observations suggest that, despite the variation in Th17 cell purity, sorted cells were primarily enriched in Th17 cells in both HIV-infected and uninfected individuals.

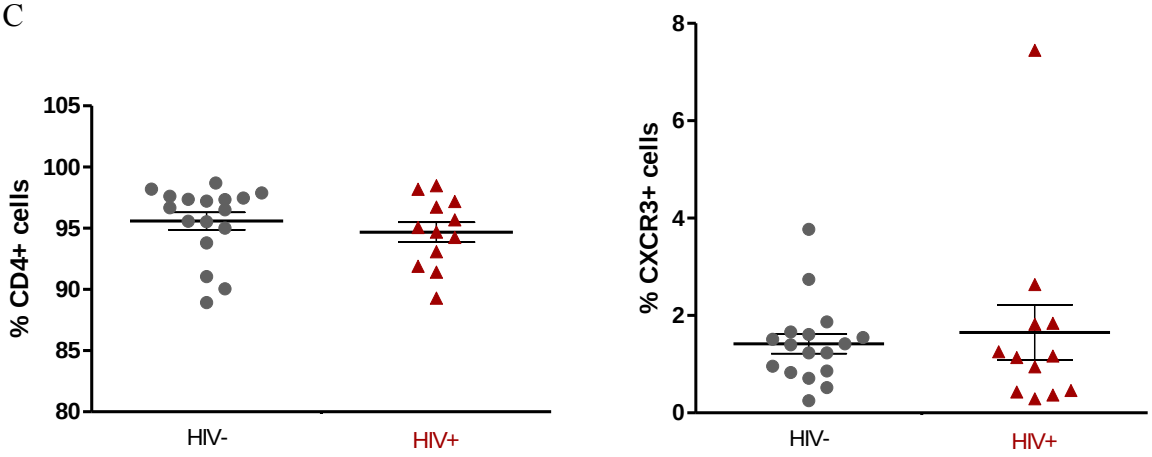
A


Th17 cells - uninfected
97.57%
CD4 - PC7
CD4+ gate
CD4+ gated
0.05%
1.03%
16.80%
82.12%
CXCR3 - Alexa Fluor 488
CCR6 - APC
Patient Th17 cells
98.19%
CD4 - PC7
CD4+ gate
CD4+ gated
0.09%
0.46%
40.38%
59.07%
CXCR3 - Alexa Fluor 488
CCR6 - APC

B


***
% Th17 cells
HIV-
HIV+

C


% CD4+ cells
105
100
95
90
85
80
HIV-
HIV+
% CXCR3+ cells
8
6
4
2
0
HIV-
HIV+

D

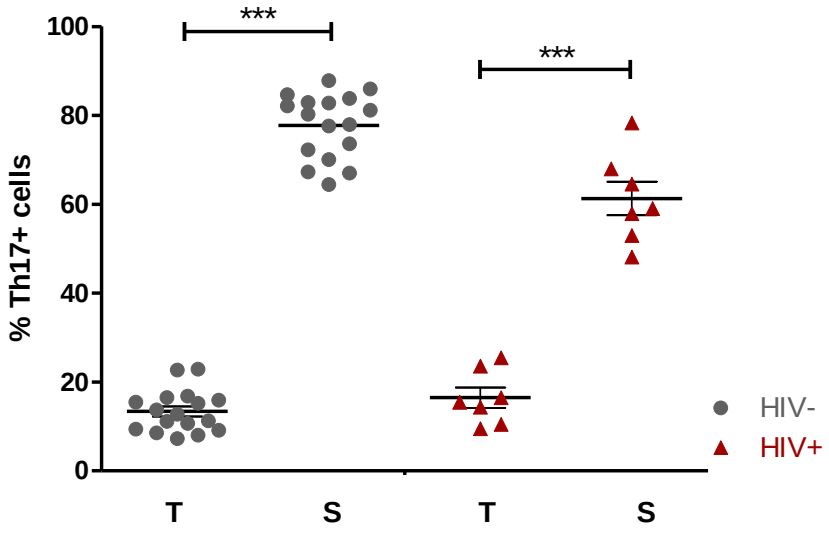
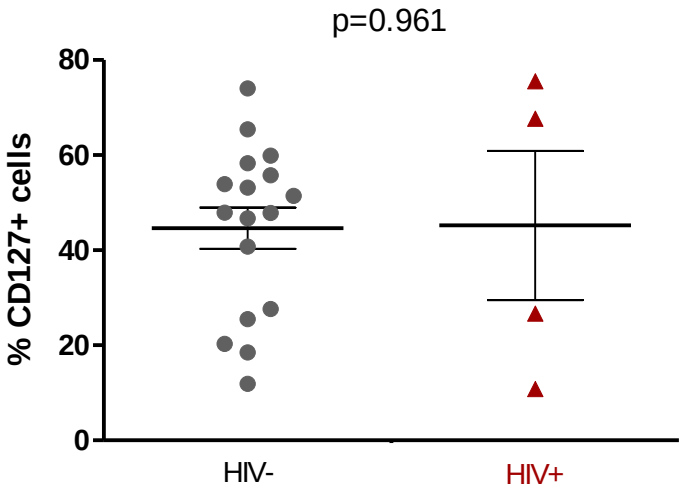

% Th17+ cells
100
80
60
40
20
0
***
***
T
S
T
S
HIV-
HIV+

Figure 8: Sorted cells are primarily representative of Th17 cells in both HIV-infected, HAART-treated individuals and uninfected individuals. A) Th17 cell purity, defined as the frequency of CD4+CXCR3-CCR6+ cells, is represented in uninfected individuals (top) and HIV-infected individuals (bottom) is demonstrated in the sample histograms and orange scatter plots, representative of one participant from each group. **B)** Th17 cell purity is summarized in uninfected individuals (gray circles) (mean=78% $\pm$1.77%, N=17) and HIV-infected individuals (red triangles) (mean=58% $\pm$5.36%, N=12) (*** $p<0.001$ by Student's Unpaired T Test). **C)** Expression of CD4 (left) and CXCR3 (right) was measured in sorted Th17 cells, both from uninfected individuals (gray circles, N=17) and HIV-infected individuals (red triangles, N=12). The mean frequency of Th17 cells expressing CD4 was 96% $\pm$0.72% in uninfected individuals and 95% $\pm$0.82% in HIV-infected individuals. The mean frequency of Th17 cells expressing CXCR3 was 1% $\pm$0.2% in uninfected individuals and 2% $\pm$0.57% in HIV-infected individuals. **D)** Th17 cell frequencies are demonstrated in total CD4+ lymphocytes (T) and sorted cells (S) in uninfected individuals (gray circles, N=17) and HIV-infected individuals (red triangles, N=7). In uninfected individuals, the mean Th17 cell frequency within total CD4+ lymphocytes and sorted cells is 14% $\pm$1.17% and 78% $\pm$1.77%, respectively, while, in HIV-infected individuals, the mean Th17 cell frequency within total CD4+ lymphocytes and sorted cells is and 17% $\pm$2.3% and 61% $\pm$3.79%, respectively. *** $p<0.001$ by Student's Paired T Test. Error bars represent SEM.

3.6 CD127 is not downregulated on Th17 cells from HIV-infected, HAART-treated subjects. HIV infection results in CD127 downregulation on CD8+ and CD4+ T cells, which becomes restored to variable degrees in patients receiving HAART [146, 127]. How CD127 becomes affected in the context of Th17 cells, however, remains unknown. CD127 expression on Th17 cells from uninfected individuals and HIV-infected, HAART-treated subjects was measured. Notably, the mean percentage of CD127+ Th17 cells and mean CD127 MFI value were comparable between both groups ($p=0.961$ and 0.4223, respectively, by Student's Unpaired T Test) (Fig. 9).


p=0.961
% CD127+ cells
80
60
40
20
0
HIV-
HIV+

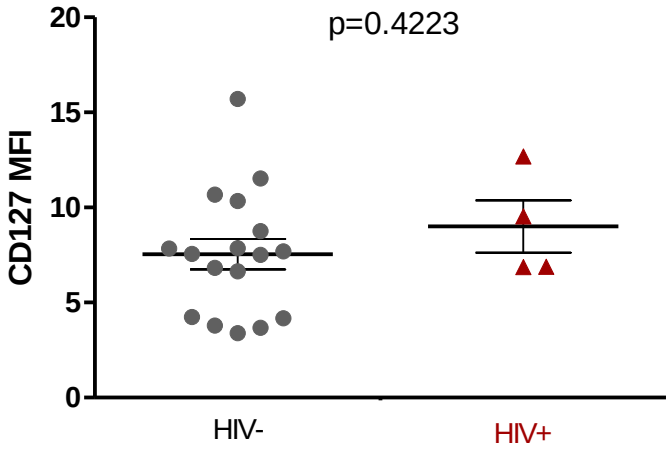
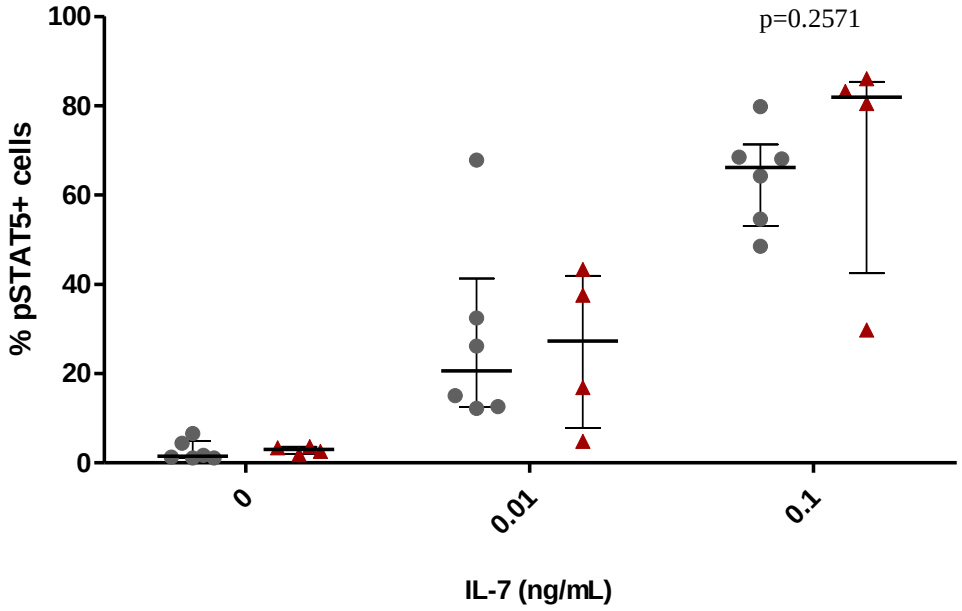

p=0.4223
CD127 MFI
20
15
10
5
0
HIV-
HIV+

Figure 9: CD127 is not downregulated on Th17 cells from HIV-infected, HAART-treated subjects. The frequencies of CD127+ Th17 cells from uninfected individuals (gray circles) (mean=45% ±4.32%, N=17) and HIV-infected subjects (red triangles) (mean=45% ±15.68%, N=4) are summarized in the top bar graph (p=0.961, Student's Unpaired T Test), while the CD127 MFI values from uninfected individuals (mean=7.54 ±0.79, N=17) and HIV-infected subjects (mean=9 ±1.38, N=4) are summarized in the bottom bar graph (p=0.4223, Student's Unpaired T Test). Error bars represent SEM.

3.7 Th17 cells from HIV-infected, HAART-treated subjects exhibit altered responses to IL-7. Responses to IL-7 become altered in both CD8+ and CD4+ memory T cells during HIV infection [126, 127, 154]. In CD4+ memory T cells, HAART completely normalizes IL-7-mediated phosphorylation of STAT5 while partially restoring expression of Bcl-2 [127]. Moreover, studies suggest that impaired IL-7 responses do not arise solely as a result of downregulated CD127 [152, 153]. In Th17 cells, IL-7 responses have yet to be studied in the context of HIV infection. Therefore, the effects of IL-7 on phosphorylation of STAT5, expression of Bcl-2, and proliferation were evaluated in Th17 cells derived from HIV-infected, HAART-treated subjects.

First of all, the median frequency of pSTAT5+ Th17 cells obtained from HIV-infected subjects was similar to that obtained from uninfected individuals when cultured in media alone (Fig. 10A). When stimulated with a lower dose (0.01 ng/mL) of IL-7, roughly 25% Th17 cells from HIV-infected subjects expressed pSTAT5, which is similar to what was observed in uninfected individuals (Fig. 10A). When cultured with a higher dose of IL-7 (0.1 ng/mL), approximately 80% Th17 cells from HIV-infected subjects expressed pSTAT5, while roughly 65% from uninfected individuals expressed pSTAT5 ($p=0.2571$ by Mann Whitney Test) (Fig. 10A). Furthermore, the MFI values of basal and IL-7-induced pSTAT5 were similar between Th17 cells from both groups of subjects (Fig. 10B). In summary, IL-7-mediated phosphorylation of STAT5 in Th17 cells from HIV-infected, HAART-treated subjects is not impaired.

A


p=0.2571
% pSTAT5+ cells
100
80
60
40
20
0
0
0.01
0.1
IL-7 (ng/mL)

B

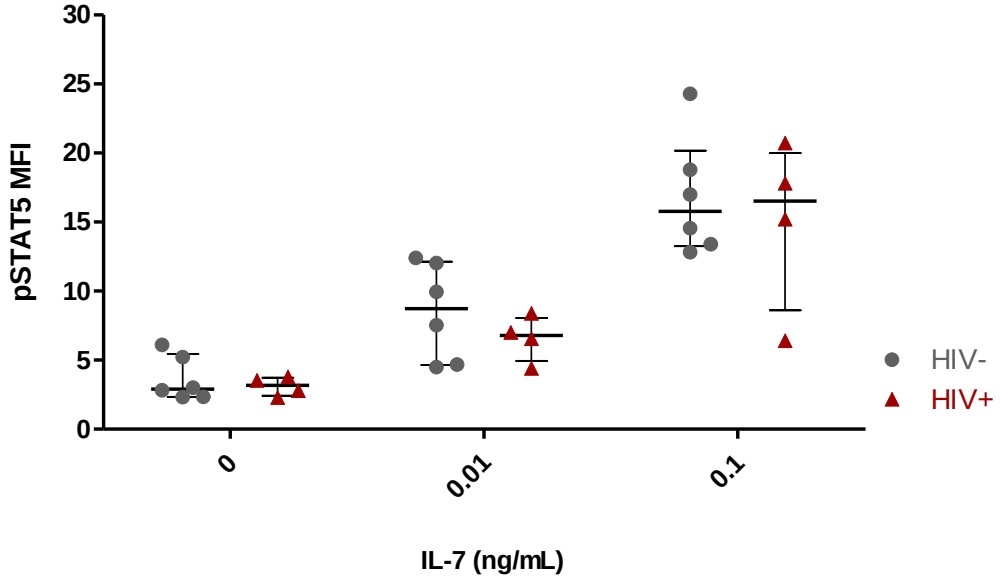
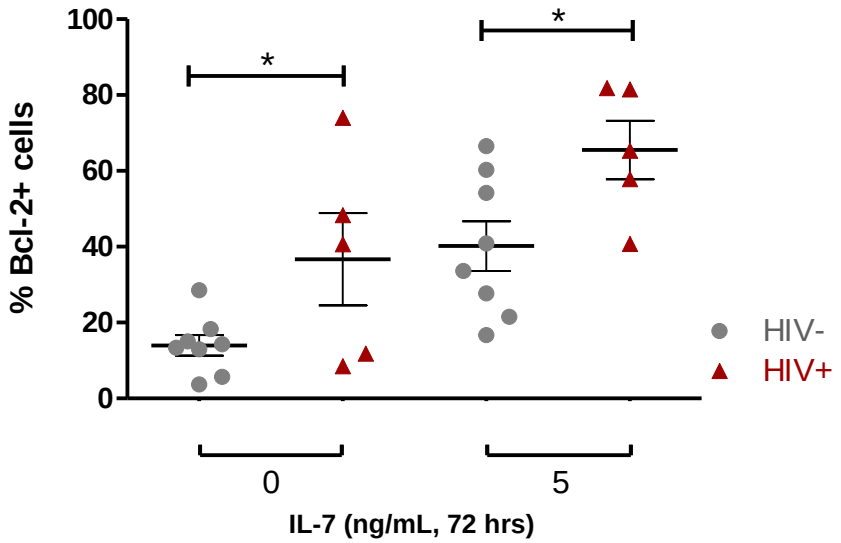

pSTAT5 MFI
30
25
20
15
10
5
0
0
0.01
0.1
IL-7 (ng/mL)
HIV-
HIV+

Figure 10: STAT5 phosphorylation in Th17 cells is not impaired in HIV-infected, HAART-treated subjects. A) The frequencies of pSTAT5+ Th17 cells are summarized. In uninfected individuals (gray circles, N=6), the median pSTAT5+ cell frequency was 2% (1.06%, 6.58%) in unstimulated cells, 21% (12.18%, 67.83%) in cells stimulated with IL-7 (0.01 ng/mL), and 66% (48.54%, 79.8%) in cells stimulated with IL-7 (0.1 ng/mL). In HIV-infected subjects (red triangles, N=4), the median pSTAT5+ cell frequency was 3% (1.86%, 3.67%) in unstimulated cells, 27% (4.84%, 43.36%) in cells stimulated with IL-7 (0.01 ng/mL), and 82% (30.49%, 86.06%) in cells stimulated with IL-7 (0.1 ng/mL). p=0.2571 by Mann Whitney Test. **B)** The pSTAT5 MFI values in Th17 cells are represented. In uninfected individuals (N=6), the median MFI value was 2.91 (2.32, 6.11) in unstimulated cells, 8.74 (4.5, 12.41) in cells stimulated with IL-7 (0.01 ng/mL), and 15.77 (12.81, 24.28) in cells stimulated with IL-7 (0.1 ng/mL). In HIV-infected subjects (N=4), the median MFI value was 3.17 (2.3, 3.8) in unstimulated cells, 6.79 (4.42, 8.4) in cells stimulated with IL-7 (0.01 ng/mL), and 16.52 (6.41, 20.73) in cells stimulated with IL-7 (0.1 ng/mL). Error bars represent IQR values.

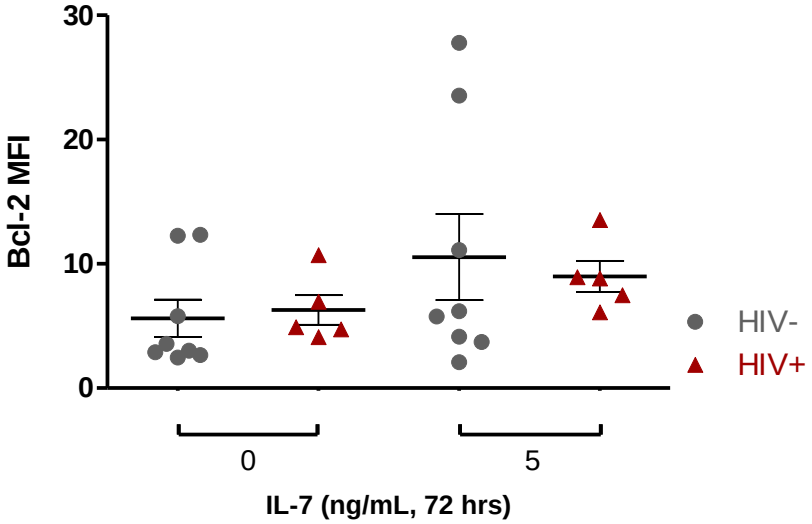
Next, following 72 hours of cell culture, the mean frequency of unstimulated Bcl-2+ Th17 cells from HIV-infected subjects was roughly 40%, which is approximately three-fold higher than what was observed in uninfected individuals ($p<0.05$ by Student's Unpaired T Test) (Fig. 11A). When stimulated with IL-7 (5 ng/mL) for 72 hours, Bcl-2 expression was observed in approximately 70% of Th17 cells from HIV-infected subjects, which is almost 50% higher than the results obtained in Th17 cells from uninfected individuals ($p<0.05$ by Student's Unpaired T Test). Furthermore, the mean Bcl-2 MFI value in unstimulated Th17 cells from HIV-infected subjects was roughly 6, which is similar to what was observed in uninfected individuals (Fig. 11B). Following IL-7 stimulation, the mean MFI value increased to approximately 9 and 11 in Th17 cells from uninfected individuals and HIV-infected subjects, respectively (Fig. 11B). Overall, the percentage of Bcl-2+ Th17 cells is increased both at the basal level and following stimulation with IL-7 in HIV-infected individuals, while the mean MFI values for Bcl-2 do not change.

Next, to determine whether the ability of IL-7 to upregulate Bcl-2 was altered in Th17 cells from HIV-infected, HAART-treated subjects, the mean relative increase in frequencies of Bcl-2+ cells (Δ % cells) following IL-7 stimulation was calculated for both groups of individuals. After 72 hours, the mean relative increase in the frequency of Bcl-2+ Th17 cells was approximately 3 in Th17 cells from both uninfected individuals and HIV-infected subjects (Fig. 11C). Furthermore, the mean relative increase in Bcl-2 MFI values in response to IL-7 (Δ MFI) was also calculated for each group. The mean relative increase in MFI was approximately 2 for both uninfected and HIV-infected individuals (Fig. 11D). Overall, these observations confirm that the ability of IL-7 to upregulate Bcl-2 in Th17 cells is not impaired during treated HIV-infection.

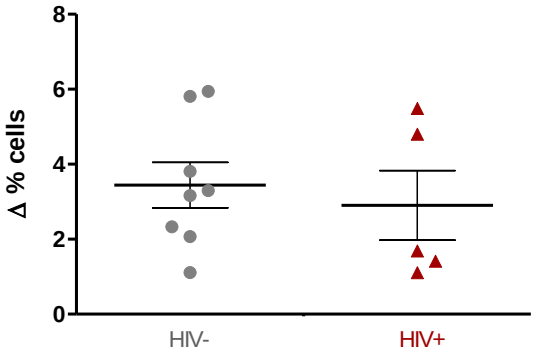
A


100
80
60
40
20
0
% Bcl-2+ cells
*
*
HIV-
HIV+
0
5
IL-7 (ng/mL, 72 hrs)

B


30
20
10
0
Bcl-2 MFI
HIV-
HIV+
0
5
IL-7 (ng/mL, 72 hrs)

C


8
6
4
2
0
Δ % cells
HIV-
HIV+

D

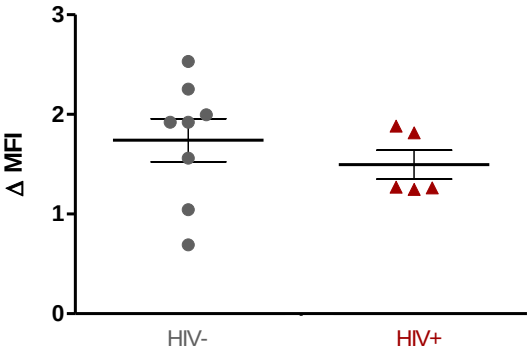
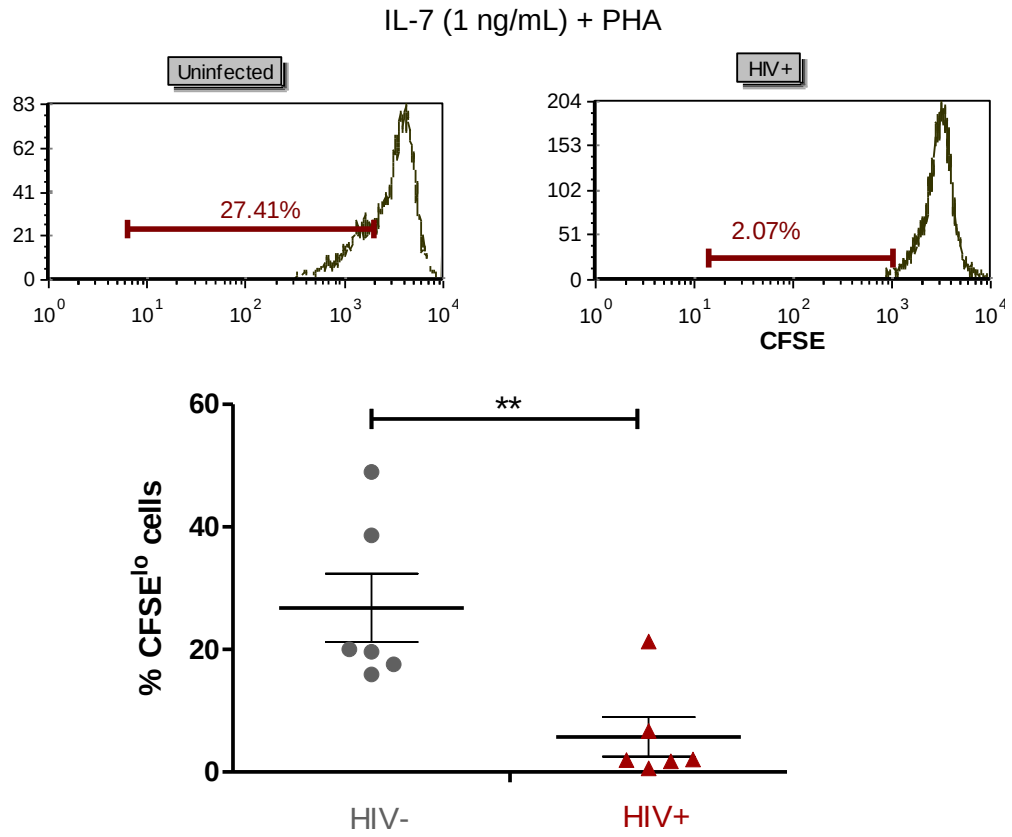

3
2
1
0
Δ MFI
HIV-
HIV+

Figure 11: Bcl-2 expression is upregulated in Th17 cells derived from HIV-infected, HAART-treated subjects. A) The frequencies of Bcl-2+ Th17 cells at the basal level and in response to 72 hours of stimulation with IL-7 (5 ng/mL) are represented in the top, left bar graph. In uninfected individuals (gray circles, N=8), the mean Bcl-2+ cell frequency was 14% $\pm$2.69% in unstimulated cells and 41% $\pm$6.55% in IL-7-stimulated cells. In HIV-infected subjects (red triangles, N=5), the mean Bcl-2+ cell frequency was 37% $\pm$12.16% in unstimulated cells, and 66% $\pm$7.73% in IL-7-stimulated cells. * $p<0.05$ by Student's Unpaired T Test). **B)** MFI values of Bcl-2 in Th17 cells at the basal level and in response to 72 hours of stimulation with IL-7 (5 ng/mL) are summarized in the bottom bar graph. In uninfected individuals (N=8), the mean MFI value was 5.62 $\pm$1.5 in unstimulated cells and 10.55 $\pm$3.45 in IL-7-stimulated cells. In HIV-infected subjects (N=5), the mean MFI value was 6.29 $\pm$1.21 in unstimulated cells and 8.98 $\pm$1.25 in IL-7-stimulated cells. **C)** The mean relative changes in frequencies of Bcl-2+ Th17 cells (Δ % cells) in response to 72 hours of stimulation with IL-7 (5 ng/mL) are represented in the bottom, left dot plot. The mean relative change in Bcl-2+ cell frequency is 3.44 $\pm$0.61 in uninfected individuals (N=8) and 2.9 $\pm$0.93 in HIV-infected subjects (N=5). **D)** The mean relative changes in the MFI values of Bcl-2 (Δ MFI) in response to 72 hours of stimulation with IL-7 (5 ng/mL) are depicted in the bottom, right bar graph. The mean relative change in MFI is 1.74 $\pm$0.22 in uninfected individuals (N=8) and 1.5 $\pm$0.15 in HIV-infected subjects (N=5). Error bars represent SEM.

In summary, in the context of treated HIV infection, the frequency of Bcl-2+ Th17 cells becomes elevated at the basal level and in response to IL-7, while the ability of IL-7 to upregulate Bcl-2 in Th17 cells is not impaired.

Finally, following five days of stimulation with IL-7 (1 ng/mL) and PHA (0.25 μg/mL), roughly 30% Th17 cells from uninfected individuals had undergone proliferation, whereas approximately 5% Th17 cells from HIV-infected, HAART-treated subjects had ($p<0.01$ by Student's Unpaired T Test) (Fig. 12A). Since virtually none of the Th17 cells from HIV-infected subjects had undergone a proliferative response, they were next stimulated with a higher dose of IL-7 (10 ng/mL) combined with PHA (0.25 μg/mL) to determine whether proliferation could be recovered using a maximal concentration of IL-7. Notably, while roughly 60% Th17 cells from uninfected individuals had undergone proliferation, approximately 20% Th17 cells from HIV-infected subjects had undergone proliferation (Fig. 12B), indicating that Th17 cell proliferation in response to IL-7 (10 ng/mL) and PHA is reduced by over 50% in the context of treated HIV infection ($p<0.01$ by Student's Unpaired T Test). Overall, Th17 cell proliferation in response to IL-7, combined with a sub-optimal dose of PHA, is severely impaired in HIV-infected, HAART-treated subjects.

A


IL-7 (1 ng/mL) + PHA
Uninfected
HIV+
27.41%
2.07%
CFSE
% CFSE^lo cells
**
HIV-
HIV+

B

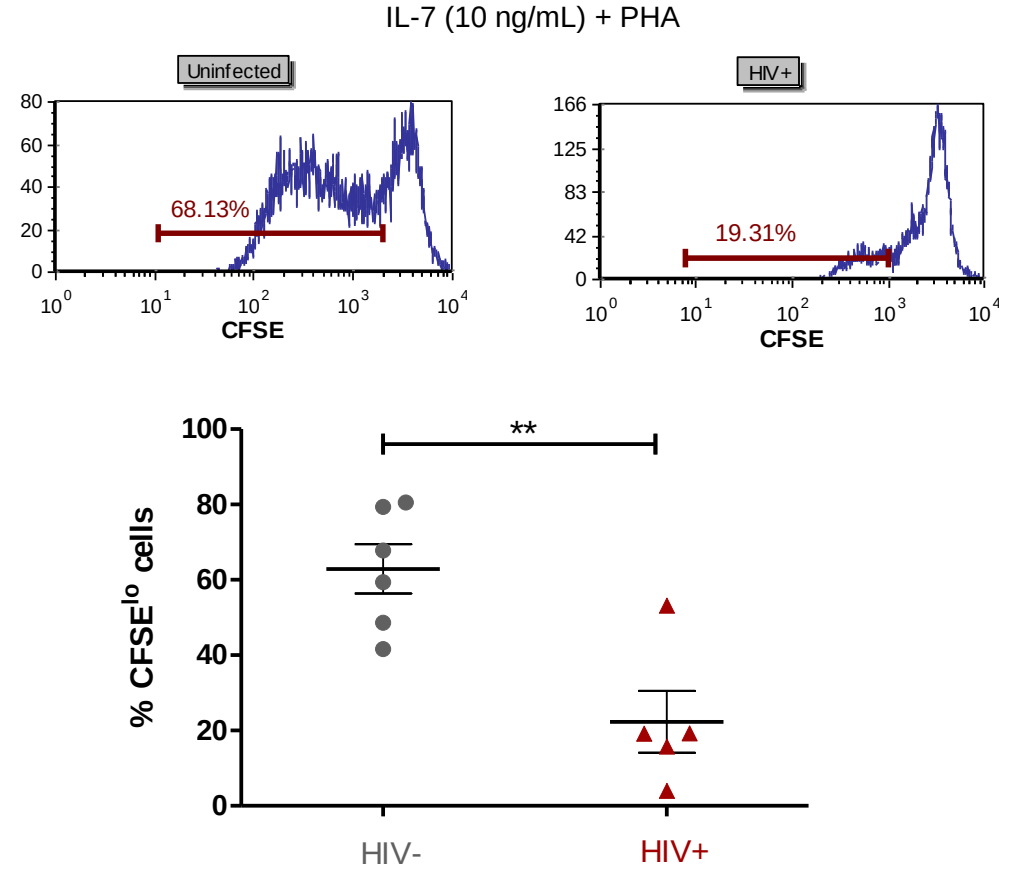

IL-7 (10 ng/mL) + PHA
Uninfected
HIV+
68.13%
19.31%
CFSE
% CFSE^lo cells
**
HIV-
HIV+

Figure 12: Th17 cell proliferation in HIV-infected, HAART-treated subjects is severely impaired. A) Th17 cell proliferation in response to IL-7 (1 ng/mL) and PHA (0.25 μg/mL) is represented. The mean frequency of proliferating Th17 cells ($CFSE^{lo}$ cells) was 27% ±5.57% in uninfected individuals (gray circles, N=6) and 6% ±3.23% in HIV-infected subjects (red triangles, N=6). ** $p<0.01$ by Student's Unpaired T Test. **B)** Th17 cell proliferation in response to IL-7 (10 ng/mL) and PHA (0.25 μg/mL) is depicted. The mean frequency of proliferating Th17 cells ($CFSE^{lo}$ cells) was 63% ±6.52% in uninfected individuals and 22% ±8.21% in HIV-infected subjects (N=5). ** $p<0.01$ by Student's Unpaired T Test. Error bars represent SEM.

3.8 Conclusions: Th17 cells from uninfected individuals express CD127 and are responsive to IL-7, as demonstrated through phosphorylation of STAT5, expression of Bcl-2, and proliferation. While CD127 expression on Th17 cells from HIV-infected, HAART-treated subjects is similar to that of Th17 cells from uninfected individuals, certain IL-7 responses are preserved while others are altered. First of all, phosphorylation of STAT5 is not impaired in Th17 cells from HIV-infected individuals. Next, Bcl-2 expression becomes upregulated at the basal level and in response to IL-7. Finally, IL-7- and PHA-mediated proliferation of Th17 cells is dramatically impaired.

4.0 DISCUSSION:

4.1 Limitations of defining and purifying Th17 cells: The definition of Th17 cells being IL-17A+ CD4+ T cells [77] provides obstacles to this area of study, as some IL-17-producing cells also exhibit functional activity of Th cells from other lineages, such as IL-17A+IFNγ+ cells, which represent Th1/Th17 cells [51], or IL-17A+IL-4+ cells, which represent Th2/Th17 cells [91]. To induce cytokine production, CD4+ T cells would first need to be stimulated with PMA and ionomycin, then fixed and permeabilized to be purified based on cytokine expression [170, 171]. As a result, it would not be possible to study the effects of IL-7 on fixed, IL-17A+ Th17 cells. To avoid the limitations of using fixed/permeabilized cells, others in our lab have generated Th17 cells from naïve CD4+ T cells *in vitro.* It was found that pSTAT3, which is upregulated by IL-6 or IL-23 [69, 64], did not revert to normal baseline levels in *in vitro*-generated Th17 cells, as they had been cultured in polarizing and expansion conditions (Fernandes et al., manuscript in preparation). Ultimately, using *in vitro*-generated cells could misrepresent the ability of any given cytokine to activate any corresponding signalling mediator downstream of its receptor.

An alternative method of obtaining Th17 cells would involve isolating these cells based on surface marker expression. As this approach would not require stimulation, it would better represent the cells that naturally occur *in vivo* [79]. As mentioned, studies have concluded that Th17 cells are identified as CCR4+CCR6+CD4+ T cells that do not express CXCR3 [80, 81]. In addition, others have further identified these cells as also being CCR10- [51] or CD161+ [79]. In this study, Th17 cells were defined as $4_3 6$ cells (CD4+CXCR3-CCR6+ memory T cells) and, functional Th17 cells were defined as IL-17A+ $4_3 6$ cells.

4.2 Sorting Th17 cells by magnetic separation: Th17 cells were sorted from PBMCs using the Human Th17 Enrichment Kit (STEMCELL Technologies, Vancouver, BC) [156]. Th17

cell purity, defined as the percentage of Th17+ ($4_3$6+) cells present within the population following magnetic separation, was determined on the isolated cells by analyzing surface expression of CD4, CXCR3, and CCR6. Th17 cell purity was always measured immediately after the isolation, as we have previously determined that CXCR3 expression becomes upregulated on up to 24% of the cells following overnight cell culture (Fig. S1).

Th17 cell purity has typically ranged between 60%-92% among 17 uninfected blood donors, averaging at approximately 80% (Fig. 1C). This wide range likely resulted from variation in Th17 cell frequencies within PBMCs, which we had found to range between approximately 3-7% in healthy individuals (Fig. 1A). Despite the fluctuation in CCR6+ cell frequencies resulting from magnetic cell separation, isolation of CD4+CXCR3- cells was always successful, as CD4 expression was evident on 96% of the cells, while CXCR3 expression was evident on 1% of the cells (Fig. 8C).

In HIV-infected subjects undergoing HAART, Th17 cell purity averaged to approximately 60%, and was thus lower than that of Th17 cells sorted from uninfected donors (Fig. 8A and 8B). Moreover, in patient-derived cells, CD4+ cells constituted approximately 95% of the population, while CXCR3+ cells constituted roughly 2% of the population (Fig. 8C). The lower degree of Th17 cell purity is likely a consequence of altered immune cell frequencies in HAART-treated subjects, as we and others have shown that Th17 cell frequencies are reduced in PBMCs by roughly 40% (Fig. 7) [81]. To determine the degree by which Th17 cells were enriched from CD4+ T cells, Th17 cell frequencies were measured in total CD4+ lymphocytes and in sorted cells. In uninfected individuals, Th17 cell purity was approximately four times higher in sorted cells, while in HIV-infected subjects, Th17 cell purity was roughly three times higher in sorted cells (Fig. 8D).

Overall, despite the variation in Th17 cell purity, Th17 cells were still enriched

within the sorted population, and the data acquired in this study is therefore primarily representative of Th17 cells.

4.3 IL-17A expression in Th17 cells: IL-17A, like other cytokines, is induced by 4-6 hours of stimulation with PMA and ionomycin [51, 54] and contained inside the cells using Brefeldin A [163]. IL-17A expression can be maximized by subjecting CD4+CCR6+ memory T cells to 3-12 days of *in vitro* expansion conditions (anti-CD3/CD28, further maximized with IL-2), followed by 4-6 hours of PMA/ionomycin stimulation. Moreover, Th17 polarizing cytokines may also be added to the culture conditions to further prime IL-17A expression [51, 80, 119]. Roughly 1% IL-17A+IFNγ- CD4+ T cells occur within PBMCs, whereas roughly 2.5-5% IL-17A+IFNγ- cells occur within blood-derived CD4+ memory T cells [48, 80, 172].

Approximately 10% CD4+CCR6+ memory T cells become IL-17A+IFNγ- (Th17) in response to PdBU and ionomycin [80]. This frequency becomes maximized to approximately 30% following expansion of CD4+CCR6+ memory T cells [119]. In CD4+CXCR3-CCR10-CCR4+CCR6+ memory T cells, PMA and ionomycin stimulation induces IL-17A expression in roughly 20% of the cells, while IFNγ becomes expressed in less than 1% of the cells [51]. Furthermore, up to 50% CD4+CXCR3-CCR4+CCR6+ memory T cells acquire an IL-17A+IFNγ- functional phenotype following *in vitro* expansion [80]. Using the same cell type, others managed to induce IL-17A expression in approximately 70% of the cells, and IFNγ expression in roughly 3% of the cells [79]. In summary, a minority of these cells have been shown to typically express IL-17A following *in vitro* stimulation, and for reasons that remain elusive.

In this study, the IL-17A+ cells within the population were identified by stimulating Th17 cells with PMA and ionomycin, with Brefeldin A included, for six hours, which is

similar to what has been done in other studies [51,54] and by the manufacturer of the Human Th17 Cell Enrichment Kit [156]. On average, roughly 10% Th17 cells expressed IL-17A (Fig. 3A), which can be expected based on data reported in other studies and by the manufacturer [51, 80, 156]. Despite the variation in Th17 cell purity obtained in this study, these minimal stimulation conditions induced IL-17A production in a small percentage of the cells, which is similar to the results others have obtained using purified, FACS-sorted Th17 cells [51,80], suggesting that the variation in Th17 cell purity did not result in the loss of functional (IL-17A+) Th17 cells.

As mentioned previously, it is unknown why the majority of Th17 cells acquire an IL-17A- functional phenotype following PMA and ionomycin stimulation. Interestingly, in one study, IL-17A production was triggered in IL-17A- Th17 cells following 48 hours of stimulation with IL-2, IL-7 or IL-15 prior to PMA/ionomycin stimulation, while IL-17A was further upregulated in IL-17A+ Th17 cells [86]. Both the IL-17A- and IL-17A+ Th17 cells were found to express equal amounts of RORC, indicating that the IL-17A- cells are Th17 lineage-committed [86]. Furthermore, Wan et al. also proposed that IL-17A expression evident *in vitro* is lower than IL-17A expression *in vivo*, most likely due to lack of appropriate stimulation conditions [86]. In accordance with Wan et al.'s findings, it can be speculated that the IL-17A- Th17 cells obtained in this study are mature Th17 cells unable to effectively respond to mitogen stimulation *in vitro*.

4.4 Expression of Th17-associated surface markers: To further evaluate the phenotype of sorted Th17 cells, CCR4, CD161, CD25, CXCR4 and CCR5 were also measured on the cells. CCR4, when expressed by CCR6+CD4+ T cells, further identifies Th17 cells [79, 80]. In addition, CD161 has been established as a Th17 surface marker [161], especially when expressed by CCR6+CCR4+CXCR3- cells [79]. CCR4 was evident on roughly 65% Th17

cells (Fig. 1D), which further confirms the notion that the cells sorted in this study were representative of Th17 cells based on phenotypic analysis. Furthermore, CD161 was expressed by roughly 30% of the Th17 cells (Fig. 1D), which is lower than what others have reported [161]. This discrepancy could be due to variation in methods of analysis, as Kleinschek et al. measured CCR6 expression on total CD161+ CD4+ memory T cells that had not been depleted of CXCR3+ cells [161]. Furthermore, the variation in Th17 cell purity observed in our study could also account for this discrepancy.

Next, high levels of CD25 expression identify T_{regs} [162]. Since a minority (roughly 10%) of Th17 cells expressed CD25 (Fig. 1D), it is most probable that $CD25^{hi}$ T_{regs} are completely absent from or present at low frequencies within the population. Finally, CXCR4 and CCR5 are co-receptors that facilitate HIV entry into the cell [173], and have been previously measured on Th17 cells by others [81]. In particular, it was found that almost 50% Th17 cells express CXCR4 while almost 20% express CCR5 [81]. In this study, the majority of Th17 cells (roughly 90%) expressed CXCR4 (Fig. 1D), which is higher than the value reported by Gosselin et al [81]. Furthermore, approximately 20% Th17 cells expressed CCR5 (Fig. 1D), which is consistent with what Gosselin et al. reported. While Th17 cells were sorted based on the CD4+CXCR3-CCR4+CCR6+ memory T cell phenotype in the study performed by Gosselin et al. [81], Th17 cells were sorted based on the CD4+CXCR3-CCR6+ memory T cell phenotype in this study, which could account for this discrepancy. Furthermore, as mentioned previously, Th17 cell purity was variable between donors.

Overall, these findings provide further insight into the phenotype of sorted Th17 cells used in this study, and suggest that the population was mainly representative of Th17 cells.

4.5 Characterization of IL-7 responses in Th17 cells from healthy individuals: This project involved characterizing CD127 expression and functional IL-7 responses in blood-

derived Th17 cells from healthy individuals, then determining whether CD127 expression or any of the above responses became dysregulated in Th17 cells from HIV-infected, HAART-treated subjects. Responses to IL-7 were characterized by measuring phosphorylation of STAT5, expression of Bcl-2, and cell proliferation, as these responses have been previously established in both CD4+ and CD8+ T cells [132, 138].

Others have reported that murine Th17 cells express CD127 [130, 131], and that Th17 cells are present within total CD127+ CD4+ memory T cells [51, 134]. One study has even reported that the majority of total, human CD4+CCR6+ memory T cells express CD127 [118]. Expression of CD127 has not yet been measured on an isolated population of human Th17 cells, however. In this study, CD127 expression was measured on human, blood-derived Th17 cells, which was found to be comparable to that of bulk CD4+ T cells (Fig. 2). Furthermore, CD127 was expressed by total, PMA/ionomycin-stimulated Th17 cells, IL-17A+ cells and IL-17A- cells (Fig. 3B). CD127 becomes downregulated on activated T cells [174], which could account for the reduction in the mean frequency of total, stimulated CD127+ Th17 cells. It is unknown, however, why the IL-17A+ cells contained the highest frequency of CD127+ cells amongst the three groups of stimulated Th17 cells. Overall, resting and activated human Th17 cells express the IL-7 receptor α component.

Next, functional IL-7 responses were evaluated in human Th17 cells. First of all, IL-7 induced STAT5 phosphorylation in human Th17 cells in a dose-dependent manner (Fig. 4). These results could be expected, as IL-7 plays a pivotal role in STAT5 phosphorylation in murine IL-17A+ Th17 cells [130]. Next, Bcl-2 was expressed by a small frequency of Th17 cells, which became upregulated following 48 and 72 hours of stimulation with IL-7 (Fig. 5). These results can be expected, as murine IL-17A+ Th17 cells have also been reported to express Bcl-2, which becomes downregulated when CD127 is inhibited *in vivo* [130].

Finally, while neither IL-7 nor PHA induced Th17 cell proliferation on their own, IL-7 induced Th17 cell proliferation in a dose-dependent manner when used in combination with a sub-optimal dose of PHA (Fig 6). As mentioned previously, using a sub-optimal dose of PHA enhanced the response elicited by IL-7 without causing proliferation on its own. Some have reported that T cells treated with higher doses of IL-7 (10-100 ng/mL) proliferate without TCR stimulation [169]. Others have reported, however, that T cell proliferation in response to IL-7 on its own occurs to a lower degree than when IL-7 is complimented with TCR stimulation [175]. Despite these reports, IL-7 (100 ng/mL) on its own was unable to induce proliferation in Th17 cells (Fig. 6B), which confirms the requirement for co-stimulation.

In summary, human Th17 cells are responsive to IL-7.

4.6 Characterization of IL-7 responses in Th17 cells from HIV-infected, HAART-treated subjects: In this study, while CD127 expression and IL-7 responses have been characterized in Th17 cells from uninfected individuals, these responses were found to be altered in Th17 cells from HIV-infected individuals receiving HAART. Since Th17 cells are not always normalized in patients receiving HAART [113,115], we hypothesized that Th17 cells exhibiting any abnormalities in IL-7 responses during treated HIV infection could, in part, account for their incomplete recovery. Accordingly, Th17 cells were only studied from HAART-treated patients.

CD127 expression on Th17 cells was found to be similar between HIV-infected subjects and uninfected individuals (Fig. 9). As HAART restores CD127 expression on CD4+ memory T cells from treated patients [127], it could be possible that similar phenomena also occur in the context of Th17 cells. Whether HIV infection initially downregulates CD127 on Th17 cells prior to initiation of treatment, however, is unknown.

While others have suggested that disturbances in IL-7 signalling occur in T cells irrespective of normal or abnormal CD127 expression [152, 153], we have proposed that IL-7 responses may also be perturbed in Th17 cells regardless of normal CD127 expression. Interestingly, Th17 cells from HIV-infected, HAART-treated subjects exhibited alterations in certain IL-7 responses. First of all, following stimulation with a higher dose of IL-7 (0.1 ng/mL), the frequency of pSTAT5+ cells appeared to be slightly higher in HIV-infected subjects, although statistical significance was not observed (Fig. 10A). Accordingly, no definite conclusions may be formulated at this time. Since pSTAT5 was only measured in Th17 cells from four different patients, increasing this sample size could lead to a statistically significant result. Interestingly, the pSTAT5 MFI values were similar in Th17 cells between HIV-infected subjects and uninfected individuals (Fig. 10B), indicating that the degree of STAT5 phosphorylation was not affected. Overall, IL-7-mediated phosphorylation of STAT5 was not impaired in Th17 cells from treated patients. Moreover, whether this particular response to IL-7 is initially suppressed in Th17 cells from untreated patients is unknown.

The percentages of Bcl-2+ Th17 cells were significantly higher in HIV-infected subjects compared to uninfected individuals, both at the basal level and following stimulation with IL-7, while MFI values of Bcl-2 expression were similar between both groups (Fig. 11A and 11B). Furthermore, the relative increase in both the frequencies of Bcl-2+ Th17 cells and Bcl-2 MFI values was not impaired in HIV-infected individuals (Fig. 11C and 11D), confirming that the ability of IL-7 to upregulate Bcl-2 is not impaired in Th17 cells during treated HIV infection. Overall, during HAART-treated HIV infection, a higher proportion of Th17 cells express Bcl-2 at the basal level and in response to IL-7.

It is unknown why Bcl-2 is upregulated in Th17 cells from patients receiving HAART. Aìro et al. proposed that HAART restores naïve CD4+ T cells by upregulating cell survival in a Bcl-2-dependent manner [176]. Alternatively, others have suggested that HIV infection persists in target cells by upregulating Bcl-2 and thus maintaining viability [144, 177]. Th17 cells have been found to be susceptible to HIV infection [81, 118, 119]. Moreover, Th17 cells from HAART-treated patients have been shown to carry integrated viral DNA, which could be indicative of latent HIV infection [81]. It is therefore possible that the increase in Bcl-2+ Th17 cells from HAART-treated patients observed in this study could be caused by the virus as a means to maintain latent infection, although this relationship would need to be established.

Finally, Th17 cell proliferation from HIV-infected subjects was dramatically suppressed (Fig. 12), which could be a result of multiple events. This can be expected, as CD8+ T cells from treated patients also exhibit impaired proliferative responses when stimulated with PHA and IL-7 [126]. Furthermore, T cell proliferation in treated patients was also shown to be impaired following combined stimulation with anti-CD3 and IL-7 [165]. While perturbations in cytokine signalling could partially account for the suppressed proliferative response, it is unclear whether signalling events induced specifically by IL-7 and/or PHA become dysregulated during HIV infection. One study has shown that IL-7-mediated proliferation is suppressed in CD4+ effector memory T cells from untreated patients [154], while another study has shown that expression of cyclins indicative of the S and G1 phases of the cell cycle are impaired in Vβ3-TCR-stimulated CD4+ T cells from HIV-infected individuals [178]. These studies independently demonstrate that HIV infection results in perturbations in both TCR- and IL-7-mediated proliferation.

4.7 Potential causes of impaired Th17 cell proliferation: While Sieg et. al had concluded that CD4+ T cells from patients failed to proliferate in response to Vβ3-TCR stimulation, it was noted that events involved in early activation did not become altered during HIV infection, indicated by comparable expression of CD25 and CD69 between cells from HIV-infected individuals and uninfected individuals [178]. In this study, it has not been confirmed whether the Th17 cells were being sufficiently stimulated in order to undergo proliferation. If signalling mechanisms resulting in activation were impaired, it could be possible that proliferation became suppressed as a result. IL-2 is a γc cytokine that promotes T cell proliferation, and its responses become enhanced through increased expression of CD25, the α component of the IL-2 receptor [179]. While high CD25 expression identifies T_{regs} [162], it also identifies activated T cells [166, 167]. In CD4+ T cells, IL-7 promotes responses to IL-2 by upregulating CD25 [180]. PHA also increases CD25 expression on T cells, which further facilitates a proliferative response [166]. During HIV infection, however, the ability of IL-7 to upregulate CD25 becomes suppressed in T cells, which was also observed with impaired proliferation [165]. Therefore, deducing the effects of IL-7 on CD25 expression in Th17 cells, from both uninfected and HIV-infected individuals, may provide further insight into the mechanisms by which proliferation becomes impaired during HIV infection.

In Th17 cells, the cell signalling events leading to proliferation are poorly characterized. In T cells, IL-7 triggers proliferation through PI3K/Akt signalling [142]. Moreover, IL-7 enhances IL-17A expression in Th17 cells in a PI3K-dependent manner [86], although the direct effects of IL-7 on phosphorylation of PI3K and Akt have not been characterized in this lineage. In CD4+ effector memory T cells, however, IL-7 was shown to induce low levels of Akt phosphorylation [132]. Notably, Akt may play a pivotal role in

Th17 cell proliferation, as it has been shown to promote Th17 cell differentiation in a TCR-dependent manner [143]. In CD4+ T cells from untreated, HIV-infected individuals, the ability of IL-2 to induce phosphorylation of Akt was shown to be impaired [181], although the particular effects of IL-7 have yet to be examined in this regard. In summary, characterizing the effects of Akt phosphorylation on Th17 cell proliferation in healthy individuals and HIV-infected, HAART-treated individuals may provide insight into the mechanisms by which Th17 cells are impaired in their ability to undergo proliferation, as has been determined in this study.

In conclusion, the impaired proliferative response observed in Th17 cells may provide insight into the mechanisms by which these cells fail to become completely restored in the context of HAART-treated HIV infection.

4.8 Overview: In this study, we have established functional roles for IL-7 in human, blood-derived Th17 cells, and elucidated how these roles become altered in the context of treated HIV infection. As we have demonstrated that Th17 cells from HIV-infected, HAART-treated subjects exhibit impaired proliferation in response to IL-7 combined with a sub-optimal dose of PHA, promising outcomes may result from targeting the specific mechanisms underlying this impairment. It is therefore crucial to first characterize the signalling events responsible for Th17 cell proliferation, and then determine how their activity may become affected in HIV-infected individuals receiving HAART. Furthermore, as these results were generated from Th17 cells obtained from the bloodstream, they could provide insight into the basic mechanisms that regulate homeostasis in this particular Th lineage, although they may or may not be reflective of such mechanisms occurring in the GALT. Therefore, it may be of interest to next determine whether similar phenomena also occur in Th17 cells derived from the GALT, and how the microenvironment in the GALT

may influence the normal, homeostatic responses to IL-7 occurring in Th17 cells from both uninfected individuals and HIV-infected, HAART-treated individuals.

Overall, as indicated by others [113, 114], normalizing and maintaining functional Th17 cells during HIV infection may in turn significantly alleviate microbial translocation and chronic inflammation, which could potentially reduce the associated risk factors of developing non-AIDS complications and thus improve quality of life. Developing therapeutics targeted towards restoring homeostatic Th17 cell proliferation could facilitate their complete restoration.

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APPENDICES

Supplementary figures

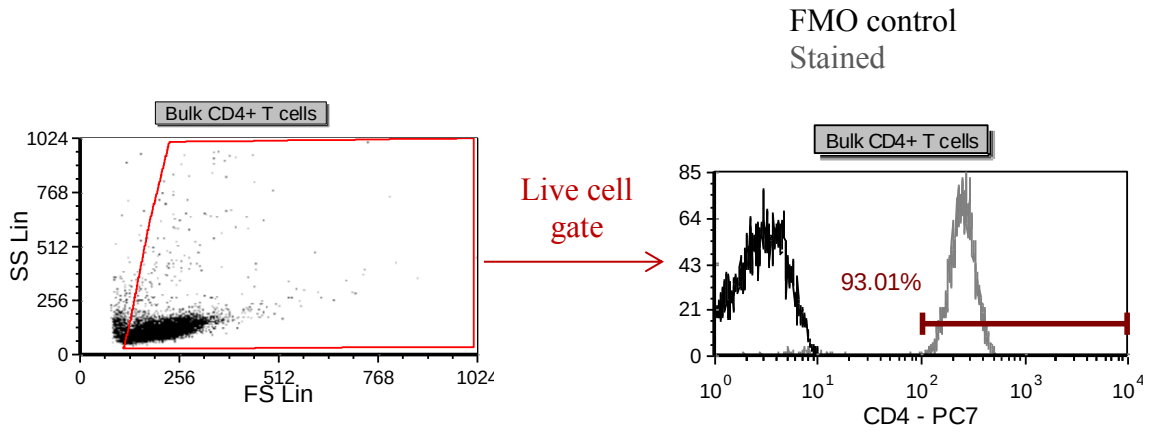
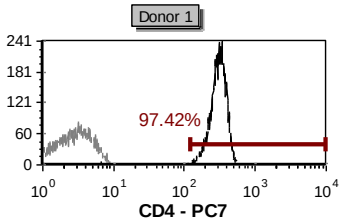
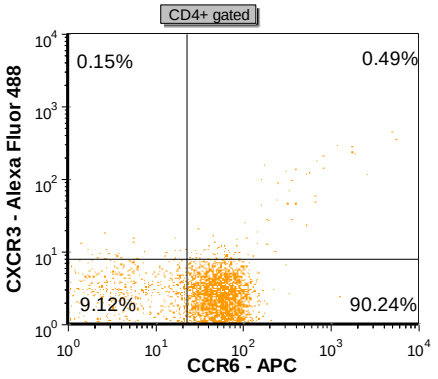


Figure S1: Verification of CD4 expression on purified, bulk CD4+ T cells. CD4+ T cell purity, defined as the percentage of CD4+ cells, is demonstrated as a representative of one donor. The mean frequency of CD4+ cells is 93% $\pm$0.88 (SEM) (N=6).

Day of enrichment





FMO control

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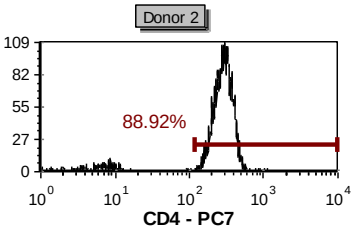
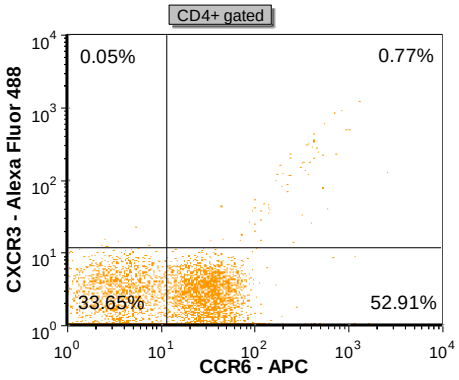
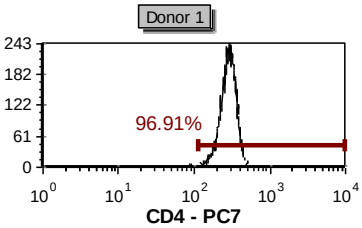
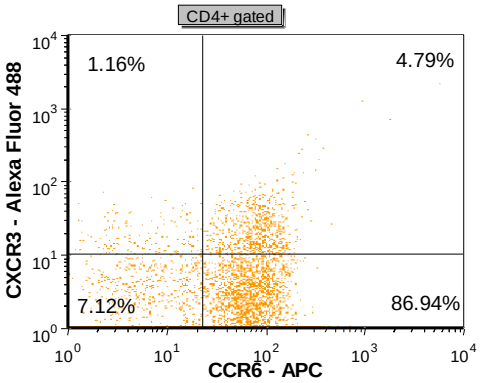
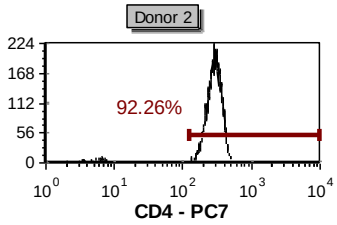




One day post enrichment







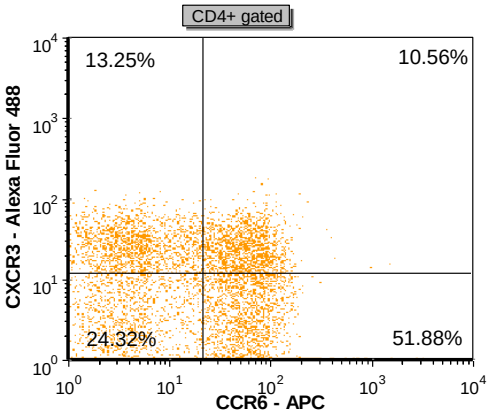


Figure S2: CXCR3 expression is upregulated on sorted Th17 cells following overnight cell culture. Sorted Th17 cells from two different individuals are represented in the histograms and orange scatter plots. Expression of CD4, CXCR3 and CCR6 were measured **A)** immediately after enrichment and **B)** one day following the enrichment.

CURRICULUM VITAE

Education:

Master of Science in Microbiology and Immunology - University of Ottawa/Ottawa Hospital Research Institute (OHRI) (September 2013 – November 2015)

Bachelor of Science in Microbiology - University of British Columbia Okanagan (UBC-O) (September 2008 - April 2013)

Academic Awards:

Graduate Studies Entrance Scholarship - University of Ottawa (September 2013)

Achievement of Dean's List recognition - University of British Columbia Okanagan (June 2013)

University of British Columbia Okanagan Entrance Scholarship (June 2008)

Kevin Molloy Scholarship (June 2008)

Research and Academic Experience:

MSc Graduate Studies– Laboratory of Dr. Jonathan Angel, University of Ottawa/OHRI (September 2013 – November 2015)

Expertise: Human immunology, HIV immunopathogenesis, peripheral blood mononuclear cell (PBMC) isolation, magnetic cell separation, mono- and poly-chromatic surface and intracellular staining, measurement of protein expression using flow cytometry (up to four fluorochromes), and Biosafety Level 3 operational practices.

Project: To characterize IL-7-mediated mechanisms of homeostasis in human Th17 cells and to determine whether these mechanisms become dysregulated as a result of HIV infection.

Undergraduate Research Assistant - Laboratory of Dr. Louise Nelson, UBC-O (May 2010 – August 2010)

Project: Assisted with the investigation of potential preventative measures of post-harvest decomposition in apples, and identification of various micro-organisms in apple orchards.

Techniques practiced: Bacterial plating and DNA quantification using PCR and macroarrays.

Communication and Leadership:

Undergraduate Teaching Assistant - Laboratory Component of Introductory Biology, UBC-O (September 2011 – December 2011, September 2012 – April 2013)

Duties: Prepared and presented lessons, marked assignments and exams, and assisted students.

Volunteer – Canadian Cancer Society (February 2011 – May 2013)

Duties: Member of planning committee for the restructuring of the volunteer unit in Kelowna, BC, facilitated a volunteer orientation webinar, helped with Health Promotions at various local events, assisted with the organization of Relay for Life and facilitated games and activities for the guests on Relay for Life Event Day.

Conference Abstracts:

"IL-7 Induces Mediators of Cell Survival and Proliferation in Th17 Cells"

Stilla, Alana M.[1,2], Côté, Sandra C.[1,2], Angel, Jonathan B.[1,2,3]

1. Department of Biochemistry, Microbiology & Immunology, University of Ottawa, Ottawa, ON, Canada

2. Chronic Disease Program, Ottawa Hospital Research Institute, Ottawa, ON, Canada

3. Division of Infectious Diseases, Ottawa Hospital-General Campus, Ottawa, ON, Canada

Canadian Association of HIV Research (CAHR) 2015: Apr. 30th – May 3rd, Toronto ON

Presented research poster, attended Annual New Investigator Workshop for new HIV researchers

Ontario HIV Treatment Network (OHTN) 2014: Nov. 25th-26th, Toronto ON

Presented research in plain language summary

Languages:

Fluent in English, intermediate level Italian and beginner level French