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Katherine Anne Lambert April 14, 2020

Ruxolitinib to Modulate CD4+ Memory T-cells in HIV+ Individuals Under Long-term Antitretroviral Therapy

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Abstract

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By Katherine A. Lambert

Despite the successful use of Antiretroviral Therapy (ART). physicians have still been unable to eradicate Human Immunodeficiency Virus (HIV) from the body of HIV+ individuals. ART is only able to target infected CD4+ T cells in which HIV is actively replicating, and not every cell infected with HIV will be actively producing more virus. These latently infected CD4+ T cells make up the HIV latent viral reservoir and prevent the ability to fully rid the body of an HIV infection. Central and stem cell memory (T_{CM} and T_{SCM}) CD4+ T cells, both of which are long-lived, younger T cell subtypes, make up a disproportionately large amount of this latent reservoir. This study analyzes the ability of ruxolitinib, a Jak-STAT inhibitor FDA approved for myelofibrosis and polycythemia vera, to cause the differentiation of these CD4+ T cells. This induced differentiation effectively pushes the latently infected cells to die. We analyzed blood samples from the A5336 AIDS Clinical Trials Group (ACTG) randomized controlled trial, in which participants received ruxolitinib for 5 weeks. Through flow cytometric analysis, we found conflicting results. We found that ruxolitinib treatment resulted in a reduction of the CD4+ T_{TM} and T_{TE} and CD8+ $T_{CM,}$ $T_{TM,}$ and T_{EM} cell subsets. We also found significant correlations between the change in size of CD4+ T_{TM} cell subset and the change in expression of Bcl-2 as well as the change in size of the CD8+ T_{CM} cell subset and the change in expression of soluble CD14. Overall, our results do not provide an overarching conclusion of the ability of ruxolitinib to change the composition of the CD4+ T cell population in a way that would facilitate the eradication of the HIV latent viral reservoir. However, the correlation between the changes in Bcl-2 expression and relative CD4+ T_{TM} cell population size could imply there are certain patients

who can reduce the reservoir. This study provides the groundwork for future analyses on the effectiveness of the drug as a possible cure for HIV infections.

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Introduction

History of Human Immunodeficiency Virus

The Human Immunodeficiency Virus (HIV)/Acquired Immunodeficiency Syndrome (AIDS) pandemic is one of the largest and most long-lived pandemics in human history. While HIV may have crossed over from chimpanzees to humans as early as the 1920s, it was not until 1981 that AIDS started making national news in the United States. On June 5th, 1981, the Center for Disease Control published a report of five homosexual men in the greater Los Angeles area who, between December 1980 and May 1981, had been hospitalized due to *pneumocystis carinii* and candidiasis in conjunction with unexplained immunodeficiency [1]. This marked the discovery of the emerging Acquired Immunodeficiency Syndrome (AIDS) epidemic in the United States of America. In 1983, Dr. Luc Montagnier, Dr. Francoise Barre-Sinoussi, and Dr. Harald zur Hausen discovered a retrovirus belonging to the Human T-Lymphotropic Retrovirus family that was isolated from an individual with symptoms of AIDS [2]. Their discovery of HIV earned them the 2008 Nobel Prize in Physiology and Medicine. One year later in 1984, Dr. Richard Gallo found a causal link between this retrovirus, later named HIV in 1986, and AIDS [3,4]. Since the beginning of the epidemic, an estimated 75 million people have been infected with HIV and 32 million people have died of HIV/AIDS related illnesses. As of 2018, 37.9 million people were living with HIV/AIDS worldwide, with 1.7 million people newly infected in 2018 [5].

The emergence of HIV is the result of at least eleven cross-species transmission events from non-human African primates infected with Simian Immunodeficiency Virus (SIV) to humans [6]. There are two different strains of HIV that emerged from simian-human cross-species transmission: HIV-1 and HIV-2. HIV-1 is responsible for the majority of HIV epidemics around the globe, while HIV-2 is mainly concentrated in Western Africa [7]. Specifically, there were three transmissions of SIV_{cpz} from central African chimpanzees

subspecies, resulting in HIV-1 groups M, N, and O, while there were eight SIV_{smm} transmissions from sooty mangabeys, creating HIV-2 groups A-H [6].

Transmission of HIV

The transmission of HIV occurs via multiple mechanisms. HIV is carried in certain bodily fluids, including blood, breast milk, vaginal fluids, rectal fluids, and semen. Exposure to these bodily fluids can lead to transmission. Transmission via blood can occur through using contaminated needles or being infused with contaminated blood products. Mother to child transmission occurs through breast feeding and before, during, or after birth. Lastly, and most commonly, HIV is transmitted by sexual contact across mucosal membranes. This includes anal and vaginal intercourse [8]. Globally, 70% of HIV transmissions come from heterosexual intercourse, with the remaining percentages from maternal-fetal transmission, men who have sex with men, and injection drug use [9].

Structure of HIV

HIV is classified as a Lentivirus. Lentiviruses have been observed in numerous species, such as cats, sheep, goats, horses, cattle, and monkeys. The HIV genome consists of two identical single-stranded RNA molecules enclosed in the core of the virus. HIV targets the immune system, specifically CD4+ cells. Glycoprotein gp120 on the surface of HIV binds to the CD4 receptor on the host cell, inducing a conformational change. This conformational change in the envelope then allows for binding to the co-receptor, either CCR5 or CXCR4, on the surface of the cell, allowing for the fusion of the host cell and HIV membranes. HIV is a member of the Retroviridae family, which includes RNA viruses that infect birds and mammals and utilize reverse transcriptase, an enzyme that synthesizes DNA from RNA. The reversely transcribed DNA can be inserted into the genome of its host cell [7]. Once the virus has entered the cell, the enzyme catalyzes proviral DNA from the single-stranded RNA that is

then integrated into the human genome. CD4+ cells include T cells, macrophages, and dendritic cells, all of which are susceptible to HIV [10].

Memory CD4+ T cells

The cell type that is the most targeted by HIV infection is CD4+ T cells. CD4+ T cells are commonly characterized as T helper cells and are critical to the functioning of the adaptive immune system. In general, all types of T cells have T cell receptors (TCR) that are the primary mediator of T cell activation, and the successful combination of a functional TCR and the emergence of the cell from the thymus results in a naïve T-cell. Naïve T cells, which can migrate to secondary lymphoid tissues, cannot initiate an immune response [11]. When naïve cells are exposed to an antigen-presenting cell (APC) in the right context, they can be activated to differentiate into more mature T cells. An APC is a cell that expresses major histocompatibility complex (MHC) class II molecules. MHC molecules bind peptide fragments from pathogens and display them on the surface of the cell, initiating an immune response. CD4+ T cells respond to MHC class II molecules and other signals from APCs, inducing their activation [12]. CD4+ T cells, after being activated and differentiated into various subtypes, play an important role in the secretion of cytokines. There are various functions of CD4+ T cells, including the activation of the innate immune system, B-lymphocytes, and CD8+ T cells. Additionally, some CD4+ T cells play a vital role in the suppression of immune reactions [13]. HIV infection leads to the rapid deterioration of the CD4+ T cell population via destruction (viral cytopathic effect and activation induced apoptosis) and decreased production.

A specific segment of the T-cell population that is vital to CD4+ T cell function is memory T cells. Memory T cells can be defined as antigen-specific T cells that remain long term post infection. These memory T cells provide a rapid response to re-infection of a specific antigen, as memory T-cells can quickly be converted into large numbers of effector T

cells when the body is re-exposed to a specific antigen [14]. There are a variety of memory CD4+ T cell subtypes. Naïve T cells are the least differentiated of all of the subtypes. Memory stem cells (T_{SCM}) have an important function in replenishing differentiated memory T cell populations. Specifically, T_{SCM} cells have the ability to differentiate into specialized cell types in response to specific signals and self-renew [15]. Central memory T cells (T_{CM} cells) are thought to also be a stem-cell like memory subset, meaning they can further differentiate into more mature cell subsets. T_{CM} cells circulate throughout lymph nodes and mucosal lymphoid organs and undergo secondary immune responses. Effector memory T cells (T_{EM} cells) and transitional memory T cells (T_{TM} cells) express homing receptors that allow them to migrate to nonlymphoid sites of inflammation and produce a variety of cytokines. T_{EM} and T_{TM} cells lose their stem-like properties and tend to be shorter lived [16]. Additionally, T_{EM} cells are more differentiated than T_{TM} cells. The most differentiated cell subset is terminally differentiated effector T cells (T_{TE} cells) [17]. Memory CD4+ T cells are preferentially targeted by HIV. Through the elimination of the memory CD4+ T cell population, the immune system loses the capability to control the immunological response to HIV replication as well as to other pathogens, leading to opportunistic infections and AIDS [13].

Memory CD8+ T cells

Unlike CD4+ T cells, CD8+ T cells are not targeted for infection by HIV, as they lack CD4 receptors needed for viral entry. CD8+ T cells, also known as cytotoxic T-lymphocytes, recognize infected cells through an MHC-I dependent mechanism. Infected cells insert peptides derived from viral proteins into their cellular membranes to mark themselves for the CD8+ T cells. Once located, the cytotoxic CD8+ T cells cause the virally infected cells to die [18]. HIV has been able to evade the normal CD8+ T cell response to its presence by rapidly mutating, interfering with cellular receptors, and down-regulating MHC-I expression in

infected cells, completely disrupting proper CD8+ T cell functioning. This leads to an overall decrease in the ability of memory CD8+ T cells to fight off HIV infection [19]. Additionally, during HIV infection, while CD4+ T cells are depleted, the CD8+ T cell count is elevated throughout the chronic phase of infection. While ART helps to restore CD4+ T cell levels, it has no significant event on CD8+ T cell count elevation [20].

The Latent Viral Reservoir

Antiretroviral therapy (ART) improves the survival of infected individuals by decreasing the replication of HIV-1. Specifically, ART targets cells that are undergoing active HIV replication. If started early enough (i.e., within 1-2 days), ART can actually be used as a preventative measure to block HIV infection after exposure. While this therapy has greatly prolonged survival of infected individuals, not every cell infected with HIV will be actively producing more virus [21]. Rather, a significant portion of infected memory CD4+ T cells are in a latent state and because they are not replicating HIV, cannot be targeted by ART or the immune system. The result of this latency is a viral reservoir that remains hidden in cells even during rigorous ART treatment; because these infected cells cannot be targeted, they remain in the body and HIV continues to persist [22, 23]. In ART treated patients who have had no detectable viremia for seven years, the viral reservoir was still detectable [24]. In fact, it is predicted that eradication of the viral reservoir via ART only could take up to 74 years [24]. Once ART is stopped, these latent cells can begin replicating virus, reestablishing the presence of large quantities of HIV in the body and causing clinical symptoms [24]. This latent viral reservoir is the reason why HIV has yet to be able to be eradicated from the body in the vast majority of cases. Evidently, ART is not sufficient to completely eliminate HIV-1 from individuals with HIV infection.

A major question in determining how to eliminate the HIV viral reservoir is understanding which cell subtypes need to be targeted. HIV frequently infects CD4+ T_{CM} and

T_{SCM} cells and both of these cell types have stem-like properties, including enhanced self-renewal/proliferation, resulting in CD4+ T_{CM} and T_{SCM} cells being major contributors to the HIV viral reservoir in individuals on ART [25]. Importantly, it has been found that proliferation of infected cells plays a vital role in maintaining the size of the HIV viral reservoir. Therefore, proliferation of infected CD4+ T_{CM} and T_{SCM} cells may enable latent HIV to remain in the CD4+ T cell population [26]. Additionally, while the CD4+ T_{SCM} cell population is only a small subset of the overall CD4+ T cell pool, they disproportionately contribute to the total HIV reservoir in patients on long-term ART [25]. Evidently, in order to eliminate the HIV viral reservoir in individuals on long-term ART, latently infected CD4+ T_{SCM} and T_{CM} cells need to be considered in cure-directed approaches.

Jak-STAT Pathway

One of the pathways that is integral to self-renewal of CD4+ T_{SCM} and T_{CM} cells is the Jak-STAT pathway. There are four families in the Jak-STAT family: Jak1, Jak2, Jak3, and Tyk2. Jak3 is primarily expressed in hematopoietic cells, while Jak1, Jak2, and Tyk2 are expressed throughout many different types of cells [27]. The Jak-STAT pathway is a tyrosine kinase pathway. Jak proteins are tyrosine kinases embedded in the cellular membrane of cells and multimerize upon the binding of ligands. Multimerization induces autophosphorylation of the intracellular domain of the cell, inducing the phosphorylation of STAT proteins, causing them to dimerize. STATs are latent transcription factors. In mammals, there are seven STAT genes: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. Once phosphorylated, STATs dimerize into their active form and enter the nucleus via the importin α−5 and the Ran nuclear import pathway. Once in the nucleus, dimerized STATs bind to specific regulatory sequences on DNA to target the transcription of specific genes [28, 29].

Furthermore, different Jak proteins respond to different ligands. The main ligands for Jak proteins include interleukins (ILs) and interferons (IFNs), both of which are cytokines involved in proper immune function and signaling [30]. Tyk2 associates with IFN-1, IL-6, IL-10, IL-12, and IL-23 families. Jak1 interacts with type I (IFN-α/B), type II (IFN- γ), IL-2, and IL-6 families. Jak2 associates with single-chain (Epo-R, GH-R, PrI-R) and IL-3 families while Jak3 exclusively interacts with with the IL-2 family [31]. As different Jak proteins interact with different ligands, multiple different cellular responses can be invoked from the Jak-STAT pathway by simply altering the ligand.

The Jak-STAT pathway induces a variety of cellular responses. Responses can include differentiation, migration, apoptosis, and cell survival. The response depends on not only the signal but also the tissue and the cellular context [32]. Numerous developmental and homeostatic processes are controlled by Jak-STAT signaling, including hematopoiesis, organismal growth, mammary gland development, and, in the context of this paper, immune cell development and stem cell maintenance [27, 29, 32]. Cell proliferation is vital to the maintenance and survival of the HIV viral reservoir. With the activation of the Jak-STAT pathway, CD4+ T cells can be induced to proliferate, allowing the maintenance and replenishment of the reservoir [33].

Cellular and Soluble Markers of HIV Infection

Various cellular and soluble markers are associated with persistent HIV infection and can help physicians and researchers better understand the severity of a patient's HIV infection while on ART. Interleukin-6 (IL-6) is a pro and anti-inflammatory cytokine and is typically associated with chronic inflammation. Importantly, as HIV can lead to chronic inflammation, high IL-6 levels are associated with HIV infection and replication [34]. Another important marker for persistent HIV infection is soluble CD14 (sCD14). CD14 is a glycosyl phosphatidyl inositole (GPI) linked receptor protein found on the surface of

monocytes, macrophages, polymorphonuclear leukocytes, and dendritic cells involved in innate immune responses. In HIV-1 infected patients, high sCD14 serum levels are associated with disease activity and viral load. Hence, patients with higher latent viral reservoirs will have higher sCD14 serum levels [35]. Moreover, Bcl-2 proteins can be used as another marker of HIV persistence. Bcl-2 is a major regulator of apoptosis, or induced cell death. When the protein Casp8p41 binds to the protein Bak, the complex becomes activated and induces apoptosis of infected cells. HIV induces higher expression of Bcl-2, which will bind to Casp8p41 and prevent apoptosis from occurring. Therefore, higher levels of Bcl-2 expression are indicative of HIV-infected cells [36].

Additionally, while it can be hard to measure underlying HIV infection while patients are on ART due to the lack of plasma viremia, other markers can be used to quantify the viral reservoir. Specifically, cell-associated HIV RNA (CA-RNA), or HIV RNA levels within the cells. Importantly, higher the levels of CA-RNA indicates residual virus transcription and a more significant viral reservoir [37]. Furthermore, another marker for the latent viral reservoir in HIV patients on ART is total HIV-1 DNA levels (totDNA). TotDNA quantifies the total HIV DNA within the cells, whether it is integrated into the host genome or not, and whether it is replication competent or not (typically thought of as the largest estimate of virus persistence). Similar to CA-RNA, high totDNA levels indicate a large HIV latent viral reservoir within the cells [38].

Ruxolitinib

Ruxolitinib is a commercially available FDA-approved Jak1/2 inhibitor. Commercially, ruxolitinib has been used to treat myelofibrosis, a myeloproliferative disease where collagen fibrosis replaces bone marrow due to the stromal overproduction of collagen. Due to the prevalence of the Jak-STAT pathway in cell proliferation, there has been an initiative to investigate the possible positive effects of ruxolitinib on HIV+ individuals

undergoing ART. Past studies have demonstrated ruxolitinib's ability to block HIV viral production and prevent IL-15-induced viral reactivation. Evidently, ruxolitinib has the ability to impact the maintenance of the HIV viral reservoir in memory CD4+ T cells in vitro and ex vivo in terms of inhibiting viral reactivation [39]. In the context of cell proliferation, ruxolitinib has the ability to inhibit IL-6, IL-7, and IL-15 mediated proliferation; IL-6, IL-7, and IL-15 are interleukins that activate the Jak1 pathway and play major roles in the maintenance of the HIV viral reservoir via cell proliferation. [40]. Ruxolitinib achieves this by interfering with the binding of ATP to Jaks. If ATP cannot properly bind to Jaks, they are unable to initiate an intracellular signaling cascade in response to the binding of their ligands. By inhibiting the functioning of the Jak-STAT pathway, ruxolitinib may prevent homeostatic proliferation of latently infected CD4+ T_{SCM} and T_{CM} cells, inducing their differentiation into shorter lived CD4+ T cells, which will die more quickly than T_{SCM} and T_{CM} cells, reducing the viral reservoir.

Objectives and Hypotheses

Experimental Objectives

Our main objective is to determine if ruxolitinib may be a promising treatment for HIV-1+ individuals on ART to decrease the size of the latent viral reservoir via the push and vanish strategy. We will achieve this by measuring the prevalence of various memory CD4+ and CD8+ T cell subsets, including T_N, T_{SCM}, T_{CM}, T_{TM}, T_{EM} and $T_{TE,}$ in a cohort of HIV-1 infected, ART-suppressed participants receiving ruxolitinib as a part of a clinical trial. In this clinical trial, sponsored by the AIDS Clinical Trials Group (ACTG) and called A5336, participants received ruxolitnib daily for 5 weeks. A decrease in the size of the CD4+ T_{SCM} and T_{CM} cell populations would indicate the potential of ruxolitinib to interfere with proliferation of these subsets and as a consequence decrease the long-lived HIV-1 viral reservoir. While changes in the memory CD8+ T cell subsets are not indicative of a change in the size of the HIV latent viral reservoir, a similar reduction in the frequency of CD8+ T_{SCM} and T_{CM} cell populations would support the hypothesis that ruxolitinib induces a change in proliferation/differentiation of T cells by inhibiting the Jak-STAT pathway. Using blood processing, flow cytometric analysis, and statistical analyses, we will determine if in vivo administration of ruxolitinib is able to induce a change in the composition of the memory CD4+ and CD8+ T cell populations in HIV-1-infected, ART-suppressed patients participating in A5336.

We also analyze the effects of ruxolitinib on the size of the latent viral reservoir by comparing changes in cellular subsets with cellular and soluble markers of HIV infection. Decreases in IL-6 concentration, sCD14 expression, Bcl-2 expression, CA-RNA levels, and totDNA levels would indicate that the HIV-1 viral reservoir may be shrinking. By comparing our analyses on the change in composition of the memory CD4+ and CD8+ T cell

populations to the changes in these cellular markers, we can again analyze if ruxolitinib is likely to have a major impact on the size of the long-lived viral reservoir.

Hypotheses

Hypothesis 1:

We hypothesize that treatment with ruxolitinib and hence inhibition of the Jak-STAT pathway will lead to a reduced frequency of CD4+ and CD8+ T_{SCM} and T_{CM} cells and will increase the frequency of CD4+ and CD8+ T_{TM}, T_{EM} and T_{TE} cells.

Hypothesis 2:

We hypothesize that significant reduction in frequencies of CD4+ and CD8+ T_{SCM} and T_{CM} cells and increase in frequencies of CD4+ and CD8+ T_{TM}, T_{EM} and T_{TE} cells will be associated with a decrease in IL-6 concentration, sCD14 expression, Bcl-2 expression, CA-RNA levels, and totDNA levels.

Methods

Patient Selection

The study is a randomized, open-label, prospective, 2-arm study that enrolled 60 participants with HIV-1 infection. Participants who were virologically suppressed on ART were included in this study. Virologic suppression was defined as a) at least two HIV-1 RNA < 50 copies/mL assays, one of which was obtained between 336 to 31 days, inclusive, prior to study entry and one obtained 337 to 730 days, inclusive, prior to study entry, b) no HIV-1 RNA values ≥ 50 copies/mL within 336 days, inclusive, prior to the study, and c) not more than one HIV-1 RNA ≥ 50 and ≤ 200 copies/mL from a result obtained between 337 and 730 days, inclusive, prior to study entry. The participant population was within an age range of ≥ 18 to < 75 years-of-age and had an adequate CD4+ T cell count (> 520 cells/mm^3 ≤ 30 days prior to entry) to minimize adverse events (AEs) associated with ruxolitinib. Additionally, pregnant women were excluded from the study due to its pregnancy category designation (C) and adverse animal studies. Moreover, Hepatitis B Virus (HBV) and Hepatitis C Virus (HCV) positive individuals were excluded from the study. Participants were randomized at a 2:1 ratio to either Arm A (treatment with ruxolitinib) or Arm B (no study treatment). Participants in Arm A were treated with ruxolitinib 10 mg BID for 5 weeks and were followed for 7 weeks after the discontinuation of ruxolitinib for a total of 12 weeks under observation. Screenings and laboratory tests taken from every individual at entry and weeks 1, 2, 4, 5, 10, and 12. All participants remained on ART throughout the study.

PBMC Selection

Blood samples from weeks 0 (baseline), 5, and 12 from every participant were processed. Blood was diluted 2 to 3 times with sterile PBS and was then layered onto Ficoll

in a 2:1 ratio. The layered samples were then spun for 30 minutes at 1850 rpm on low acceleration and no break to allow the separation of Peripheral Blood Mononuclear Cells (PBMCs) from red blood cells (RBCs), polymorphonuclear cells (PMNs) and plasma. RBCs and PMNs gather below the Ficoll layer and plasma settles above the Ficoll layer. PBMCs collect above the Ficoll layer, creating a buffy coat below the plasma. The PBMC layer was then collected, transferred to a new 50 mL tube, topped off with PBS, and spun at 1850 rpm for 10 minutes. The supernatant was then dumped off and the cell pellet was resuspended with 5mL of ACK Lysing Buffer to cause the lysis of red blood cells. After 5 minutes, the tube was again filled to the 50 mL mark with PBS and then spun for 10 minutes at 1850 pm. The supernatant was dumped and the cell pellet was resuspended in 50 mL of PBS and washed for 20 minutes at 200g. The supernatant was aspirated and conicals from the same animal were combined. The cells were counted and frozen for subsequent analyses.

Flow Cytometric Analysis

We used flow cytometric analysis to analyze the components of each PBMC sample. Flow cytometric analysis achieves this by flashing light on cells as they pass through a narrow channel, one cell at a time. Sensors detect the types of light that are refracted or emitted from the cells, which have antibodies bound to either cell surface or intracellular markers. The emitted and/or refracted light is then recorded. The antibodies used are conjugated to different fluorophores with specific light excitation and emission wavelengths. All of the recorded data is integrated to build a comprehensive representation of the overall sample. The antibodies we used were as follows: Live/Dead (Amcyan), CCR7 (FITC), CD45RO (APC), CD3 (APC-Cy7), CD4 (BV650), CD8 (BV711), CXCR5 (PE-eFlour610), CD27 (PE-Cy7), CD122 (PE), CD95 (PV605), PD-1 (BV421), HLA-DR (PerCP5.5), and Ki67 (AF700).

Cell Gating Strategies

Multiple different cell populations were gated in order to quantify the composition of the T cell sample based on cellular markers. Singlets were gated under FSC-H and FSC-A, with the gate surrounding the highest concentration of cells. From the singlets, lymphocytes were determined by plotting SSC-A vs FSC-A. Lymphocytes were then separated into the live CD3+ subset and then CD4+ and CD8+ T cell populations by plotting CD4 vs CD8 (Figure 1A). The CD4+ and CD8+ T cell populations were gated as well. In the CD4+ population, non-TFH and TFH cells were gated using PD-1 and CXCR5 (Figure 1B). Within both the CD4+ non-TFH and CD8+ T cell populations, we gated different subsets of memory T cells by using CD45RO, CCR7, CD27, CD95, and/or CD122 cellular markers, in various combinations. Each subset was initially divided based on CD45RO and CCR7. T_{CM} cells were gated as CD45RO+ CCR7+, and then further specified as CD27+. On the other hand, T_{TTE} cells were gated as CD45RO- CCR7-, and then specified as CD27-. Naïve and T_{SCM} cell subsets were defined as CD45RO- and CCR7+. Within their combined subset, naïve cells were defined as CD95- and CD122-, while T_{SCM} cells were labeled CD95+ and CD122+. Lastly, T_{TM} and T_{EM} cells were gated as CD45RO+ and CCR7- (Figure 1B, 1C). From this population, T_{EM} cells were gated as CD27- and T_{TM} were gated as CD27+.

Statistical Analyses

A variety of statistical analyses will be used to analyze the data. Within the treated participants, we will use a repeated measures ANOVA test to perform a longitudinal comparison between weeks 0 to 5 to 12. These tests show if there were significant changes within the subsets between weeks 0, 5, and 12. Results with $p<0.05$ are considered to be significant.

Simple linear regression analyses are utilized to analyze the difference in subset population concentrations of memory T cells between baseline and week five. Simple linear regression analyses were used to account for the variance in the baseline memory T cell

population composition at baseline among the participants. ANOVA tests were then run to determine if the slopes of the control and treated participants that were calculated from the simple linear regression analyses for each subset were significantly different. Values with $p<0.05$ are considered to be significant.

Lastly, Pearson correlation tests are used to analyze the relationship between the change in memory CD4+ T-cell subsets and cellular markers from baseline to week five. The only cellular subsets used in these analyses were ones that were found to be significantly different than the control samples in the previous ANOVA tests on the results of the simple regression analyses. The cellular markers utilized include IL-6 concentration, CA-RNA levels, Bcl-2 expression, totDNA levels, and sCD14 expression. Data for these markers were quantified and provided by the ACTG as a part of the clinical trial.

Results

Patient Sample Description

To provide more context for the study, the patient sample needs to be described. Various parameters can describe our patient population. The age distribution of the participants ranges from 19 to 67 years of age, with the average age being 46 years of age. Moreover, out of the 60 patients, 12 patients identified as female, while 48 identified as male. Additionally, the participants identified racially as follows: 29 as Black Non-Hispanic, 22 as White Non-Hispanic, 6 as Hispanic, 1 as multiracial, and 2 did not provide identification. After calculating BMIs, 13 of the patients qualified as normal, 18 as overweight, and 29 as obese. Out of the 60 patients, 7 were not included in our analyses. These patients were not included for the following reasons: missing more than 6 doses of ruxolitinib, discontinuing treatment prior to week 4/5, being ineligible after randomization, ending study prematurely at week 2, and having virologic failure.

Overall trends in CD4+ and CD8+ T cell populations from baseline, week 5, and week 12

To begin our analyses, we investigated the overall trends of the total CD4+ and CD8+ T cell populations. We used ANOVA analyses to compare the changes in relative contributions of the CD4+ and CD8+ T cell populations to the overall T cell population from baseline to week 5 to week 12. Significant results would indicate that there were significant differences between the means of the baseline, week 5, and week 12 cell frequencies. For comparison, we ran the analyses on the control CD4+ (Figure 2A) and CD8+ T cell populations (Figure 2C) as well. There were no significant results for either the control or ruxolitinib CD4+ (Figure 2B) or CD8+ T cell populations (Figure 2D).

Overall trends in memory CD4+ T cell populations from baseline, week 5, and week 12

Similar to the ANOVA analyses done for the overall CD4+ and CD8+ T cell populations (Figure 2), we ran ANOVA analyses for each of the memory CD4+ T cell subset

populations. Significant results indicate that there is a strong difference between the means of the baseline, week 5, and week 12 frequencies of one of the CD4+ T cell subset populations. Within the CD4+ T cell population subsets, there were two significant results. Statistically significant changes occurred within the CD4+ T_{SCM} cell population (p=0.0454, Figure 3D) and the T_{TM} cell population (p=0.0267, Figure 3H) in the ruxolitinib group, but not in the controls.

Overall trends in memory CD8+ T cell populations from baseline, week 5, and week 12

We conducted the same ANOVA analyses done for the overall CD4+ and CD8+ T cell populations as well as the memory CD4+ T cell subset populations for the memory CD8+ T cell subset populations. Again, significant results would indicate that there is a clear difference between the means of the baseline, week 5, and week 12 frequencies within a memory CD8+ T cell subset population. In the CD8+ T cell subset populations, there were two statistically significant results. Specifically, there were significant changes within the ruxolitinib CD8+ T_N cell population (p=0.0089, Figure 4B) and the ruxolitinib CD8+ T_{SCM} cell population (p=0.0306, Figure 3D). Similar changes were not seen in the controls.

Changes in CD4+ and CD8+ T cell populations from baseline to week 5

Before analyzing the subsets of the CD4+ and CD8+ T cell populations, we wanted to determine if there were any overall changes to the sizes of the total CD4+ and CD8+ T cell populations between baseline and week 5. We analyzed the change from baseline to week 5 because the ruxolitinib was administered to the patients only for 5 weeks, making the first 5 weeks the critical time period. After obtaining the slope values from the linear regression analyses for both the control and ruxolitinib groups, the ANOVA analyses, which compared the slopes of the control and ruxolitinib groups for both the CD4+ and CD8+ T cell populations, found no significant changes in the sizes of the CD4+ and CD8+ T cell populations sizes from baseline to week five (Figure 5A, 5B).

Changes in memory CD4+ T cell populations from baseline to week 5

In order to determine if treatment with ruxolitinib resulted in a change to the overall makeup of the memory CD4+ T cell population, we compared changes in subset composition percentages from baseline to week 5 between the control and treated individuals. Two significant results have been obtained from running ANOVA analyses on the results of simple linear regression analysis. Specifically, the CD4+ T_{TM} cell population decreased significantly from baseline to week five in comparison to the control populations ($p=0.0355$ Figure 6D). Furthermore, the CD4+ T_{TE} cell population similarly decreased significantly from baseline to week five in comparison to the control population ($p=0.0014$, Figure 6F).

Changes in memory CD8+ T cell populations from baseline to week 5

The same analyses were run for the memory CD8+ T cell subset population. In a similar pattern to the CD4+ T_{TM} cell population size, the CD8+ T_{TM} cell population size decreased significantly from baseline to week 5 in comparison to the control population ($p=0.0360$, Figure 7D). Unlike the CD4+ T cell results, the CD8+ T_{EM} and T_{CM} cell populations also had statistically significant changes in their population sizes by the end of the five-week treatment period. The relative CD8+ T_{EM} cell population size decreased significantly from baseline to week five when compared to the control population ($p=0.0126$, Figure 7C). Moreover, while both the control and the patient population CD8+ T_{CM} cell populations decreased in their relative size from baseline to week five, the patient population CD8+ T_{CM} cell population decreased significantly less than the control group ($p=0.0126$, Figure 7E).

Correlations between the change in frequency of memory CD4+ and CD8+ T cell subsets and the change in levels of IL-6, CA-RNA, and totDNA from baseline to week 5

Spearman correlation analyses were run to look for any associations between significant changes in memory CD4+ and CD8+ T cell subset populations from Figures 6 and

7 and changes in HIV associated cellular and soluble markers. Spearman correlation analyses revealed no significant relationships between the memory CD4+ and CD8+ subsets from Figures 6 and 7 and the change in levels of IL-6, CA-RNA, and totDNA from baseline to week 5 in the ruxolitinib group.

Correlation between the change in frequency of memory CD4+ and CD8+ T cell subsets and the change in the percentage of cells expressing Bcl-2 from baseline to week 5

In the Spearman correlation analyses, a significant result was found for the relationship between the change in CD4+ T_{TM} cell frequency and the change in the percentage of cells expressing Bcl-2 from baseline to week 5 in the ruxolitinib group. The Spearman correlation analysis revealed a moderately negative correlation between the two variables (r=-0.4346, Figure 8). While only a moderate correlation exists between the variables, a two-tailed t-test revealed the relationship to be statistically significant (p=0.0208, Figure 8). No significant relationships were found between the change in the frequency of CD4+ T_{TE} and CD8+ T_{TM}, T_{EM}, and T_{CM} cells and the change in percentage of cells expressing Bcl-2 from baseline to week five.

Correlation between the change in frequency of memory CD4+ and CD8+ T cell subsets and the change in the expression of sCD14 from baseline to week 5

Furthermore, in additional Spearman correlation analyses, a significant result was found for the relationship between the change in CD8+ T_{CM} cell frequency and the change in the expression of sCD14 from baseline to week 5 in the ruxolitinib group. The Spearman correlation analysis revealed a moderately positive correlation between the two variables (r=0.3901, Figure 9). Again, while only a moderate correlation exists between the two variables, an unpaired t-test revealed that the relationship is statistically significant (p=0.0364, Figure 9). No significant relationships were found between the change expression

of sCD14 and the change the frequencies of CD4+ T_{TM} and T_{TE} and CD8+ T_{EM}, T_{TM}, and T_{CM} cellular subsets.

Discussion

In the context of our hypotheses, the results we obtained allowed us to further refine our understanding of the effect of a 5-week treatment with ruxolitinib on T cell subset frequencies in HIV-infected, ART-suppressed participants. Our first hypothesis was largely rejected by the data. We hypothesized that treatment with ruxolitinib would lead to a reduced frequency of CD4+ and CD8+ T_{SCM} and T_{CM} cells and an increased frequency of CD4+ and CD8+ T_{TM}, T_{EM} and T_{TE} cells. In terms of the memory CD4+ T cells subsets, there were no significant changes in the T_{SCM}, T_{CM}, and T_{EM} cell populations, and the T_{TM} and T_{TE} cell populations decreased in relative size, all of which contradicted our first hypothesis. Our first hypothesis was also rejected by the majority of the memory CD8+ T cell subset results; T_{SCM} and T_{TE} cell populations did not change significantly in relative size and T_{TM} and T_{EM} cell populations both decreased in relative size. Our first hypothesis was supported by the decrease in frequency of the CD8+ T_{CM} cell population.

Our second hypothesis was again largely rejected by the results of the analyses. In our second hypothesis, we anticipated that significant reduction in frequencies of CD4+ and CD8+ T_{SCM} and T_{CM} cells and increase in frequencies of CD4+ and CD8+ T_{TM}, T_{EM} and T_{TE} cells would be associated with a decrease in IL-6 concentration, sCD14 expression, Bcl-2 expression, CA-RNA levels, and totDNA levels. As our cell populations that showed significant changes in relative size from baseline to week 5 almost all contradicted our first hypothesis, the only memory T cell subset that could be utilized to test our second objective was the CD8+ T_{CM} cell population. There were no correlations between the relative decrease in CD8+ T_{CM} cell population with significant changes in IL-6 concentration, Bcl-2 expression, CA-RNA levels, and totDNA levels, which rejects the hypothesis. On the other hand, the significant, positive correlation between the change in CD8+ T_{CM} cell population size and the change in sCD14 expression does support our second hypothesis.

A variety of conclusions can be drawn from this study. Primarily, when focusing on the change in relative sizes CD4+ and CD8+ T cell populations to the overall T cell pool from baseline to week 5, there were no significant results, indicating that ruxolitinib did not change the total size of the T cell populations. While there were no significant changes in the contributions of CD4+ T_{SCM}, T_{CM}, and T_{EM} cell subsets to the overall CD4+ cell population, the CD4+ T_{TM} and T_{TE} cell subsets contradicted our first hypothesis; rather than the CD4+ T_{TM} and T_{TE} cell subsets increasing in their contribution to the overall CD4+ T cell pool, they significantly decreased. A multitude of possibilities could explain these different results. First, patients were treated over the course of five weeks. While blood was taken at weeks 2 and 4 as well, we only utilized samples from baseline and week 5. It is possible that the changes we expected to see in our hypothesis could have already occurred earlier on in the treatment. For instance, ruxolitinib could have pushed the CD4+ T_N, $T_{SCM,}$ and T_{CM} cell subsets to become T_{EM}, T_{TM}, and T_{TE} cell subsets earlier on in the treatment period; our results could be showing the end result after the initial differentiation push that ruxolitinib induced. Altogether, our study may have missed the crucial period in which ruxolitinib causes the initial cellular differentiation that leads to a depletion of the HIV latent viral reservoir to occur. Another possible explanation is that the inhibition of the Jak-STAT pathway does not lead to the push in differentiation of the CD4+ cell type. It is always important to remember how interconnected all of the cellular pathways are. For instance, the Jak-STAT pathway also interacts with receptor tyrosine kinase (RTK)/Ras/MAPK, TGF-β, and phosphoinositide 3-kinase (PI3K) pathways; these pathways all influence each other, resulting in significant cross talk. Hence, while ruxolitinib may be causing a change in the Jak-STAT pathway signaling, other pathways may be compensating for its inhibition [40]. This could prevent differentiation of less differentiated CD4+ T cell types and could also be causing a decrease in the presence of more differentiated CD4+ cell types.

Additionally, it is important to compare the CD4+ T cell results to the CD8+ T cell results. Similar to the CD4+ results, the decrease in CD8+ T_{TM} and T_{EM} cell concentration contradicts our hypothesis, as we expected both subsets to increase. On the contrary, the decrease in the presence of CD8+ T_{CM} cells confirms our hypothesis; we expected ruxolitinib to cause the T_{CM} cells to differentiate, therefore shrinking the presence of T_{CM} cells. Additionally, while the proportional size of the CD4+ T_{CM} cell subset did not significantly decrease, it was smaller in proportion to the controls. The question is then raised as to why this difference was significant in the CD8+ population but not the CD4+ population. One possibility, similar to first explanation above used for the CD4+ results, is that we are again seeing the end result of the treatment, and that, while the CD8+ T_{CM} cell population has not recovered by the end of five-week treatment period, the CD4+ T_{CM} cell population has. This raises the prospect that the CD8+ and CD4+ populations respond differently to the inhibition of the Jak-STAT pathway. Again, cross talk between multiple cellular pathways may have led to the unexpected results in the CD8+ T_{TM} and T_{EM} cell subsets.

The results of the ANOVA analyses of the cellular subset trends between weeks zero, five, and twelve do not appear to correlate with the results from the regression analyses describing the changes in subset concentration from baseline to week 5. While the CD4+ T_{TM} cellular subset yielded significant results in both the ANOVA analyses of the trends from week zero to twelve and the regression analysis for the change in subset concentration from week zero to five, there were no other paired significant results between the two tests. This could be due to the different types of testing measures used to describe the data as well as the addition of the twelfth week time point in the cellular subset trends analyses.

Moreover, analyzing the various cellular markers brought interesting results. Importantly, there were no correlations between the change in the concentrations of IL-6, CA-RNA, and totDNA with any of the cellular subsets that had produced significant changes

from the regression analyses. It should be noted that these results do not indicate that there were no a significant changes in the concentrations of IL-6, CA-RNA, and totDNA from week zero to five; these results only concern the relationship between the change in the concentrations of IL-6, CA-RNA, and totDNA with the changes in composition of the CD4+ and CD8+ T cell populations. On the other hand, there were two significant relationships that emerged from our study. First, a smaller decline in the CD4+ T_{TM} cell subset proportion was associated with a larger decrease in Bcl-2 expression (or, alternately, a greater decline in CD4+ T_{TM} cells was associated with a smaller decrease in Bcl-2). Importantly, as Bcl-2 leads to the inhibition of apoptosis, and HIV infection leads to an increased expression of Bcl-2, this relationship could confirm our hypothesis that pushing CD4+ T_{SCM} and T_{CM} cells to differentiate into T_{TM} cells leads to a decrease in the HIV latent viral reservoir. Second, a larger increase in the CD8+ T_{CM} cell subset proportion was associated with a larger increase in sCD14 expression (or, alternately, a greater decline in CD8+ T_{TC} cells was associated with a greater decrease in sCD14 expression). Again, while this relationship involves the CD8+ subset, it could confirm our overarching hypothesis that pushing CD4+ T cells to becoming shorter lived CD4+ T cells results in the depletion of the HIV latent viral reservoir. As HIV infection induces sCD14 expression, the larger increase in central memory type cells could be indicative of a larger HIV latent viral reservoir.

Our main objective of determining if ruxolitinib is an effective treatment for HIV-infected individuals on ART to decrease the size of the latent viral reservoir was not fully met. Due to the various limitations of our study, our results cannot fully prove or disprove the effectiveness of ruxolitinib on decreasing the size of the HIV latent viral reservoir. To fully meet this objective, steps discussed above towards improving the overall study need to be conducted.

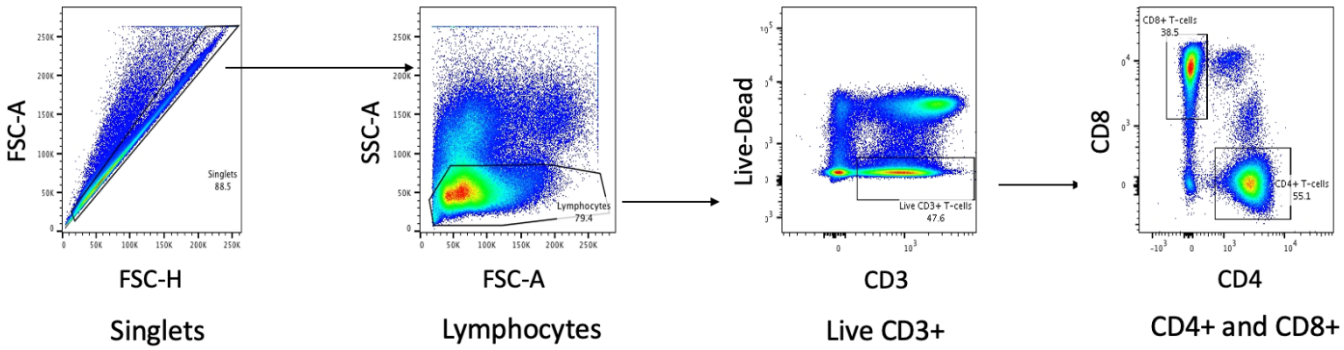
The results of this study also hold various clinical implications for goal of curing HIV-1 infections. Importantly, our study demonstrates that using known Jak-STAT pathway inhibitors, such as ruxolitinib, have the ability to change the overall makeup of the memory CD4+ and CD8+ T cell populations. While our study is preliminary, the significant changes observed at the end of the ruxolitinib trial period offer evidence that inhibiting the Jak-STAT pathway could be a possible way to decrease the size of the HIV latent viral reservoir. Again, our study lends important information towards the duration of ruxolitinib treatment. With further analyses on earlier blood samples from weeks 2 and 4, we can determine when ruxolitinib is generating its main effects on the memory CD4+ T cell subset populations. In summary, there is still a lot to be explored in terms of the relationship between ruxolitinib, the Jak-STAT pathway, and the HIV latent viral reservoir, and more studies need to be performed to accurately gauge ruxolitinib's effects.

Future Directions

Overall, our study has multiple future directions. Importantly, additional analyses need to be run including the week twelve data. Vital results could be seen after the treatment has ceased, and they were not fully explored in the ANOVA analyses on the trends from baseline, week 5, and week 12. Furthermore, blood samples from weeks two and four of the participants needs to be analyzed and then compared to the baseline samples to truly determine the impact ruxolitinib has on the HIV latent viral reservoir. While we observed mostly unexpected changes to the cellular subsets in the CD4+ and CD8+ T cell populations, we do not know if the observed results are the direct result of ruxolitinib or if different trends were observed at earlier weeks. Additionally, after analyzing the changes to the CD4+ and CD8+ T cell subsets at the week two and four time points, unpaired t-tests need to be rerun to analyze the relationships with the cellular markers for HIV infection. Establishing stronger relationships between the presence of certain cellular markers HIV infections and memory T cell subsets is important to understanding how we can measure HIV eradication. Lastly, to truly understand how ruxolitinib interferes with cell signaling, analyses need to be run to analyze the impact of inhibiting the Jak-STAT pathway on the functioning of interrelated pathways. If other pathways' signaling is being altered in response to Jak-STAT inhibition, they may need to be controlled via different mechanisms to see if Jak-STAT inhibition itself can lead to HIV eradication via the push and vanish approach.

Figures

A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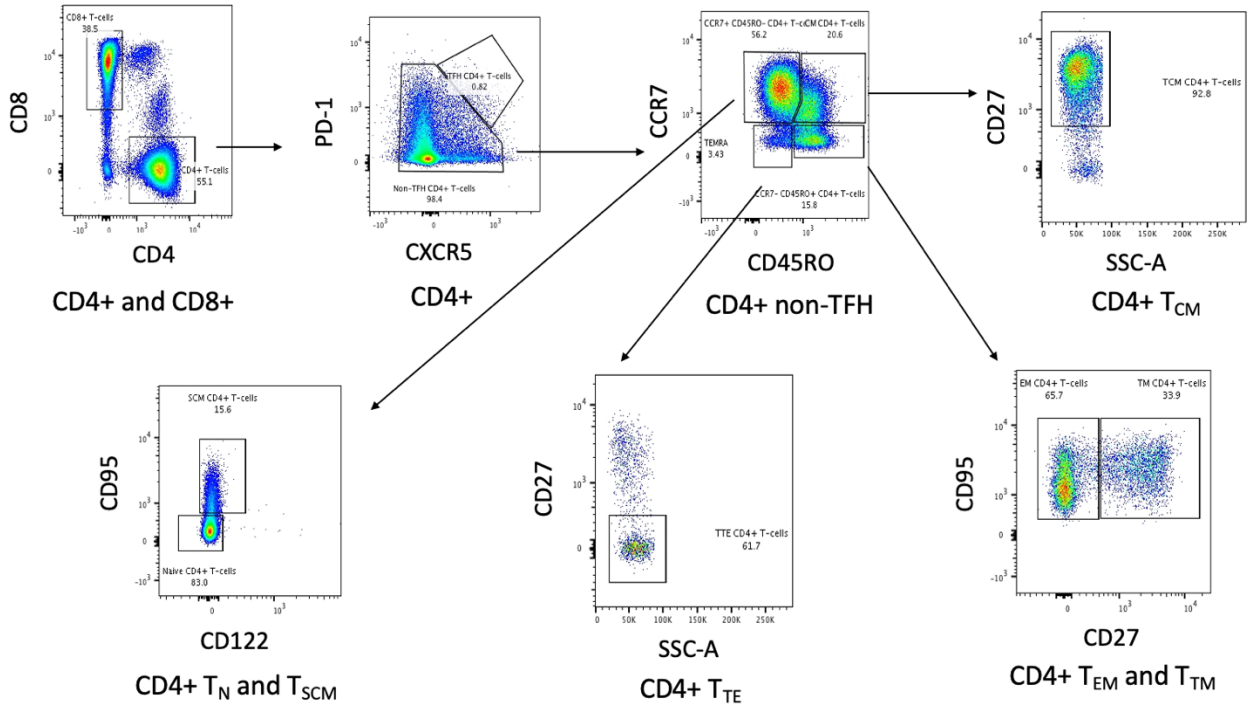
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CD95
CD122
CD4+ T_N and T_{SCM}
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C.

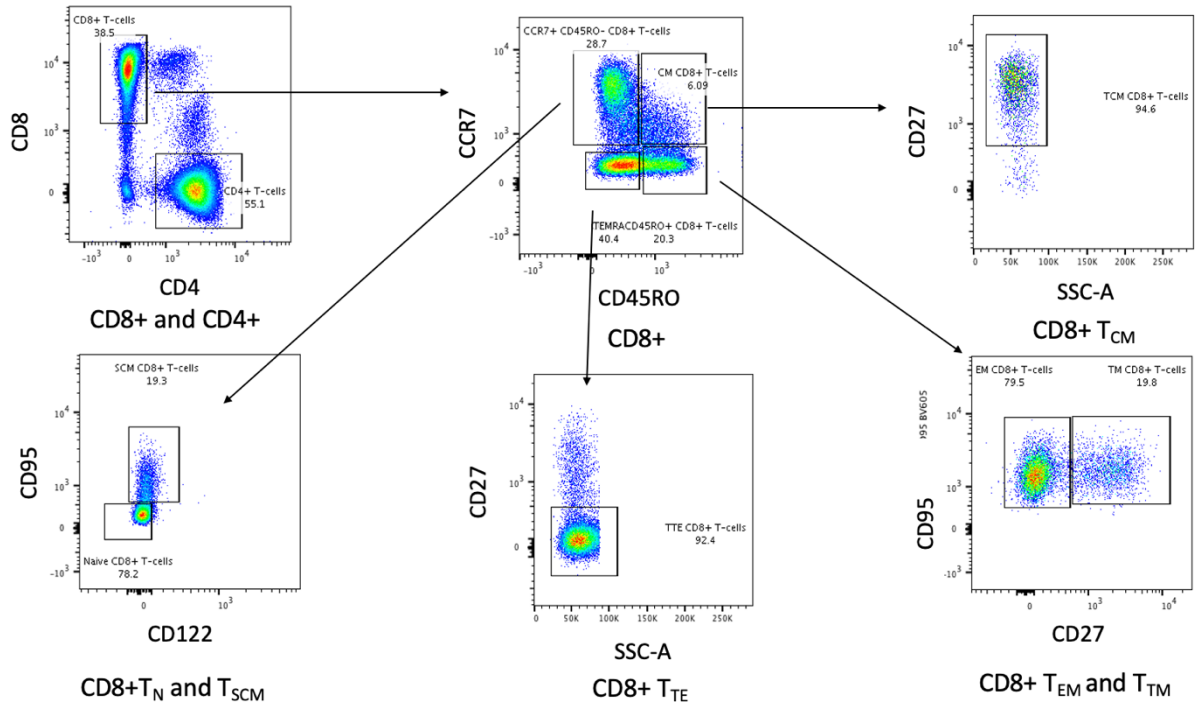


Figure 1. Gating Strategies for memory CD4+ and CD8+ cell subsets. **(A)** Gating of CD4+ and CD8+ T cells from singlet results from flow cytometric analysis. **(B)** Gating of memory CD4+ T cell subsets from the total CD4+ T cell population. **(C)** Gating of memory CD8+ T cell subsets from the total CD8+ T cell population.

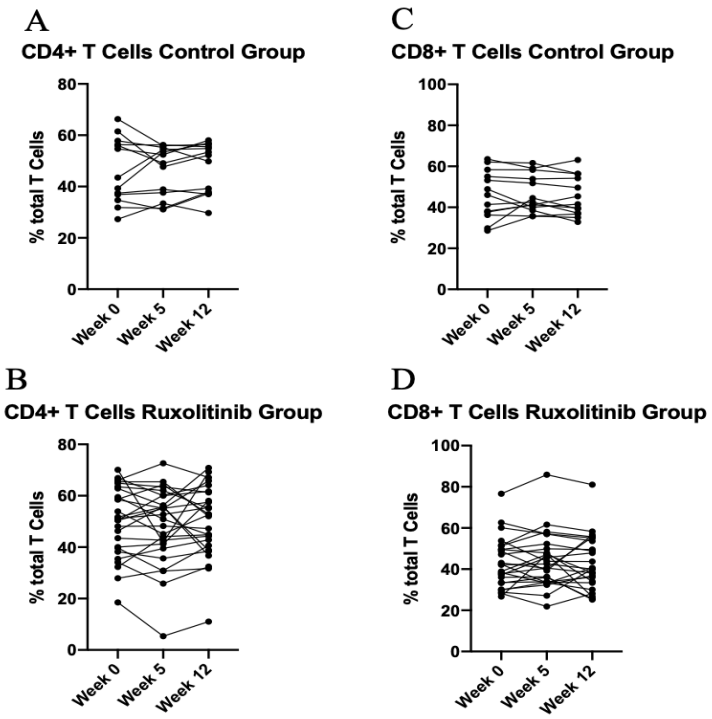


Figure 2. Frequency Trends for CD4+ and CD8+ T Cells from Baseline to Week 12. ANOVA analyses were performed on the **(A)** control participants' CD4+ T cell, **(B)** Ruxolitinib participants' CD4+ T cell, **(C)** control participants' CD8+ T cell, **(D)** Ruxolitinib participants' CD8+ T cell percentages of total T cell population from baseline to week 12. No significant results were found in the analyses.

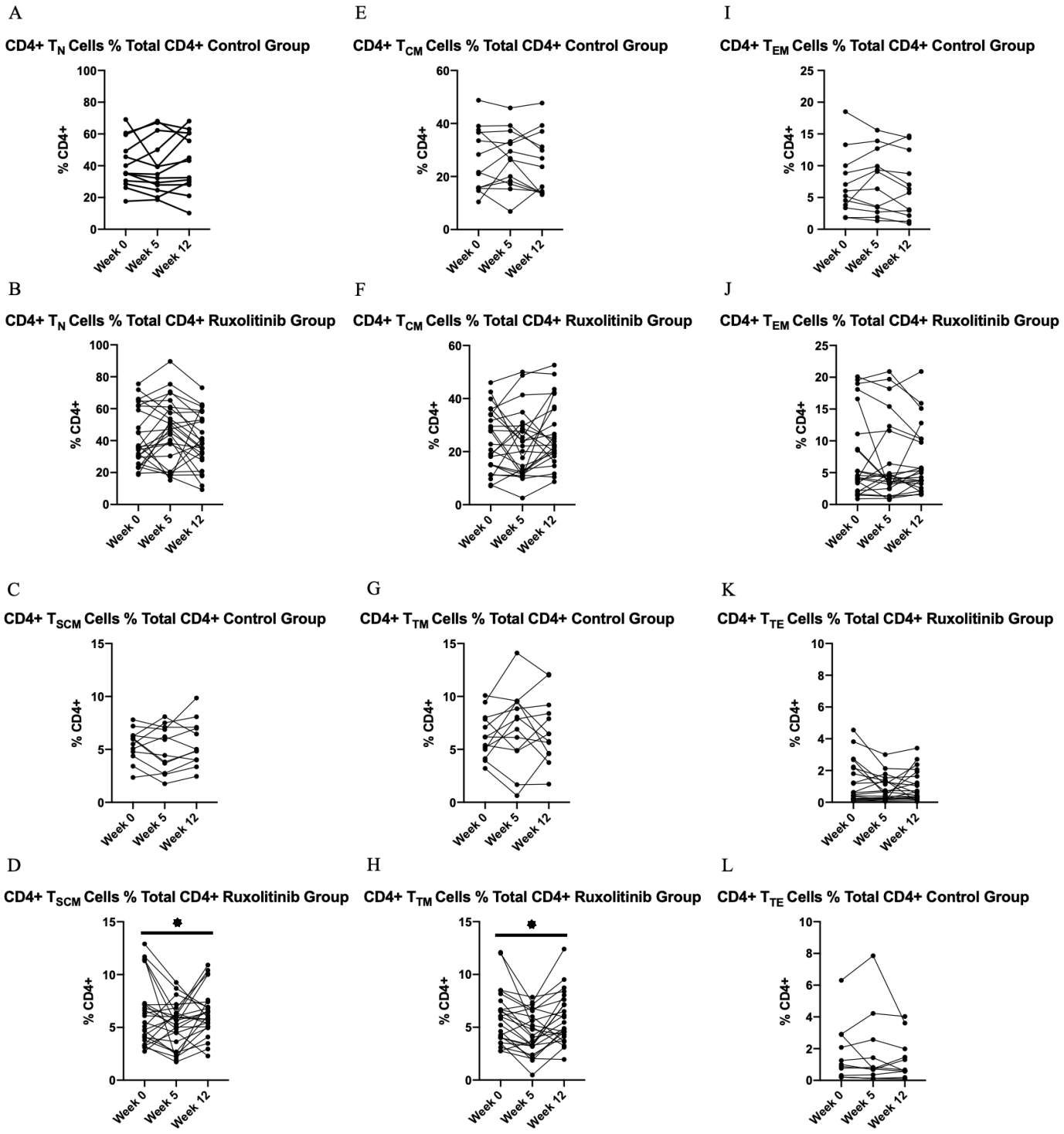


Figure 3. Frequency Trends for Memory CD4+ T Cell Subsets from Baseline to Week 12. (A) ANOVA analyses were performed on the control participants' CD4+ T_N, **(B)** ruxolitinib participants' CD4+ T_N, **(C)** control participants' CD4+ T_{SCM}, **(D)** ruxolitinib participants' CD4+ T_{SCM}, **(E)** control participants' CD4+ T_{CM}, **(F)** ruxolitinib participants'

CD4+ T_{CM}, **(G)** control participants' CD4+ T_{TM}, **(H)** ruxolitinib participants' CD4+ T_{TM}, **(I)** control participants' CD4+ T_{EM}, **(J)** ruxolitinib participants' CD4+ T_{EM}, **(K)** control participants' CD4+ T_{TE}, and **(L)** ruxolitinib participants' CD4+ T_{TE} percentages of CD4+ T cell population from baseline to week 12. Two populations yielded significant results. **(D)** The overall ruxolitinib participants' CD4+ T_{SCM} cell subset trend was significant with p=0.0454, and **(H)** The overall ruxolitinib participants' CD4+ T_{TM} cell subset trend was significant with p=0.0267.

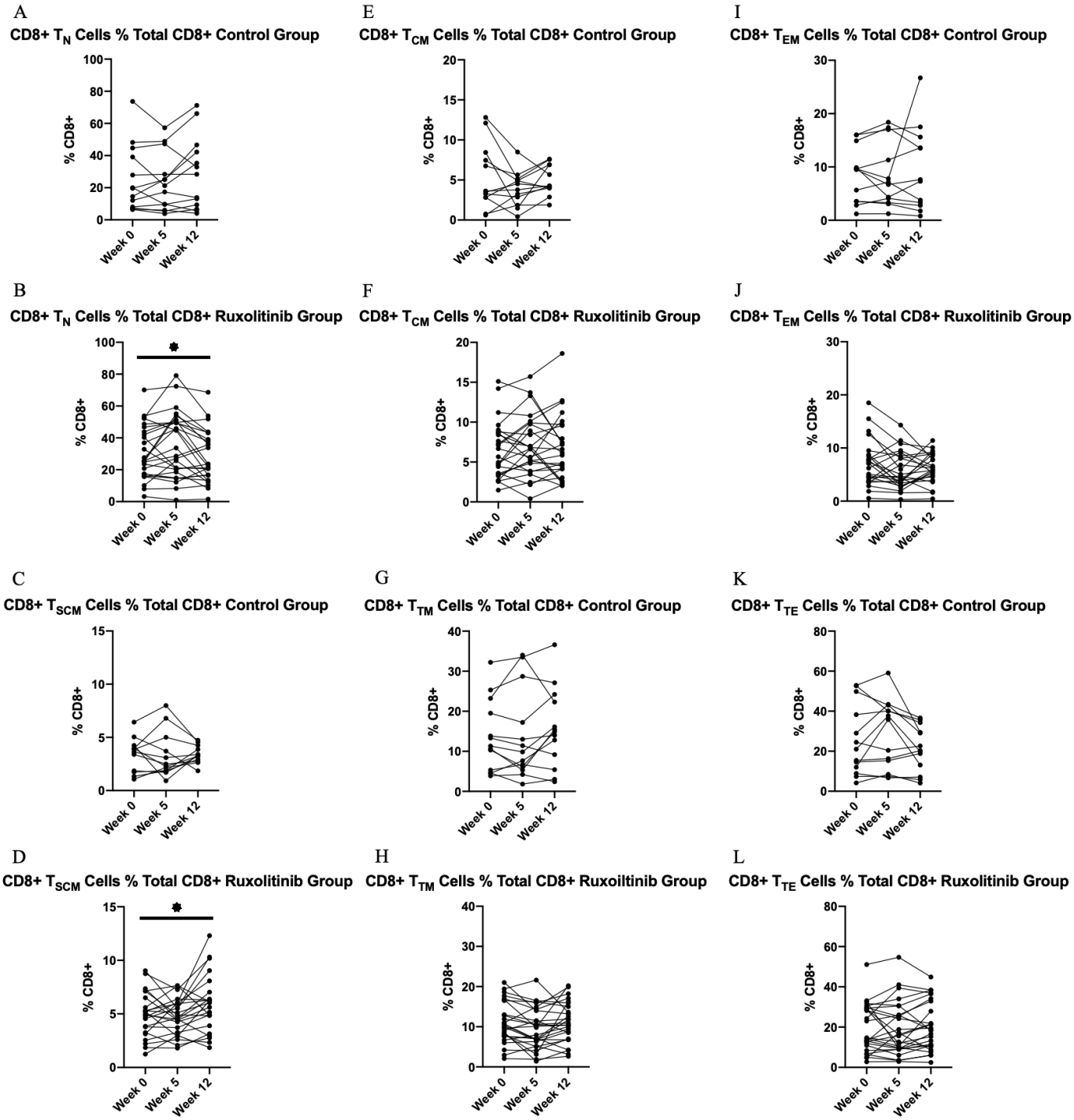
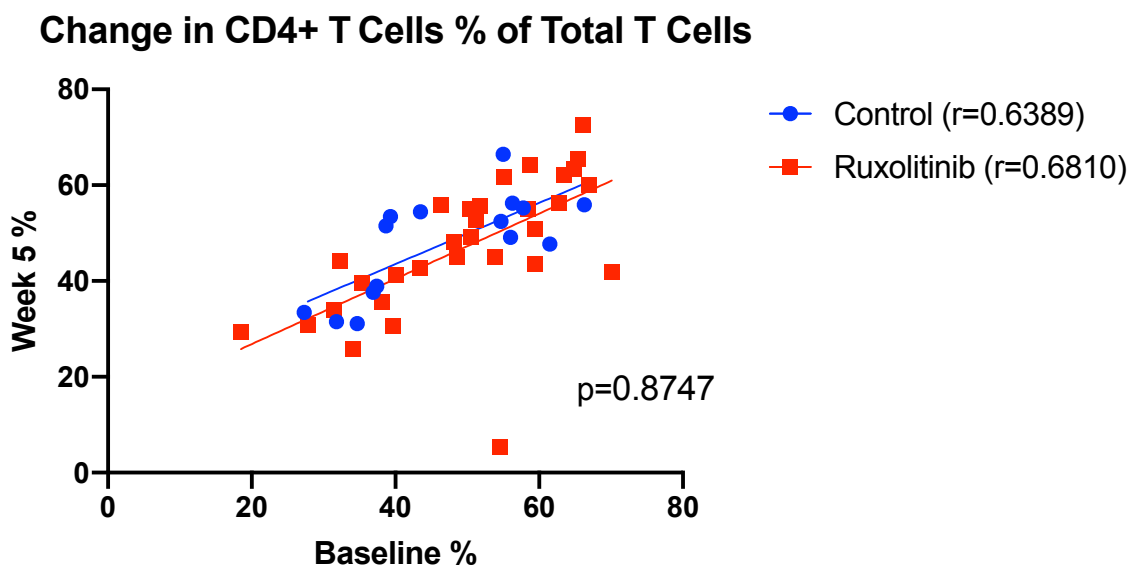


Figure 4. Frequency Trends for Memory CD8+ T Cell Subsets from Baseline to Week 12. (A) ANOVA analyses were performed on the control participants' CD8+ T_N, **(B)** ruxolitinib participants' CD8+ T_N, **(C)** control participants' CD8+ T_{SCM}, (D) ruxolitinib participants' CD8+ T_{SCM}, **(E)** control participants' CD8+ T_{CM}, **(F)** ruxolitinib participants' CD8+ T_{CM}, **(G)** control participants' CD8+ T_{TM}, **(H)** ruxolitinib participants' CD8+ T_{TM}, **(I)**

control participants' CD8+ T_{CM}, **(J)** ruxolitinib participants' CD8+ T_{EM}, **(K)** control participants' CD8+ T_{TE}, and **(L)** ruxolitinib participants' CD8+ T_{TE} percentages of CD8+ T cell population from baseline to week 12. Two of the analyses yielded significant results; **(B)** The ruxolitinib CD8+ T_N cell subset had a p=0.0089 and **(D)** the ruxolitinib T_{SCM} cell subset had p=0.0306.

A



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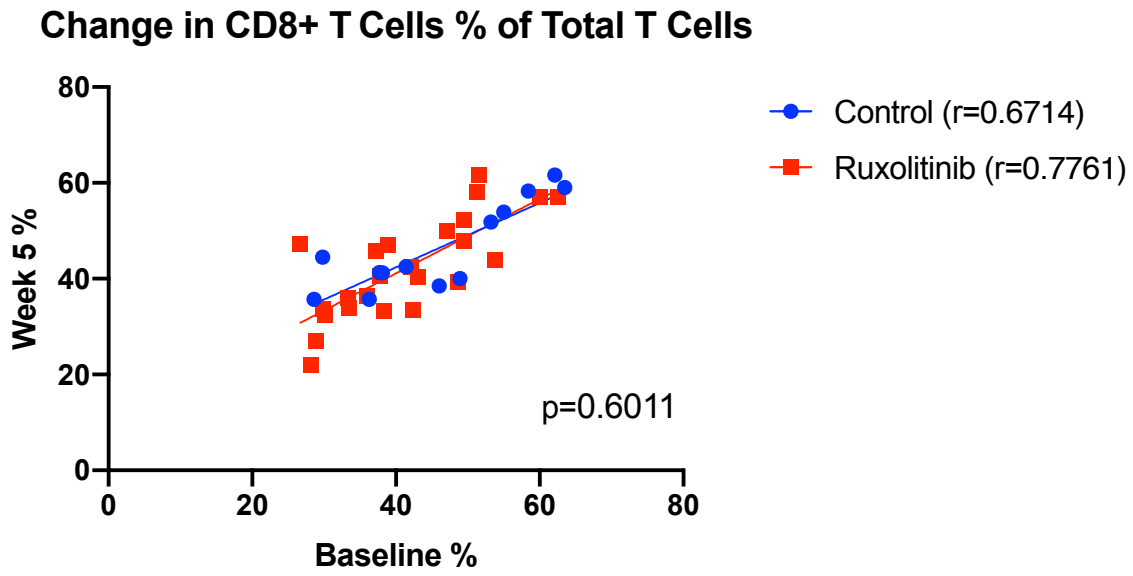

Figure 5. Change in CD4+ and CD8+ T Cell Percentages of the Total T Cell Population from Baseline to Week 5. **(A)** Regression analyses were performed on the control and Ruxolitinib participant groups to analyze the change in the overall size of the CD4+ T cell

population and **(B)** the CD8+ T cell population relative to the overall T cell population. Unpaired t-tests on the regression lines found no significant results for either population.

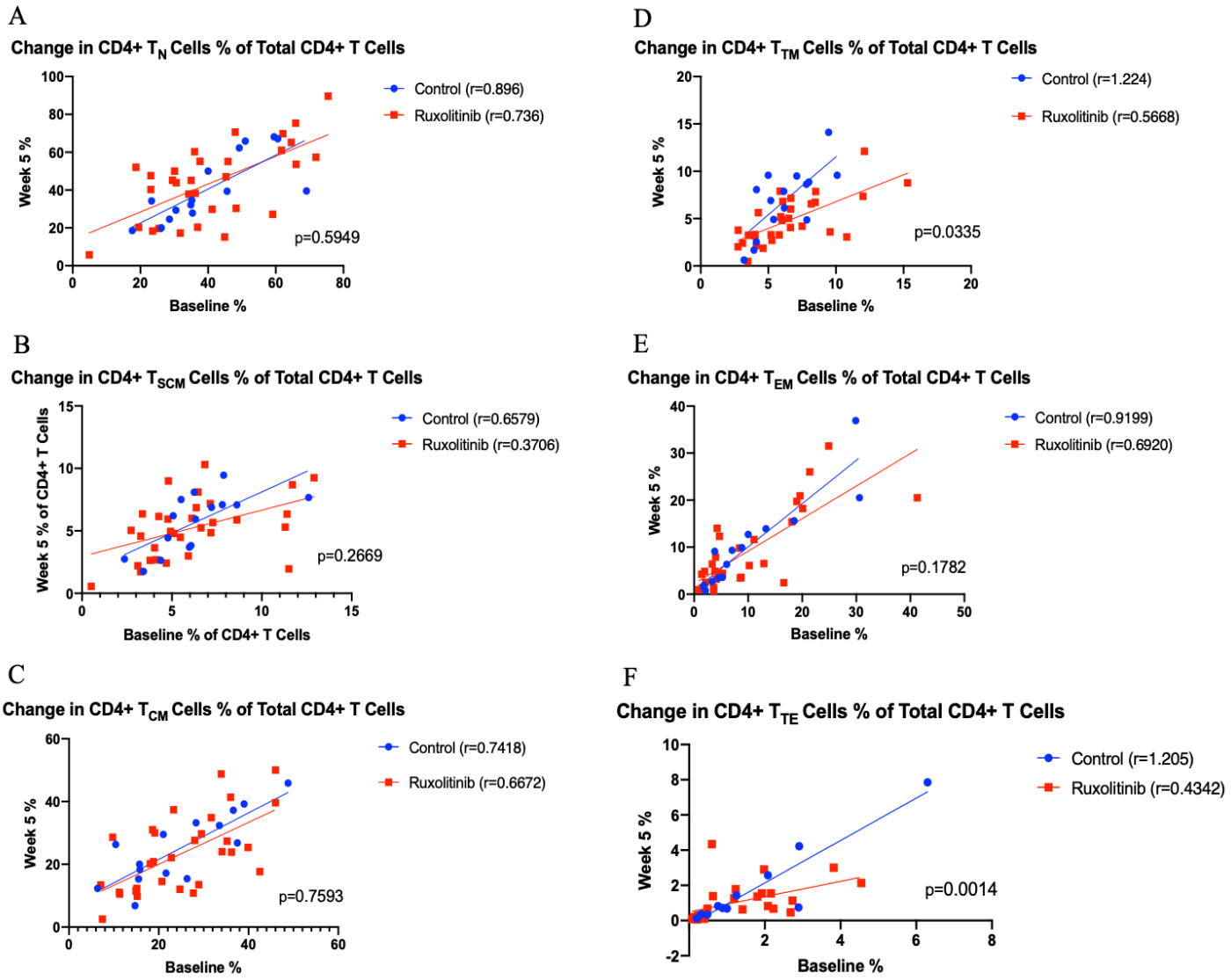

Figure 6. Change in Memory CD4+ T Cell Subset Percentages from Baseline to Week 5. **(A)** Regression analyses were performed on the CD4+ T_N, **(B)** T_{SCM}, **(C)** T_{CM}, **(D)** T_{TM}, **(E)** T_{EM}, and **(F)** T_{TE} cell subset populations with the data from baseline and week 5 for both the control and ruxolitinib participant groups. The ANOVA tests analyzed if there were significant differences between the control and ruxolitinib group regression lines for each of the cellular subsets. The ANOVA tests found two significant results. **(D)** The CD4+ T_{TM} cell subset was found to be significant with p=0.0355 and **(F)** the CD4+ T_{TE} cell subset was found to be significant with p=0.0014.

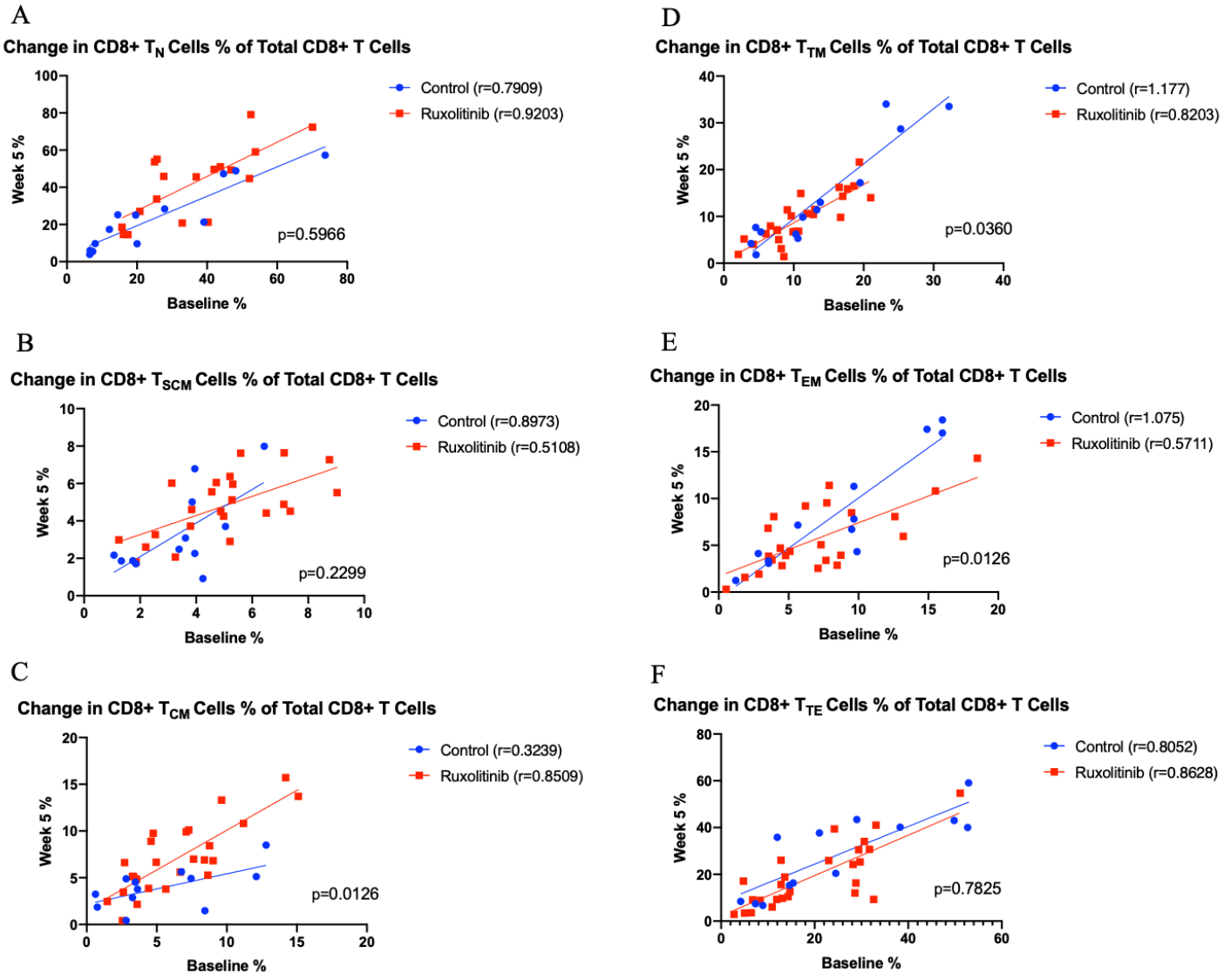

Figure 7. Change in Memory CD8+ T Cell Subset Percentages from Baseline to Week 5. **(A)** Regression analyses were performed on the CD8+ T_N, **(B)** T_{SCM}, **(C)** T_{CM}, **(D)** T_{TM}, **(E)** T_{EM}, and **(F)** T_{TE} cell subset populations with the data from baseline and week 5 for both the control and ruxolitinib participant groups. ANOVA tests analyzed the if there were significant differences between the control and ruxolitinib group regression lines for each of the cellular subsets. The ANOVA tests found three significant results. **(C)** The CD8+ T_{CM} cell subset was found to be significant with p=0.0126, **(D)** the CD8+ T_{TM} cell subset was

found to be significant with p=0.0360, and **(E)** the CD8+ T_{EM} cell subset was found to be significant with p=0.0126.

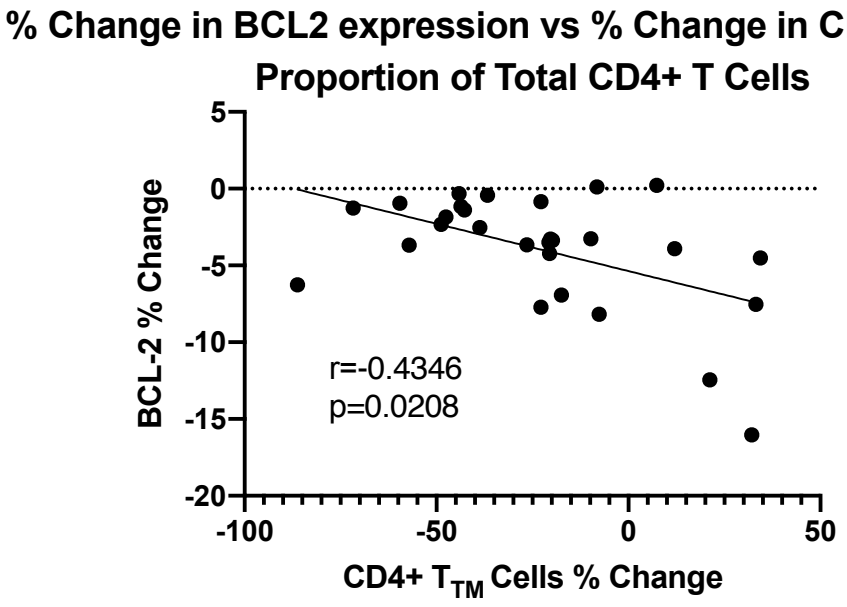

Figure 8. Correlation Between the Percentage Change in BCL2 Expression and CD4+ T_{TM} Cell Percentage from Baseline to Week 5. Spearman correlation analyses revealed a moderate correlation of r=-0.4346. An unpaired t-test found the correlation to be significant with p=0.0208.

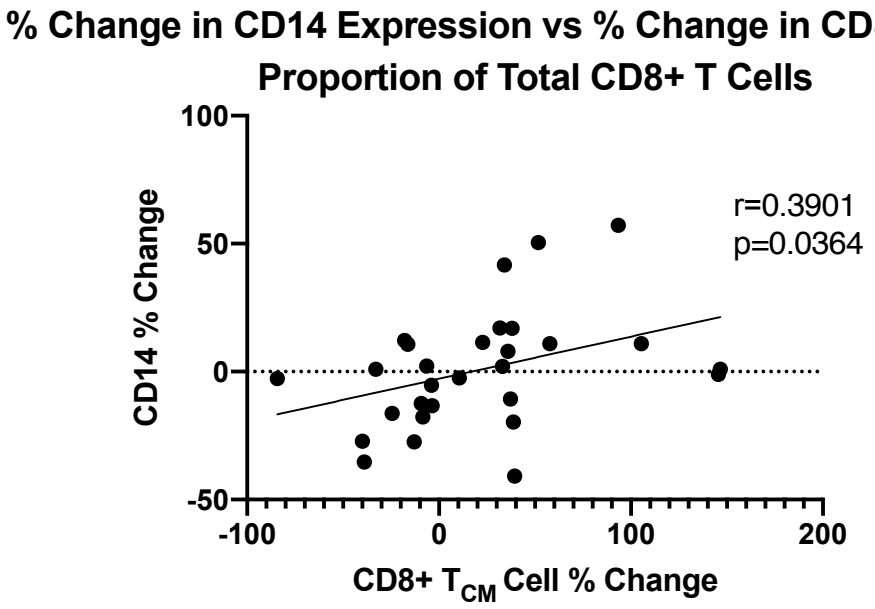

Figure 9. Correlation Between the Percentage Change in sCD14 Expression and CD8+ T_{CM} Cell Percentage from Baseline to Week 5. Spearman correlation analyses revealed a moderate correlation of r=0.3901. An unpaired t-test found the correlation to be significant with p=0.0364.

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