

Exploration of HIV-specific Immune Responses Following Dipyridamole Treatment

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University of Pittsburgh, 2022

Dipyridamole is a vasodilator used to prevent heart attacks and strokes. HIV-associated reduction in extracellular adenosine levels allows for continuous immune activation in people living with HIV (PLWH). Dipyridamole inhibits the cellular uptake of adenosine via equilibrative nucleoside transporters to increase extracellular adenosine in the inflammatory milieu. We previously completed a randomized, double-blind, placebo-controlled study that showed 12 weeks of dipyridamole treatment resulted in a significant decrease in CD8+ T cell activation. Given the anti-inflammatory effects of dipyridamole, we sought to evaluate whether treatment also affected HIV-specific immune responses. We obtained cryopreserved peripheral blood mononuclear cells (PBMCs) from a subset of participants with available baseline and week 12 samples from the clinical trial. Using flow cytometry, we evaluated polyfunctional responses (defined as expression of 2 or more effector cytokines following peptide pool stimulation) to gag and env peptide pools (15mer peptides with overlapping 11 amino acids) and compared the change in response from baseline to week 12 between participants receiving dipyridamole versus placebo. We assessed whether changes in polyfunctional responses were associated with virologic, immunologic, and purinergic parameters. We evaluated virally suppressed participants with HIV (N=9 in dipyridamole arm and N=13 in placebo arm). We found a statistically significant decrease in HIV-specific CD4+ T-cell polyfunctional responses following dipyridamole treatment. Specifically, a significant decrease was observed in CD4+ T cell expression of TNF α and CD107a. Additionally, there was a trend towards decreasing polyfunctional Gag-specific CD8+ T cell responses.

Furthermore, IFN γ expression was significantly decreased in HIV-specific CD4⁺ and CD8⁺ T cells. Polyfunctional responses were not associated with HIV viral persistence. However, we observed a direct correlation between T cell cycling and HIV-specific immune responses. Moreover, plasma levels of inosine inversely correlated with Gag-specific CD4⁺ T cell response. Although the decrease in HIV-specific immune responses was not associated with measures of HIV persistence, additional studies should focus on how modulation of the adenosine pathway can affect HIV-specific responses and the subsequent implications in HIV cure research.

Table of Contents

1.0 Introduction.....	1
1.1 The Epidemiology, Pathogenesis, and Clinical Manifestations of HIV.....	1
1.1.1 Epidemiology of HIV	1
1.1.2 HIV Virology and Pathogenesis.....	3
1.1.2.1 Natural Control of HIV Infection	7
1.1.3 The Natural History of HIV	7
1.1.3.1 Transmission Routes	9
1.1.3.2 Prevention, Treatment, and Curative Strategies	9
1.2 The Epidemiological Impact of Non-AIDS Co-morbidities.....	12
1.3 HIV-associated Inflammation and the Adenosine Pathway	13
1.3.1 An Overview of the Adenosine Pathway	13
1.3.2 Homeostatic Inflammatory Control Through the Adenosine Pathway	16
1.3.3 Linking the Adenosine Pathway and HIV-associated Inflammation.....	17
1.4 Adenosine Modulation Through Dipyridamole Use	18
1.4.1 Supporting Evidence for Dipyridamole Use in HIV Infection.....	18
2.0 Specific Aims	21
2.1 Aim 1: Determine the Effect of Dipyridamole on HIV-specific T Cell Responses .	21
2.2 Aim 2: Evaluate if Polyfunctional HIV-specific T cell Responses are Associated with Virologic, Immunologic, and Purinergic Parameters	21
3.0 Materials and Methods.....	23
3.1 Phase I/II Clinical Trial Samples and Biosafety	23

3.2 CD4+ and CD8+ T Cell Cytokine Selection	23
3.3 Relevance of HIV Consensus Sequence Peptides Gag and Env	24
3.4 Validation of Positive Control Stimulus	25
3.5 Polyfunctional Intracellular Cytokine Staining Assay	27
3.6 Flow Cytometric Analysis of CD4+ and CD8+ Cytokine Expression.....	29
3.7 Statistical Analysis.....	29
4.0 Results	30
4.1 HIV-specific Immune Responses to Dipyridamole Treatment	30
4.1.1 Single Cytokine Expression and Polyfunctionality of HIV-specific T Cells.	31
4.2 Virologic, Immunologic, and Purinergic Parameters and HIV-specific Immune Responses Following Dipyridamole Treatment	38
4.2.1 HIV Persistence	39
4.2.2 Immune Activation and Inflammation	39
4.2.3 Adenosine Metabolites	40
4.2.4 Ectoenzyme Expression	41
5.0 Discussion.....	42
6.0 Public Health Statement.....	45
7.0 Future Directions	46
Appendix A Polyfunctional ICS Assay Schematic.....	47
Bibliography	48

List of Tables

Table 1. Antibody Distribution of FMO Controls and All-stain Sample.....	28
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List of Figures

Figure 1. Shift in CD4+ T Cell Population Due to PMA/Ionomycin Stimulation Compared to Media Control	26
Figure 2. Flow Cytometry Gating Strategy for HIV-specific T Cell Responses	30
Figure 3. Distribution of HIV-specific CD4+ T Cell Responses	32
Figure 4. Distribution of HIV-specific CD8+ T Cell Responses	33
Figure 5. TNFα Expression in HIV-specific T Cells Following Dipyridamole Treatment ..	34
Figure 6. IFNγ Expression in HIV-specific T Cells Following Dipyridamole Treatment....	35
Figure 7. CD107a Expression in HIV-specific T Cells Following Dipyridamole Treatment	36
Figure 8. IL-2 and Granzyme B Expression in HIV-specific T Cells Following Dipyridamole Treatment	37
Figure 9. Polyfunctional HIV-specific T Cell Responses Following Dipyridamole Treatment	38
Figure 10. T Cell Cycling Directly Correlated with HIV-specific Immune Responses	40
Figure 11. Inosine Inversely Correlated with Gag-specific CD4+ T Cell Responses	41
Figure 12. Workflow of ICS Assay Employed to Assess HIV-specific T Cell Responses.....	47

1.0 Introduction

There has been a valiant effort to eradicate human immunodeficiency virus (HIV) as it relentlessly continues to cause epidemics worldwide. While a diagnosis is not immediately fatal, with the help of medications, HIV is undeniably still a threat to individuals around the globe. The first identified case of HIV occurred in the early 1980s even though it is postulated that the virus was circulating prior to its genomic identification. In 1981, multiple cases of *Pneumocystis carinii* and Kaposi's Sarcoma were reported in the United States among a group of gay men (1, 2). Within a year, a general severe immune deficiency was observed among 270 gay men and 121 had succumbed to the disease (3). This disease, coined acquired immunodeficiency syndrome (AIDS), took countless, innocent lives and induced fear within communities nationwide. Fortunately, scientific advancements in antiretroviral therapy (ART) have significantly prolonged life with HIV and diminished the number of lives lost to AIDS. However, in 2020, approximately 1.5 million people contracted HIV globally making HIV/AIDS an ongoing, major public health issue (4).

1.1 The Epidemiology, Pathogenesis, and Clinical Manifestations of HIV

1.1.1 Epidemiology of HIV

For the past two decades, there has been a unified effort to fight the HIV/AIDS pandemic. The rates of HIV infection vary by region and within countries (5-7). Internationally, most regions have experienced a decline in newly acquired HIV infection and AIDS-related deaths due to

increased funding and resources for HIV health services (4). In 2014, the UNAIDS proposed a 5-year plan to reach 90% efficacy across HIV status awareness, treatment and viral suppression (8). Eight individual countries achieved those targets and eleven countries reached 73% viral load suppression among all PLWH. Unfortunately, this target was not globally achieved. By 2020, approximately 84% people knew their HIV status, 73% were on treatment, and 66% were virally suppressed (4). While percentages were high, a major reason that the global targets were missed was due to an underinvestment in low-to-middle income countries. In 2020, funding to these countries was 29% lower than the United States \$26 billion budget. Consequently, Eastern Europe and central Asia experienced an increase in HIV diagnoses and AIDS-related mortality likely due to lack of funding and availability of resources (4).

Additionally, gender inequalities, HIV criminalization, social factors, and ART challenges contribute to our inability to control HIV epidemics. There is a remarkable variation in epidemic settings and sociocultural norms across countries. In sub-Saharan Africa, women and girls accounted for approximately 25% of all HIV infections in the past year. Women in these areas are less likely to access and adhere to treatment due to gender-based inequalities (9). When tending to sexual health concerns, many women experience domestic violence or stigma that deters them from seeking HIV health services. Alternatively, in the past year, men and boys contributed to approximately 57% of all HIV infections in most countries. The stigmatization of HIV and lack of healthcare services prevent men from accessing care for HIV infections (10, 11). Thus, improving sexual and reproductive health services is a pillar to preventing acquisition of HIV for all genders.

There is significantly slower progress in controlling HIV infections within areas where HIV status, sexual orientation, and certain behaviors are criminalized. As a result, these individuals are most at risk for contracting HIV. Some countries marginalize people based on sexuality and

gender identity, such as men who have sex with men and transgender women. For example, same-sex male relationships are illegal in Malawi, and many lesbian, gay, bisexual and transgender (LGBT) people experience extreme violence and discrimination (12). Eliminating the discrimination and stigma of PLWH is difficult but necessary at controlling HIV/AIDS epidemics in these areas. Kavanagh et al 2021 reported a positive correlation between better HIV infection outcomes and nondiscriminatory laws against sexuality (13). Finally, behaviors existing outside of the clinic are disproportionality affected by HIV infection. As of 2020, people who inject drugs (PWID) were 35 times more likely to acquire HIV compared to those that don't, and sex workers were at a 26 times greater risk than other adults (14). Unfortunately, both groups of individuals are ubiquitously criminalized and, thus, have inadequate access to therapy.

Whether or not HIV discrimination exists within a region, some countries do not have access to ART (15, 16). For example, some regions lack reliable and affordable healthcare systems making it difficult for PLWH to receive HIV testing and adhere to ART (17). These health disparities disproportionately affect marginalized communities as well. Alternatively, in areas where antiretrovirals are routinely administered, there has been a rise in antiretroviral resistance associated with long-term use (18). Further research and funding into preventative and curative strategies is imperative to control the HIV/AIDS pandemic.

1.1.2 HIV Virology and Pathogenesis

HIV is similar to other viruses in the Lentivirus genus and Retroviridae family through disease course, persistent viral replication, and latency. While HIV-1 and HIV-2 differ by geographical region and virulence, both virus strains are structurally similar (19). An HIV-1 virus particle has two identical copies of single-stranded RNA, encased in a capsid, surrounded by a

lipoprotein-dense and glycoprotein membrane. Various glycoprotein heterodimer complexes comprised of a covalently bound surface gp120 and transmembrane gp41 span the viral envelope. The gp41 subunit facilitates fusion and the gp120 complex directly binds to CD4 on the target cell surface (20). Once bound, this envelope complex undergoes a conformational change to expose a gp120 domain to allow for chemokine binding (21). This chemokine ligand acts a co-receptor on the HIV-1 virus particle that is a determinant of HIV-1 tropism. For example, CXCR4 and CCR5 are HIV-associated chemokine receptors that are expressed on activated T-lymphocytes, macrophages, and dendritic cells (DCs) (22). This chemokine receptor-ligand bond, in conjunction with gp120 bound to CD4, anchors the virus particle. This allows gp41 to penetrate the target cell membrane, via genetic hairpin formation, for fusion and entry of the viral capsid into the cell. The viral capsid contains viral core antigens, two copies of HIV RNA combined with a nucleoprotein, reverse transcriptase, integrase and protease. During entry, the viral capsid delaminates and frees the viral RNA, and other contents, into the cytoplasm of the target cell. The accompanied reverse transcriptase and integrase ensure adequate transcription and translation within the host cell. In the cytoplasm, the ribonuclease H active site of the reverse transcriptase complex begins reverse transcription of viral RNA to DNA. At the primer-binding site, reverse transcriptase begins minus-strand polymerization to create an RNA/DNA hybrid double helix. Following this formation, the ribonuclease H site further breaks down the final RNA strand, and the polymerase active site on reverse transcriptase produces a complementary DNA strand to form a double helix that is recognized by the host cell's nucleus. Subsequently, integrase cleaves the 3' ends of the proviral DNA to be incorporated into the cell genome. Once inside the nucleus, the cell machinery takes over and transcribes the viral DNA into viral messenger RNA (mRNA). During this process, early synthesis of HIV proteins Tat and Rev stimulate viral transcription and facilitate the expression of

viral structural genes. Synthesis of the structural proteins gag, pol, and env and replication enzymes meet the migrating mRNA in the cytoplasm to form new virions. The gag gene encodes the structural proteins of viral core and matrix. The env genes are responsible for producing viral glycoproteins gp120 and gp41. Specifically, proteases cleave a large gp160 molecule into the viral core proteins p24, p9, and p7, matrix protein p17, and envelope proteins gp120 and gp41. Finally, the pol gene encodes viral enzymes required for replication, such as reverse transcriptase, integrase, and protease. Other accessory proteins and regulatory genes involved during the late stages of viral replication include: the vpu protein that's necessary for the correct release of virus particles; the vpr protein that's postulated to arrest the cell cycle; the vif gene that enhances the infectiveness of progeny virions; and the nef protein that is involved in cell signaling and downregulating the CD4 receptor on the cells surface to allow for viral budding (23). All necessary viral products come together to assemble immature HIV-1 viral particles that migrate to the cell's membrane. Finally, these viral particles bud from the cell's membrane and consequently acquire host cell proteins, cholesterol, and phospholipids in their viral envelope during their exit. Virions leave the cell, mature, and circulate to infect other immune cells.

The pathogenesis of HIV has proved to be complex and versatile making it difficult to overcome. During cellular immunity, macrophages and DCs, in the local environment, process and present viral antigens to lymphocytes. Thus, these antigen presenting cells (APCs) trigger the adaptive response via T cells and B cells. Primary HIV infection causes a dramatic decrease in CD4⁺ T cells and initial increase in CD8⁺ T cell populations (24, 25). Primary viral control is achieved by cytotoxic CD8⁺ T cells that directly kill HIV-infected cells to lower viremia (26-28). During acute stages of infection, the number of HIV-specific CD8⁺ T cells in circulation can reach up to 10% of total responding cytotoxic T cells (29, 30). In turn, the survival and establishment of

HIV escape variants is partially dictated by selective pressure exerted from cytotoxic lymphocytes (31).

As HIV infection persists, humoral immunity drives the production of antibodies that directly bind and neutralize HIV or initiates antibody-dependent cellular cytotoxicity (ADCC) mediated by macrophages and NK cells (32, 33). However, the features and functions of HIV allow the virus to prevail over host immunity and ART (34). HIV-associated immunologic dysfunction occurs through T cell expansion, senescence, and depletion because of persistent immune activation and chronic inflammation (35-38). Cytotoxic CD8⁺ T cell clearance is restricted by a downregulation of MHC Class I expression and antigen presentation caused by HIV (39). Specifically, CD8⁺ T cells maintain the ability to secrete antiviral cytokines, such as IL-2, IFN γ , TNF α , MIP-1 β and CD107a, however, they experience a reduction in cytolytic function following chronic HIV infection (40). Expression of exhaustion marker programmed cell death protein 1, PD-1, on CD8⁺ T cells positively correlates with impaired proliferation, cytokine production, survival, and cell turnover following viral peak in HIV infection (41-44). HIV persistence drives CD8⁺ T cell exhaustion and senescence that can be associated with disease progression (45-48). While ART decreases viral loads and stabilizes CD4⁺ T cell populations, treatment does not completely restore CD4⁺ T cell compartments to pre-infection levels (49-53). Thus, a patient's CD4/CD8 ratio may be used as a health parameter to characterize rates of disease progression, response to treatment, and mortality during viral suppression (54). The characterization of HIV-associated immune dysfunction allows for a better understand of the necessary components to achieve viral control.

1.1.2.1 Natural Control of HIV Infection

Long-term nonprogressors are PLWH who have the ability to maintain stable CD4 counts for many years (55). Approximately 1 in 300 PLWH are elite controllers who experience no detectable viremia with HIV diagnosis without the use of ART (56-58). Studies show that naturally controlling HIV infection can occur through Nef-defects, weakened viral replicative fitness and HIV-specific CD8+ T cell responses (59-62). Research surrounding controllers involves the mechanisms behind durable viral control mediated by HIV-specific CD8+ T cells (63, 64). Specifically, CD8+ T cells in elite controllers display sustained proliferation, polyfunctionality, and cytotoxicity overtime compared to progressors (65-70). Also, the magnitude of Gag-specific CD8+ T cell responses strongly correlates with CD8-mediated viral suppression in controllers (71). Thus, enhancing CD8+ T cell numbers in ART-controlled PLWH could be a valuable therapeutic target to decrease disease burden.

1.1.3 The Natural History of HIV

The natural history of HIV occurs in three main stages: (1) acute infection, (2) seroconversion, and (3) chronic infection. Each stage of the disease presents differently in some patients, but there is a clear immunological and clinical trend observed in most PLWH.

Patients presenting with influenza-like symptoms is the hallmark of acute retroviral syndrome observed in some individuals with primary HIV infection (72). The characteristics and duration of symptoms make HIV difficult to diagnose, thus, patient-focused medicine becomes vital to detect infection early. Within 2-4 weeks from exposure, acute infection presents as fever, malaise, myalgia, arthralgia, rash, pharyngitis, lymphadenopathy, and diarrhea that typically only last for 7-10 days. Occasionally, some infected individuals experience neurological

manifestations, such as facial and oculomotor nerve palsies. Altogether, these symptoms could resemble mononucleosis or aseptic meningitis as well (73). From the initial exposure, HIV viremia is detectable in the blood, using real-time polymerase chain reaction (RT-PCR) amplification or 4th generation antigen tests, at approximately 10-33 days or 18-45 days, respectively (74, 75). These initial symptoms arise due to the innate immune response to high levels of circulating HIV-1. CD4+ T cell counts decrease as they serve as primary HIV targets.

Following initiation of humoral immunity, newly infected individuals undergo antibody seroconversion at approximately 3-5 weeks from HIV exposure. Most patients become asymptomatic due to a drop in HIV viremia from an effective innate and adaptive immune response (76). Eventually, viral loads stabilize within 6 weeks (72). During this stage, antibody tests can detect HIV antibodies in the blood at approximately 23-90 days post-exposure.

The time from seroconversion to late-stage disease varies among patients with chronic HIV infection. The CD4+ T cell count declines gradually over the span of 6-8 years without treatment. Persistent HIV replication causes chronic immune activation and inflammation resulting in the destruction of lymphoid tissues. Specifically, dismantling gut mucosa and draining lymph nodes favors HIV advancement into other tissues (77, 78). The multifactorial HIV-associated immune dysfunction leaves PLWH more susceptible to opportunistic infections, such as cytomegalovirus (CMV) (79). Without ART, chronic HIV infection causes patients to experience severe immune impairment and ultimately progress to AIDS. Many patients experience severe weight loss, fever, respiratory disease, and gastrointestinal issues. While each individual experiences HIV infection differently, patients inevitably succumb to AIDS-related deaths, such as neoplastic diseases and progressive encephalopathy(80). Fortunately, the development of ART has improved the quality of life for PLWH.

1.1.3.1 Transmission Routes

During the 1980s, many people feared they would contract HIV by shaking hands with someone who was infected. While the stigma surrounding PLWH still exists, our knowledge surrounding HIV transmission routes has improved. HIV transmission occurs horizontally via blood, breast milk, semen/vaginal fluids and vertically from mother to child during pregnancy and delivery. Common routes include direct contact to skin damage, such as a cut or needle injection and crossing mucosal barriers via abrasions during sexual intercourse. Transmission is dependent on the concentration of HIV in the fluid, host susceptibility, and the virus isolate. For example, the surface area of the cervix increases the likelihood of HIV transmission (81). However, gender is not the only determining factor of acquiring HIV. The risk level of an individual is determined by their sexual behavior, environment, and socioeconomic status. In addition, HIV infection rates vary around the globe. Regardless of the situation, prevention strategies and HIV risk reduction counseling are integral to decrease transmission rates.

1.1.3.2 Prevention, Treatment, and Curative Strategies

Ending the HIV/AIDS pandemic involves effective prevention, treatment, and cure strategies that are applicable at local, regional, and national levels. It is imperative to tailor preventative strategies to accommodate for the diversity among people at high risk of acquiring HIV infection in different regions of the world. Overtime, preventing new HIV infections had declined due to increased condom use, use of clean syringes, blood supply screening, and behavioral interventions. Unfortunately, there have been some drawbacks in these prevention methods. For example, consistent condom use is difficult to achieve in most populations (82). Recent biomedical advances have allowed another opportunity to prevent HIV transmission via pre-exposure prophylaxis (PrEP). Currently, Truvada and Descovy are the two main PrEP options

offered to at-risk populations. Comprised of emtricitabine and tenofovir, these oral medications are taken once daily and approximately 95% effective at preventing HIV infection (83-86). However, PrEP prevention is impacted by the lack of awareness, adherence and access to care (87). Public health campaigns, such as PrEP4Love in Chicago, increase PrEP and HIV awareness by disseminating information to millions of individuals (88). Fortunately, these high resource areas have the capacity to develop a structured, effective system to caring for PLWH and vulnerable populations. Improving adherence and accessibility to PrEP will involve adapting HIV care services to accommodate for the unmet needs in different sub-populations. For example, young MSM of color experience stigma and medical mistrust which prevent them from seeking PrEP services (89, 90). Another barrier to adherence lies within the lack of options of PrEP. Most individuals may forget or experience pill fatigue while on oral PrEP. Thus, clinical research has shifted focus towards HIV prevention methods that mimic contraceptive options. For example, long-acting injectables have been FDA-approved and clinical trials are investigating the safety and efficacy of medicated products, such as vaginal rings and enemas, at preventing HIV transmission (91, 92). Finally, another approach to preventing HIV transmission is achieving undetectable plasma viremia via ART. There are currently six classes of ART that target different stages of the HIV life cycle. Depending on the patient, some clinicians implement highly active antiretroviral therapy (HAART), or the combination use of three or more antiretroviral drugs, to control HIV infection. Several studies have demonstrated that ART-associated viral suppression prevents HIV transmission during sexual activity (93-95). Current issues with ART include adherence, drug toxicities, side effects, and drug resistance (96, 97). Unfortunately, these prevention modalities alone have not been successful at completely controlling global HIV epidemics. Vaccine development and effectiveness has been difficult to achieve because of global genetic diversity

and HIV strain variation (98, 99). The HIV Vaccine Trials Network (HVTN) is the world's largest international effort to evaluate HIV vaccines. Specifically, multiple sites around the United States, including Pittsburgh, are conducting a clinical trial that utilizes various vaccine strategies, including mRNA technologies. (NCT05217641, clinicaltrials.gov). In the HIV treatment and cure field, therapeutic vaccine development efforts have not been successful mainly due to viral immune escape and replication competent HIV-1 that latently resides in infected memory CD4+ T cells. Other cure strategies, such as the use of latency reversing agents (LRA) utilizing the "shock and kill" method, were unable to eradicate the HIV latent reservoir (100). Gene therapy and stem cell transplantation, which was done in the "Berlin patient" Timothy Brown, are not scalable (101-103). Early treatment, occurring after birth or during primary HIV-1 infection, temporarily allows some babies, children, and adults to achieve long-term viral remission. However, they eventually experience viral rebound, because ART cannot eliminate the viral reservoir (104-107). Additionally, some patients receiving broadly neutralizing antibodies (bNAbs), during ART interruption, experienced viral rebound due to viral escape via resistance mutations (108, 109). Since genomic mutations rapidly occur in HIV replication cycles, targeting human proteins may be a more effective route for vaccine development. Specifically, $\alpha 4\beta 7$ is an integrin expressed on the surface of lymphocytes with the ability to be incorporated on the surface of viral particles during budding (110, 111). Studies using anti- $\alpha 4\beta 7$ antibody therapy showed a reduction in SIV transmission and pathogenesis in the blood and gut-associated tissues in Rhesus macaques (112-114). Currently, this therapeutic approach is being employed in human clinical trials (NCT02788175, clinicaltrials.gov).

1.2 The Epidemiological Impact of Non-AIDS Co-morbidities

While there is no cure for HIV infection, the introduction to effective ART regimens allows for viral suppression and a reduction of AIDS progression in PLWH. The implementation of ART has allowed us to assess the full spectrum of someone's life with HIV, however, it doesn't fully restore the functionality of the antiviral immune response (115-117). While AIDS-related deaths have decreased overtime, recent clinical data has shown an increase in non-AIDS comorbidities across the lifespan resulting in early mortality (118, 119). Globally, virally suppressed individuals are experiencing cardiovascular disease, liver disease, non-AIDS cancers, and other common diseases associated with aging at earlier rates compared to people living without HIV (PLWOH)(120). The duration of HIV infection and ART-use has been linked to an increased risk of developing multimorbidity (121). This accelerated immunologic aging is believed to be due to chronic immune activation and inflammation caused by long-term HIV infection (122). Other behavioral factors, such as smoking, likely contribute to an increased risk of developing non-AIDS co-morbidities as well (123). Thus, there is a significant need to develop therapeutic interventions that target factors contributing to HIV-associated inflammation to reduce the prevalence of non-AIDS comorbidities.

1.3 HIV-associated Inflammation and the Adenosine Pathway

1.3.1 An Overview of the Adenosine Pathway

Adenosine is an immunomodulatory molecule that suppresses inflammatory responses by relaying information to the immune system about tissue damage or acute inflammatory changes in the host immune response (124). Extracellular adenosine arises mainly from two sources: 1) metabolism of extracellular adenine nucleotides to adenosine by membrane-bound ecto-enzymes and 2) metabolism of intracellular adenine nucleotides to adenosine, followed by transport of adenosine to the extracellular compartment by equilibrative nucleoside transporters (ENTs) (125).

Whether produced intracellularly or extracellularly, ATP is the main, but not the only, precursor for adenosine generation. In addition to its critical role as an energy source and as a substrate for kinases, ATP, like adenosine, acts extracellularly as a signaling nucleotide to regulate biological processes (126). In contrast to adenosine, ATP acts as a damage associated molecular pattern (DAMP), and stressed cells release ATP into the extracellular space in response to hypoxia, ischemia and inflammation (127). Extracellular ATP is catabolized into adenosine 5'-monophosphate (5'-AMP) by a family of enzyme ectonucleotidases with triphosphate dephosphorylase-1 (CD39) being the most important in most cells and organ systems. Subsequently, 5'-AMP is converted to adenosine mainly by ecto-5'-nucleotidase (CD73) but also by tissue non-specific alkaline phosphatase (128). On most cells, including lymphocytes and endothelial cells, the surface expression of CD39 and CD73 is regulated by external stimuli that ultimately influence the concentration of adenosine in the local environment to mediate paracrine signaling (129).

Under normal physiological conditions, nucleoside or purine transporters maintain constitutively low levels of extracellular ATP and adenosine(130). Depending on the receptor subtype, the accumulation of adenosine in the extracellular milieu triggers either stimulation or inhibition of adenylyl cyclase, which regulates the intracellular production of adenosine 3',5'-cyclic monophosphate (cAMP). In immune cells, intracellular cAMP inhibits effector functions and prevents recruitment of newly activated immune cells to the site of inflammation (131, 132). ENTs control excessive levels of extracellular adenosine by transporting adenosine into cells to be metabolized into inosine via adenosine deaminase (ADA) (133). Although less potent than adenosine, inosine also inhibits pro-inflammatory cytokine production by neutrophils, macrophages, and lymphocytes in addition to other effects (134). Together, the intricate mechanisms that regulate adenosine production, transport, and metabolism provide a delicate homeostatic system that subdues inflammation while preventing excessive immunosuppression.

Purinergic receptors mediate the effects of extracellular ATP and adenosine by activating a cascade of intracellular events depending on the cell type (135, 136). P1 receptors recognize adenosine, whereas P2 receptors bind to extracellular nucleotides (127). For many P2 receptor subtypes, ATP is the dominant endogenous agonist. Both types of purinergic receptors are expressed by various cell types and vary in function (137). P1 purinergic receptors determine the function of adenosine via four G protein-coupled receptor subtypes: A₁, A_{2A}, A_{2B}, and A₃. Each receptor subtype differs in tissue distribution, pharmacological profile, and effector coupling, but can be found on neutrophils, monocytes, macrophages, DCs, and T lymphocytes (138). The A₁ receptor is ubiquitous throughout the human body and typically has a pro-inflammatory effect (139). A₃ receptors have a similar signaling pathway as A₁ receptors (140). A₂ receptors exhibit an anti-inflammatory role (141). Specifically, activating the A_{2A} adenosine receptor (A_{2A}AR),

expressed on neutrophils, monocytes, macrophages, NK cells and T-cells, suppresses cytotoxicity and TNF α , IL-6, and IL-12 production (142). Overall, the A₁ and A_{2A} receptors have a high affinity for adenosine whereas A_{2B} and A₃ receptors have low binding affinity. The differences in binding affinity suggests that recruitment of immune cells with P1 receptor expression depends on the progression of extracellular adenosine accumulation (143). When activated, the A₁ and A₃ receptors expressed on immune cells counteract A₂ receptors by inhibiting adenylyl cyclase and cAMP formation (144). Although not as potent, inosine can also activate A₁, A_{2A} and A₃ receptors (134).

P2 receptors consist of two main subtypes, P2X and P2Y receptors. P2X receptors are ATP-gated ionotropic channels that are generally involved in pro-inflammatory processes. Specifically, P2X₇ stimulation causes ATP release, which triggers a positive feedback loop to amplify the ATP signal while recruiting appropriate cells to the area (145). Although ATP can serve as a DAMP, all cells are capable of non-lytic ATP release via large conductance channels, exocytosis, plasma membrane carriers and P2 receptors (146). P2Y receptors are G-protein coupled receptors implicated in a broad range of functions, including facilitating platelet aggregation, vasodilation, cell migration and immune responses (147). For example, the P2Y₂ receptor is most likely responsible for recruiting neutrophils, DCs, eosinophils and macrophages and signal the release of pro-inflammatory factors at inflamed sites (148). The P2X₇ receptor has been extensively studied and is integral to innate immunity, pro-inflammatory cytokine activation, antigen presentation, and lymphocyte proliferation and differentiation (149-151).

1.3.2 Homeostatic Inflammatory Control Through the Adenosine Pathway

The concentrations of extracellular ATP and adenosine are intrinsically regulated during inflammation and immune responses, which modulates the functions of myeloid and lymphoid cells (135). Immune cells express purinergic receptors to regulate their immunological responses via purinergic mediators, ATP and adenosine (152). During acute inflammatory responses, high levels of extracellular ATP act as a DAMP and trigger pro-inflammatory effector functions in a setting of low extracellular adenosine levels (128). A₁ receptors work synergistically with high ATP levels and are predominantly activated due to the lower extracellular adenosine concentration (153, 154). In neutrophils (155), monocytes (156, 157), and macrophages (158, 159), high concentrations of extracellular ATP promote cell migration, cytotoxicity, apoptosis, and pro-inflammatory cytokine secretion. However, high levels of ATP inhibit phagocytosis through P2X₇ receptor activation, whereas low ATP levels stimulate phagocytosis (160). Excessive ATP release influences DC function and subsequent lymphocyte activation (135). Extracellular ATP promotes DC migration (161) and maturation through prolonged antigen exposure (154, 162) as well as T lymphocyte migration and proliferation (163).

As inflammation persists, the concentration of extracellular adenosine increases as a result of the breakdown of more ATP from collateral damage of surrounding healthy tissue. Immune cells in the most injured areas produce an adenosine-rich environment to inhibit themselves and other local immune cells while allowing neighboring cells to continue eliminating the pathogen (164). The protective increase in extracellular adenosine inhibits the effector functions of neutrophils, macrophages, DCs and T lymphocytes (135). Specifically, A_{2A} receptor activation by adenosine attenuates inflammatory functions of neutrophils (165). Thus, a rise in extracellular adenosine and A_{2A} receptor expression provides a negative feedback mechanism to prevent further

tissue damage by neutrophils (166). High extracellular adenosine concentrations block monocyte and macrophage recruitment, suppress macrophage phagocytic function, and inhibit pro-inflammatory cytokine secretion (167, 168). Moreover, studies show that adenosine modulates the actions of DCs including: 1) antigen capture in favor of Th2-mediated responses; 2) expression of co-stimulatory molecules; and 3) migration to lymph nodes (141, 169, 170). For example, adenosine binding to A₁ and A₃ receptors causes chemotaxis of circulating immature DCs (171). Also, adenosine and A_{2B} receptor signaling has been observed to play a role in inducing APCs to secrete immunosuppressive cytokines, such as TGF- β and IL-10, to create an immunosuppressive microenvironment (141).

Adenosine plays an autonomous role in resolving inflammation by inhibiting activated T cell effector functions (172, 173). Both helper CD4⁺ and cytotoxic CD8⁺ T lymphocytes express A_{2A} receptors and increase adenosine receptor expression when activated by DCs. A_{2A} receptor agonists interfere with T cell receptor signaling to inhibit T cell proliferation and effector function (173), and increase cAMP production which contribute to these inhibitory effects (174, 175). Also, not only does adenosine produce suppressive effects, but it can produce anergic T cells. Studies show an expansion of activated T cells lacking effector function in adenosine-rich environments (176, 177). These characteristics, combined with the short half-life of adenosine *in vivo*, allows for efficient paracrine and autocrine adenosine signaling among immune cells (178).

1.3.3 Linking the Adenosine Pathway and HIV-associated Inflammation

While there are various contributors to HIV-associated chronic inflammation, such as microbial translocation and NLRP3 inflammasome activation, evidence suggests that the adenosine pathway plays a crucial role in regulating inflammation in this infection (179, 180).

Preliminary animal models demonstrate a potentially significant relationship between the adenosine pathway and HIV infection. In our study comparing CD39 and CD73 expression on CD4+ and CD8+ Tregs, we found higher frequencies of ectonucleotidase co-expression as well as higher tissue gut mucosal tissue levels of adenosine in the non-progressive model of simian immunodeficiency virus (SIV) infection in African green monkeys (AGM) compared to progressive model in pigtailed macaques (PTM). When examining the functionality of adenosine *ex vivo*, we found that adenosine significantly suppressed cytokine production of CD4+ and CD8+ T cells in both AGM and PTM (181). These findings suggest a potential role for adenosine in AGM from progressing to AIDS and their control immune activation and inflammation despite SIV replication. Furthermore, HIV infection is associated with decreased frequency of CD73+ expressing T cells leading to lower extracellular adenosine in peripheral blood and a decreased ability to regulate higher levels of inflammation (179, 180). Our review article further explores the relationship between the adenosine pathway and HIV-associated inflammation as well as the therapeutic potential of modulating the adenosine pathway to curb chronic inflammation caused by HIV (<https://d-scholarship.pitt.edu/42602/1/Hixson%20et%20al%202021.pdf>).

1.4 Adenosine Modulation Through Dipyridamole Use

1.4.1 Supporting Evidence for Dipyridamole Use in HIV Infection

Therapeutically targeting the adenosine pathway could alleviate HIV-associated inflammation and reduce the prevalence of non-AIDS co-morbidities in PLWH (182). Our review outlines potential pharmacotherapeutics that target different components of the adenosine

pathway. Specifically, dipyridamole is a Food and Drug Administration (FDA)-approved nucleoside transport inhibitor that blocks the cellular uptake of adenosine via ENTs, thus, increasing extracellular adenosine levels in locally inflamed areas (183). Downstream, dipyridamole prevents the breakdown of cAMP by inhibiting phosphodiesterase to increase cAMP levels (184, 185). Currently, patients with peripheral vascular disease and stroke utilize Dipyridamole to help prevent future thrombotic events (186). Moreover, various animal studies have indicated the potential use of dipyridamole to induce an anti-inflammatory response(187, 188). Ramakers et al 2011 confirmed this effect in a human clinical trial (189). Based on the association between the adenosine pathway and SIV infection observed in human primate studies, we completed a Phase I/II clinical trial that randomized 40 ART-controlled PLWH to 12 weeks of dipyridamole versus placebo to investigate the effects of specifically targeting adenosine in HIV infection. At the end of 12 weeks, all participants received dipyridamole until completion. The primary objective was to evaluate the effect of oral dipyridamole on immune activation and inflammation in HIV-1 infected, ART-treated individuals compared to placebo (190).

Primary analysis of soluble markers showed a trend toward decreased levels of sCD163, a marker for macrophage activation, in the dipyridamole arm ($P = 0.09$). No differences were observed in plasma levels of sCD14, IL-6, CRP, and CXCL10. Moreover, initial data showed that dipyridamole decreased CD8+ T cell activation compared to placebo ($P = 0.03$). In the pooled analyses, where participants were combined based on their first 12 weeks of receiving dipyridamole, there was a significant decrease in CD4+ T cell activation ($P = 0.006$) and a trend towards decreased CD8+ T-cell activation ($P = 0.058$) in blood. Furthermore, we expanded our analysis of dipyridamole treatment to include gut mucosal responses due to the differential and distinct expression patterns of HIV-specific CD8+ T cells in gut mucosal tissue and blood (191).

In a sub-study, rectosigmoid biopsies were assessed to determine the effect of dipyridamole on mucosal immune cells (192). Those receiving dipyridamole had a median 70.2% decrease from baseline in the T regulatory (Treg) population (CD4⁺ CD25^{HI} FOXP3⁺) ($P = 0.007$) and an 11.3% increase in CD8⁺ T cells ($P = 0.05$). There were also trends toward decreased CD4⁺ and CD8⁺ T cell activation. These findings indicate a need for further analysis into HIV-specific T cell responses following dipyridamole treatment. Thus, we will be able to assess how adenosine modulation influences the immunological profile of virally suppressed PLWH.

2.0 Specific Aims

2.1 Aim 1: Determine the Effect of Dipyridamole on HIV-specific T Cell Responses

Hypothesis: Dipyridamole will decrease HIV-specific CD4+ and CD8+ T cell responses in virally suppressed PLWH.

Experimental Approach. Polyfunctional intracellular staining (ICS) assays are highly reproducible and a reliable method to monitor HIV-specific T cell responses (HIV and CD8 T cell 2001). The assessment of HIV-specific effector T cell responses will occur through an optimized polyfunctional ICS assay followed by flow cytometry. The change in HIV-specific immune responses will be calculated from baseline to week 12 of dipyridamole versus placebo.

2.2 Aim 2: Evaluate if Polyfunctional HIV-specific T cell Responses are Associated with Virologic, Immunologic, and Purinergic Parameters

Hypotheses:

1. A decrease in HIV-specific immune responses following dipyridamole treatment will inversely correlate with residual viremia.
2. A decrease in HIV-specific immune responses following dipyridamole treatment will directly correlate with decreased immune activation and inflammatory markers.
3. A decrease in HIV-specific immune responses following dipyridamole treatment will inversely correlate with levels of adenosine metabolites.

4. A decrease in HIV-specific immune responses following dipyridamole treatment directly correlate with ectoenzyme expression.

Experimental Approach. Polyfunctional T cell response data will be compared to all corresponding data for participants from our clinical trial (190). Existing baseline and week 12 percentage change values for HIV persistence (total integrase single-copy assay (iSCA) and HIV-1 DNA), activation (HLA-DR+38+ and Ki-67+), inflammation (sCD14, sCD163, IL-6, CXCL10, and CRP), and adenosine metabolites (adenosine, inosine, and 5'cAMP) will be assessed.

3.0 Materials and Methods

3.1 Phase I/II Clinical Trial Samples and Biosafety

We obtained peripheral mononuclear cells (PBMCs) from virally suppressed PLWH with CD4 counts $\geq 350/\mu\text{L}$ and plasma HIV-1 RNA levels < 50 copies/mL for ≥ 12 months. A written informed consent, including use of biological sample use for future immunologic testing, was reviewed and signed by all participants. Participants were randomized 1:1 to 100mg of oral dipyridamole taken 4 times daily or matching placebo capsules for 12 weeks. Following the 12-week administration, all participants received open-label dipyridamole for an additional 12 weeks until study completion at 28 weeks. Due to limited availability, only 22 of 40 samples were used for investigation of HIV-immune responses in the presence of dipyridamole versus placebo. Within this set of samples, 9 were treated with dipyridamole and 13 were placebo samples. My analysis specifically focused on week 0 and 12 samples for each participant. All samples were processed within a Biosafety Level 2 Plus (BSL2+) laboratory.

3.2 CD4+ and CD8+ T Cell Cytokine Selection

Polyfunctional CD4+ and CD8+ T cell responses and activation levels associate with better viral control during HIV infection (193). Typically, viral infections stimulate a Th-1 CD4+ T cell effector response. These CD4+ T cells promote a pro-inflammatory response, via secretion of cytokines IL-2, TNF α , and IFN γ , to clear infection. Additionally, a phenotypic subset of CD4+ T

cells, ThCTL, has been implicated to have cytolytic function against CMV (194-196), influenza (197), HIV (198, 199), and other chronic infections (200, 201). ThCTL CD4⁺ T cells function by directly killing infected targets (202), and can mimic Th-1 responses via expression of IFN γ , TNF α , and IL-2 (194-203). Interestingly, expression of CD107a highlights transcriptional similarities between cytolytic CD4⁺ cells and CD8⁺ T cells or NK cells (198). Nemes et al 2010 found that approximately 50% of HIV-1 Gag-specific CD4⁺ T cells express CD107a during infection (204). Also, elite controllers demonstrated higher levels of CD107a expression from HIV-specific CD4⁺ T cells which could be predictive of disease progression (199). While evidence shows Granzyme B expression from CD4⁺ T cells, it is not a reliable marker because non-cytotoxic cells are capable of secreting it during infections as well (205). Thus, we selected IL-2, TNF α , IFN γ , and CD107a for our CD4⁺ T cell polyfunctional analysis.

HIV-specific CD8⁺ T cells have a prominent role in controlling viral replication in non-progressors but can reach immunosenescence overtime in progressors due to HIV persistence (47). HIV-specific CD8⁺ T cell responses exhibit production of CD107a, Granzyme B, IL-2, IFN γ , and TNF α (40, 206-208). Thus, these five cytokines were selected for our CD8⁺ T cell polyfunctional analysis.

3.3 Relevance of HIV Consensus Sequence Peptides Gag and Env

Targeting lentiviral proteins is a plausible, yet difficult, functional HIV cure strategy. Several simian immunodeficiency virus models describe the effectiveness of using HIV viral proteins in vaccine development (209, 210). Martins et al 2017 observed that vaccines without gag, env, or both were unable to control viral replication and insufficient at reducing viremia (209).

Sacha et al 2007 demonstrated that lower viremia was significantly associated with the number of gag epitopes recognized by CD8⁺ T cells in SIV-infected rhesus macaques (210). A caveat to this strategy lies within HIV evolutionary mutations in Gag and Env T cell epitopes (211).

Use of HIV viral proteins to promote T cell immune responses have been described in strategies to overcome HIV-associated immune dysfunction (212, 213). Stimulation with HIV peptides, gag and env, can induce CD8⁺ T cell-mediated antiviral responses in rectal and blood samples from chronically infected (212). Moreover, *in vitro* stimulation of Gag-specific CD8⁺ T cells displayed higher rates of proliferation and polyfunctional cytokine responses that significantly correlated with greater inhibition of viral replication in CD4⁺ T cells (213). Thus, we selected gag and env peptide pools, 15-mer peptides overlapping by 11 amino acids, to be used in the polyfunctional ICS assay.

3.4 Validation of Positive Control Stimulus

Validation experiments were performed to determine a positive control stimulant. Common stimulants, phytohemagglutinin (PHA) and phorbol 12-myristate 13-acetate (PMA) with ionomycin, were compared through the polyfunctional ICS assay developed for this project. Preliminary data suggests functional differences exist between PHA and PMA (214). While PMA/ionomycin induces higher TNF α and IFN γ levels, it altered cell morphology and membrane expression of CD4⁺ T cells. Thus, cell activation via PHA was more precise during intracellular cytokine (214).

During our validation experiments, we observed lower activation levels of cytokines using PHA compared to PMA/ionomycin. At times, cytokine percentages were closer to our negative

control values. Alternatively, PMA/ionomycin consistently induced a more robust cytokine response compared to PHA. In some samples, Granzyme B percentages were lower than expected during PMA/ionomycin stimulation but did not reach the threshold of our negative control.

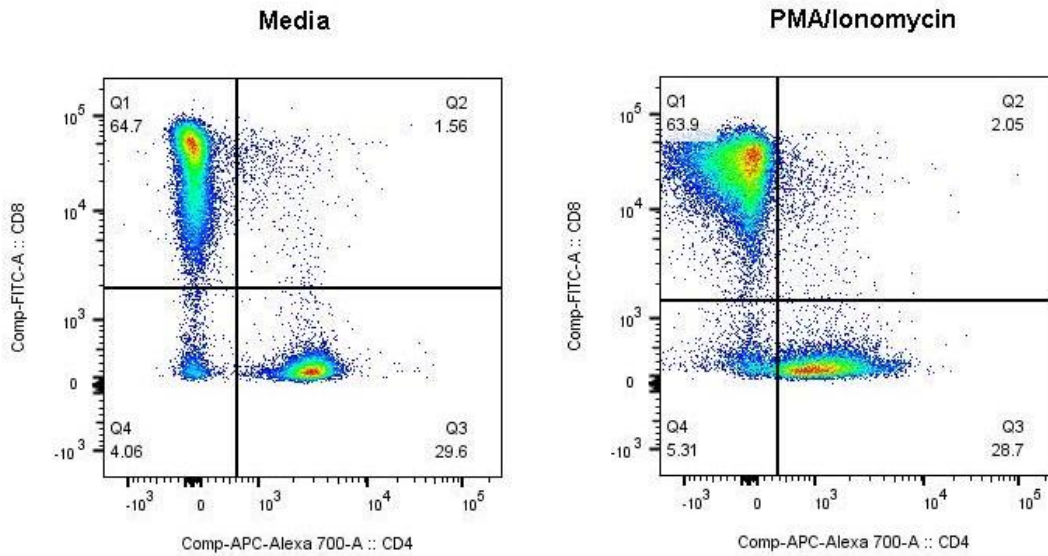


Figure 1. Shift in CD4⁺ T Cell Population Due to PMA/Ionomycin Stimulation Compared to Media Control

Also, PMA/ionomycin caused the CD4⁺ T cell population to shift closer to CD8⁺ T cell population during CD3⁺ T cell gating (Figure 1). However, this shift in morphology was gated appropriately and did not influence CD4⁺ T cell polyfunctionality. To ensure an effective response, we chose to proceed with PMA/ionomycin as our positive control.

3.5 Polyfunctional Intracellular Cytokine Staining Assay

Cryopreserved PBMCs, obtained from participants in our study, were thawed in complete RPMI 1640 media with 10% FBS, 1X HEPES, 1X penicillin-streptomycin, and 1X L-glutamine. Following centrifugation at 400 x g for 5 minutes, cells were immediately washed with Dulbecco's phosphate-buffered saline (D-PBS) solution (Gibco, USA). Cell counting was performed at a 1:20 dilution on an Orflo[®] Moxi Flow Cell Counter. Approximately 1-2 million cells were distributed per well in a 96-well plate (Corning, USA). Cells were activated during a 4-hour incubation at 37°C in the presence of brefeldin A (BFA), monensin, and CD28/CD49d monoclonal antibodies (all from BD Biosciences). The duration of cell activation was determined to optimize cytokine accumulation levels (215). Specifically, BFA and monensin block protein transport from the endoplasmic reticulum (ER) to the Golgi and trans-golgi function, respectively (216, 217). This prevents the secretion of intracellularly synthesized cytokines effector T cells. Also, CD28/CD49d co-stimulatory antibodies facilitate cell activation during incubation. CD28 is a cell adhesion molecule that functions as a ligand for CD80 and CD86 antigens expressed on peripheral blood CD3⁺ T cells that are involved in T cell activation (218-222). CD49d is an integrin alpha subunit on the $\alpha 4\beta 1$ lymphocyte homing receptor, expressed primarily on T and B lymphocytes, that recognizes and binds to the alpha chain of very-late antigen (VLA)-4 necessary for cell adhesion (223-225). Supplemental Figure 1 represents the work flow of the ICS assay. Cells incubated solely in media served as negative controls to identify influences from experimental controls. Gag and env were experimental controls to assess HIV-specific T cell responses. Finally, PMA/ionomycin served as the positive control and served to establish the validity of the experiment. Each condition was divided into 3 fluorescence minus one (FMO) controls per well. Table 1 displays the contents of the FMO controls and all-stain cocktail.

Table 1. Antibody Distribution of FMO Controls and All-stain Sample

FM1	FM2	FM3	All
IgG e450	TNF α	-	TNF α
IgG BV510	-	CD107a	CD107a
IgG BV711	IFN γ	-	IFN γ
IgG PE	Granzyme B	-	Granzyme B
Rat IgG2a APC	-	IL-2	IL-2

FMO controls were utilized to set the upper boundary for background signal of a cytokine for accurate gating of the all-stain sample. Following incubation, centrifugation and resuspension, the live/dead cells were stained using Invitrogen© Live/Dead Aqua stain FVS 780, resuspended in DMSO, for 10 minutes at room temperature (RT; 21°C to 23°C) and protected from light. Cells were washed with BD Pharmingen™ Flow Cytometry Staining buffer to removed excess Live/Dead stain. Cell lysis and permeabilization was completed using 1X BD FACSLyse and 1X BD PermWash, respectively. To assess polyfunctional cytokine production, cells were stained for 30 minutes at RT with the following monoclonal antibody cocktail: CD3-PE-Cy7, CD4-Alexa Fluor 400, CD8-FITC, Granzyme B-PE, CD107a-BV510, IFN γ -BV711, TNF α -e450, and IL-2-Rat APC (all BD Biosciences). Cells were washed with 1X BD PermWash to remove excess antibodies. Prior to flow acquisition, samples were fixed using 0.5% paraformaldehyde prior to flow cytometry analysis.

3.6 Flow Cytometric Analysis of CD4+ and CD8+ Cytokine Expression

Flow cytometry completed on a BD Biosciences LSR Fortessa Cell Analyzer within 24 hours after staining. Isotype and unstained negative compensation controls were performed every two weeks per laboratory standard operating procedures. BD Biosciences antibodies, with their corresponding isotypes, used in each experiment included: CD3-PE-Cy7, CD4-Alexa Fluor 400, CD8-FITC, Granzyme B-PE, CD107a-BV510, IFN γ -BV711, TNF α -e450, and IL-2-Rat APC. Polyfunctionality is defined as the expression of 2 or more effector cytokines following peptide pool stimulation. Flow data was acquired using FACS Diva software and analyzed on Flow Jo 10.8.1, Graphpad Prism 9.3.1, and Simplified Presentation of Incredibly Complex Evaluations (SPICE) 6.1.

3.7 Statistical Analysis

Statistical analysis was performed in Graphpad Prism 9.3.1. Mann-Whitney U tests were used to determine changes in cytokine expression from HIV-specific T cells following dipyridamole treatment. Spearman's Rank-Order Correlation tests were employed to analyze associations between HIV-specific immune responses and virologic, immunologic, and purinergeric parameters.

4.0 Results

4.1 HIV-specific Immune Responses to Dipyridamole Treatment

Following the decreased in T cell activation from our primary analysis, we wanted to determine the influence of dipyridamole treatment on HIV-specific T cell responses. The employed ICS assay allowed for an analysis of single cytokine expression and polyfunctionality of CD4+ and CD8+ T cells in PBMCs of ART-treated samples from our study.

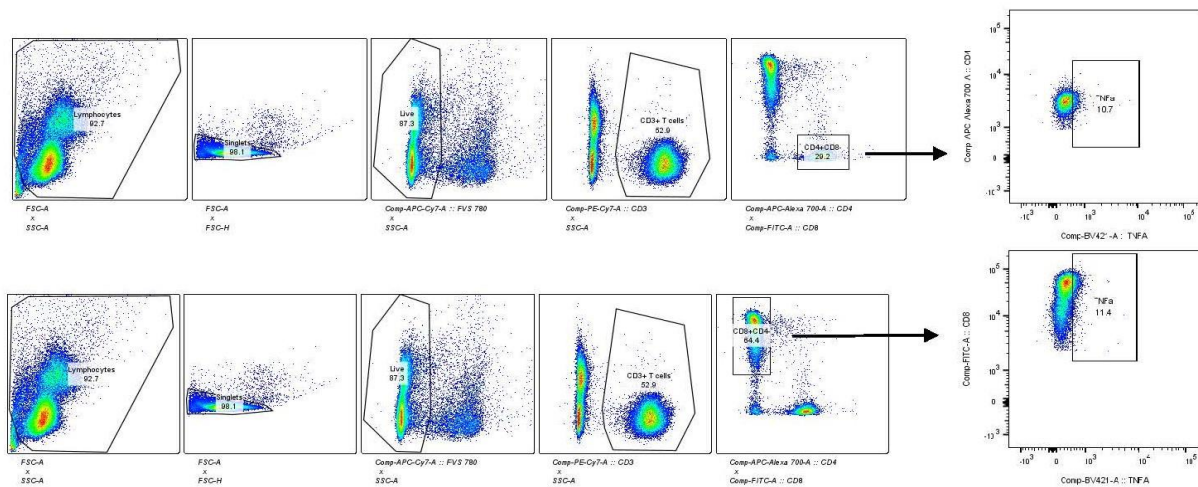


Figure 2. Flow Cytometry Gating Strategy for HIV-specific T Cell Responses

An antibody cocktail was developed to capture HIV-specific T cell responses using flow cytometry. The gating strategy for CD4+ and CD8+ T cell cytokine responses is depicted in Figure 2. Negative and positive controls, media and PMA/ionomycin respectively, were used to assess changes in HIV-specific T cell responses, from baseline to week 12, upon antigen stimulation with

HIV peptide pools, gag and env. During analysis, we observed a shifted CD4+ T cell population under PMA/ionomycin stimulation compared to the other controls (Figure 1). However, these changes did not affect our results.

4.1.1 Single Cytokine Expression and Polyfunctionality of HIV-specific T Cells

The changes in HIV-specific CD4+ and CD8+ T cell responses, following 12 weeks of dipyridamole treatment versus placebo, were calculated and statistically analyzed. The percent change of cytokine expression from each participant, across dipyridamole treatment and placebo groups, was calculated by subtracting the negative control values from HIV peptide-induced cytokine expression levels to obtain the net positive cytokine-specific signal. Following single cytokine expression analyses, Boolean gating was used to assess changes in polyfunctional responses of HIV-specific CD4+ and CD8+ T cells. Figure 3 represents the distribution of TNF α , IFN γ , IL-2, and CD107a expression, alone or in combination with one another, in HIV-specific CD4+ T cells.

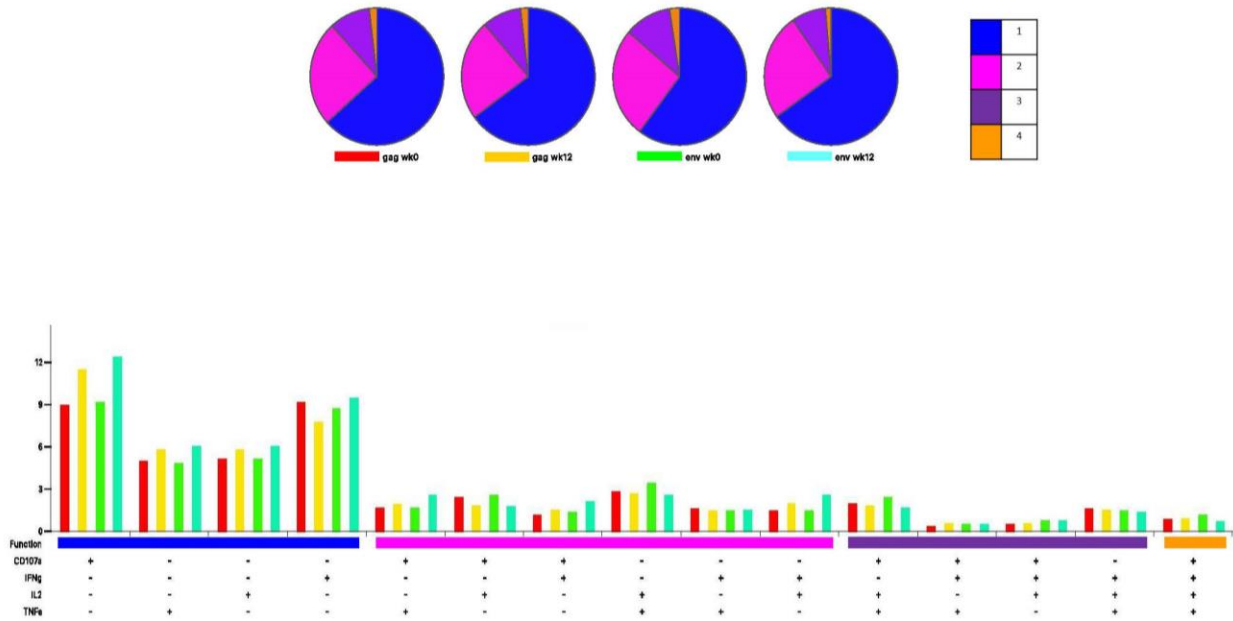


Figure 3. Distribution of HIV-specific CD4+ T Cell Responses

Additionally, baseline and week 12 samples for HIV-specific CD8+ T cell were distributed based on single and polyfunctional expression of TNF α , IFN γ , IL-2, CD107a, and Granzyme B. (Figure 4). Collectively, this cytokine gating strategy was applied to CD4+ and CD8+ T cells for all participant samples, at baseline and week 12, to assess potential changes in HIV-specific T cell responses following dipyridamole treatment.

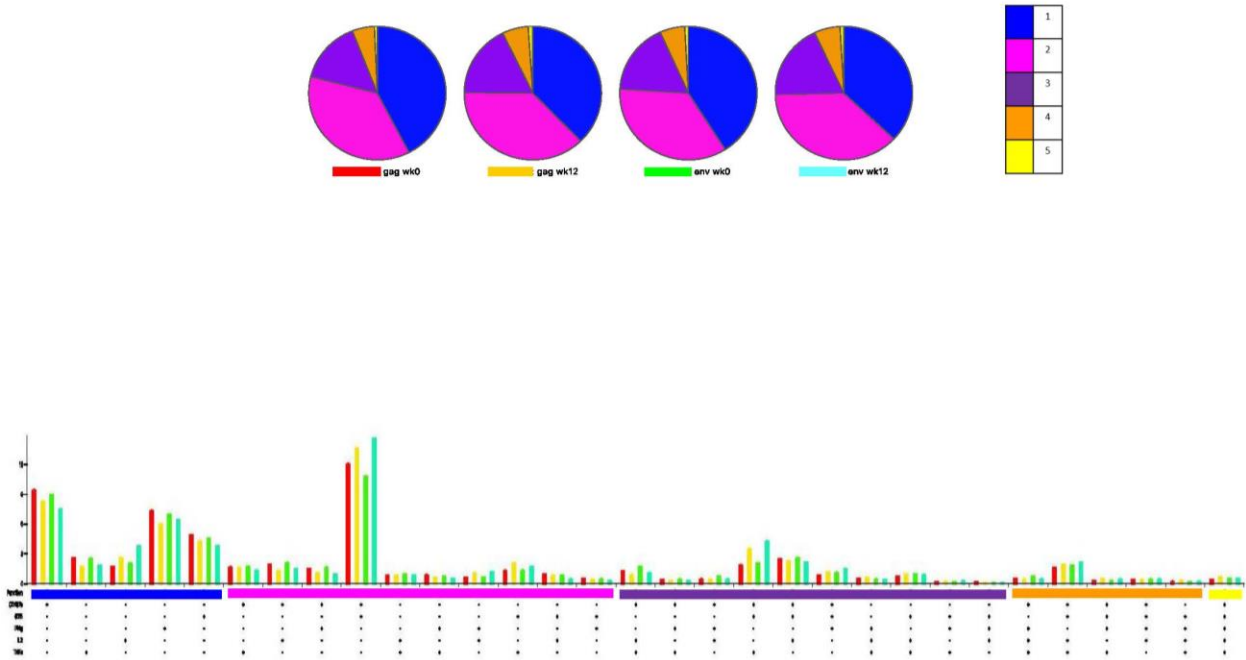


Figure 4. Distribution of HIV-specific CD8+ T Cell Responses

Quantitative analyses were required to accurately determine if dipyridamole influenced HIV-specific immune responses in our population. We found that dipyridamole significantly decreased TNF α expression of HIV-specific CD4+ T cells (median_{gag} -62.50% change vs -10.00% in placebo; median_{env} -51.52% change versus -23.33% in placebo; Figure 5A).

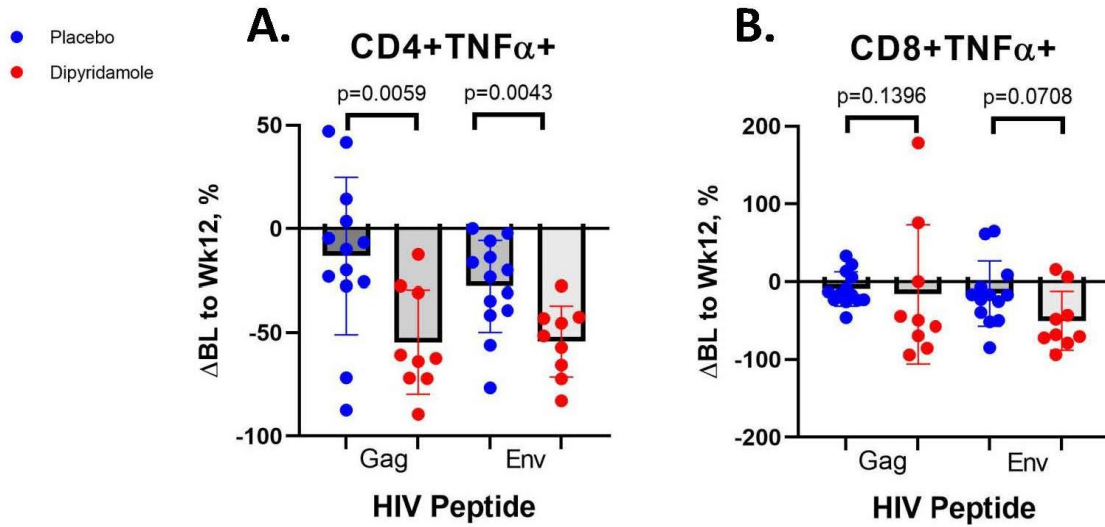


Figure 5. TNF α Expression in HIV-specific T Cells Following Dipyridamole Treatment

Also, there was a trend towards decreasing TNF α expression of HIV-specific CD8+ T cells following dipyridamole treatment (median_{gag} -49.40% vs -15.79% in placebo; median_{env} -68.33% vs -17.24% in placebo; Figure 5B).

Expression of IFN γ , among HIV-specific CD4+ and CD8+ T cells, significantly decreased following dipyridamole treatment (Figure 6).

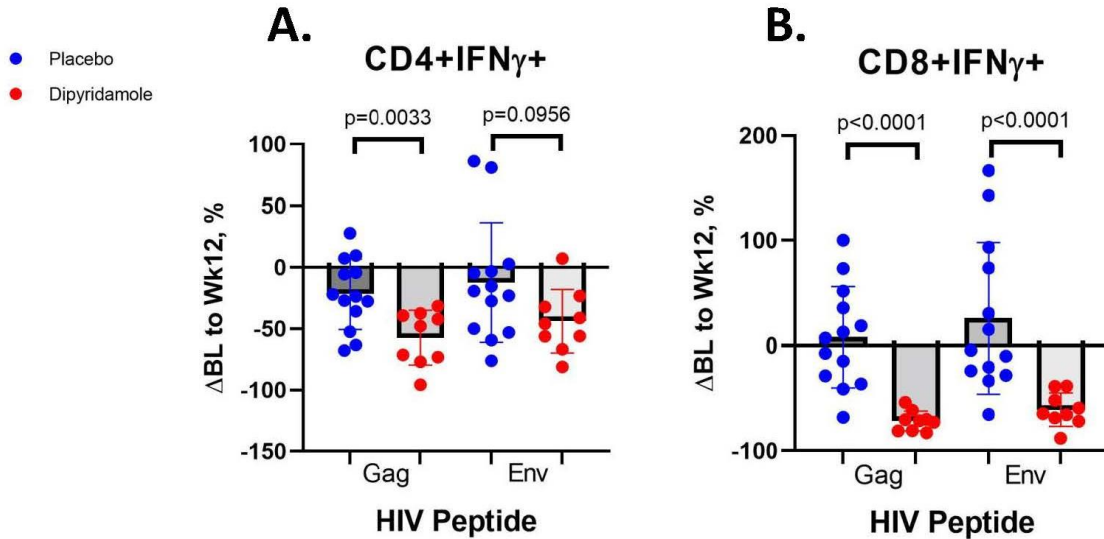


Figure 6. IFN γ Expression in HIV-specific T Cells Following Dipyridamole Treatment

A median_{gag} decrease of -47.83% change vs -23.61% and median_{env} decrease of -45.95% vs -19.23% was observed for IFN γ expression from HIV-specific CD4+ T cell when comparing treatment and placebo groups (Figure 6A). Additionally, there was a median_{gag} decrease of -71.43% change in dipyridamole vs 6.90% in placebo and median_{env} decrease of -64.71% in dipyridamole vs -4.350 in placebo of IFN γ expression for HIV-specific CD8+ T cells (Figure 6B).

Furthermore, dipyridamole treatment influenced HIV-specific CD4+ T cell expression of CD107a (Figure 7).

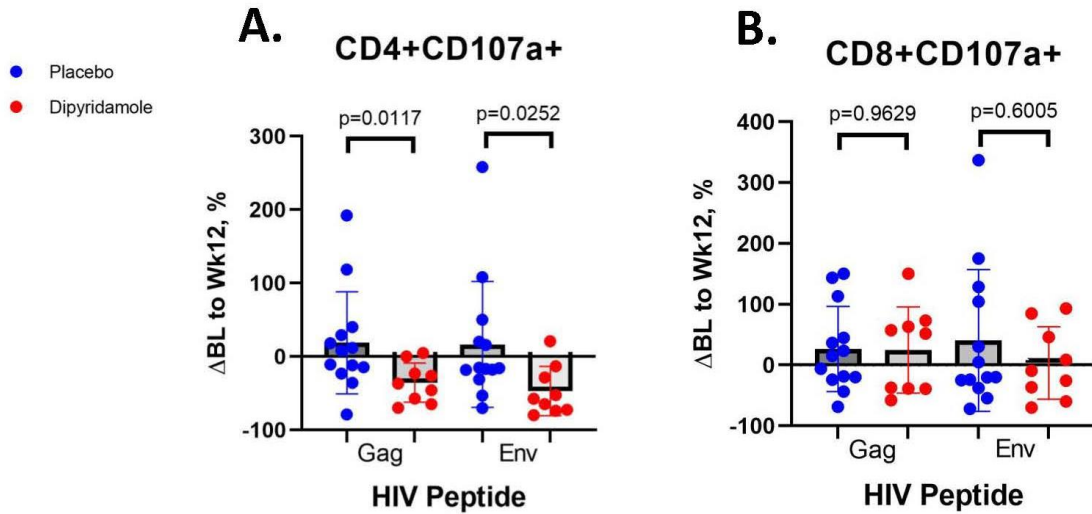


Figure 7. CD107a Expression in HIV-specific T Cells Following Dipyridamole Treatment

We observed a significant decrease in CD4+CD107a+ expression from the treated to placebo groups (median_{gag} -36.84% change vs 8.00% in placebo; median_{env} -57.50% vs -16.00% in placebo; Figure 7A). However, there was no significant change in CD107a expression among HIV-specific CD8+ T cells (median_{gag} 51.85% change vs 15.00% in placebo; median_{env} -9.520% vs -20.00% in placebo; Figure 7B).

Dipyridamole treatment did not influence expression of IL-2 or Granzyme B among HIV-specific T cells (Figure 8).

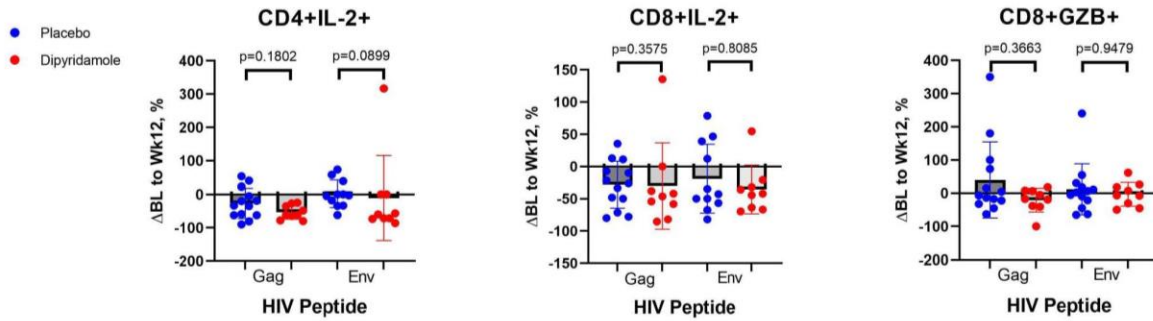


Figure 8. IL-2 and Granzyme B Expression in HIV-specific T Cells Following Dipyridamole Treatment

There was a trend towards decreasing IL-2 expression in HIV-specific CD4⁺ T cells when comparing treatment versus placebo groups (median_{gag} -61.29% change vs -33.33% in placebo; median_{env} -60.00% change vs -2.630% in placebo; Figure 8A). Alternatively, there was no significant change in IL-2 expression among HIV-specific CD8⁺ T cells following dipyridamole treatment (median_{gag} -46.43% change vs -26.09% in placebo; median_{env} -42.22% change vs -42.86% in placebo; Figure 8B). Also, we observed that dipyridamole treatment did not significantly change Granzyme B expression from HIV-specific CD8⁺ T cells (median_{gag} -17.39% change vs -6.780% in placebo; median_{env} -6.90% change vs 2.330% in placebo; Figure 8C).

HIV-specific polyfunctional T cell responses were influenced by dipyridamole treatment compared to placebo as well (Figure 9).

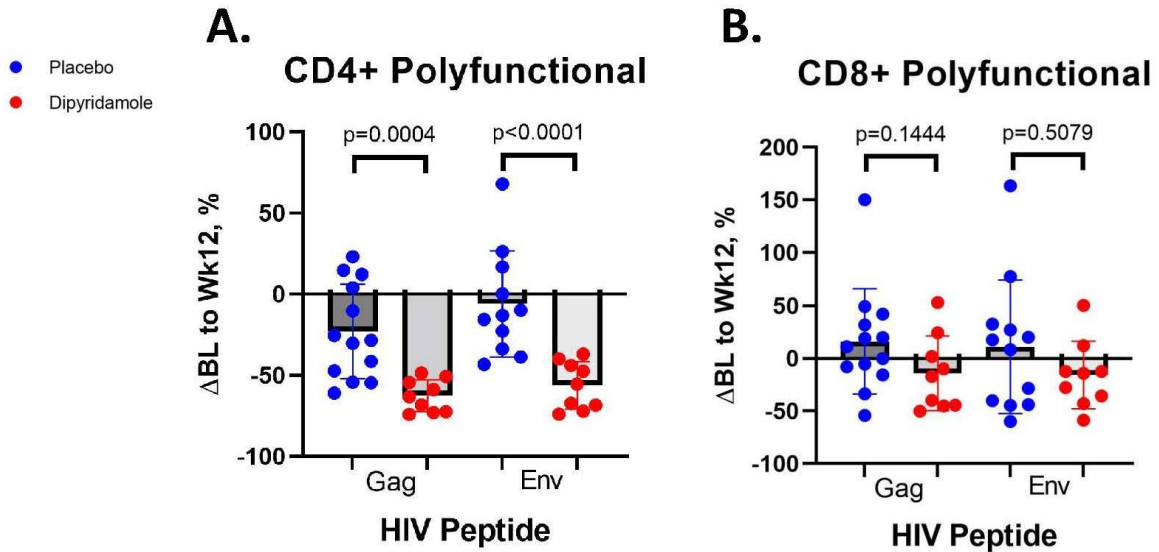


Figure 9. Polyfunctional HIV-specific T Cell Responses Following Dipyridamole Treatment

We observed a significant decrease in HIV-specific CD4+ T cell polyfunctional responses in the treatment group versus placebo (median_{gag} -63.19% change vs -28.41% in placebo; median_{env} -58.38% change vs -13.07% in placebo Figure 9A). Alternatively, HIV-specific CD8+ T cell polyfunctional responses, following dipyridamole treatment, did not significantly change (median_{gag} -17.06% change vs 11.15% in placebo; median_{env} -14.16% change vs 13.00% in placebo; Figure 9B).

4.2 Virologic, Immunologic, and Purinergic Parameters and HIV-specific Immune Responses Following Dipyridamole Treatment

Various biological endpoints were analyzed between dipyridamole and placebo groups in our clinical trial. Following the polyfunctional analysis, we wanted to assess the association

between HIV-specific immune responses and HIV persistence, immune activation, inflammation, adenosine pathway metabolites, and ectoenzyme expression.

4.2.1 HIV Persistence

Participants were virally suppressed on ART and had low viral loads(190). Residual viremia of cell-associated HIV RNA and DNA was measured in peripheral blood for all participants. The percent change of residual viremia, from baseline to week 12, was analyzed against the percent change of HIV-specific T cell responses upon gag and env stimulation. There was no significant association between HIV-specific polyfunctional CD4+ and CD8+ T cell responses and residual viremia.

4.2.2 Immune Activation and Inflammation

T cell activation, cell cycling, and inflammatory markers were measured in our primary analysis. We defined T cell activation as co-expression of CD38+ and HLADR+ and T cell cycling as intracellular Ki-67+ expression. The percent change, from baseline to week 12, was calculated for each activation and inflammatory marker and compared to the percent change in HIV-specific CD4+ and CD8+ T cell responses.

There was no correlation between T cell activation and HIV-specific immune responses. However, T cell cycling directly correlated with HIV-specific CD4+ and CD8+ T cell responses (Figure 10).

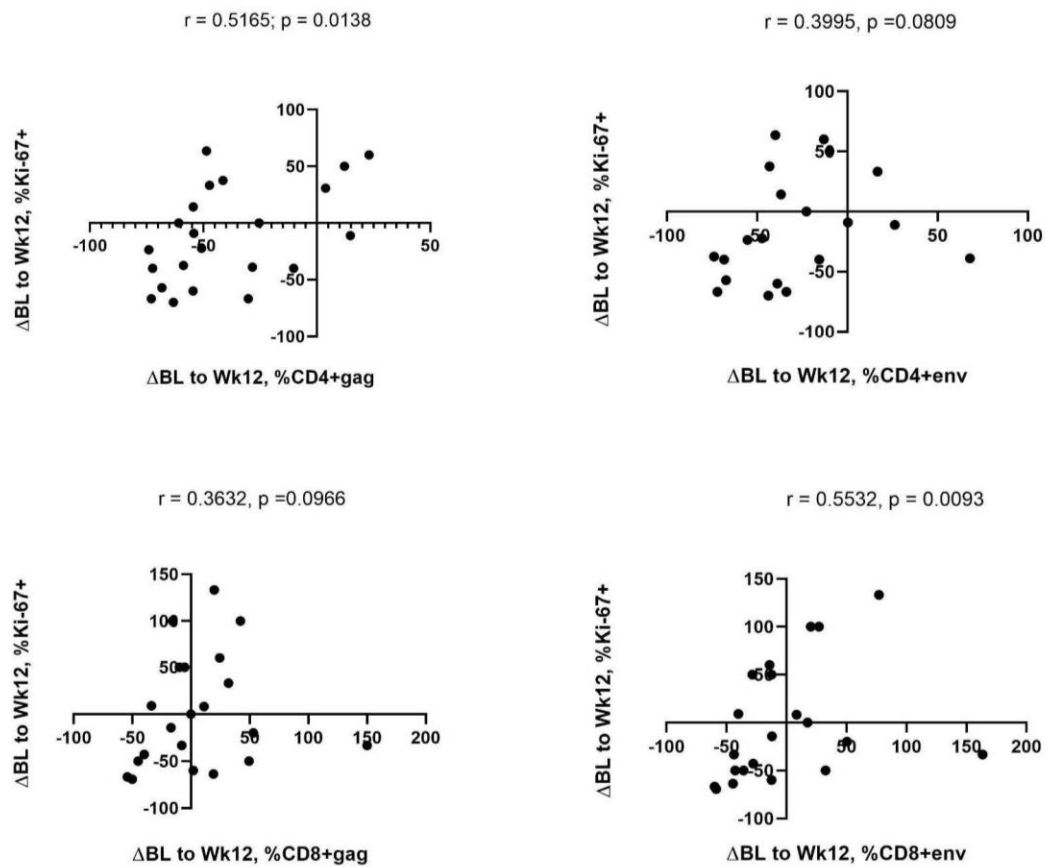


Figure 10. T Cell Cycling Directly Correlated with HIV-specific Immune Responses

Plasma levels of sCD14, sCD163, IL-6, CXCL10, and CRP were obtained from all participants at baseline and week 12. There was no correlation between inflammation and HIV-specific immune responses.

4.2.3 Adenosine Metabolites

To confirm the mechanism of dipyridamole action, purine levels were measured in plasma at baseline and week 12 for all participants. Inosine was used a surrogate for adenosine due to its

short half-life in the blood. Interestingly, we found the percent change of inosine levels, from baseline to week 12, inversely correlated with Gag-specific CD4+ T cell responses (Figure 11).

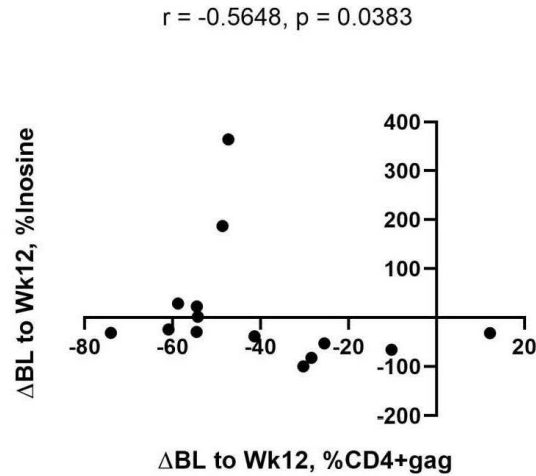


Figure 11. Inosine Inversely Correlated with Gag-specific CD4+ T Cell Responses

However, there was no correlation between other HIV-specific T cell responses and inosine. Additionally, we found no correlation between adenosine or 5'cAMP and HIV-specific immune responses.

4.2.4 Ectoenzyme Expression

Ectoenzyme expression of CD39+, CD73+, and CD39+CD73+ on CD4+ and CD8+ T cells was obtained in our primary analysis. Since dipyridamole does not influence ectoenzyme expression, we analyzed CD39+, CD73+ and co-expression levels at baseline and week 12 separately against HIV-specific immune responses. This enabled us to determine whether pre- and post-dipyridamole levels were associated with the change in polyfunctionality. We did not observe correlations between ectoenzyme expression and HIV-specific immune responses.

5.0 Discussion

We assessed whether HIV-specific T cell responses changed in accordance with the dipyridamole-induced decrease in T cell activation seen in our primary analysis. Single cytokine expression analysis showed a significant decrease of TNF α and CD107a expression of HIV-specific CD4⁺ T cells following dipyridamole treatment (Figures 5A and 7A). Also, IFN γ expression significantly decreased among HIV-specific CD4⁺ and CD8⁺ T cells (Figure 6). There was a trend towards decreasing IL-2 expression among HIV-specific CD4⁺ T cells, but not CD8⁺ T cells (Figure 8). We did not observe significant changes in CD107a, IL-2 or Granzyme B from HIV-specific CD8⁺ T cells (Figures 7B and 8B,C). This could be due to HIV-associated cytolytic dysfunction of CD8⁺ T cells seen in chronic infection (226). However, single cytokine expression analysis is not sufficient to evaluate the breadth of HIV-specific T cell responses following dipyridamole treatment. Here we observed a significant decrease in polyfunctional HIV-specific CD4⁺ T cell responses, and a decreasing trend for Gag-specific CD8⁺ T cell responses (Figure 9). Overall, dipyridamole decreased T cell activation and subsequently decreased T cell effector function in virally suppressed PLWH. Further research is necessary to determine the impact of modulating the adenosine pathway to improve HIV outcomes.

Finally, there was no significant association between HIV-specific T cell responses and residual viremia, inflammation and ectoenzyme expression. However, T cell cycling directly correlated with HIV-specific CD4⁺ and CD8⁺ T cell responses (Figure 10). Alternatively, we did not observe a correlation between T cell activation and HIV-specific immune responses. While adenosine and 5'-cAMP did not correlate with HIV-specific immune responses, we reported an inverse correlation between levels of inosine, a surrogate for adenosine levels, and Gag-specific

CD4+ T cells in the blood (Figure 11). Overall, the lack of association could be due to a decreased sample size and missing or censored data that influenced our statistical analyses. A higher sample size could produce a more accurate representation of HIV-specific immune responses and their interaction with virologic, immunologic, and purinergic parameters. Additionally, some of the changes in fluorescence with antibodies, used to detect cytokine production, were rather marginal or limited. Future studies may require more optimization of the assay.

Adenosine modulation remains a plausible route to manage HIV-associated inflammation. While we described various adenosine-targeted therapeutics in HIV infection (182), other disease treatment studies are demonstrating beneficial outcomes when targeting the adenosine pathway (227). For example, targeting adenosine receptors has shown to produce lung-protective properties in patients with influenza-induced acute lung injury (228). Additionally, various adenosine receptor therapeutics are used to treat inflammatory, respiratory, cardiovascular, and central nervous diseases (229). Another route to modulate adenosine production is mediated through targeting ectoenzyme expression. In mouse models, CD73 inhibition improved memory deficits in early Alzheimer's and motor behaviors in Parkinson's disease (230, 231). However, it's important to note that there is a potential for unwarranted side effects related to autoimmunity and exacerbation of disease due to the ubiquitous nature of adenosine and adenosine receptors (232).

Furthermore, adenosine modulation is shown to be advantageous in cancer immunotherapies. High levels of extracellular adenosine production exist in the tumor microenvironment and tumor cells express various adenosine receptors (233). Sek et al 2018 describe methods of targeting the adenosine pathway, through inhibition of CD73 and A_{2A}R, to enhance efficacy of checkpoint inhibitors, chemotherapy, and Chimeric Antigen Receptor (CAR) T cell therapy (234). However, elucidating CAR T cells ability to function in ART-suppressed

PLWH is an obstacle in clinical trials (235). Despite possible benefits of modulating the adenosine pathway in HIV-associated inflammation and non-AIDS associated diseases, our results show that this could also lead to decreases in HIV-specific immune responses which could affect efforts in achieving HIV remission off ART. Additional studies need to be conducted to further evaluate how to properly balance the beneficial and potential detrimental effects of targeting the adenosine pathway in HIV.

6.0 Public Health Statement

The persistence of HIV-associated immune dysfunction during the ART era has increased the prevalence of non-AIDS co-morbidities and the complexity of treating PLWH. Aside from a few rare instances, there is no cure for HIV infection. This challenge remains due to HIV's ability to escape immune evasions and the seemingly irreversible nature of viral latency. While dipyridamole decreased T cell activation and HIV-specific immune responses, modulating the adenosine pathway is an effective way to limit inflammation and potentially reduce the burden of non-AIDS comorbidities. Current strategies employed for other disease treatments and cancer immunotherapies demonstrate improved outcomes following adenosine pathway modulations. Thus, we can use these strategies to aid the development of treatment strategies in virally suppressed PLWH.

7.0 Future Directions

Future studies in HIV vaccine development should focus on enhancing the strength of polyfunctional responses as well as capturing a complete immunological signature of protection. Moreover, adenosine modulation remains an attractive approach to treating disease. Clinical trials for other diseases, such as influenza and cancer, have demonstrated substantial benefits by targeting the adenosine pathway through ectoenzyme expression and adenosine receptor binding capabilities. However, adapting these immunotherapies that target the adenosine pathway must be handled delicately to avoid further immune dysfunction caused by chronic HIV infection.

Appendix A Polyfunctional ICS Assay Schematic

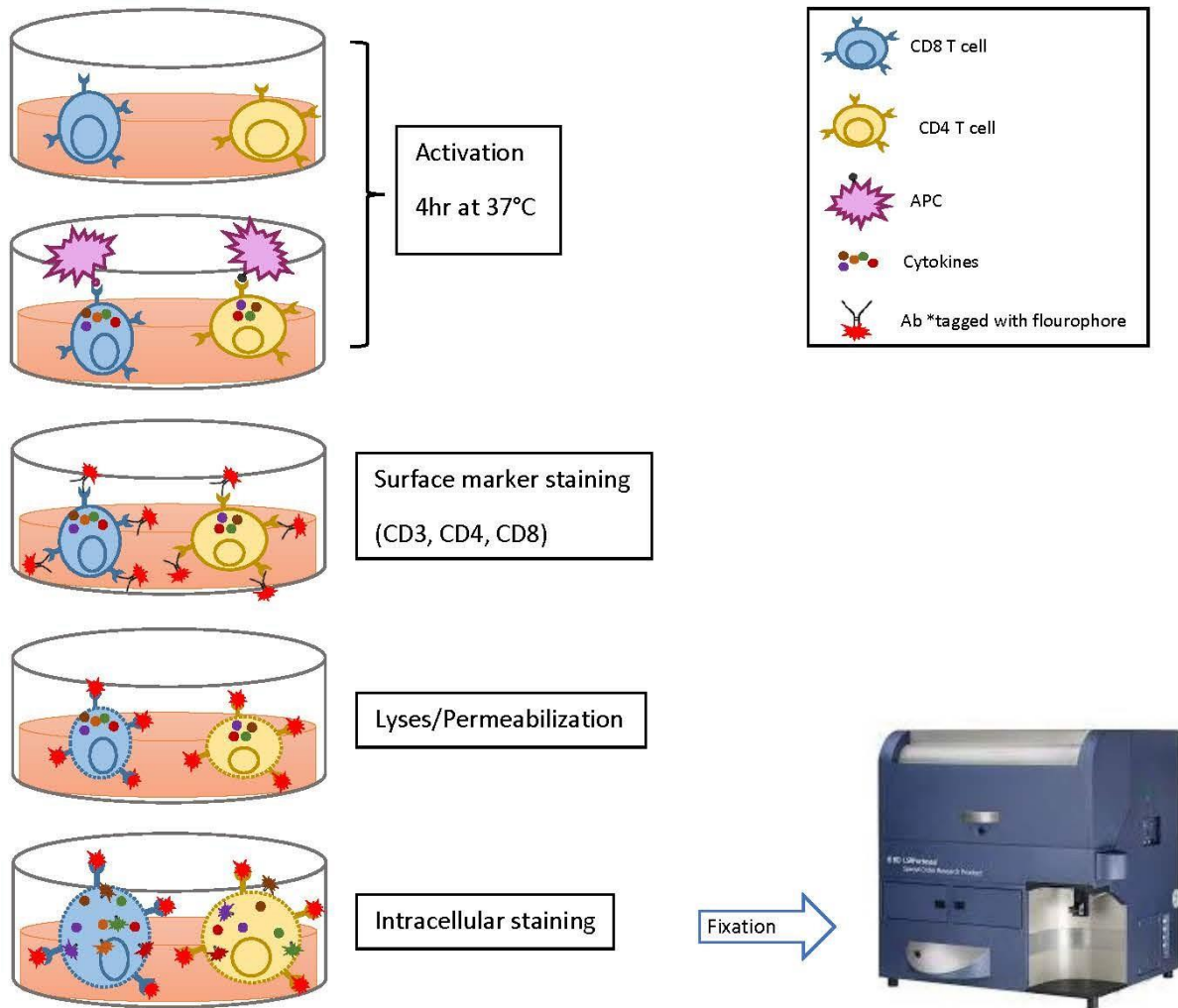


Figure 12. Workflow of ICS Assay Employed to Assess HIV-specific T Cell Responses

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