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Follicular Dendritic Cells, Resting CD4+ T Cells and Human Immunodeficiency Virus Expression

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Doctor of Philosophy

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ABSTRACT

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Many events associated with Human Immunodeficiency Virus (HIV) infection/replication occur in and around the germinal centers (GCs) of secondary lymphoid tissues where follicular dendritic cells (FDCs) reside, suggesting that this microenvironment may contribute unique signaling that is important to viral progression. My research focused on characterizing signaling, both positive and negative, contributed by FDCs that affects HIV infection and replication. Specifically, I determined if FDC signals could induce the expression of latent HIV in T cells and if so, to characterize the signaling pathways involved. Moreover, I also examined the ability of FDCs to produce inhibitory signals that might block active virus expression. I approached these problems using FDCs from tonsils and coculturing these with primary $CD4^+$ T cells or latently-infected Jurket cells with a GFP reporter. Results indicated that FDCs dramatically augmented HIV production of these cells. FDC signaling was costimulatory in nature and was mediated by soluble TNFα. However, when *ex vivo* latently infected T cells were treated with PMA/ionomycin or IL2/IL7, little virus expression was observed until FDCs were added, which greatly increased virus production. The transcription factor NFAT is important for the reactivation of latent HIV. Inhibition studies as well as ELISA suggested that JAK/STAT signaling pathway was involved in virus reactivation.

Because FDCs produce prostaglandins (PGs) E_2 and I_2, I determined the effect of PGE_2 and PGI_2 analogs on HIV infected T cells. Results indicated that both the PGE_2 and PGI_2 analogs inhibited proliferation and activation-induced cell death of HIV infected T cells in a dose- and time-dependent manner. Additionally, it was shown that indomethacin and CAY10404, cyclooxygenase and cyclooxygenase-2 inhibitors, partially restored HIV production in the presence of FDCs, suggesting that FDC-produced PGE_2 and PGI_2 may inhibit virus replication. Thus, FDCs produce PGs that can block virus gene expression in T cells, which may be ideal for viral persistence. Therefore, FDC signaling appears to both promote and inhibit HIV production. A better understanding of FDC signaling and regulation in GCs may suggest new treatment strategies that would be beneficial to infected subjects.

Key words: Follicular dendritic cell, HIV, resting $CD4^+$ T cell, prostaglandin, costimulatory

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Chapter 1 Follicular Dendritic Cells Mediated Reactivation of Latent HIV in $CD4^+$ T Cells

1.1 Abstract

A major problem in the eradication of HIV/AIDS is the establishment of reservoirs where the virus escapes drug and immune system attack. Latently infected, resting $CD4^+$ T cells are a reservoir that when re-exposed to activating stimuli, such as antigens, begins to produce HIV. In this study, we determined whether FDC signals could induce the expression of latent HIV in T cells and if so, characterize the signaling pathways involved. We approached these problems using primary FDCs obtained from the tonsils of HIV-uninfected subjects and coculturing these with primary $CD4^+$ T cells or $CD4^+$ HIV-susceptible T cell lines, including those with fluorescent reporters of virus expression. Virus gene expression was determined using p24 production and the expression of green fluorescence protein (GFP) as assessed by flow cytometry. Cellular activation was determined using flow cytometry and tritiated thymidine incorporation (^{3}H-TdR). Results indicated that FDCs increased the activation state of $CD4^+$ T lymphocytes provided they were initially stimulated with a suboptimal concentration of PHA or other primary signal. Moreover, the incorporation of ^{3}H-TdR also increased in the presence of FDCs or their supernatant after a primary activation signal was provided. Collectively these data indicated that FDC signaling was costimulatory in nature and suggested that it was due to a soluble mediator(s). Virus production in resting $CD4^+$ T cells was significantly increased in the presence of FDCs and PHA. FDCs also increased virus production in infected cell lines as assessed by increased production of the reporter, GFP. The addition of soluble Tumor Necrosis Factor Receptor (TNFR) inhibited over 50% of the FDC-mediated increase in virus production in mitogen stimulated T cells indicating that the soluble mediator in this setting included TNFα. However, when *ex vivo* HIV infected resting T cells were treated with phorbol myristate acetate

(PMA) in the presence of the calcium ionophore, ionomycin, little virus expression was observed until FDCs were added, which greatly increased virus production. Inhibition studies indicated that FDC costimulatory signaling in this latter system utilized the Nuclear Factor of Activated T cells (NFAT) for virus reactivation. Similarly, *ex vivo* HIV infected resting T cells plus IL-2/IL-7 induced 2.9% virus expression but that this was increased to 48.4% when FDCs were added. Inhibition studies as well as ELISA suggested that JAK/STAT signaling pathway was involved in the viral reactivation.

1.2 Introduction

1.2.1 Latently infected resting CD4$^+$ T cell reservoir

HIV reservoirs are a significant problem in curing HIV/AIDS. Highly active antiretroviral therapy (HAART), which is the combination of three or more antiretroviral drugs, reduces the plasma level of HIV below the detection limit [1]. HAART can prevent new rounds of infection and the progression of HIV disease is partially reversed. However, even though it was initially hoped that HAART could cure AIDS, it is now clear that some HIV escapes treatment and persists in stable reservoirs [2-4]. Viral reservoirs are defined as cells or tissue sites in which replication-competent virus both accumulates and persists with greater kinetic stability than the main pool of replicating virus [5, 6]. At least three principal reservoirs are known to exist in humans: 1) latently-infected, resting CD4$^+$ T cells [7-10], 2) macrophages [11, 12] and 3) follicular dendritic cells (FDCs) [13-15].

The best-characterized reservoir is the latently infected, resting memory CD4$^+$ T cell, which represents about 0.01% of all resting CD4$^+$ lymphocytes [16, 17]. This population of lymphocytes is thought to arise when virus-infected CD4$^+$ T cells revert from an activated to a resting state [18]. HIV persists as stably integrated but transcriptionally silent provirus [19, 20]. In the resting or quiescent condition, these cells are estimated to survive for many years, unaffected by immune responses or antiretroviral drugs. Importantly, however, when these cells become activated, they begin to produce HIV, thereby contributing to the amount of virus produced, the reignition of virus infection and consequently the rate of disease progression [21, 22]. It is unknown how latently infected resting T cells are physiologically reactivated in infected subjects and whether this reactivation increases the progression to AIDS.

1.2.2 The germinal center

The germinal center (GC) creates a unique microenvironment where activated GC B and T cells surround a network of FDCs [23]. GC lymphocytes migrate in response to chemokine signals both into and out of this specialized tissue site [24]. Chemokines bind to receptors that belong to a large family of seven-transmembrane, G protein-coupled receptors on the surface of leukocytes. Chemokine receptors are coupled to heterotrimeric Gαβγ proteins. Agonist binding to the receptor catalyzes the exchange of GTP for GDP on the Gα subunit, which results in the dissociation of the Gα and Gβγ subunits [25]. The Gα and Gβγ subunits independently activate multiple downstream effectors, one or more of which lead to cell migration [26]. However, chemokine binding to cognate receptors does not always result in cell migration. The regulator of G protein signaling (RGS) family of proteins associates with specific Gα subunits, greatly increasing their native GTPase activity, resulting in the inhibition of the signaling potential of G protein-coupled receptors [27]. Estes, et. al., previously showed that FDCs significantly increase the expression of CXCR4 on $CD4^+$ T cells, making them susceptible to infection by X4-tropic HIV [29]. However, the GC $CD4^+$ T cell migration to stromal cell-derived factor-1 (CXCL12, the CXCR4 ligand) is impaired because of the FDC-mediated high expression of RGS13 and RGS16 [28]. Thus, even though the FDCs are able to increase expression of CXCR4 in surrounding $CD4^+$ T cells, the cells cannot respond to migration signals through this receptor. FDCs also produce CXCL13 that serves as a chemoattractant to both T and B cells. Therefore, in the HIV setting, GC T cells are chemotactically attracted to FDCs bearing highly infectious HIV. Meanwhile, due to the increased expression of co-receptor CXCR4, GC T cells become more susceptible to infection by X4 tropic virus, while at the same time losing their ability to migrate to CXCR4 signals that might allow them to exit this dangerous microenvironment. Collectively

these events suggest that GC T cells may have a greater likelihood than other T cells to become infected by the FDC reservoir of HIV.

1.2.3 FDCs in HIV/AIDS

FDCs are located in the lymphoid follicles (activated or secondary follicles are also called GCs) of all secondary lymphoid tissues where they trap and retain immune complexes, including HIV complexes, for years [30, 31]. FDC-trapped antigens, including HIV, are comprised of antigen and specific antibody and /or complement proteins [32]. It is estimated that HIV is trapped on FDCs at the enormously high concentration of 1.5×10^{8} copies of viral RNA per gram of lymphoid tissue, making the FDCs one of the largest of the human HIV reservoirs [33]. FDCs trap and retain antigens (and viruses) in the form of immune complexes (ICs) on the external surface of their dendritic processes and these retained antigens are released slowly over time. When retroviral-ICs are trapped during disease, the antigens previously trapped on FDCs are lost and cannot be trapped again, which greatly impairs the specific antibody responses to these antigens [34, 35].

FDCs provide both primary and secondary signals to lymphocytes. Primary signals include presentation of native antigen in IC form to B cells and antigen-independent signals that induce B cells to respond to specific chemotactic signals [34-36]. FDCs protect lymphocytes from apoptosis [37-40]. FDCs can also provide secondary signals, which increase the proliferation and activation state of both B and T lymphocytes [41-43]. However, the signals involved are not fully understood. The microenvironment surrounding FDCs is one of intimate cell interactions and a high state of activation, making it highly conducive to HIV transmission and replication.

1.2.4 Current strategies to reduce latent HIV

Early treatment is a promising approach to reduce the number of latently infected T cells. Some groups report that the number of infected cells decreases to significantly lower levels if treatment is initiated during acute rather than chronic infection [44]. However, reports also indicate that viral rebound occurs in almost all patients following cessation of treatment, even when treatment was initiated in the acute infection stage [45, 46]. It is still unclear why some but not all patients are able to control infection following treatment during acute infection.

Treatment intensification has also been studied by addition of agents, such as Enfuvirtide, additional protease inhibitors or Raltegravir, to an already suppressive regimen [47-49]. However, none of these studies show any decline in cell-associated HIV DNA or low level viremia.

One strategy for the elimination of the latently infected T cell reservoir is to convert these cells into activated cells. Activation of latent T cells would induce virus production and subsequent cell death but additional rounds of infection resulting from the activation of latent virus production would be blocked by HAART treatment. HIV genes are silent and turned off in a latently infected cell. Histone deacetylase inhibitors (HDACi) can modify gene expression by changing the acetylation state of genes. Many HDACi have been in advanced clinical development for cancer treatment. In a small pilot study, a relatively weak HDACi, valproic acid, showed promising effects [50-52]. However, retrospective studies failed to demonstrate any benefit from this treatment. Vorinostat, a potent HDACi, is licensed for treatment of T cell lymphoma and has shown significant activity in "turning on" HIV genes *in vitro* [53, 54].

In addition to treatment using HDACi, the cytokine IL-7 is currently undergoing clinical trials to reduce the size of the latent reservoir [55]. This cytokine can effectively activate latently

infected T cells *in vitro* and it has been shown to be well tolerated in patients with HIV infection [56, 57]. However, one concern with this cytokine is that it may induce the proliferation of latently infected T cells without activating them, thereby increasing the potential pool [58]. Other compounds, such as prostratin also show promising results in reversing latency *in vitro* but it has not yet been tested in human studies [59]. Because latently infected T cells are rare (1 per $1x10^6$ cells) and because none of the strategies mentioned specifically targets only HIV infected cells, the current strategies may have effects on uninfected cells resulting in toxicity. The risk benefit of these strategies will need to be well evaluated.

1.2.5 FDCs and latently infected $CD4^+$ T cells

We recently reported that FDCs increase the replication of HIV in $CD4^+$ T lymphocytes in a TNFα-dependent manner [15]. Moreover, previous research indicates that FDCs provide costimulatory signaling that increases the activation state of both T and B lymphocytes [60-62]. We reasoned that in addition to affecting the activation state of non-infected T lymphocytes, FDCs might also activate resting, provirus containing $CD4^+$ T cells thereby resulting in the production of HIV. In this study, we report that $CD4^+$ T cells with latent HIV can migrate to FDCs. Moreover, FDCs can activate resting, infected $CD4^+$ T cells to produce HIV but this is done in a costimulatory manner that requires a weak primary signal (e.g. mitogen) to be given first. Interestingly, the low dose primary signal would not otherwise lead to significant virus production. These data suggest that HIV reservoirs may interact *in vivo* and lead to the generation of additional virus that could contribute to disease progression.

1.3 Materials and Methods

1.3.1 Virus generation and viral infection

HIV-1_{IIIB} was propagated in H9 cells as previously described [15]. Virus was harvested at the time of peak p24 and/or reverse transcriptase production, pooled, filtered through a 0.20 µm membrane, and stored in aliquots in liquid nitrogen until used. Viral stock typically contained 1 µg/mL of p24, $1x10^6$ cpm reverse transcriptase activity and when $TCID_{50}$ analysis was performed, $1x10^4$-10^5 $TCID_{50}$/mL. Resting $CD4^+$ T cells were infected by resuspending them in 1mL of HIV-1_{IIIB} stock virus and incubating at 37°C for 2 hrs, after which the cells were washed to remove the infecting inoculum.

Defective HIV (DHIV) was produced in 293T cells as described [78]. Primary naive $CD4^+$ T cells were infected by spinoculation: 10^6 cells were infected with DHIV containing 500 ng/mL p24 for 2 hrs at 2900 rpm and 37°C in 1 mL.

1.3.2 Flow cytometric analysis

Cell surface antigens (Ags) were detected using the following mAbs: anti-human CD3-PC5 (UCHT1), anti-human CD4-PC5 (13B8.2), anti-human CD8-FITC and anti-CD69-PE (TP1.55.3; Immunotech, Marseille, ME); anti-human CD25-PE (Immunotech); anti-human CD45RO-PE (UCHL1; Immunotech); anti-human HLA-DR-FITC (Becton Dickinson). Mouse isotype-matched control IgG1 (679.1 Mc7) and IgG2a (U7.27; Immunotech) were also used. Cells were incubated for 30 min on ice first with Fc-blocking reagent (Miltenyi Biotec, Auburn, CA) and then with the specific primary monoclonal antibodies (mAbs) listed above. Cells were then washed in cold phosphate buffered saline (PBS), and $1x10^5$ $CD4^+$ T cells were analyzed using a FACSVantage SE equipped with the FACSDiVa option (BD Biosciences, San Jose, CA)

for immunofluorescence analysis. Positive and negative gating was established using mouse isotype-matched control Abs to define background fluorescence.

To assess active HIV gene expression, intracellular p24-gag was measured using flow cytometry [63]. Cells (2.5×10^5) were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences, San Diego, CA) for 30 min at 4°C. Cells were then washed with Perm/Wash Buffer (BD Biosciences, San Diego, CA) and stained with a 1:40 dilution of anti-p24 antibody (AG3.0, Southwest Foundation for Biomedical Research, San Antonio, TX) in 100 μL Perm/Wash Buffer for 30 min at 4°C. Cells were washed with Perm/Wash Buffer and incubated with Alexa Fluor 488 (1:100 dilution) goat anti-mouse IgG (H + L) in 100 μL Perm/Wash Buffer for 30 min at 4°C. Cells were then washed with Perm/Wash Buffer and analyzed by flow cytometry. In all experiments, positive HIV p24-gag labeling was determined by comparison to uninfected cells tested in parallel.

1.3.3 FDC and FDC supernatant collection

Human FDCs were isolated from tonsillar tissue as previously described [13]. Briefly, tonsils were cut with sterile scalpels into 1-2 mm cubes and incubated for 45 min at 37 ° C in a digestion cocktail of Blendzyme (Roche Applied Science; Indianapolis, IN), and DNase I (Sigma, St. Louis, MO). The cells were separated from the tissues by gently pipetting the tissue up and down in a 10 mL sterile pipette and collecting the released cells. These steps were repeated two times. The resulting single cells were applied to a discontinuous (15%, 35% and 50%) pre-formed Percoll density gradient (GE Healthcare). FDCs were present in the 1.050-1.060 g/mL density band and were collected, washed and incubated with goat, ChromePure IgG (Jackson ImmunoResearch Laboratories) for 1 hr to block non-specific Fc receptor binding. The FDCs

were then labeled with 100μl mouse IgM, anti-human FDC mAb, HJ2, (250 μl hybridoma supernatant; kindly provided by Dr. M. Nahm, University of Alabama at Birmingham) on ice overnight. After removing the unbound mAb by washing, the cells were incubated for 1 hr on ice with a secondary goat $F(ab')_2$ anti-mouse IgM-FITC (γ chain specific, 25 μg). FDCs were sorted on $HJ2^{Hi}$ events (this population typically ranged from 1 to 3% of the total population post-Percoll). FACS sorted FDCs are typically ≥ 95% pure as assessed by flow cytometry.

To obtain FDC-supernatant, FACS-isolated FDCs were cultured for 6 days at a concentration of 1 x 10^6 cells per milliliter in complete tissue culture medium (CM) consisting of RPMI 1640 supplemented with HEPES buffer (20 mM); nonessential-amino-acid solution (1X); L-glutamine (2 mM); 10% heat-inactivated, defined fetal bovine serum (FBS) (all from HyClone Laboratories, Logan, UT); and gentamycin (50 μg/ml; Life Technologies, Gaithersburg, MD). At the end of the culture period, the supernatant fluid was collected, centrifuged (300 x g for 10 min) to remove any remaining cells, filtered through a 0.20 μm filter and then used immediately or stored at –80˚C. As a control, supernatant fluid was also obtained from equal numbers of FACS-sorted HJ2-negative cells from the same tissue. In all experiments, an FDC to $CD4^+$ T cell ratio of 1:10 was used for optimal FDC-lymphocyte interactions [28].

1.3.4 $CD4^+$ T cell isolation

Primary $CD4^+$ T cells were obtained from human tonsils or peripheral blood acquired from non-HIV infected subjects. When cells were obtained from tonsils, the tissue was cut into small cubes and the cells were mechanically released by gentle repeat pipetting using a 25 mL pipette. $CD4^+$ T lymphocytes were then isolated by MACS using a $CD4^+$ T cell negative isolation kit (Miltenyi Biotec) as directed by the manufacturer. Alternatively, when specific sub-

populations of $CD4^+$ T cells were needed, the MACS-enriched cells were labeled using T cell subset-specific antibodies followed by FACS. Isolation of peripheral blood mononuclear cells (PBMC) used similar enrichment/purification techniques after first applying whole blood to Ficoll-Paque Plus (GE Healthcare) and centrifuging (300 × g) at room temperature to obtain the mononuclear cells. The resulting resting $CD4^+$ T cell preparations were≥98% pure as assessed by flow cytometry.

1.3.5 Latently infected Jurkat cells with GFP reporter

Enhanced Green Fluorescence Protein (EGFP) reporter containing latent T cell clones were a kind gift from Dr. Olaf Kutsch (University of Alabama at Birmingham). The recombinant HIV-1, based on the T-cell-tropic NL4-3 strain, was engineered to express EGFP while preserving all viral nucleotide sequences and potential *cis* elements of the native virus. In this reporter system, EGFP fluorescence is tightly linked to HIV-1 protein production and can be used as a quantitative marker for HIV-1 expression on a single-cell basis by flow cytometry [79].

1.3.6 Ex vivo HIV infected resting $CD4^+$ T cells

We used the method of Bosque and Planelles to create an *ex vivo* model of latent HIV expression [63]**.** Peripheral blood mononuclear cells (PBMCs) were obtained from non-HIV infected human donors. Naive $CD4^+$ T cells were isolated using a naive T-cell isolation kit (Miltenyi Biotec, Auburn, CA) as directed by the manufacturer. The purity of the sorted population was always greater than 95% and expressed the phenotype $CD4^+CD45RA^+CD45RO^-$ $CCR7^+CD62L^+CD27^+$. These negatively selected T cells were activated using anti-CD3/CD28 coated beads (Dynal/Invitrogen, Carlsbad, CA), in medium containing TGF-β1 (10 ng/mL), IL-

12 (2 μg/mL) and IL-4 (1 μg/mL) for three days. The proliferating cells were expanded in CM medium containing IL-2 (30 IU/mL) and this media was replaced each 2 days. At day 7, the cells were infected with replication-defective DHIV/X4 [63]. DHIV/X4 is an envelope defective molecular clone derived from HIV-1_{NL4-3}, which is pseudotyped with the HIV-1_{LA1} envelope glycoprotein. Pseudotyping, in the presence of the defective envelope gene allows virus to be produced that can infect cells one time only. Once infected, the cells were continually cultured in the presence of IL-2 (30 IU/mL) and changing of the media and IL-2 every 2 days. At day 10 after infection, the cells were quiescent as estimated by the lack of intracellular $p24^{gag}$ expression and the vast majority of proviruses enter a latent state. Controls consisted of cells that were not infected by HIV.

1.3.7 Proliferation assay

Irradiated FDCs (1000 rad, $1x10^4$ cells) were cultured with resting $CD4^+$ T cells ($1x10^5$) in the presence or absence of a sub-optimal concentration of PHA (5 ng/mL) for 48 hrs at 37°C. Replicate conditions were performed in triplicate wells of a 96-well plate in a final volume of 200 μL. One μCi of ^{3}H-thymidine (Perkin Elmer, 9.25mBq) in 50 μL CM was added during the last 12 hrs of culture after which the contents of each well were harvested onto glass fiber filters using a PHD Cell Harvester (Cambridge Technology). The incorporated tritiated thymidine was detected using a Tri-Carb 2910TR liquid scintillation counter (Perkin Elmer) and the radioactivity was expressed as the mean counts per minute per sample ± the S.E.M. as indicated.

1.3.8 p24 Ag capture ELISA

Cell culture supernatants were assayed for viral p24 using an HIV p24 antigen ELISA (ZeptoMetrix Corporation, NY), according to the manufacturer's instructions. Briefly, viral antigen in the specimen was specifically captured onto immobilized antibody during specimen incubation. The captured p24 was then incubated with a high-titered human anti-HIV antibody conjugated with biotin. Following a subsequent incubation with Streptavidin-Peroxidase, color developed as the bound enzyme reacted with the substrate. Each reaction was terminated by the addition of Stop Solution (2 N H_2SO_4). Absorption was measured at 450 nm using a Vmax Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA). The absorbance of the samples was compared to a standard curve to determine the picogram of p24 per milliliter present.

1.3.9 T Cell migration assay

The ability of T cells to migrate to a chemoattractant was evaluated using a 24-well, 5 μm pore size Transwell system (Costar, Cambridge, MA). Purified resting T cells were washed once in chemotaxis medium (RPMI 1640 containing HEPES buffer (20 mM) and gentamycin (50 μg/ml; Invitrogen Life Technologies, Gaithersburg, MD)) and then adjusted to a concentration of 5×10^6 cells/ml in the same medium. An aliquot (100 μl) of the above cell suspension containing 5×10^5 cells was placed in the top well of the Transwell apparatus. FDCs, adjusted to 5×10^4 cells in chemotaxis medium (600-μl total volume), were added to the bottom of the Transwell system. After 5 hr incubation at 37°C in a 5% CO_2 atmosphere, the cells from the top and bottom of the Transwell cultures were collected and counted on a hemocytometer using light microscopy. Results were expressed as percent specific migration, which was calculated as follows: Specific migration (%) = [cells in the bottom well ÷ (cells in the bottom well + cells in the top well)]

×100. In some experiments, an aliquot (100 µl) of purified resting $CD4^+$ T cells was placed in the top well of the Transwell while specific chemokines, prepared at the indicated concentrations (600-µl total volume) were added to the bottom well of the Transwell system and cultured and assessed as described immediately above.

1.3.10 Assessment of Signal Transduction and Activator of Transcription (STAT) activity

The activity of STAT 1α, 3, 5A and 5B were quantified by ELISA using the TransAM STAT Transcription Factor Assay kit (Active Motif North America, Carlsbad, CA), according to the manufacturer's instructions. Briefly, cellular nuclear extracts were prepared using a Nuclear Extract kit (Active Motif North America, Carlsbad, CA) and incubated in 96-well plates coated with immobilized oligonucleotides containing the STAT consensus binding site (5′-TTCCCGGAA-3′). STAT1α, 3, 5A and 5B binding to the target oligonucleotide was detected by incubation of each well with primary antibody specific for the activated form of STAT. Visualization of STATs was detected using an horseradish peroxidase conjugated anti-IgG and Developing Solution followed by quantitation in a microplate reader at 450 nm with a reference wavelength of 655 nm. Specific binding was determined by subtracting the background binding from the binding to the consensus DNA sequence.

1.3.11 Statistical Analysis

Analysis of data was performed using a two-tailed, Student's t-Test. Values of $p \leq 0.05$ were considered significant. Unless specifically stated, the error bars in each figure indicate the standard error of the mean (SEM).

1.4 Results

1.4.1 FDCs provide co-stimulatory activation signals to resting CD4$^+$ T cells

To determine whether FDCs could provide primary or secondary signaling that could activate quiescent T lymphocytes, resting CD4$^+$ T cells (Figure 1) were harvested from the tonsils of HIV uninfected subjects and cocultured with FDCs (Figure 2, 1 FDC per 10 T cells) in the presence or absence of sub-optimal concentrations of phytohemagglutinin (PHA) to provide a primary activation signal (Figure 3). In the absence of PHA, FDCs did not increase ^{3}H-thymidine incorporation; however, when PHA was present, FDCs increased the amount of ^{3}H-thymidine incorporation five-fold above that achieved without FDCs. To determine whether FDCs required a primary signal to induce virus production in HIV-infected resting CD4$^+$ T cells, we cultured *in vitro* infected CD4$^+$ T lymphocytes as above and monitored the production of virus (Figure 4). As we observed with ^{3}H-TdR incorporation, FDCs did not increase virus production by themselves but in the presence of a primary signal, increased it by 5-fold more above that seen in T cells and mitogen alone. To determine any changes in the phenotype of resting CD4$^+$ T cells cocultured with FDCs in the presence or absence of PHA, the cells were examined after three days of culture for the expression of the activation markers, CD25, CD69 and HLA-DR using flow cytometry (Figure 5). While T cells cultured with PHA clearly showed a higher mean fluorescence intensity, the addition of FDCs and mitogen dramatically increased the expression of these activation markers. To better appreciate the effects of FDCs on the expression of individual activation markers, cells were cultured as before but stained separately for CD25, CD69, HLA-DR and CD45RO after one day of incubation (Figure 6). Although some increase in CD69, HLA-DR and to a lesser degree CD25 expression occurred when PHA was present in the cultures, the addition of FDCs significantly increased the expression of CD25, CD69, and HLA-

DR even after only 24 hrs confirming the activation of the resting $CD4^+$ T cells in the presence of a primary signal. Collectively these data indicate that FDC signaling of resting $CD4^+$ T cells requires a primary signal and is thus co-stimulatory in nature.

1.4.2 Migration of resting T cells to FDCs

We reasoned that if FDCs could activate resting T cells to produce virus in an *in vivo* situation, then it would be necessary for these T cells to gain access to the lymphoid follicles where FDCs reside. To determine whether resting $CD4^+$ T cells could migrate to FDCs, we used a Transwell migration assay (Figure 7). Over 35% of the resting $CD4^+$ T cells migrated to FDCs compared to lymphocyte migration to cell-culture medium alone. Moreover, $CD4^+$ T cell migration to cells obtained from the same lymphoid tissue as the FDCs but specifically devoid of FDCs was not significantly different from that observed using control medium alone ($p > 0.05$). Because FDCs produce CXCL13 [29], we also determined whether resting $CD4^+$ T cells could migrate to FDC-produced CXCL13 (Figure 8). Resting $CD4^+$ T cells were pretreated with anti-CXCR5 (10 μg/mL) for 30 min to block these receptors before performing the migration studies with FDCs. Resting $CD4^+$ T migration to FDCs was unaffected when irrelevant IgG was present in the cultures but reduced to background migration in the presence of anti-CXCR5.

1.4.3 FDC-mediated signaling augments HIV production through a TNFα dependent process

FDCs increase HIV production in activated $CD4^+$ T cells in a TNFα dependent manner [60]. To determine if FDC-mediated augmentation of HIV production in resting T cells was also dependent on TNFα secretion, *in vitro* infected resting $CD4^+$ T cells were cultured with or without FDCs and PHA. In some cultures, human TNFR-immunoglobulin (hTNFR-Ig), a potent

TNFα blocking reagent, was also added (Figure 9). In the presence of PHA, FDCs significantly increased HIV production by the T cells ($p < 0.05$). The addition of hTNFR-Ig to cultures reduced much of the FDC-mediated virus production but not all of it suggesting that while TNFα played an important role, other factor(s) also contributed.

1.4.4 FDCs activate latent HIV gene expression and virus production

Because of the rare nature of latently infected resting memory $CD4^+$ T cells *in vivo*, we began our efforts to determine if FDCs could reactivate latent HIV expression using an HIV-based GFP reporter system developed by Dr. Olaf Kutsch (Figure 10) [65]. The addition of FDCs alone to the T cell clones did not result in GFP expression; however, when a primary signal was present, (anti-CD3/CD28), FDCs almost doubled GFP expression (38.5%). As observed before, the depletion of FDCs from other lymphoid tissue derived cells failed to significantly increase GFP expression confirming that the inducing signal was produced by FDCs. Interestingly, FDC supernatant was able to partially substitute for FDCs in inducing increased GFP expression.

To determine whether FDCs could activate the production of latent HIV from primary T cells, we used the approach of Bosque and Planelles where primary $CD4^+$ T cells were infected *ex vivo* with defective virus and cultured until the cells no longer expressed HIV p24 [63]. Because FDC signaling was co-stimulatory in nature, we selected three different primary activation signals for use, anti-CD3/CD28 bound to beads, phorbol-12-myristate-13-acetate (PMA)/ionomycin, and IL-2/IL-7 (Figure 12-15). When primary signaling was induced using bead-bound anti-CD3/CD28, a potent stimulator of T cells that is a surrogate of antigen, only a small degree of viral p24 expression was noted when FDCs were present in the cultures (Figure

12). Many times, little if any additional activation was noted when bead-bound CD3/CD28 was used as the primary activation signal (Figure 11). In contrast, when sub-optimal concentrations of PMA and ionomycin (Figure 13) or IL-2 and IL-7 (Figure 14) were used instead of anti-CD3/anti-CD28, significantly greater p24 production was noted in the presence of FDCs. A smaller amount of p24 expression was noted when FDC supernatant was used in place of FDCs. Different concentrations of PMA/ionomycin (10, 50, 100 ng/mL/1, 5, 10 μM) and IL-2/IL-7 (30, 90, 150 U/50, 100, 150 ng/mL) were added to the *ex vivo* HIV infected resting T cells with or without FDCs, and the reactivation were analyzed by flow cytometry (Figure 15-20). The use of PMA/ionomycin (10 ng/mL/1 μM) or IL-2/IL-7 (30 U/50 ng/mL) as the primary signal provides a sensitive system in which FDC-mediated reactivation in the *ex vivo* HIV infected resting T cells can be assessed readily.

Because FDCs produce TNFα, we also added recombinant TNFα (10 μg/ml) to the *ex vivo* HIV infected resting T cells to determine whether it would cause any increased production of HIV p24 in the absence of FDCs (Figure 21, 22). When PMA/ionomycin were used as the primary signals, the addition of TNFα resulted in only a 2% increase of p24. When FDCs were added with or without TNFR-Ig no significant difference in p24 production was observed indicating that TNFα did not play a role in the FDC-mediated virus reactivation observed using PMA and ionomycin as primary signals. When the latently infected T cells were treated with IL-2 and IL-7 plus TNFα, there was no apparent induction of p24. However, the addition of FDCs to the IL-2 and IL-7 treated cells resulted in almost 50% of the cells expressing detectable p24 and this was not decreased by the addition of TNFR-Ig (Figure 22). Collectively these data indicate that when sub-optimal signaling of the *ex vivo* HIV infected resting T cells was induced,

the addition of FDCs substantially increased virus production and that this was independent of TNFα.

Bosque and Planelles [63] found that the transcription factor, Nuclear Factor of Activated T cells (NFAT) was responsible for the activation of viral p24 production when *ex vivo* HIV infected resting T cells were stimulated with PMA/ionomycin, however, the production of HIV p24 in their system without FDCs was significantly lower than in ours where FDCs were added. To determine the involvement of NFAT in FDC-mediated reactivation of latent HIV, we cultured $CD4^+$ T cells with latent HIV in the presence of PMA/ionomycin with and without FDCs and Cyclosporin A (CsA), a potent Calcineurin inhibitor (Figure 23). As before, the addition of FDCs to the PMA/ionomycin stimulated cells increased p24 production in the cells by 60-fold but this was reduced to background levels when CsA was added. These data indicated that NFAT alone was responsible for the FDC-induced reactivation of latent HIV when primary signaling occurred by PMA/ionomycin.

IL-2 and Il-7 signal through type 1 cytokine receptors that utilize the common gamma chain (γc). Because this family of receptors rely on Janus kinases (Jaks) and signal transducers and activators of transcription (STATs) for signal transduction, we next sought to determine the involvement of the Jak-Stat pathway in *ex vivo* HIV infected resting T lymphocytes treated with IL-2/IL-7 and cultured with FDCs. Jak3 is known to be involved in T cell signaling through the γc and in transduction of signal to phosphatidylinositol 3-kinase (PI3K) that can activate Akt and in turn, NF-κB. To determine whether NF-κB activation was involved in FDC-mediated induction of latent virus expression in IL-2/IL-7 signaled $CD4^+$ resting T cells, we used Leflunomide and Wortmannin, specific inhibitors of Jak3 and PI3K, respectively (Figure 24). As expected, Leflunomide markedly inhibited FDC-mediated latent virus p24 production. To

determine if a higher dose of Leflunomide would completely block FDC-mediated p24 production in the latent T cells, we added a 10-fold increase of this inhibitor and found no significant decrease beyond that seen in figure 24 (data not shown). To determine if PI3K signaling were involved and therefore potentially activating NF-κB, we treated the cells with Wortmannin to block this pathway (Figure 24). No significant change in the ability of FDCs to induce p24 production was observed.

Lastly we examined the level of STAT 1, 3, 5A and 5B activation during FDC-mediated virus p24 production when IL-2/IL-7 were used as the primary activation signal (Figure 25). The addition of IL-2/IL-7 to the latently infected primary T cells increased the activation levels of all the STATs examined excepting STAT 1. It was interesting to note that while STAT 1 and STAT 3 levels were very similar in the T cells cultured by themselves, the same cells demonstrated significant increases in the amount of STAT 5A and 5B activation. The addition of FDC supernatant as a source of FDC "factors" clearly increased the levels of STAT activation and this was most readably observed with STAT 5A and 5B. We also noted the increased amount of absolute activation above baseline with both STAT 5A and 5B in the presence of FDC supernatant. The addition of Leflunomide decreased STAT activation to or below the levels observed with the *ex vivo* HIV infected resting T cells cultured with IL-2/IL-7. Collectively our data indicated that FDCs increased the expression of HIV p24 in *ex vivo* HIV infected resting T cells using a costimulatory mechanism. Furthermore, the FDC-mediated effect was seen regardless of the primary signaling using IL-2/IL-7, PMA/ionomycin, or the T cell receptor and coreceptor surrogates anti-CD3/CD28.

1.4.5 AAT treated FDCs cannot induce latency reactivation in ex vivo HIV infected resting T cell model

Alpha-1-antitrypsin (AAT) is a naturally occurring serine protease inhibitor with a concentration of 1.5 to 3.5 mg/ml in humans [66, 67]. We have shown that AAT inhibited the activation of NFkB in $CD4^+$ T cells [77]. It was also found that AAT inhibited CD32, CD21 and TNFα expression, which are directly linked to FDC activation (Zhou and Burton, unpublished). we next focused on determining whether AAT-treated FDCs would be inhibited in their ability to reactivate latent HIV expression in $CD4^+$ T cells. The *ex vivo* HIV infected resting T cells were cultured with PMA/ionomycin. FDCs were isolated and incubated for 24 or 48 hr with PBS or AAT (5 mg/ml) to assess the ability of the FDCs to activate latent virus expression (Figure 26). Activated FDCs incubated in PBS activated 27.3% of the cells to express viral p24 compared with 4.5% in the absence of FDCs. FDCs treated with AAT prior to coculture induced 3.7% and 0.9% virus expression when treated for 24 or 48 hr respectively. As seen in Figure 22, a contribution of TNFα has been ruled out in the FDC-mediated latency reactivation, because the inclusion of soluble TNFR failed to noticeably affect the system.

1.5 Discussion

It was previously shown that FDCs induce a heightened state of lymphocyte activation that affects both activated B and T cells [60, 61]. FDC-signaling molecules affecting B cells include CD40, BAFF, CXCL13, CD21-ligand and IL-6. Less is known about FDC signals that affect T cells; however, we recently found that FDCs produce TNFα that, in turn, activates T cell NFκB [60]. Additionally, it is known that FDC signaling crosses species lines, is not MHC restricted and is costimulatory in nature; as evidenced by the observation that a primary signal (e.g. mitogen, antigen) must be present to observe the FDC secondary signal.

Latently infected T cells are thought to circulate throughout the body and are found in secondary lymphoid tissues with the same frequency that they are present in the blood [17]. In order to express HIV genes and produce virus, infected resting $CD4^+$ T cells must first become activated. Because FDCs do not process or present antigens to T cells, the initial activation of the T cell (i.e. antigen presentation) could occur as an antigen-specific T cell enters the germinal center (GC) and interacts with antigen-specific germinal center B cells, which are known to be excellent antigen presenting cells (APCs). Alternatively, the initial activation of T cells could also occur in the T cell dependent areas of the secondary lymphoid tissues by conventional dendritic cells (DC). After DC-mediated antigen activation, these T cells would then express CXCR5 and migrate into the GC where they would come in contact with FDCs and their signals.

We previously reported that FDCs can increase virus production in activated $CD4^+$ T cells [15]. Here we examine the ability of FDCs to activate resting T cells and we report that FDCs can provide signals that result in activation of latent HIV but that this ability is costimulatory in nature therefore requiring a primary signal existing in the microenvironment. While FDCs are able to induce virus reactivation in a costimulatory manner in *ex vivo* HIV

infected resting T cells, this reactivation includes additional signals based upon the primary signal utilized (i.e. whether PHA, anti-CD3/CD28, PMA/ionomycin, IL-2/IL-7 are used to make the cells susceptible to FDC mediated costimulation). Because PHA and CD3/CD28 can induce significant reactivation of latently infected $CD4^+$ T cells themselves, this may obscure some of the FDC-mediated contributions. Thus, to better understand the extent and nature of FDC signaling involved, PMA/ionomycin or IL-2/IL-7 were used to induce a much smaller degree of primary signaling than would be provided by either PHA or anti-CD3/CD28.

It is also important to note that FDCs produce the chemokine CXCL13 that is responsible for attracting B and T lymphocytes into the lymphoid follicles. We examined whether the resting $CD4^+$ T cells could migrate to FDCs and found that they could indicating that these cells have the potential to interact with FDCs in the *in vivo* setting suggesting that the FDC costimulation leading to latent virus expression is likely plausible in vivo. The observation that FDCs participate in chemoattraction is not novel as they were previously implicated in the transmission of a state of "migratory competence" to murine B lymphocytes, even though the exact nature of the signaling is not defined [80]. Moreover, it has been reported that FDCs upregulate the Regulators of G-protein Signaling (RGS) 13 and 16 in human T cells [29]. In these latter two instances, it is worth noting that the FDC signaling involved appears to be primary and not costimulatory in nature.

Because FDCs produce on average 178 picograms TNFα per milliliter in culture and secretion of TNFα results in the nuclear translocation of NFκB in infected $CD4^+$ T cells [60], we first examined the role of FDC-TNFα on mitogen-activated primary HIV infected $CD4^+$ T cells. Next we examined the ability of FDCs to activate latent virus and the signaling involved using latently infected Jurkat cells with GFP reporter and *ex vivo* HIV infected resting $CD4^+$ T cells. In

this study, TNFα induces both mitogen-activated primary HIV infected cells and latently infected cell lines to express virus. Importantly, it reveals a previously unknown FDC signal that can affect latency reactivation. Addition of a soluble TNFα receptor, hTNFR-Ig, to block soluble TNFα, decreased the signaling, but not to the level of resting T cells alone suggesting the potential for additional signals on top of TNFα. However, adding a similar quantity of recombinant TNFα to cultures of *ex vivo* HIV infected resting T cells failed to recapitulate the presence of FDCs or their supernatant. This is the first time that we found other signal(s) besides TNFα is involved in FDC increasing HIV production of $CD4^+$ T cells. The differences between the latent T cell line and *ex vivo* HIV infected resting T cells may be attributed to the use of a Jurkat cell line, because Jurkat and primary T cells share some but not all T cell signaling pathways [68].

Both NFκB and NFAT bind to the κB/NFAT binding sites that are present in the HIV LTR and that are a stringent requirement for viral reactivation [69]. It has been shown that memory cells contain high levels of NFATc1 and NFATc2, whereas naïve cells contain very low levels of these transcription factors. Even though both memory and naïve T cells rapidly induce IL-2 transcription upon T cell receptor ligation, NFAT is the responsible transcription factor for memory cells, whereas NFκB is the one for naïve cells [70]. The addition of the inhibitor cyclosporine A (CsA) completely blocked reactivation, confirming our prediction that in memory cells NFAT, but not NFκB, would be essential for viral reactivation.

The γc cytokines increase susceptibility of resting $CD4^+$ T cells [73, 74]. The cytokines that support viral infection, including IL-2, IL-4, IL-7 and IL-15 [74], bind to the γc cytokine receptors. Upon ligation of cytokines to γc cytokine receptors, JAK3 associates with the receptor chains, become phosphorylated, and recruit STAT5. Phosphorylated STAT5 then enters the

nucleus to regulate gene transcription [75]. STAT5 was shown to bind and activate the viral LTR in both stimulated and unstimulated primary $CD4^+$ T cells [76]. These observations are consistent with the increased STAT5 activity of our *ex vivo* HIV infected resting T cell cultured with IL-2/IL-7 and FDC supernatant.

In all, we demonstrate that FDCs are capable of activating of latent virus production in *ex vivo* HIV infected resting $CD4^+$ T cells. Furthermore, the transcription factor NFAT is important for this reactivation of latent virus. Cytokines produced by FDCs might also be involved in the viral reactivation. While these data do not show that FDC reactivate latently infected resting $CD4^+$ T cells *in vivo*, they are supportive of this activity. It is not well confirmed whether a multitude of other cells and other factors *in vivo* could influence the reactivation of latent T cells observed in our *ex vivo* coculture system. Further studies should be performed using human tissues as well as the humanized mouse model inoculated with human $CD4^+$ T cells bearing latent provirus. These results shed valuable new light on our understanding of the natural progression of latently infected $CD4^+$ T cell reservoirs, potentially providing a means for designing novel therapeutic interventions against HIV/AIDS.

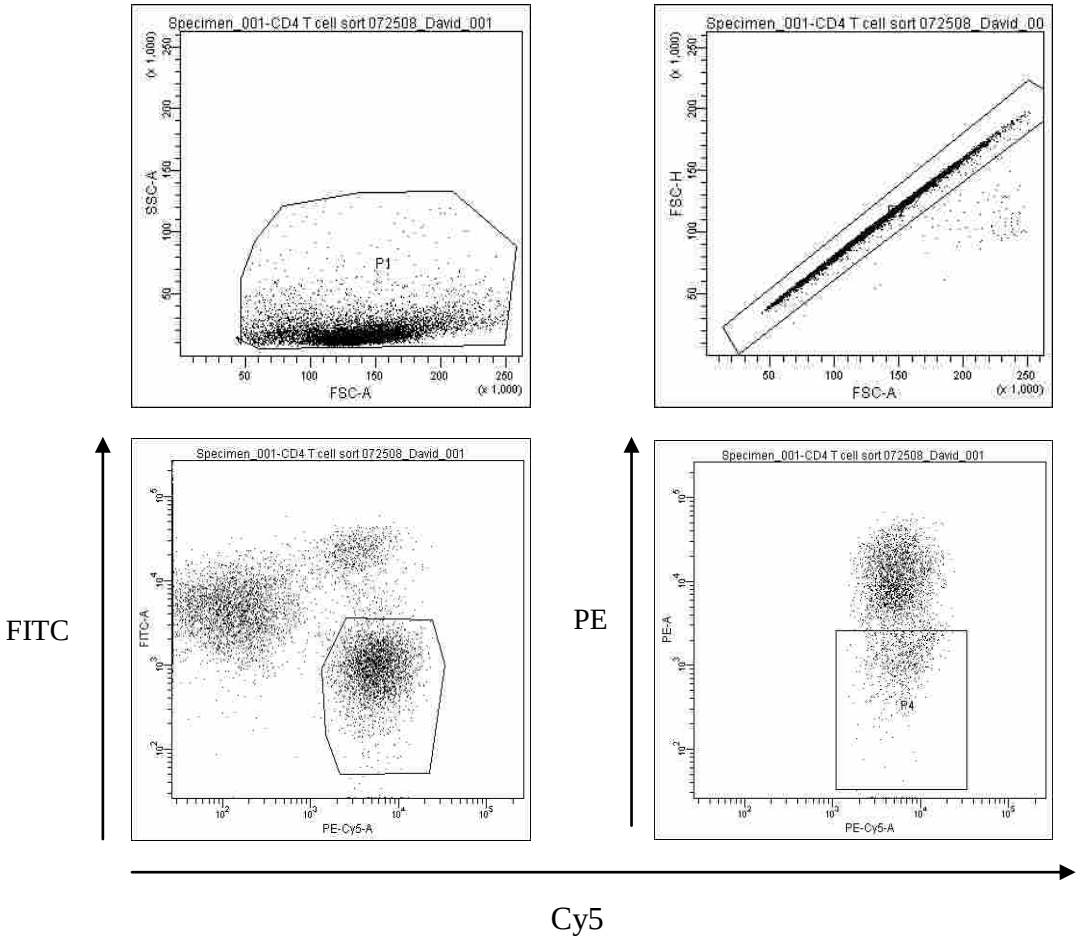


Figure 1 Resting $CD4^+$ T cell sorted by flow cytometry. Cells were first incubated with goat Fc block for 30 min, then stained with anti-CD3-Cy5, anti-CD25-PE, anti-CD69-PE, anti-CD8-FITC, anti-HLA-DR-FITC for another 30 min. Resting T cells were sorted as $CD3^+$, $CD8^-$, $CD25^-$, $CD69^-$, HLA-DR^- as seen in gate R4 in the lower right hand panel.

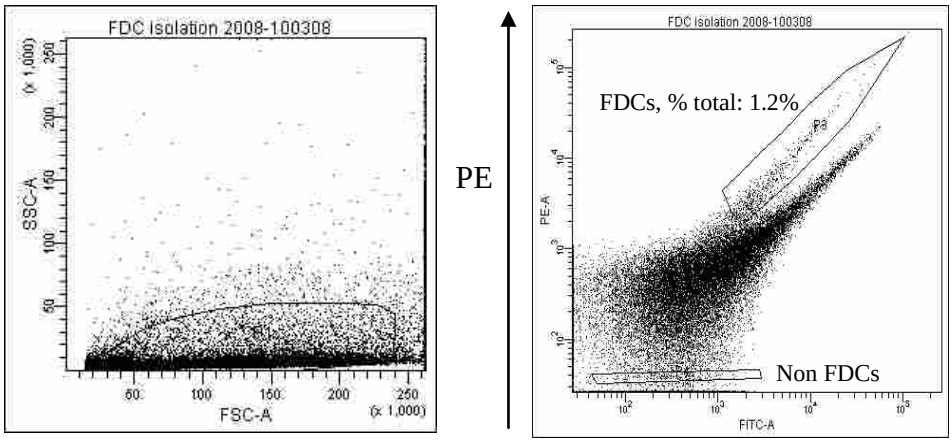


Figure 2 FDC sorted by flow cytometer. FDC were first stained with HJ2 overnight, then incubated with secondary antibody, rabbit-anti mouse IgM-PE for 1 hr. FDC were sorted as PE high with total percentage around 1-2%.

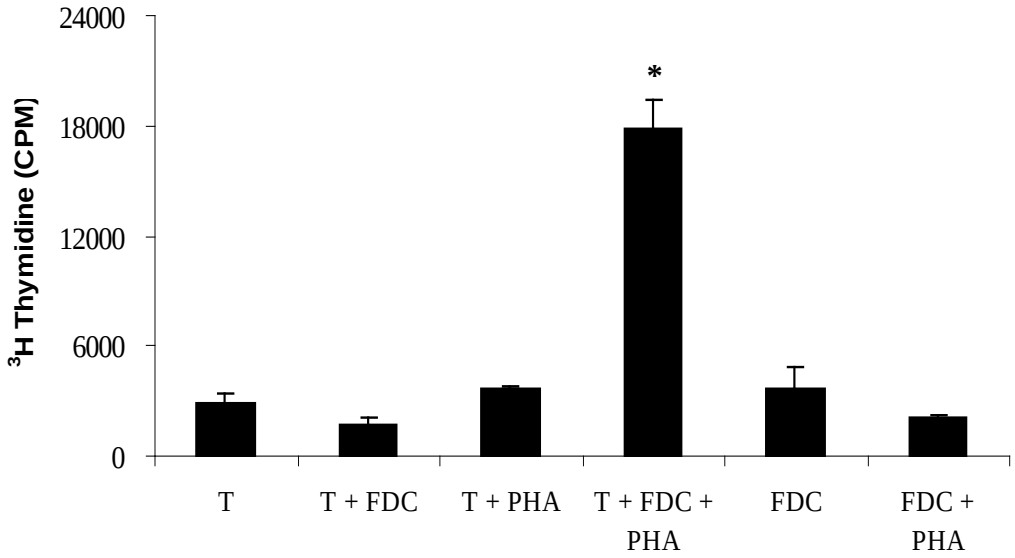


Figure 3 FDCs provide a co-stimulatory activation signal to resting T cells. FDCs ($1x10^4$) were cocultured for 48 hrs with resting T cells ($1x10^5$) in the presence or absence of a sub-optimal primary activation stimulus (i.e. the mitogen PHA). Tritiated thymidine (^{3}H-TdR) incorporation was used as a means of examining cell activation following the addition of radioactive thymidine during the last 12hr of cell culture. The data are expressed as CPM plus or minus the SEM of three independent experiments. *, $p < 0.05$ compared to T+PHA.

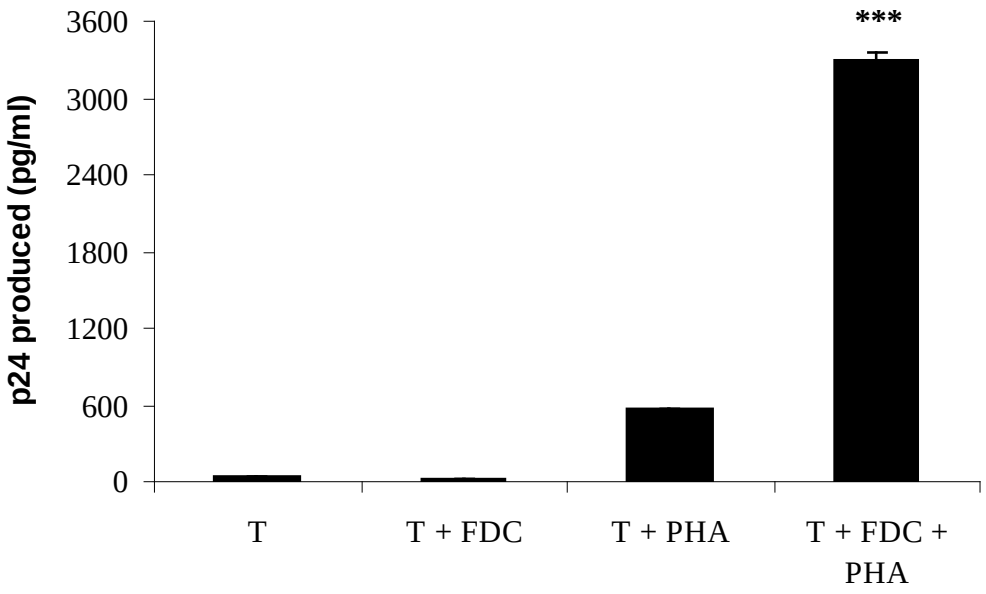


Figure 4 FDCs provide a co-stimulatory activation signal to resting T cells. Resting T cells infected *in vitro* with HIV_{IIIB} were cultured with FDCs and/or PHA. After 3 days, cells were washed and resuspended in RPMI with 20% FCS and IL-2 (20U/ml). The culture supernatant was collected at the end of 7 days and assayed for p24 produced per ml culture fluid. The data represent the mean plus/minus the SEM of three independent experiments. ***, $p < 0.001$ compared to T+PHA.

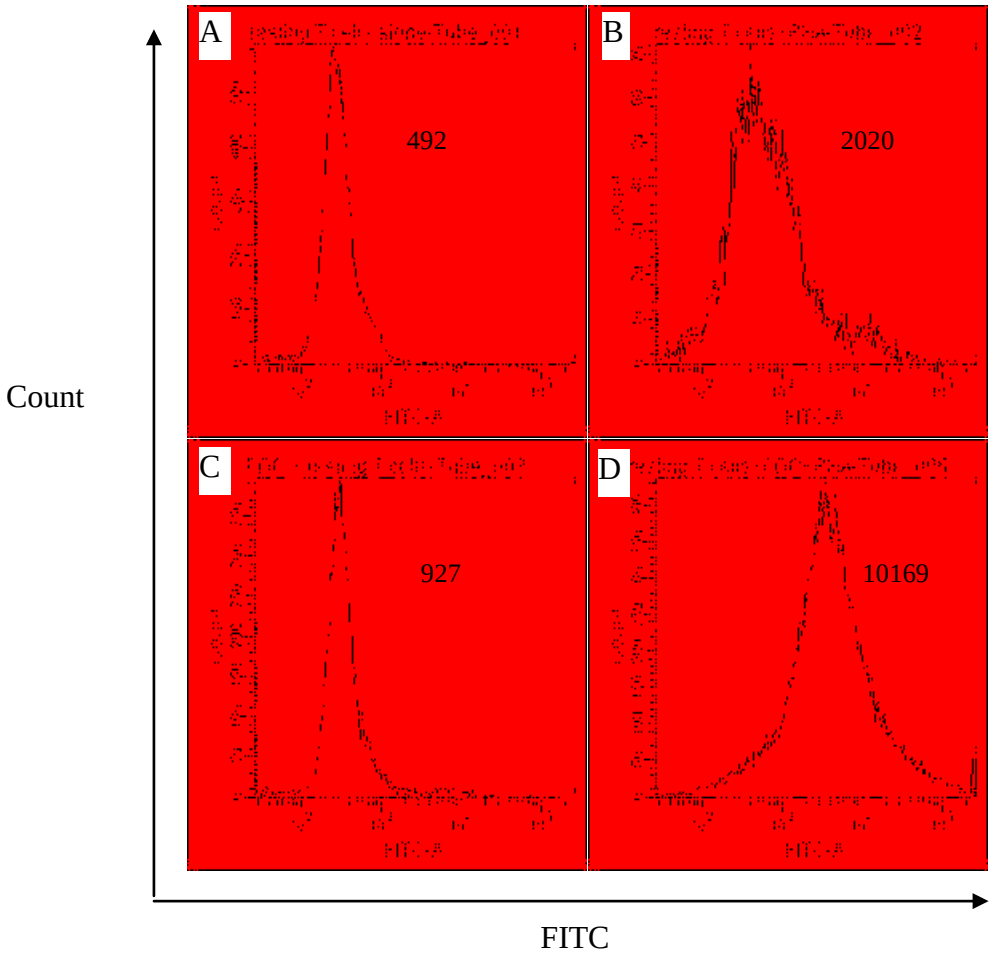


Figure 5 FDCs increase the activation phenotype of resting T cells. Resting T cells (5×10^5) were cocultured alone (A) or with (B) PHA (5μg/ml); (C) FDC (5×10^4); (D) FDC (5×10^4) and PHA (5 μg/ml) for 3 days, and then labeled with pooled FITC-conjugated antibodies directed against the activation markers CD25, CD69 and HLA-DR. The cells were then analyzed by flow cytometry. The data depicted are representative of three independent experiments.

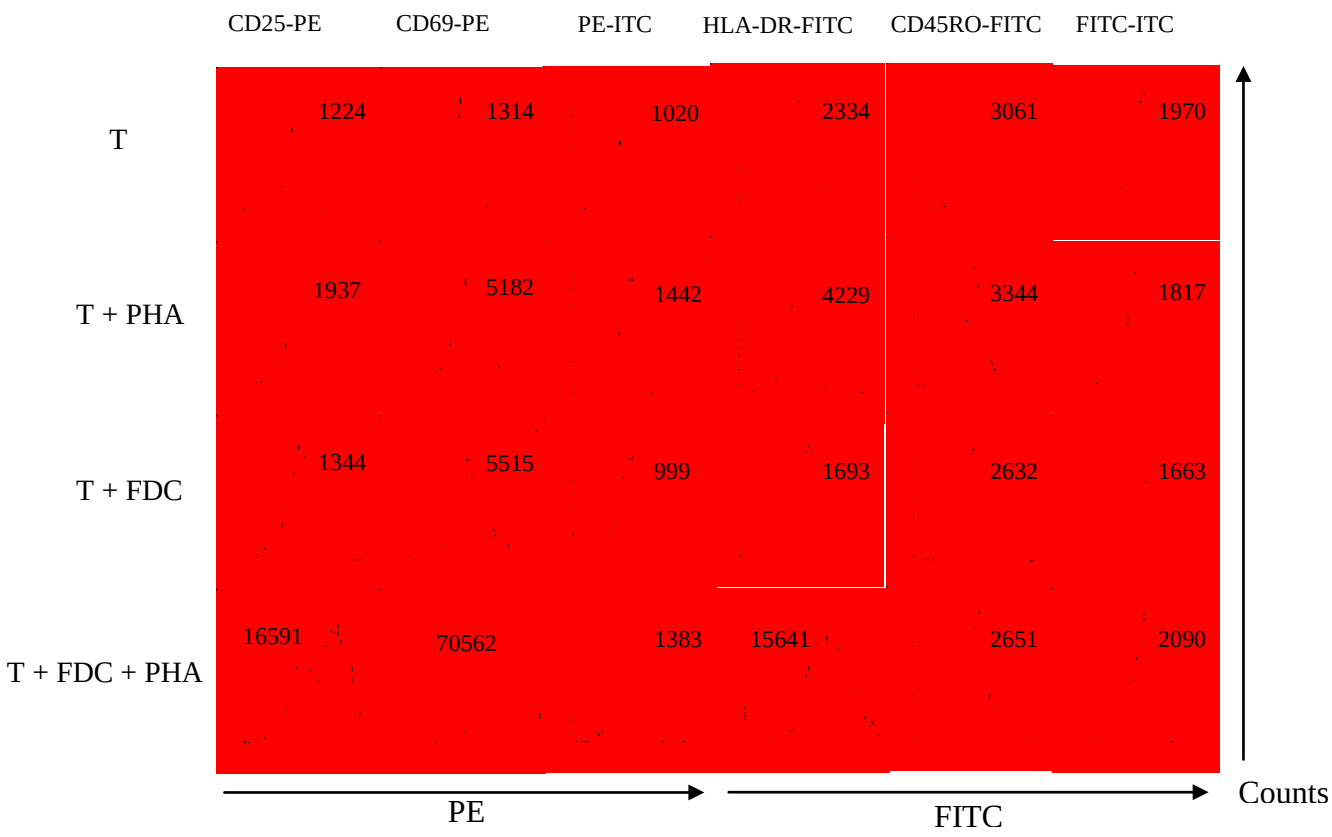


Figure 6 FDC-mediated effects on the expression of CD25, CD69, HLA-DR and CD45RO on resting T cells. Resting T cells (5×10^5) were cultured ± PHA (5μg/ml) ± FDC (5×10^4). After 24 hrs, the cells were stained with anti-CD25-PE, anti-CD69-PE, ITC-PE, anti-HLA-DR-FITC, anti-CD45RO-FITC or ITC-FITC respectively and then analyzed by flow cytometry. The data depicted are representative of three independent experiments.

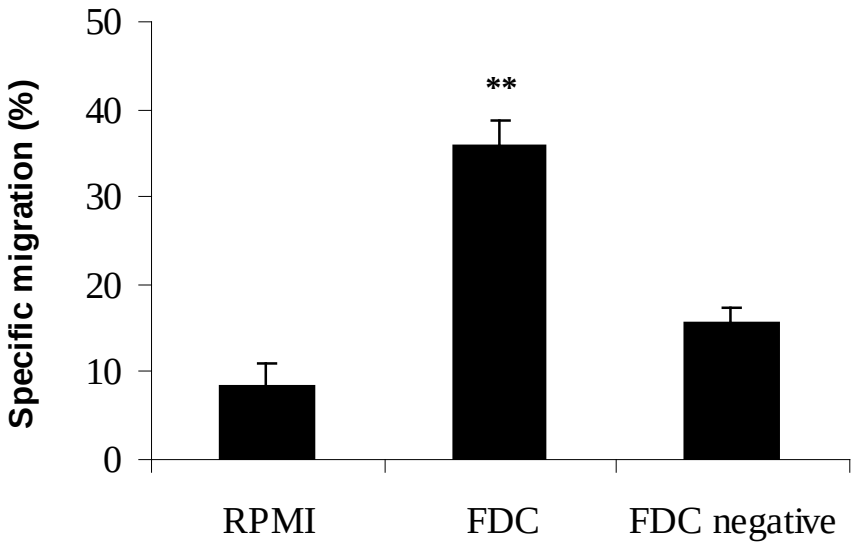


Figure 7 Resting CD4 T cells migrate efficiently to FDCs. Purified resting T cells were added to the top chamber of the Transwell and assessed for their ability to migrate to RPMI medium alone, FDCs, or HJ2-negative cells (FDC negative) obtained from the same tissue as the FDCs. After 5 h of culture, the specific migration of the resting T cells was determined by counting the number of live cells in the upper and lower chambers. The data represent the mean plus/minus the SEM of three independent experiments. **, $p < 0.01$ compared to medium control.

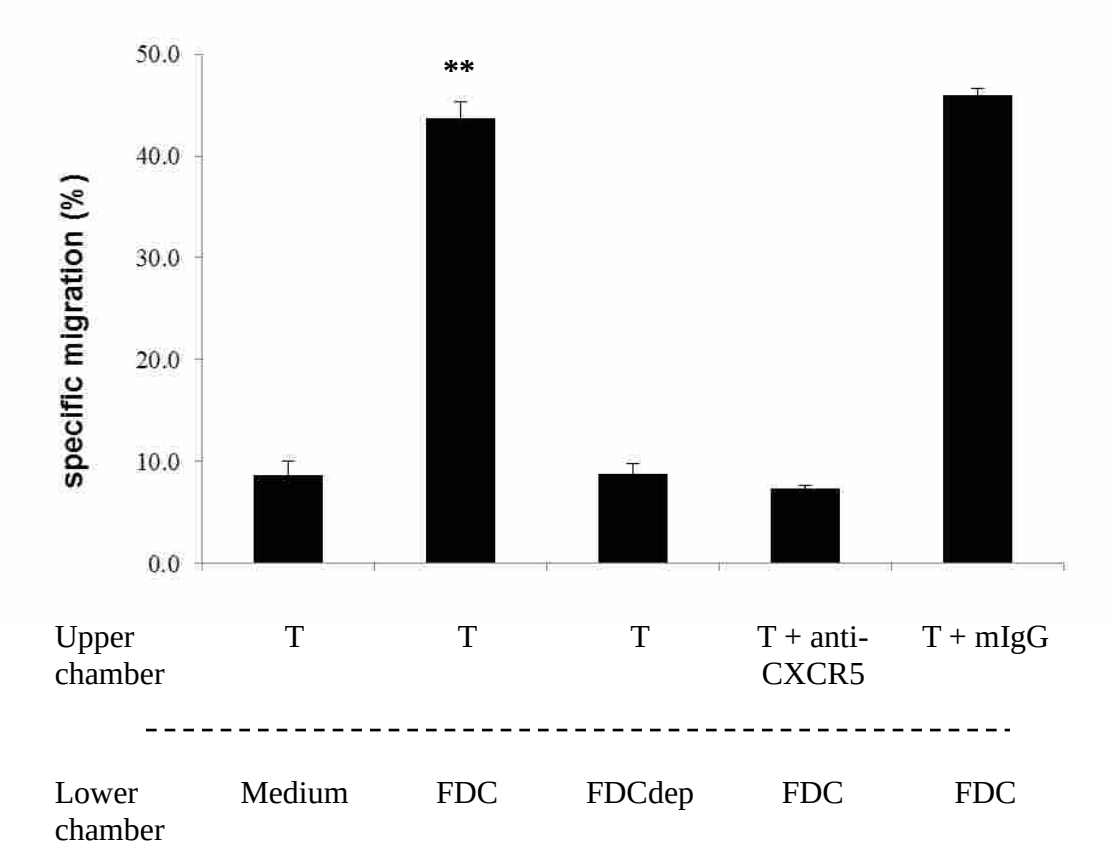


Figure 8 Resting T cells migrate efficiently to FDCs and the migration is mediated by CXCR5. Purified resting T cell migration to FDCs, FDC negative cells or medium was determined after 5 hrs of culture in a Transwell migration system. T cells were pretreated with either anti-CXCR5 (10 μg/ml) or control Ab (10 μg/ml) for 30 min on ice before performing the migration studies with FDCs. The data represent the mean plus/minus the SEM of three independent experiments. **, $p < 0.01$ compared to medium control.

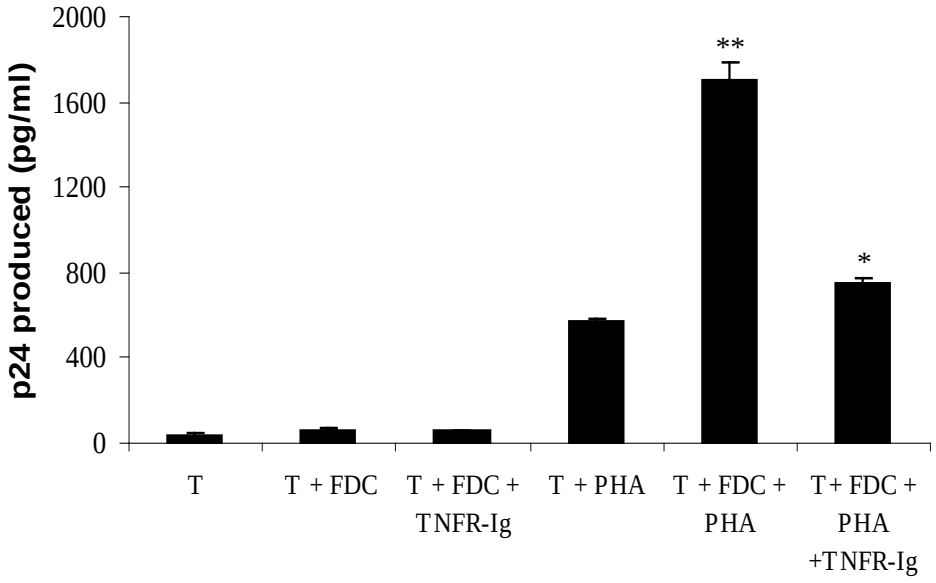


Figure 9 FDC-mediated augmentation of HIV production in resting $CD4^+$ T cells uses TNFα. Resting $CD4^+$ T cells were infected with HIV_{IIIB} for 2 hrs at 37°C, washed extensively and cultured for 6 days (5×10^5 cells/ml) ± FDCs (5×10^4 cells/ml) ± PHA (5μg/ml) ± hTNFR-Ig (10 μg). Supernatant was harvested and p24 ELISA was performed. The data were represented as the mean plus/minus the SEM of three independent experiments. Note that the FDCs were pretreated with hTNFR-Ig for 30 min and then incubated with resting T cells or PHA. *, $p < 0.05$; **, $p < 0.01$ compared to T+PHA.

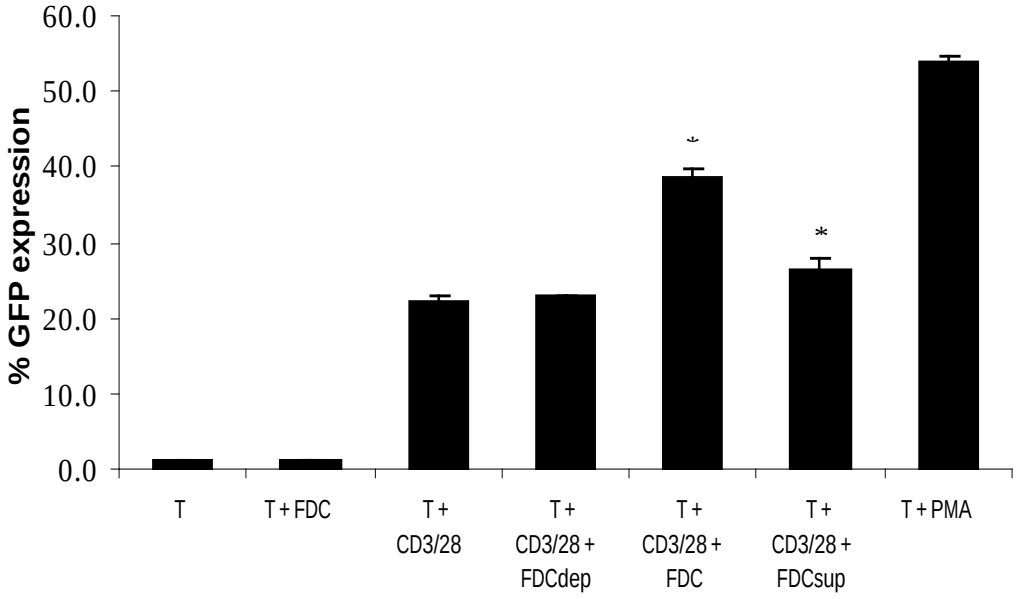


Figure 10 FDCs activate GFP expression in latently infected reporter Jurkat T cells. Latently infected Jurkat reporter cells (5×10^5 cells/ml) were cultured with or without FDCs (5×10^4 cells/ml) in a 48 well plate coated with αCD3/αCD28. After 3 days' incubation, the cells were harvested and analyzed for EGFP expression using flow cytometry. Phorbol-12-myristate-13-acetate (PMA) was used as a positive control to induce EGFP expression. The data are expressed as the percent of cells that express EGFP plus/minus the SEM of three independent experiments. *, $p < 0.05$ compared to T+CD3/28.

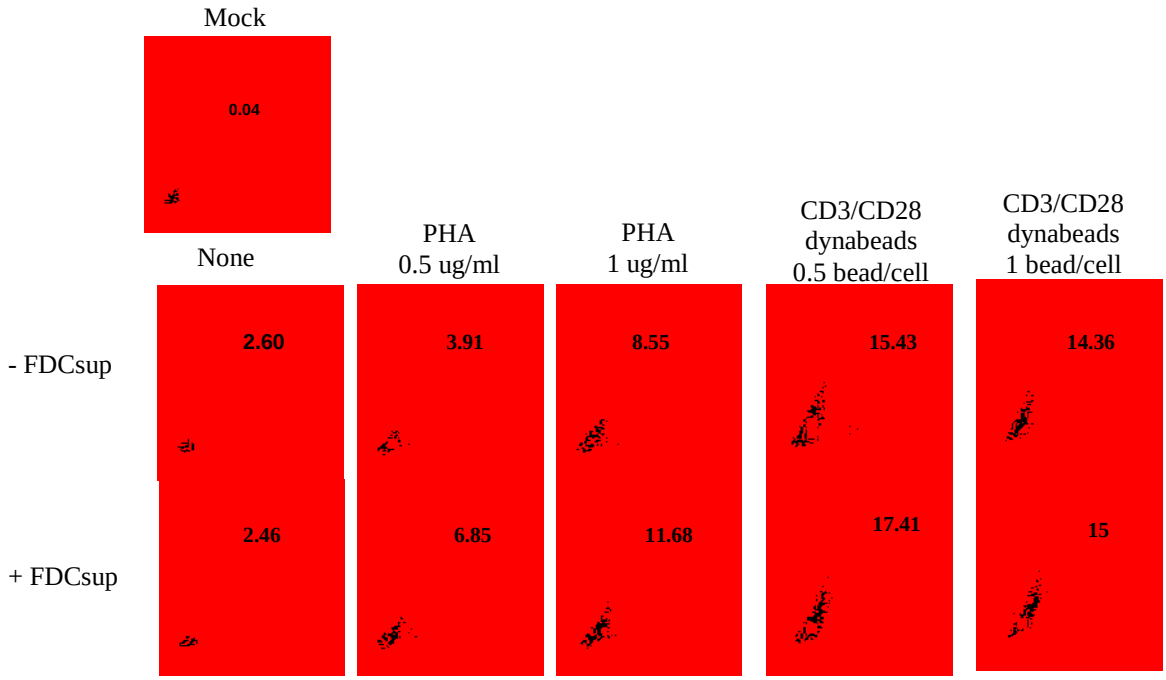


Figure 11 FDCs reactivate latent HIV in ex vivo HIV infected resting T cells. Resting T cells were prepared according to the Bosque and Planelles protocol (Blood, 113:58-65, 2009). Cells were cultured ± FDC supernatant (10%) with either PHA or anti-CD3/CD28-coated dynabeads as described in Methods. After 3 days' incubation, the cells were harvested and analyzed for intracellular p24 by flow cytometry. The outlined boxes indicate the region in which cells that express p24 are detected and the numbers indicate the percent cells that express viral p24. The data depicted are representative of three independent experiments.

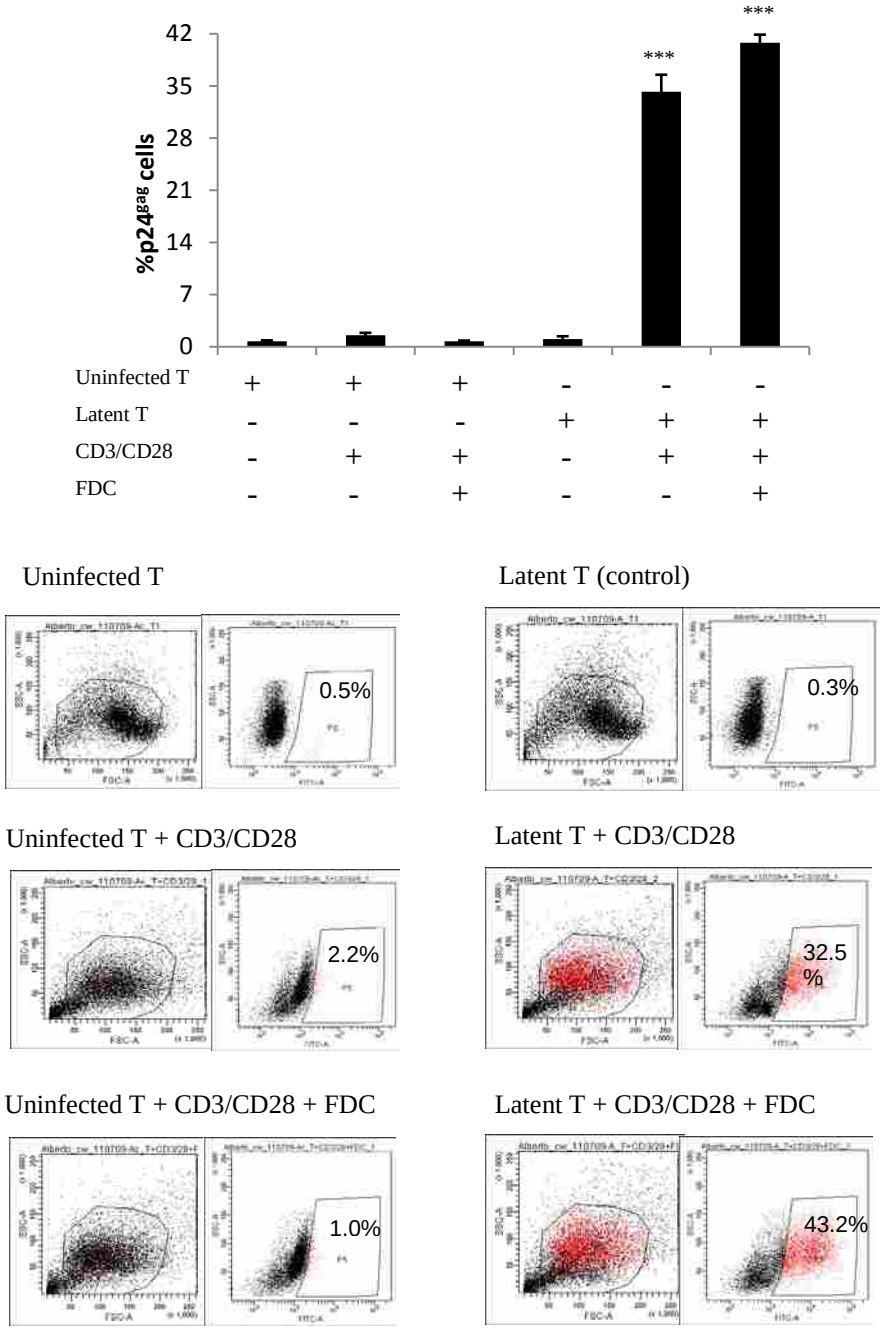


Figure 12 FDCs reactivate latent HIV in *ex vivo* defective virus infected resting T cells with CD3/CD28 providing a primary activation signal. Resting T cells were cultured ± FDCs with anti-CD3/CD28-coated dynabeads (1 cell per bead). After 3 days' incubation, the cells were harvested and analyzed for intracellular p24 by flow cytometry. The outlined boxes indicate the region in which cells that express p24 are detected and the numbers indicate the percent cells that express viral p24. The data depicted are representative of four independent experiments. ***, $p < 0.001$ compared to the control.

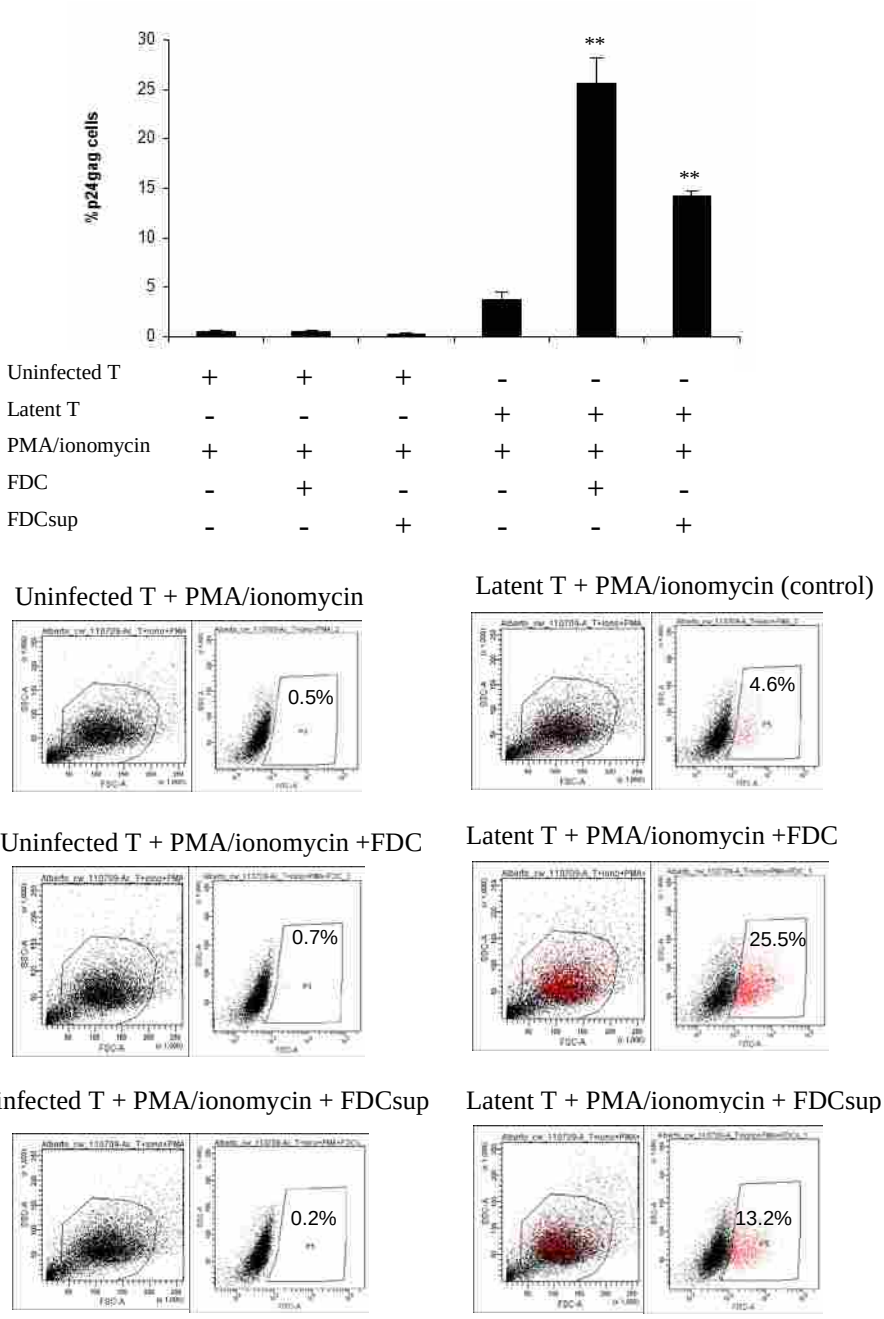


Figure 13 FDCs reactivate latent HIV in ex vivo defective virus infected resting T cells with PMA/ionomycin providing the primary activation signal. Resting T cells were cultured ± FDC or FDC supernatant (10%) in the presence of PMA and ionomycin (10 ng/ml, 1µM). After 3 days' incubation, the cells were harvested and analyzed for intracellular p24 by flow cytometry. The outlined boxes indicate the region in which cells that express p24 are detected and the numbers indicate the percent cells that express viral p24. The data represent the mean plus/minus the SEM of four independent experiments. **, $p < 0.01$ compared to the control.

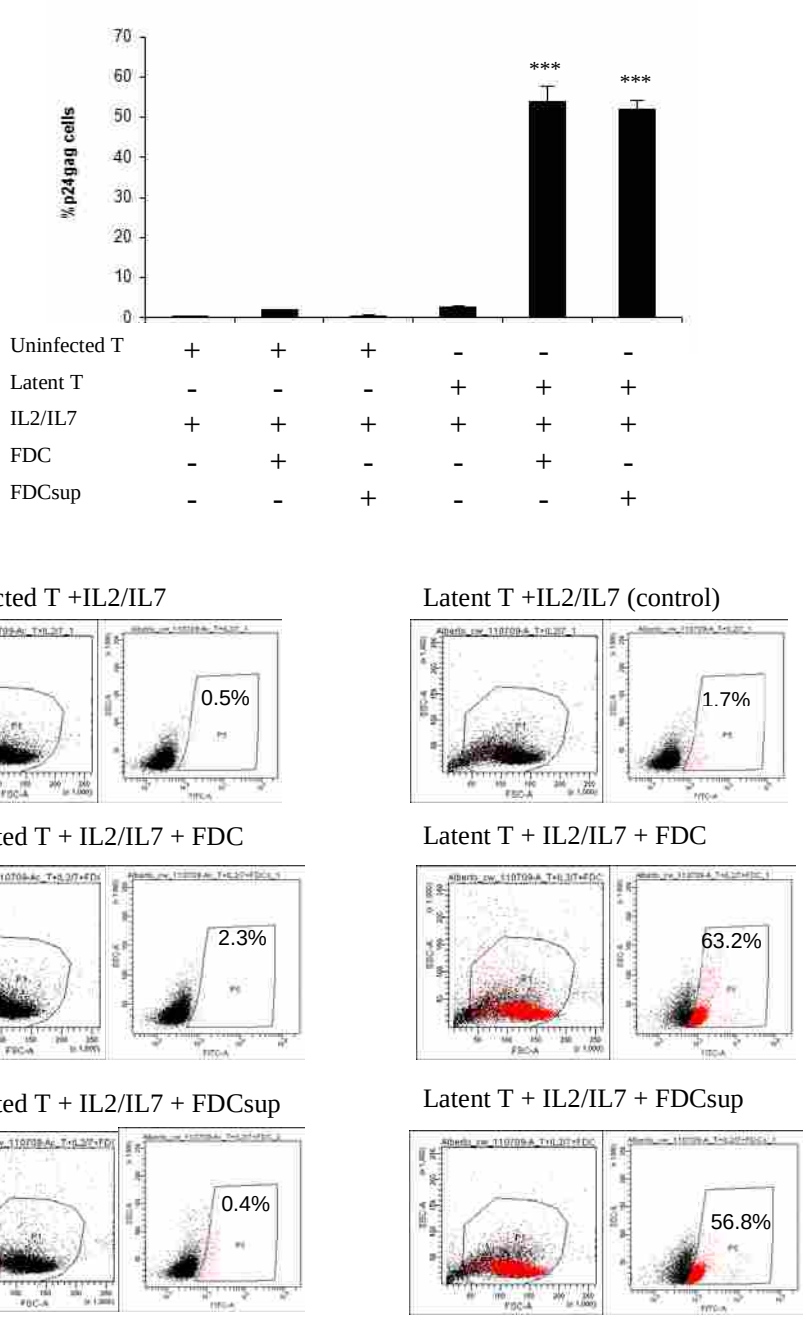
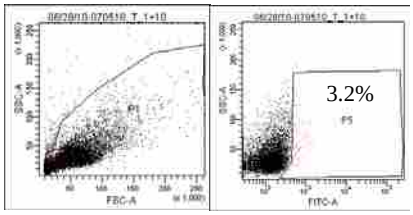
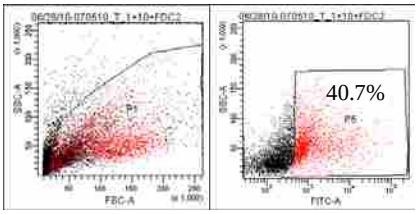


Figure 14 FDCs reactivate latent HIV in ex vivo defective virus infected resting T cells with IL-2/IL-7 providing the primary signal. Latently infected T cells were cultured ± FDC or FDC supernatant (10%) in the presence of IL-2/IL-7 (30U/ml/50ng/mL). After 3 days' incubation, the cells were harvested and analyzed for intracellular p24 by flow cytometry. The outlined boxes indicate the region in which cells that express p24 are detected and the numbers indicate the percent cells that express viral p24. The data represent the mean plus/minus the SEM of four independent experiments. ***, $p < 0.001$ compared to the control.

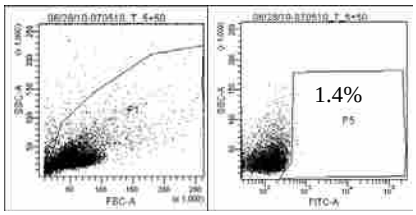
Latent T + PMA/ ionomycin (10 ng/ml/1.0 μM)



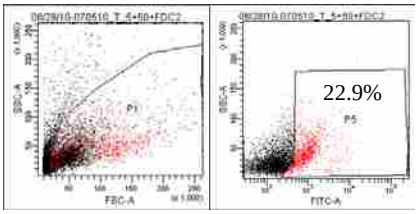
Latent T + PMA/ionomycin (10 ng/ml/1.0 μM) +FDC



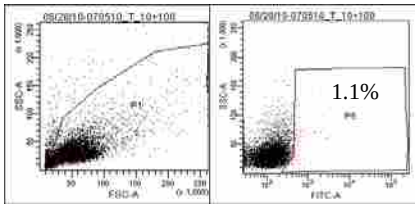
Latent T + PMA/ionomycin (50 ng/ml/5.0 μM)



Latent T + PMA/ionomycin (50 ng/ml/5.0 μM) +FDC



Latent T + PMA/ ionomycin (100 ng/ml/10 μM)



Latent T + PMA/ionomycin (100 ng/ml/10 μM) +FDC

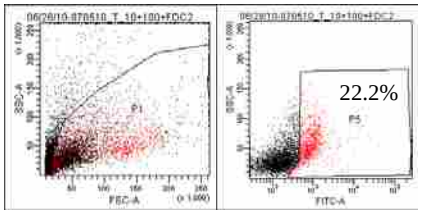


Figure 15 Dose curve of PMA/ionomycin concentration with or without FDCs. *Ex vivo* HIV infected resting T cells were cultured ± FDC in the presence of various concentrations of PMA/ionomycin (10, 50 or 100 ng/ml/ 1, 5 or 10 μM). After 3 days' incubation, the cells were harvest and analyzed for intracellular p24 by flow cytometry. The outlined boxes indicate the region in which cells that express p24 are detected and the numbers indicate the percent cells that express viral p24. The data depicted are representative of three independent experiments.

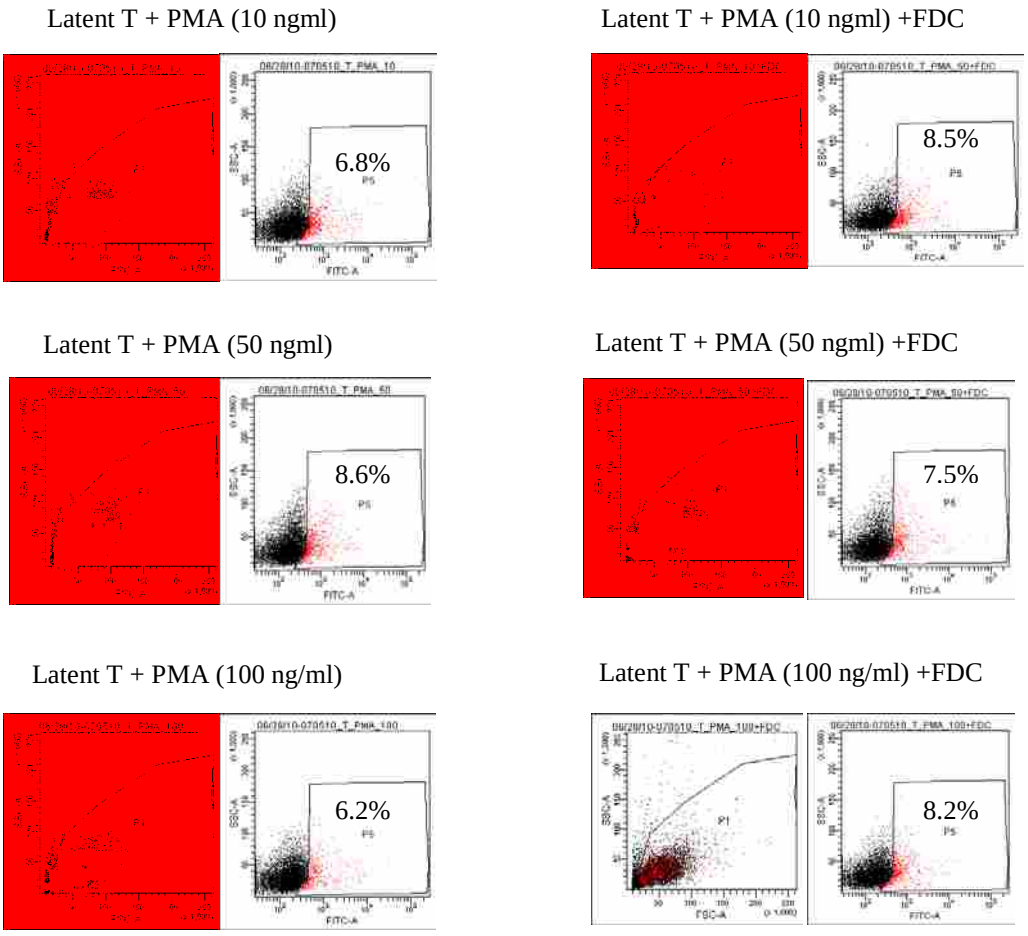


Figure 16 Dose curve of PMA concentration with or without FDCs. *Ex vivo* HIV infected resting T cells were cultured ± FDC in the presence of various concentrations of PMA (10, 50 or 100 ng/ml). After 3 days' incubation, the cells were harvest and analyzed for intracellular p24 by flow cytometry. The outlined boxes indicate the region in which cells that express p24 are detected and the numbers indicate the percent cells that express viral p24. The data depicted are representative of three independent experiments.

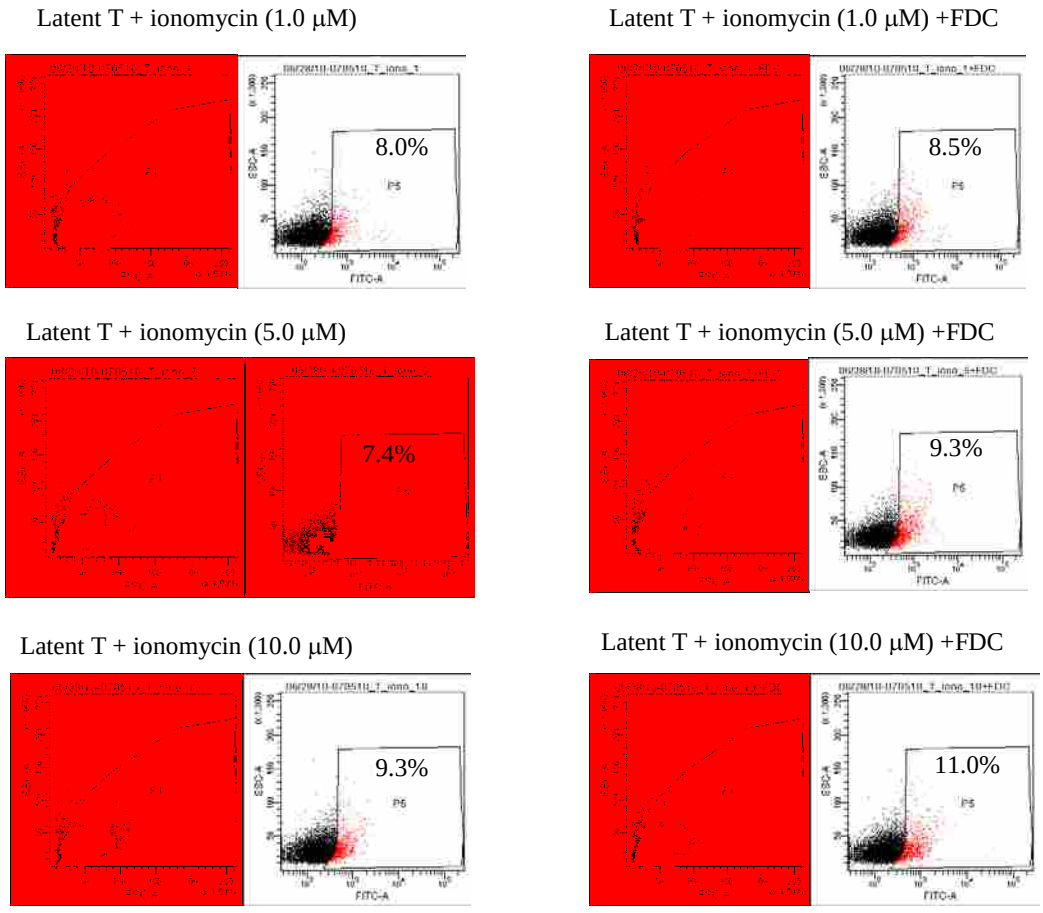


Figure 17 Dose curve of ionomycin concentration with or without FDCs. *Ex vivo* HIV infected resting T cells were cultured ± FDC in the presence of different concentrations of ionomycin (1, 5 or 10 μM). After 3 days' incubation, the cells were harvest and analyzed for intracellular p24 by flow cytometry. The outlined boxes indicate the region in which cells that express p24 are detected and the numbers indicate the percent cells that express viral p24. The data depicted are representative of three independent experiments.

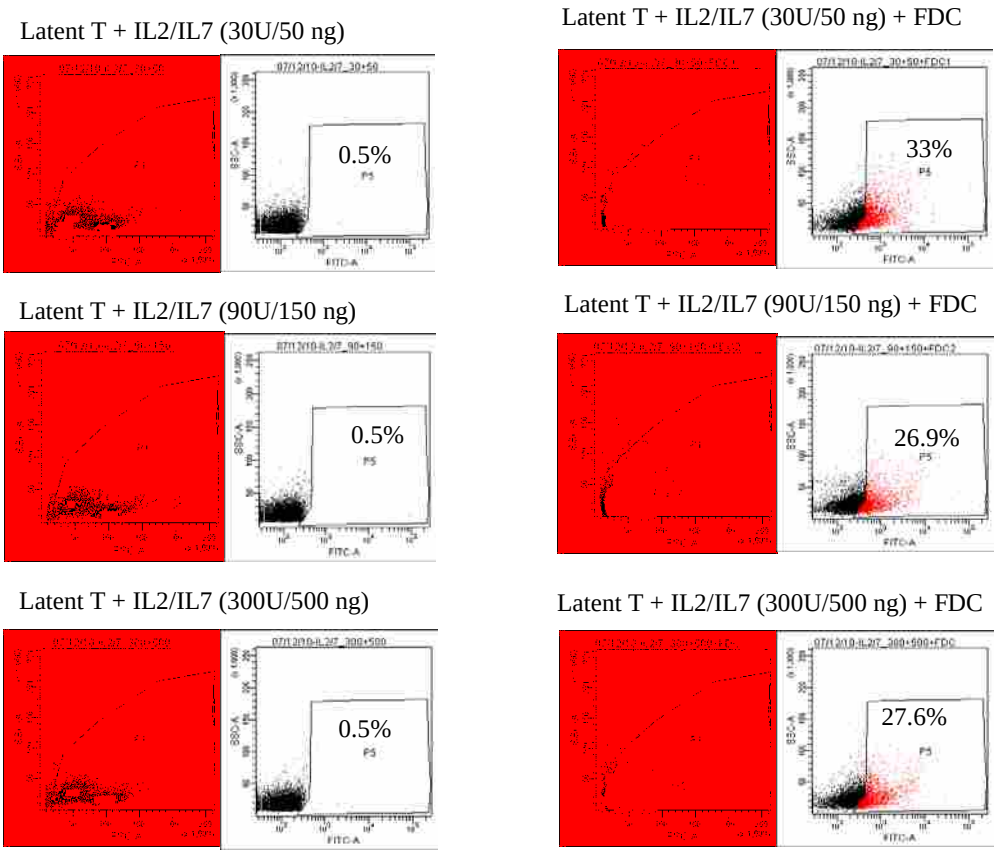


Figure 18 Dose curve of IL2/IL7 concentration with or without FDCs. *Ex vivo* experimental latently infected T cells were cultured ± FDC in the presence of various concentrations of IL2/IL7 (30, 90 or 300 U/ml/ 50, 150 or 500 ng/ml). After 3 days' incubation, the cells were harvest and analyzed for intracellular p24 by flow cytometry. The outlined boxes indicate the region in which cells that express p24 are detected and the numbers indicate the percent cells that express viral p24. The data depicted are representative of three independent experiments.

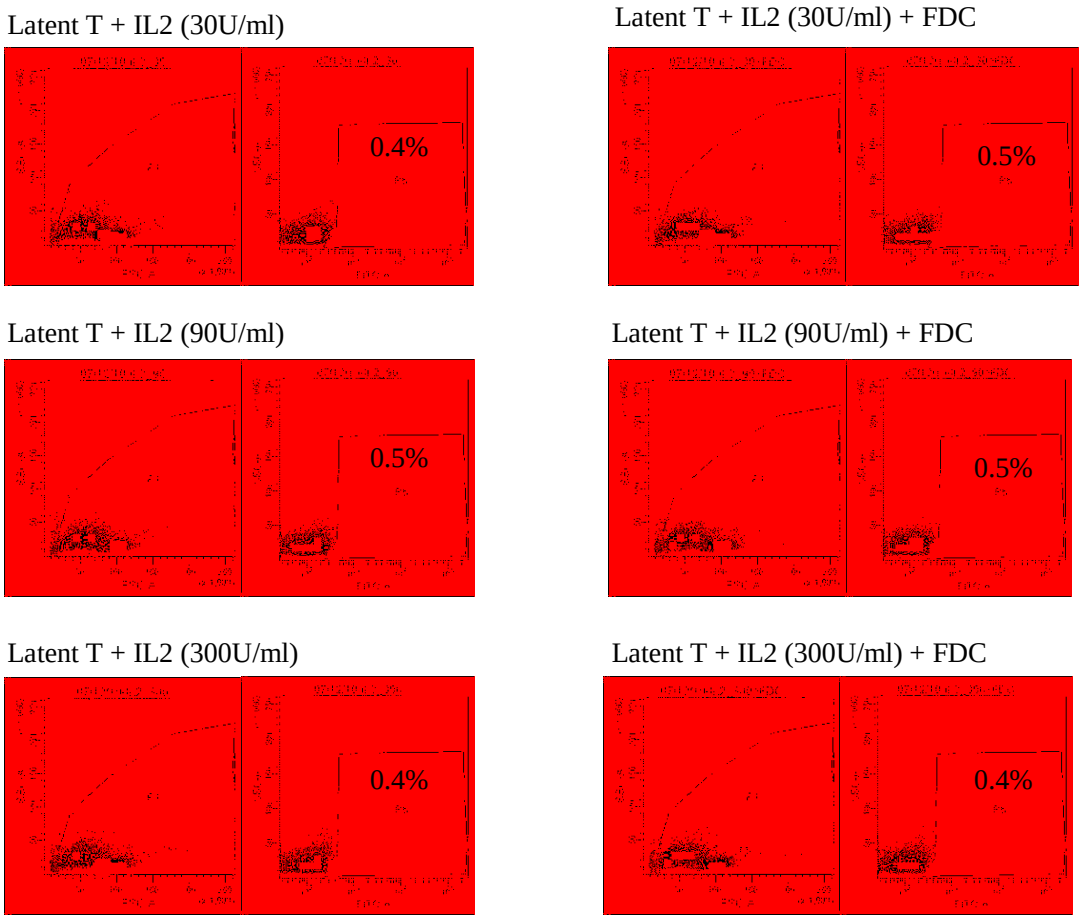


Figure 19 Dose curve of IL-2 concentration with or without FDCs. *Ex vivo* HIV infected resting T cells were cultured ± FDC in the presence of different concentrations of IL-2 (30, 90 or 300 U/ml). After 3 days' incubation, the cells were harvest and analyzed for intracellular p24 by flow cytometry. The outlined boxes indicate the region in which cells that express p24 are detected and the numbers indicate the percent cells that express viral p24. The data depicted are representative of three independent experiments.

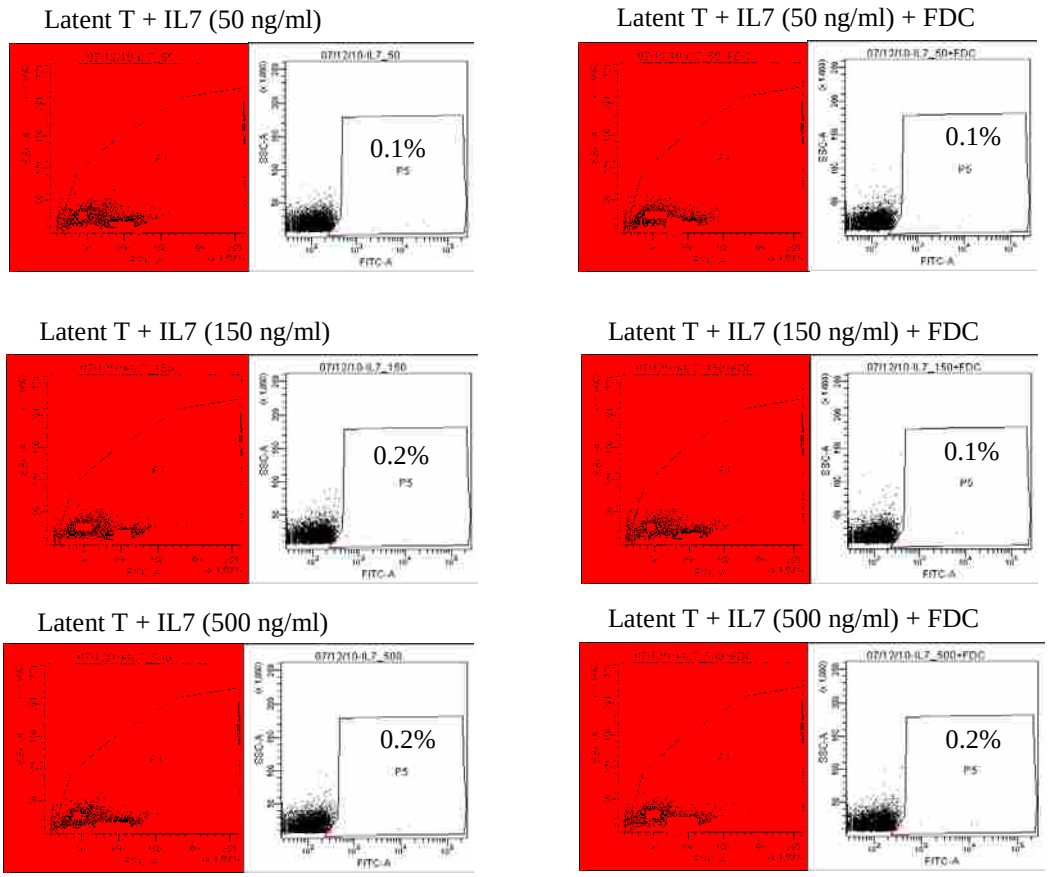


Figure 20 Dose curve of IL7 concentration with or without FDCs. *Ex vivo* HIV infected resting T cells were cultured ± FDC in the presence of various concentrations of IL7 (50, 150 or 500 ng/ml). After 3 days' incubation, the cells were harvest and analyzed for intracellular p24 by flow cytometry. The outlined boxes indicate the region in which cells that express p24 are detected and the numbers indicate the percent cells that express viral p24. The data depicted are representative of three independent experiments.

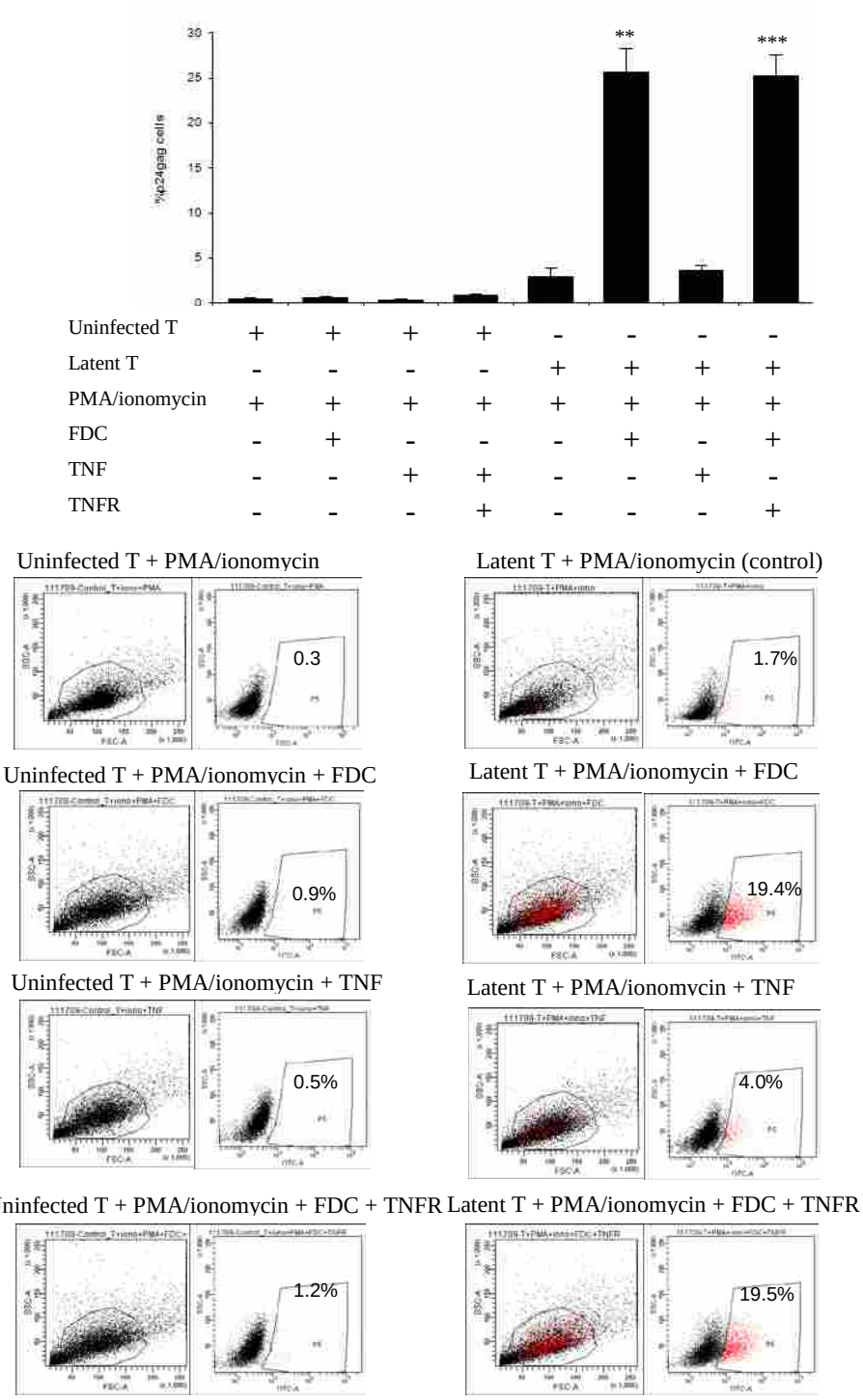


Figure 21 FDC-mediated HIV augmentation is not TNFα dependent in *ex vivo* HIV infected resting T cells. FDCs were incubated with TNFR-Ig (10 µg/ml) on ice for 30 min, and then washed out. Resting T cells were cocultured with TNFR treated or untreated FDCs in the presence of PMA/ionomycin for 3 days. Then the cells were harvested and analyzed for intracellular p24 by FACS. The outlined boxes indicate the region in which cells that express p24 are detected and the numbers indicate the percent cells that express viral p24. The data represent the mean plus/minus the SEM of four independent experiments. **, $p < 0.01$; ***, $p < 0.001$ compared to the control.

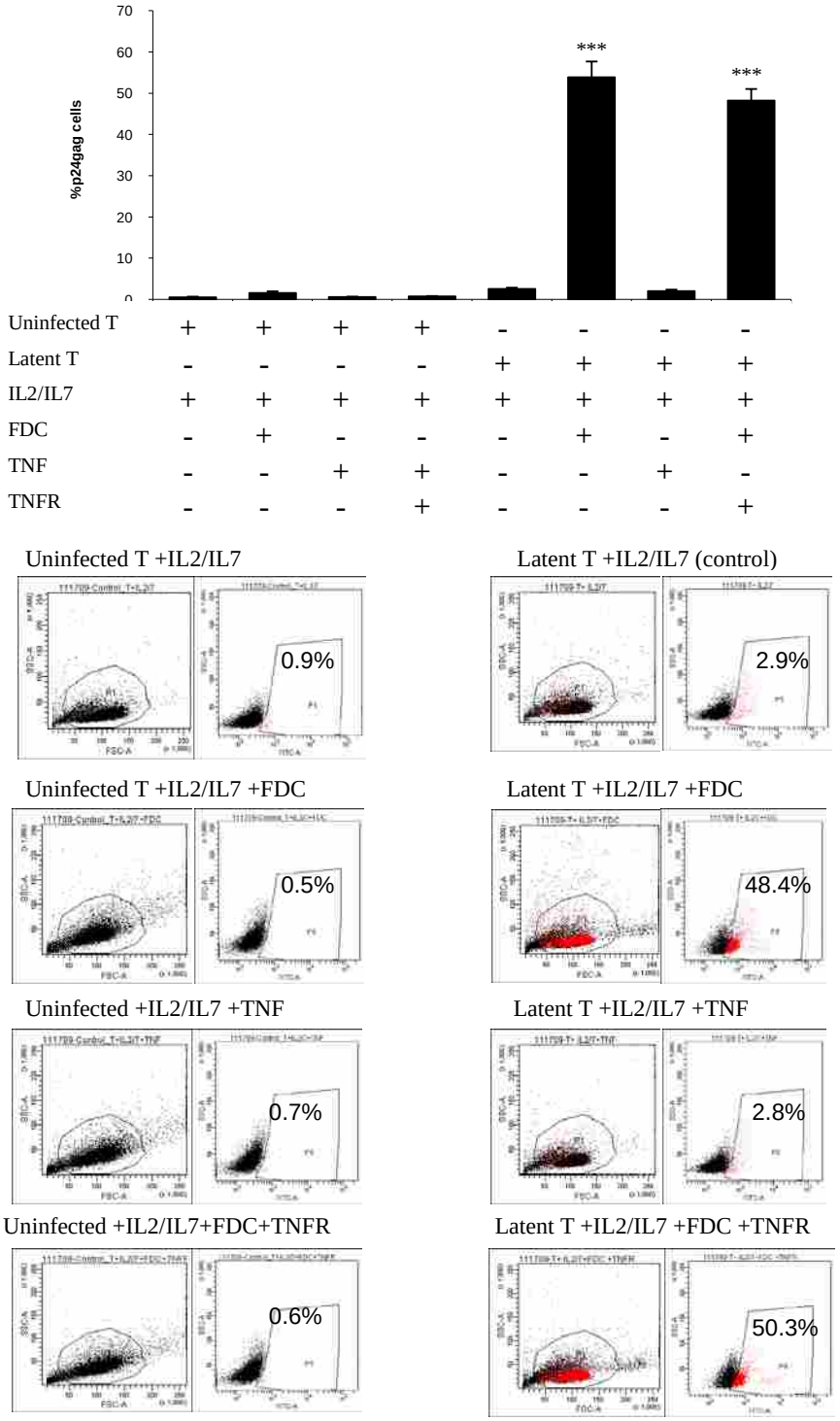


Figure 22 FDC-mediated HIV augmentation is not TNF-α dependent in ex vivo HIV infected resting T cells. FDCs were incubated with TNFR-Ig (10 μg/ml) on ice for 30 min, and then washed out. Resting T cells were cocultured with TNFR treated or untreated FDCs in the presence of IL2/IL7 for 3 days. Then the cells were harvested and analyzed for intracellular p24 by FACS. The outlined boxes indicate the region in which cells that express p24 are detected and the numbers indicate the percent cells that express viral p24. The data represent the mean plus/minus the SEM of four independent experiments. ***, $p <$ 0.001 compared to the control.

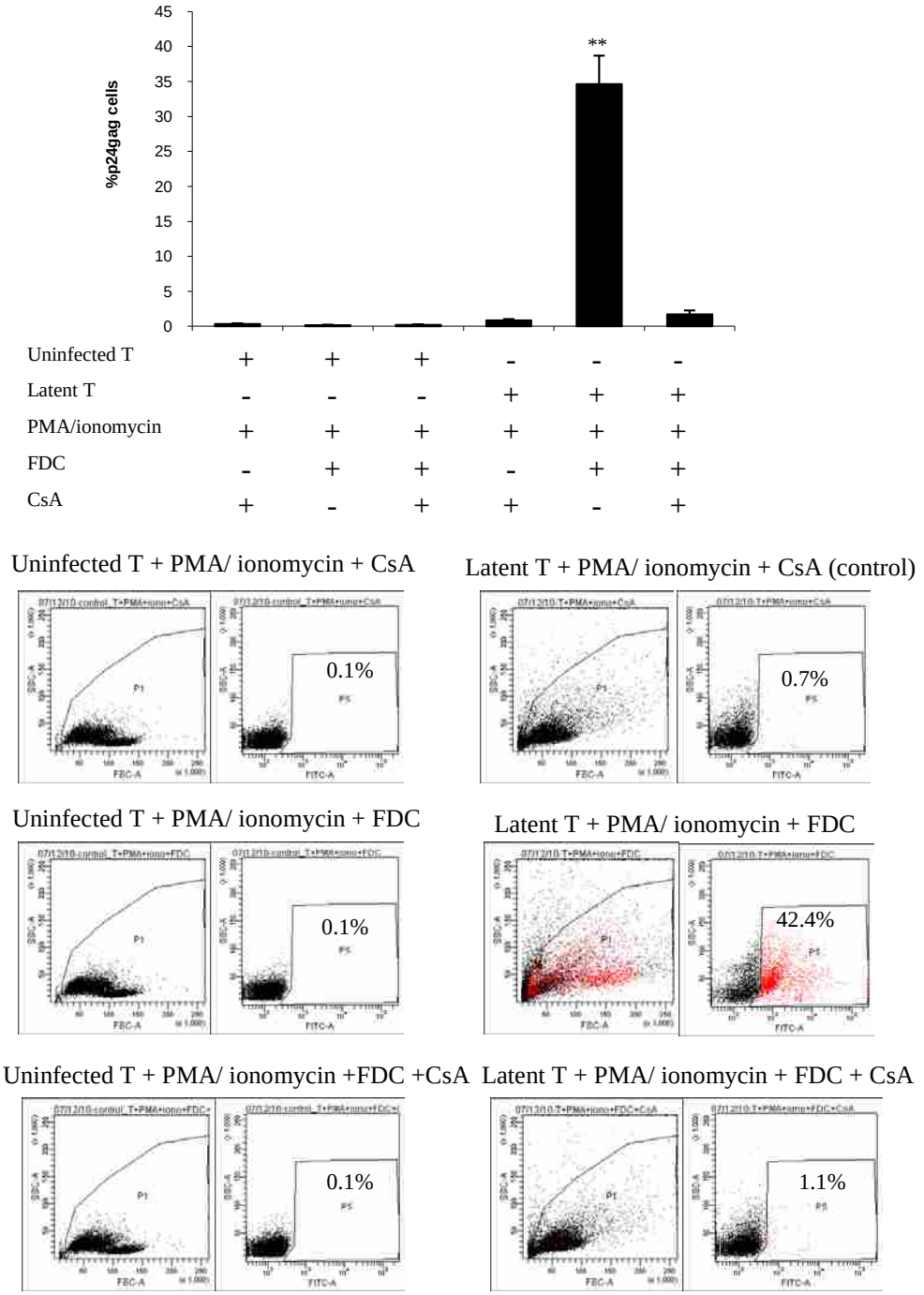


Figure 23 CsA completely abolished viral reactivation. *Ex vivo* HIV infected resting $CD4^+$ T cells were cultured with PMA/ionomycin ± FDC in the presence of calcineurin inhibitor CsA (500ng/ml). After 3 days' incubation, the cells were assessed for intracellular p24 gag expression by flow cytometry. The outlined boxes indicate the region in which cells that express p24 are detected and the numbers indicate the percent cells that express viral p24. The data represent the mean plus/minus the SEM of four independent experiments. **, $p < 0.01$ compared to the control.

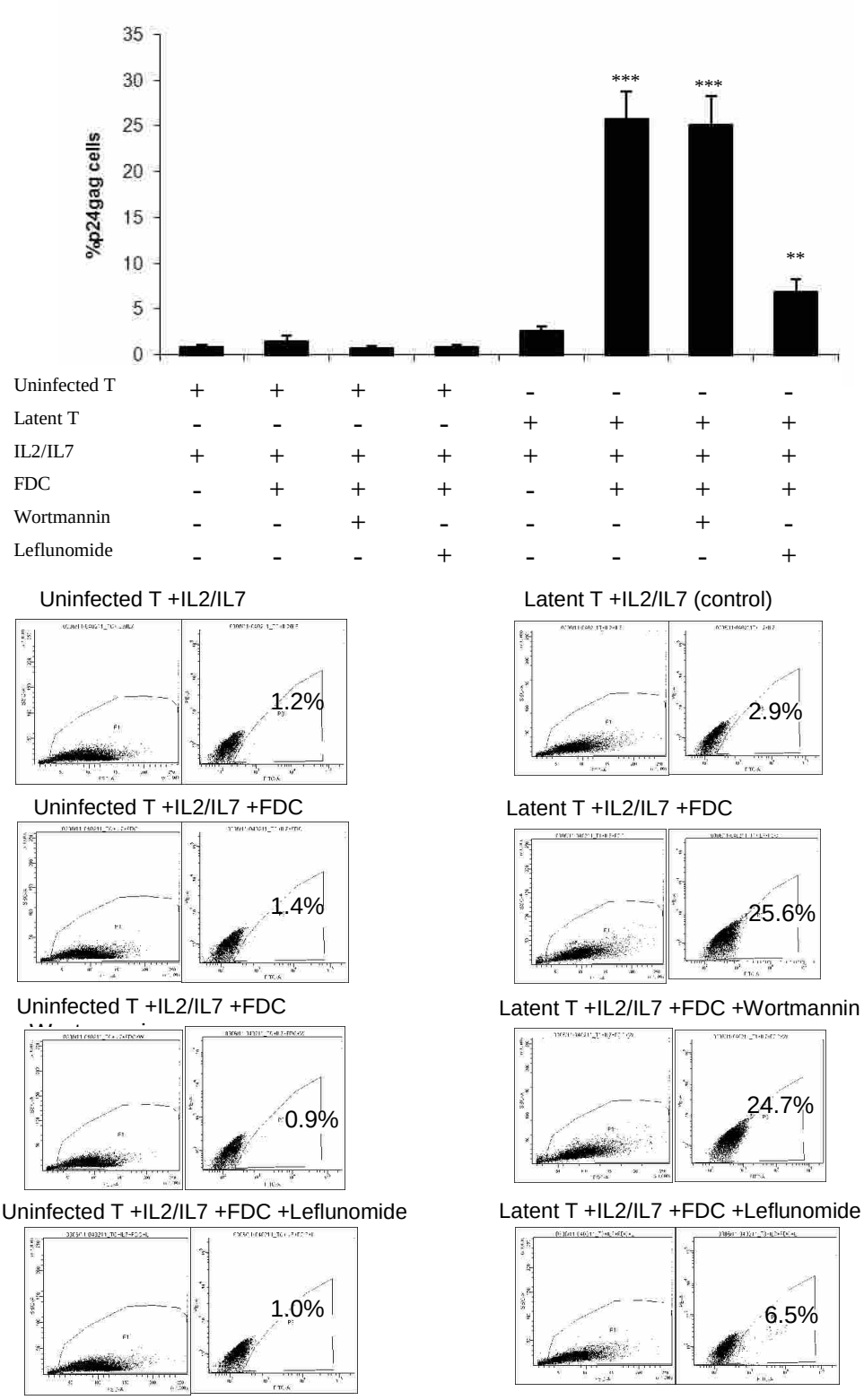


Figure 24 Leflunomide block viral reactivation. *Ex vivo* defective HIV infected resting $CD4^+$ T cells were cultured with IL2/IL7 ± FDC in the presence of the JAK3 inhibitor, Leflunomide (125ng/ml), or the PI3K inhibitor, Wortmanin (250nM). After 3 days' incubation, the cells were assessed for intracellular p24 gag expression by flow cytometry. The outlined boxes indicate the region in which cells that express p24 are detected and the numbers indicate the percent cells that express viral p24. The data represent the mean plus/minus the SEM of four independent experiments. **, $p < 0.01$; ***, $p < 0.001$ compared to the control.

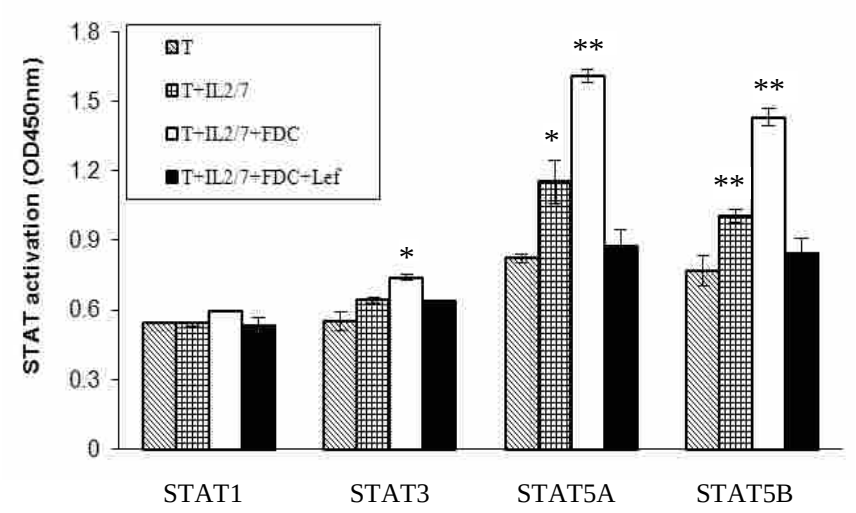


Figure 25 Leflunomide blocks viral reactivation by the JAK-STAT pathway. *Ex vivo* defective HIV infected resting $CD4^+$ T cells were cultured with IL2/IL7 ± FDC supernatant (10% vol/vol) in the presence or absence of Leflunomide (125ng/mL) for 3 days. The nuclear proteins were extracted and subjected to ELISA for the measurement of STAT1, STAT3, STAT5A and STAT5B. The data represent the mean plus/minus the SEM of three independent experiments. *, $p< 0.05$; **, $p < 0.01$ compared to the T cell control.

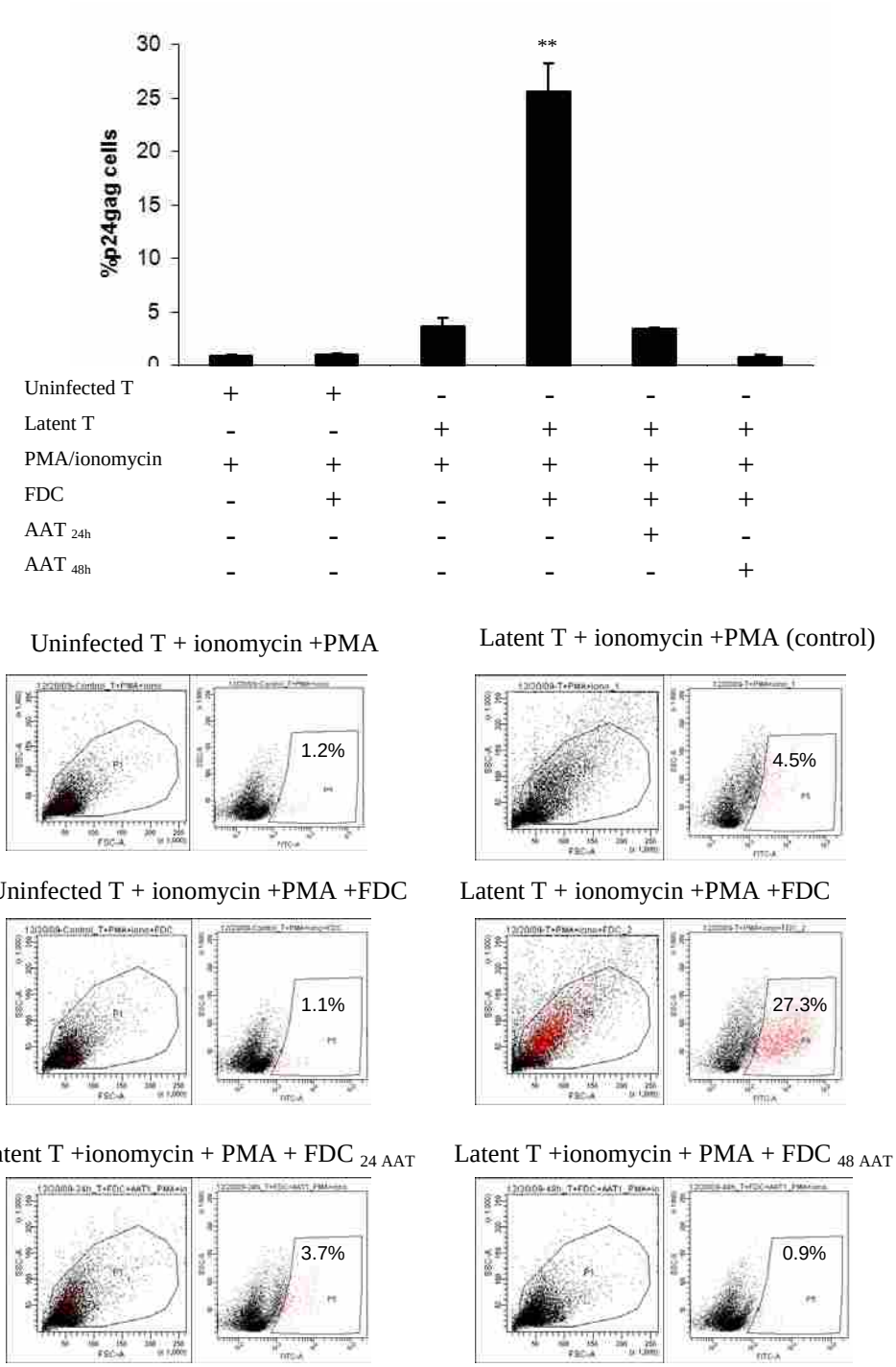


Figure 26 AAT inactivated FDCs do not activate virus expression in ex vivo defective HIV infected CD4 T cells. FDCs were cultured with phosphate buffered saline (PBS) or AAT (5mg/ml) for 24 or 48 hrs and then they were washed and cultured with latently infected primary CD4 T cells in the presence of PMA/ionomycin (10 ng/ml/1µM). After three days, the cells were tested for intracellular p24 by flow cytometry. The outlined boxes indicate the region in which cells that express p24 are detected and the numbers indicate the percent cells that express viral p24. The data represent the mean plus/minus the SEM of four independent experiments. **, $p < 0.01$ compared to the control.

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Chapter 2 Follicular Dendritic Cells, HIV and Prostaglandins

2.1 Abstract

Current antiviral therapy for HIV/AIDS, can reduce the amount of virus in the blood to undetectable levels. Nonetheless, virus still persists in long-term "reservoirs" such as the follicular dendritic cell (FDC) and the latently infected T cell, creating barriers to eradication. Because FDCs produce prostaglandins (PGs), we postulated that these molecules might inhibit virus replication of HIV-infected $CD4^+$ T cells. To test this hypothesis, we cocultured *ex vivo* infected $CD4^+$ T cells with FDCs, their supernatant and with PGs and monitored the production of HIV. We also examined the affect of PGs on activation-induced cell death (AICD). We found that PGE_2 and PGI_2 analogs inhibited cellular proliferation and AICD of HIV-infected $CD4^+$ T cells in a dose- and time-dependent manner. PGs did not affect HIV integration, but inhibited virus transcription in infected T cells. Indomethacin and CAY10404, cyclooxygenase and selective COX-2 inhibitors, respectively, partially restored HIV production in the presence of FDCs. Therefore we conclude that FDCs appear to block HIV production in $CD4^+$ T cells by producing PGs and these may be able to contribute to the establishment of HIV latency in T cells. A better understanding of the effect of FDC-prostaglandins in HIV infection may lead to novel therapies to interfere with virus production.

2.2 Introduction

2.2.1 Prostanoids

Prostanoids are oxygenated 20-carbon fatty acids, subdivided into prostaglandins (PG), which contain a cyclopentane ring, and thromboxanes (Txs), which contain a cyclohexane ring. PGs are classified into types A to I, where types A, B and C do not occur naturally, but are produced only in extraction procedures. Prostaglandin D (PGD), E (PGE), F (PGF), and I (PGI) are naturally existing prostaglandins.

Prostaglandins (PGs) are lipid mediators derived from endogenous arachidonic acids, and are released from the cell membrane by phospholipase A2 or diacylglycerol lipase upon various physiological and pathological stimuli [7]. Through the sequential actions of the cyclooxygenase (COX) enzymes, arachidonic acid is first converted to PGG_2, which is then peroxidized to PGH_2 (Figure 1). COX includes two isoforms, COX-1 and COX-2. COX-1 is constitutively expressed [1], and is a key player in the regulation of important human physiology including diverse processes such as normal renal function and platelet aggregation [2, 3]. COX-2, on the other hand, is induced in cell types that are treated with mitogens (polyclonal cell activators) and proinflammatory agents at sites of inflammation [4-6]. Following COX enzymatic activities, PG synthesis is then completed by cell specific synthases [7].

2.2.2 PGE_2 and PGI_2

Prostaglandin E_2 (PGE_2) participates in immunity by inhibiting T cell proliferation [8], protecting from T-lymphocytes from T cell receptor (TCR)-mediated AICD [9] and “deviating” T cells toward T helper 2 responses by inhibiting the production of T helper 1 cytokines [10]. PGE_2 is a proinflammatory molecule and regulates production of many cytokines, including IL-6,

IL-10 and TNFα [11, 12]. The diverse and sometimes functionally opposing effects of PGE_2 suggested the existence of multiple cellular and tissue receptor subtypes. The existence of four receptors, EP_1, EP_2, EP_3 and EP_4, was confirmed by molecular cloning. EP_1 receptor most likely mediates G_q activation, which leads to a phospholipase C (PLC) dependent calcium release and protein kinase C (PKC) activity [13]. EP_2/EP_4 receptor activates G_s, which stimulates adenylate cyclase activity, leading to cyclic AMP (cAMP) production [14] and thus activation of the cAMP-dependent protein kinase, also known as protein kinase A (PKA) (Figure 2). The PGE_2 induced cAMP production impairs mitogen activated protein (MAP) kinase activation through direct inhibition of the MAP kinase kinase activator Raf [15]. EP_3 activation generally inhibits adenylate cyclase via activation of the pertussis toxin-sensitive G-protein G_i [16]. Because of decreased cAMP levels, the activation of the EP_3 receptor results in MAP kinase activation [17].

PGI_2, which is released by healthy endothelial cells, inhibits platelet aggregation and induces local vasodilatation [52]. On the other hand, PGI_2 counteracts TxA_2, which is a vasoconstrictor and initiator of platelet aggregation. These two opposing effects of PGI_2, suggest a mechanism of cardiovascular homeostasis between the two in relation to vascular damage. The cellular signaling initiated by this class of compounds is not well understood. However, it was show that the interaction of PGI_2 with its receptor IP stimulates G_s [18]. This stimulation, in turn, signals adenylyl cyclase to produce cAMP, followed by activation of PKA [19].

2.2.3 FDCs and GC

FDC-trapped antigens are periodically released into the microenvironment of the germinal center (GC) where they are endocytosed, processed and presented to antigen-specific GC T cells by antigen specific GC B cells. These interactions result in the generation of the

germinal center reaction that produces a population of antibody forming cells and B memory cells [20-24]. Thus, trapped antigens on FDCs form a repository of immunogenic immune complexes that is important for the long-term maintenance of humoral immunity to T cell dependent antigens. In addition, FDCs contribute signaling that increase the lifespan of both normal B lymphocytes and lymphomatous lymphocytes [25-28]. However, in HIV disease, FDCs are converted into a dangerous repository of infectious viral particles.

In contrast to the minute quantities (i.e., picogram amounts) of soluble antigens typically present on FDCs, HIV (as immune complexes) is trapped in enormous quantities estimated at ~$1x10^8$ copies of viral RNA per gram of lymphoid tissues [29]. This repository of virus is adjacent to GC $CD4^+$, HIV-coreceptor $^+$ T cells. FDC-trapped virus readily infects these and other $CD4^+$ T cells, and remarkably, this occurs even in the presence of high levels of neutralizing antibody [30]. Furthermore, FDCs maintain the infectious nature of HIV for months and years in the complete absence of viral infection and/or replication [31, 64]. In addition, FDCs increase the expression of the HIV co-receptor CXCR4, which dramatically increases the sensitivity of these T cells to infection by X4-tropic HIV even with limiting amounts of virus [32, 33]. Therefore, FDCs not only serve as an important reservoir of infectious virus but also contribute to a unique microenvironment that is ideal for HIV transmission and persistence.

2.2.4 FDC and PGs

Throughout the natural course of HIV/AIDS, a main site of virus infection is in and around the GCs of secondary lymphoid tissues where FDCs reside. The GC is a microanatomic site for the generation of memory B cells and plasma cells with high affinity antigen receptors, surrounded by the follicular mantle zone [34]. The GC is organized into two main compartments

termed the dark and the light zones. The dark zone is rich in actively dividing centrobasts that differentiate into centrocytes in the light zone [35, 36]. In addition to the major cellular component of the light zone (i.e., centrocytes), it also contains FDCs [37, 38]. In addition T cells are found in this location and represent approximately 10% of the total cells in the light zone [39]. Although FDCs play central roles in B cell survival, proliferation, and differentiation into memory cells, the regulatory role of FDCs upon T cells is unclear [40-43]. PGE_2 inhibits mature T cell proliferation and protects T cells from AICD [44, 45]. Heinen E. previously reported that FDCs within lymphocyte clusters are capable of producing PGE_2 [46]. Human FDCs and an FDC-like cell line, HK, express functionally active COXs, PGE and PGI synthases [47], indicating that FDCs are fully equipped to produce the major PGs. Therefore, we postulate that FDC-produced PGs could inhibit HIV replication in $CD4^+$ T cells.

The research presented in this dissertation indicates that PGE_2 and the PGI_2 analog, Beraprost can inhibit the proliferation and AICD of *ex vivo* HIV-infected T cells. The ability of indomethacin or CAY10404 to partially restore HIV production in the presence of FDCs lends support to the hypothesis that FDC-mediated inhibition is facilitated by production of PGs. These findings are consistent with the observation that GC T cells have a high frequency of infection without virus gene expression. FDCs appear to block HIV production in $CD4^+$ T cells by producing PGs and these may be able to contribute to the establishment of HIV latency in T cells. A better knowledge of FDC contributions to virus transmission and disease progression will provide us a better understanding of HIV pathogenesis and may aid in the design of potential treatments that can specifically target HIV reservoirs.

2.3 Materials and Methods

2.3.1 Virus preparations

Viral stocks for infection were prepared by propagating HIV-1_{IIIB} in neoplastic H9 cells. Virus was harvested at the time of peak p24 and/or reverse transcriptase production, pooled, filtered through a 0.20 µm membrane, and stored in aliquots in liquid nitrogen until used. Our HIV_{IIIB} preparations typically contained 1 µg/mL of p24 and $1x10^6$ cpm reverse transcriptase activity [65].

2.3.2 Flow cytometric analysis

Cell surface antigens were detected using the following monoclonal antibodies: anti-human CD3-PC5 (UCHT1), anti-human CD4-PC5 (13B8.2), anti-human CD8-FITC and anti-CD69-PE (TP1.55.3; Immunotech, Westbrook, ME); anti-human CXCR4-PE (12G5; BD PharMingen, San Diego, CA); mouse IgM, anti-human FDC (HJ2; gift from Dr. M. Nahm, University of Alabama, Birmingham, AL). Mouse isotype-matched IgG1 (679.1 Mc7) and IgG2a (U7.27; Immunotech) were also used as controls. Isolated CD4+ T cells from human blood were incubated for 30 min on ice first with Fc-blocking reagent (Miltenyi Biotec, Auburn, CA) and then with specific mAbs. The cells were then washed in cold PBS, and 10,000 $CD4^+$ T cells were analyzed using a FACSVantage SE flow cytometer equipped with the FACSDiVa option (BD Biosciences, San Jose, CA). Positive gating was established using isotype-matched control Abs to define background fluorescence.

2.3.3 HIV infection of CD4+ T cells

$CD4^+$ T cells were centrifuged and the medium was removed, after which the cells were resuspended in complete medium (with 20% fetal bovine serum) containing HIV-1_{IIIB} stock virus at a concentration of 50,000 cpm reverse transcriptase activity per $1x10^6$ cells. The virus-cell suspension was incubated at 37°C for 2 hr, after which the cells were washed to remove the infecting inoculum. The resulting infected cells were cultured with or without PGs or FDCs under the conditions described for each experiment.

2.3.4 FDC isolation

Human FDCs were isolated from tonsillar tissues as previously described [48]. Briefly, tonsils were cut into small pieces and incubated with a digestion cocktail of Blendzyme (Roche Applied Science; Indianapolis, IN), and DNaseI (Sigma, St. Louis, MO) for 45 min. Tissue and cells were separated by pipetting the tissue multiple times through a 25ml sterile pipette and collecting the medium which contained the released cells. This step was repeated twice. The resulting single cells were applied to pre-formed discontinuous (15%, 35% and 50%) Percoll gradients (GE Healthcare). The low density tonsillar cells (1.050-1.060 g/ml) were collected after centrifugation, washed and incubated with heat-aggregated goat ChromePure (Jackson ImmunoResearch Laboratories) IgG for 15 min to block non-specific Fc receptor binding. The FDCs were then labeled with mouse IgM, anti-human FDC mAb, HJ2, (250 μl hybridoma supernatant; kindly provided by Dr. M. Nahm, University of Alabama at Birmingham) on ice overnight followed by secondary goat $F(ab')_2$ anti-mouse IgM-FITC (γ chain specific, 25 μg) incubation for 1h. FDCs were sorted on $HJ2^{Hi}$ events (this population typically ranged from 1 to 3% of the total population post-Percoll). To obtain FDC-supernatant, FACS isolated FDCs were

cultured in CM for 6 days at a concentration of 1 x 10^6 cells per milliliter. At the end of the culture period, the supernatant fluid was collected, centrifuged (300 x g for 10 min) to remove cell debris, filtered through a 0.20 μm filter and then used immediately or stored at -80˚C. As a control, supernatant fluid was also obtained from equal numbers of FACS-sorted HJ2-negative cells. In all experiments, an FDC to $CD4^+$ T cell ratio of 1:10 was used for optimal FDC-lymphocyte interactions [32].

2.3.5 $CD4^+CD69^+$ T cell isolation

Primary $CD4^+$ T cells were obtained from human tonsils or the peripheral blood. When cells were obtained from the tissues, human tonsils were cut into small pieces and cells were mechanically separated from tissue by repeat pipetting. Cells were washed and then labeled using the Miltenyi $CD4^+$ T cell negative isolation kit as per the direction insert. After negative selection using an autoMACS, the enriched cells were labeled with anti-CD3-PC5, anti-CD8-FITC and anti-CD69-PE antibodies followed by FACS sorting. Isolation of peripheral blood mononuclear cells used similar enrichment/purification techniques after first applying whole blood to Ficoll (GE Healthcare) and centrifuging (300 × g) to obtain the mononuclear cells. The resulting $CD4^+CD69^+$ T cell preparations were ≥98% pure as assessed by flow cytometry.

2.3.6 ELISA for HIV P24

Culture supernatants were assayed for p24 production by an HIV-1 p24 antigen ELISA (ZeptoMetrix Corporation, NY), according to the manufacturer's instructions. Briefly, viral antigen in the specimen was specifically captured onto the immobilized antibody during specimen incubation. The captured antigen was then reacted with a high-titered human anti-HIV-

1 antibody conjugated with biotin. Following a subsequent incubation with streptavidin-peroxidase, color developed as the bound enzyme reacted with the substrate, followed by addition of Stop Solution (2 N H_2SO_4). Absorption was measured at 450 nm using a Vmax Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA). The absorbance of the samples was compared to a standard curve to determine the picograms p24 per milliliter of culture fluid.

2.3.7 ELISA for PGE_2

Cell-free FDC and macrophage supernatants were obtained from different individuals and were assayed for PGE_2 using an ELISA kit (R&D Systems, Minneapolis, MN) as instructed in the direction circular. The optical density of each reaction was determined on a Vmax kinetic microplate reader (Molecular Devices, Sunnyvale, CA) at 450 nm with wavelength correction at 570 nm. Each well was assayed in duplicate and averaged. With the generation of a standard logarithmic curve, the direct concentration in pM PGE_2 could be determined. Values were expressed in pM prostaglandin on triplicate samples.

2.3.8 Integrated virus analysis

Genomic DNA from 10^6 cells was isolated using a DNeasy blood and tissue kit (QIAGEN, Valencia, CA). Integrated viral DNA was detected by a modified nested Alu-LTR polymerase chain reaction (PCR) as previously described [19, 20]. The first round of amplification was carried out in a 50 μl reaction mixture containing 250 ng DNA template, 3.3×buffer, 0.2mM dNTP, 1.2mM MgOAc, 25 pmol PBS primer and 25 pmol Alu 164 primer, and 1.6U rTth polymerase XL. The amplification reaction was as follows: 94°C for 5 min, 18 cycles of 94°C for 30s, 66°C for 30s, 70°C for 5 min, and a final extension at 72°C for 10 min.

The reaction products were analyzed using an ABI Prism 7500 Instrument (Applied Biosystems, Foster City, California). These reactions contained 2×Quantitee, 300 nM forward primer, 300 nM reverse primer, 100 nM primer probe and 100-500ng template DNA from the first round PCR and was performed in a 50 μl volume. After the initial incubation at 50°C for 2 min and 95°C for 10 min, 40 cycles of amplification were carried out at 95°C for 15 s, followed by 60°C for 1 min.

2.3.9 RNA isolation and real time, quantitative PCR assay for multiple spliced HIV

Immediately after cell culture, an equal number of T cells were centrifuged and resuspended in 800 μl RNA STAT60 (Tel-Test, Friendswood, TX). To each of the samples, 160 μl of chloroform (Fisher Scientific, Pittsburgh, PA) was added and the solution was then vortexed and centrifuged at 13,000 x g for 15 min at 4 °C. An equal volume of the aqueous phase (350 μl) from each sample was transferred to a fresh tube containing the same volume of isopropanol, and the contents were vortexed, incubated at -20°C for 30 min, and centrifuged at 13,000 x g for 30 min at 4 °C. After discarding the supernatant fluid, the RNA pellet was washed once in 75% ethanol and air dried, and the samples were resuspended in a volume of 20 μl in RNase/DNase-free water (Invitrogen Life Technologies). RNA from the samples was subjected to DNase treatment (Ambion, Austin, TX) to remove contaminating DNA. The isolated RNA was then reverse transcribed into cDNA using random primers (Invitrogen) and SuperScript III reverse transcriptase (Invitrogen).

Quantitation of the cDNA template was determined using real-time, quantitative PCR (TaqMan) with an ABI Prism 7500 instrument (Applied Biosystems, Foster City, Calif.) using the following primers and probe (Applied Biosystems): forward primer, 5'-CGAAGAGCTCATCAGAACAGTCA-3'; reverse primer, 5'-TTGGGAGGTGGGTCTGCTT-3'; and probe, 5'-CTTCTCTATCAAAGCAGACCCACCTCC-3' (FAM-TAMRA). The quantitative PCR conditions were as follows: 1 cycle at 50°C for 2 min and 1 cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The primers were used at 300 nM each, and the probe was used at 250 nM. The housekeeping gene, glyceraldehyde phosphate dehydrogenase (*GAPDH*), was used as a normalization control.

2.3.10 Statistical Analysis

Analysis of the data was performed using a one-tailed Student's t-Test. Values of $p \leq 0.05$ were considered significant. Unless specifically stated, the error bars indicate the S.E.M.

2.4 Results

2.4.1 PGE_2 and Beraprost inhibit HIV-infected $CD4^+$ T cell virus production and cell proliferation but not cell survival

Because FDCs are capable of producing PGs (especially PGE_2 and PGI_2) [46, 47], we first determined whether PGE_2 and the stable PGI_2 analog, Beraprost affected HIV production by *ex vivo* infected tonsillar $CD4^+CD69^+$ T cells (Figure 3). Both PGE_2 and Beraprost significantly inhibited HIV production in a dose dependent manner. For instance, at 0.1μM PGE_2, HIV production was inhibited by 42%, compared to HIV production in the absence of PGE_2. As the concentration of PGE_2 increased to 10 μM, HIV production decreased by 60%. Similar results were obtained using Beraprost at concentrations ranging from 0.1μM to 10 μM. As expected, the stable but inactive metabolite 6-keto-$PGF_{1\alpha}$, did not affect HIV-1 production.

We next determined the effect of PGE_2 and the stable PGI_2 analog, Beraprost on HIV-infected tonsillar T cell tritiated thymidine (^{3}H-TdR) incorporation as a measure of synthesis of new DNA (reflective of activation/proliferation). Both PGE_2 and Beraprost inhibited HIV-infected T cell incorporation of ^{3}H-TdR. The inhibition by PGE_2 appeared greater than that observed with Beraprost when compared at the same molar concentration (Figure 4). Because both PGE_2 and PGI_2 bind to distinct receptors to repress the Mitogen Activated Protein Kinase (MAP) pathway by inducing upregulation of cAMP and the subsequent activation of protein kinase A (PKA) (52), we added the adenylate cyclase and PKA antagonists, SQ22536 and H89 respectively, and found they partially reversed the inhibitory effect of PGs on T cell proliferation (Figure 5). The control, 6-keto-$PGF_{1\alpha}$, did not display any inhibitory effect on T cell proliferation, indicating the specificity of PGE_2 and Beraprost.

In vivo, FDCs, T cells and the antigen presenting GC B cell reside in close proximity within the lymphoid follicle. Because T cells may receive continued stimulation from B-cell presented Ags in the GC, we reasoned that this could induce AICD and determined the ability of PGE_2 and PGI_2 to modulate this effect. AICD was induced in HIV-infected tonsillar $CD4^+$ T cells as described and the level of AICD was assessed by flow cytometry (Figure 6). The inhibition of AICD by both PGE_2 and Beraprost was dose-dependent and as before, the inactive PG 6-keto-$PGF_{1\alpha}$ failed to show any modulating effect.

Because both PGE_2 and Beraprost were able to repress the activation/proliferation of HIV-infected T cells, we also analyzed the activation markers, HLA-DR, CD25, CD45RO and CD95 to determine whether the PGs induced any phenotypic changes in the HIV-infected T cells (Figure 7). As expected, the addition of PGE_2 or Beraprost decreased the expression of activation markers (HLA-DR, CD25, CD45RO and CD95) on the infected T cells and this occurred in a dose dependent manner. As shown in Figure 7, the addition of 0.1μM PGE_2 to the cultures resulted in a decrease of HLA-DR expression by 30%, CD25 expression by 25% and CD45RO expression by 45% and these values increased with increasing PGE2 dose. Both PGE_2 and Beraprost induced only slight decreases in CD95 expression. Thus it appears that both PGE_2 and Beraprost not only increase cell viability but also decrease the activation state of the HIV-infected T cells.

2.4.2 Effect of PGE_2 and Beraprost on HIV integration and transcription in HIV-infected T cells

Because PGE_2 and Beraprost alter the activation state of infected $CD4^+$ T lymphocytes, we next determined whether these PGs affected either HIV infection itself or virus replication.

The effects of PGE_2 and Beraprost on HIV integration were determined by quantitative Alu-LTR PCR (Figure 8A). The addition of PGE_2 or Beraprost immediately after a two-hr incubation with infectious HIV had no detectable effect on viral integration. In contrast, when we assessed active viral infection as determined by the presence of multiply spliced HIV RNA transcripts, these were inhibited by about 20% with Beraprost and 30% by PGE_2 (Figure 8B).

2.4.3 Effect of FDC-produced PGs on HIV production

Because FDCs within lymphocyte clusters are capable of producing PGE_2 [46], and FDCs strongly express functionally active PGE synthase [47], we examined the amount of PGE_2 production from human FDCs obtained from different tonsil donors (Figure 9A). Tonsillar FDCs produced between 270 and 810 pM PGE_2 per milliliter of tissue culture fluid after 6 days of incubation suggesting that FDC-produced PGE_2 is present in sufficient quantity to potentially decrease the amount of virus production that is possible in the GC. We further reasoned that if FDC-produced PGs decreased virus production in cultures of infected $CD4^+$ T cells, then the addition of PG inhibitors would result in an increase in the amount of virus production in the presence of FDCs. We therefore cultured infected T cells with FDCs in the presence of indomethicin or CAY10404 to block PG production by the FDCs (Figure 9B). The addition of either of these inhibitors increased HIV production by infected $CD4^+$ T cells, compared to cultures without indomethicin or CAY10404. Collectively, these results suggest that FDC produced PGs have the potential to exert an inhibitory affect upon virus production and the agents that decrease the production of these molecules by FDCs may have potential to be therapeutic.

2.5 Discussion

FDCs are in the lymphoid follicles of all secondary lymphoid tissues. In these sites, they interact with lymphocytes by contacting them directly or by producing diffusible molecules. Human FDCs express PGE synthase and PGI synthase in addition to COXs [47, 53]. Furthermore, FDCs within lymphocyte clusters have been reported to produce PGE_2 [46]. PGs have been suggested as inflammatory and vascular modulators of immunity [54-56], but their effects on HIV latency have not been previously studied. Based upon the results presented above, our data suggest that FDC-produced PGs can decrease HIV production while potentially increasing the lifespan of T cells that are continually undergoing antigen activation.

Significant information is now available about the interactions between FDCs and B lymphocytes. The observation that FDCs trap antigens externally and are not able to process or present antigens to T cells led to the rationale that FDCs and T cells were unlikely to have significant interactions. As a result, an understanding of FDC-T cells interactions is in its infancy. FDC-T cell interactions appear to involve both direct cell-to-cell contacts and the production of diffusible cytokines. Butch et al. reported that FDCs inhibited T cell proliferation by two distinct mechanisms. One mechanism involves cellular interactions between CD54 and CD106. Interestingly, CD54 and CD106 are also critical for B cell binding to FDCs. The other mechanism involves FDC production of cytokines. FDCs can either induce or inhibit B cell proliferation depending on the B cell activating agent used [62, 63] suggesting the ability to participate in the fine regulation of the GC response to antigen stimulation.

Much of the understanding we have about the interplay between FDCs and T cells comes from the study of HIV. Our observation that PGE_2 and Beraprost inhibited HIV-infected T cell

activation/proliferation extends the previous report that PGs are involved in FDC-mediated inhibition of T cell proliferation [57]. Basselin et al. reported that in HIV-1 transgenic rats, arachidonic acid metabolism was significantly elevated [58]. These findings may help explain why T cells constitute only a minor population of the GC cells (5%~10%) compared with B cells (90%) in the GC. This may also explain why GC T cells are defective in IL-2 production in response to Ag stimulation [59]. Castan et al. showed that 70%-90% GC T cells express the inhibitory receptor for CD28, CTLA-4 [60], indicating that the number of GC T cells is tightly regulated by FDC and B cells. T cells do not produce PGs [61], but the possibility that T cells produce PGs as a result of interaction with FDCs can not be ruled out. It should be noted that in our studies FDCs produced between 270 and 750 pM PGE_2 per milliliter of tissue culture fluid. The potency of the FDC-PG signal to adjacent T cells may further be strengthened beyond that seen *in vitro* because FDCs "embrace" adjacent cells potentially concentrating and focusing this signal. Moreover, *in vivo*, other PGE_2-producing cells, including macrophages (especially tingible body macrophages) could act in concert with the FDCs in creating a microenvironment that is especially suited to the persistence of HIV-infected T cells in the GC. Consistent with this hypothesis is the observation that FDC-T cell interactions induce the expression of Regulators of G-protein Signaling 13 and 16 [33] that block the ability of GC T cells to migrate to CXCL12 signals present outside the GC thereby inhibiting their "escape" from this location. Of further support for the postulate that FDCs could contribute to the persistence of HIV infection in the GC is the observation that FDCs increase T cells production of the HIV coreceptors CXCR4 and this increased expression makes these cells highly susceptible to infection by X4-tropic virus [32]. The production of PGs by FDCs seems therefore to be one more means whereby FDCs can contribute to virus pathogenesis to the detriment of the host.

Additional studies are underway to determine whether cellular interactions are involved in the FDC inhibition of HIV infected T cells. Insight into mechanisms used by FDCs that regulate HIV infected T cells can lead to a better understanding of latent T cell reservoir and may further aid in the design of better intervention strategies target this reservoir.

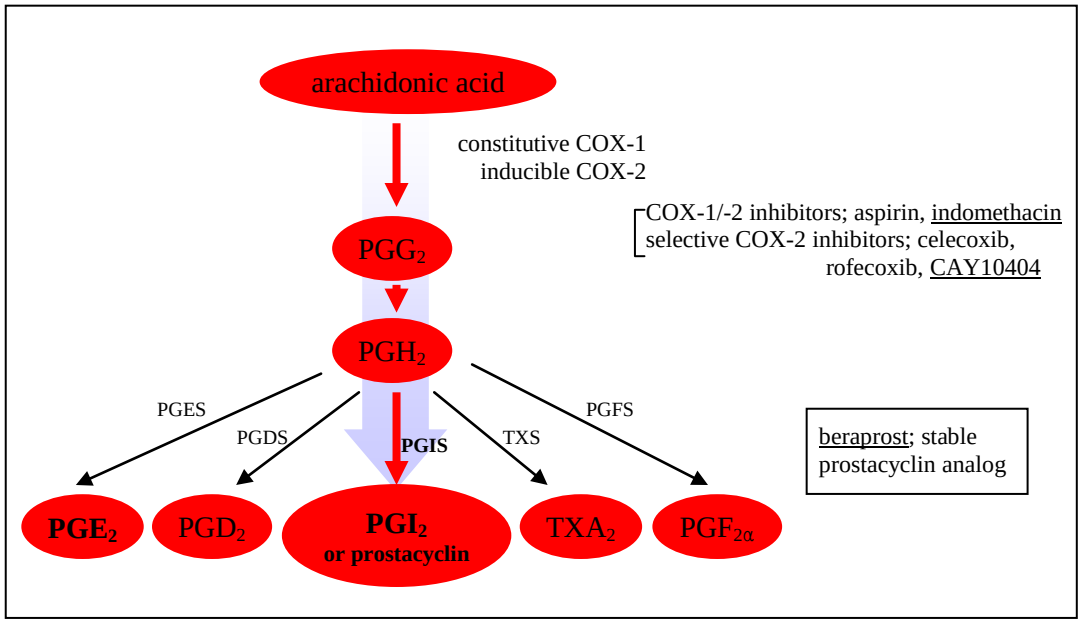

Figure 1 Biosynthetic pathways of prostanoids.
COX: cyclooxygenases; PG: prostaglandin; PGES: prostaglandin E synthase; PGDS: prostaglandin D synthase; PGIS: prostaglandin I synthase; TX: thromboxane; TXS: thromboxane synthase; PGFS: prostaglandin F synthase.

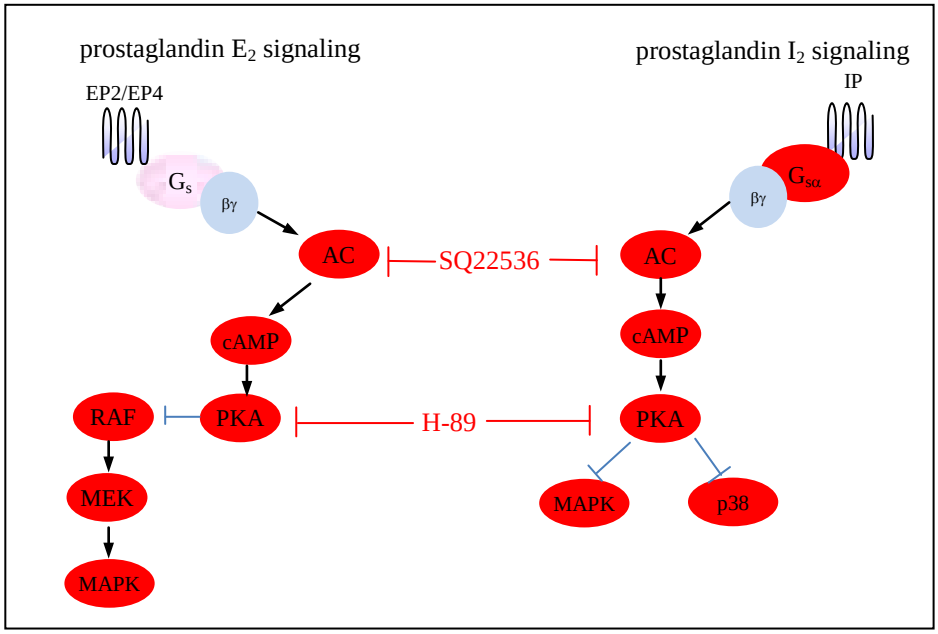

Figure 2 The signaling pathway of PGE_2 and PGI_2.
EP2/EP4: PGE receptors; IP: PGI receptor; AC: adenylate cyclase; cAMP: cyclic adenosine monophosphate; PKA: protein kinase A; MAPK: mitogen activated protein kinase; MEK: MAP kinase kinase. SQ22536: AC inhibitor; H-89: PKA inhibitor.

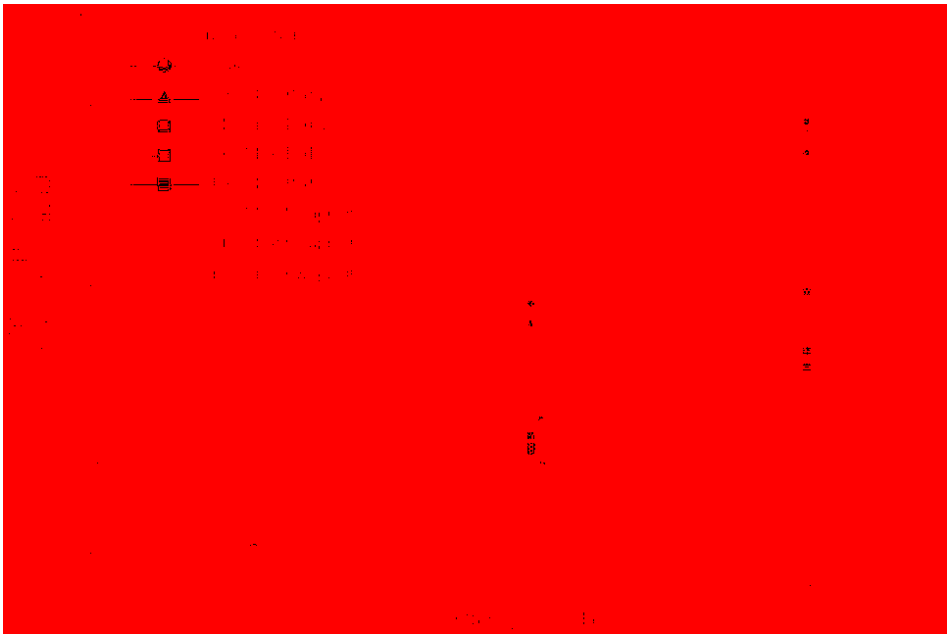

Figure 3 PGE_2 and Beraprost inhibit HIV production of T cells. Tonsillar $CD4^+$ $CD69^+$ T cells were infected with HIV for 2 hrs. Then 4 X 10^5 cells were stimulated with 5μg/ml of PHA in the presence or absence of 0.1, 1.0 or 10 μM concentration of PGE_2 or Beraprost up to 9 days assayed for p24 produced. Error bars represent mean ± SEM of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with the control (vehicle).

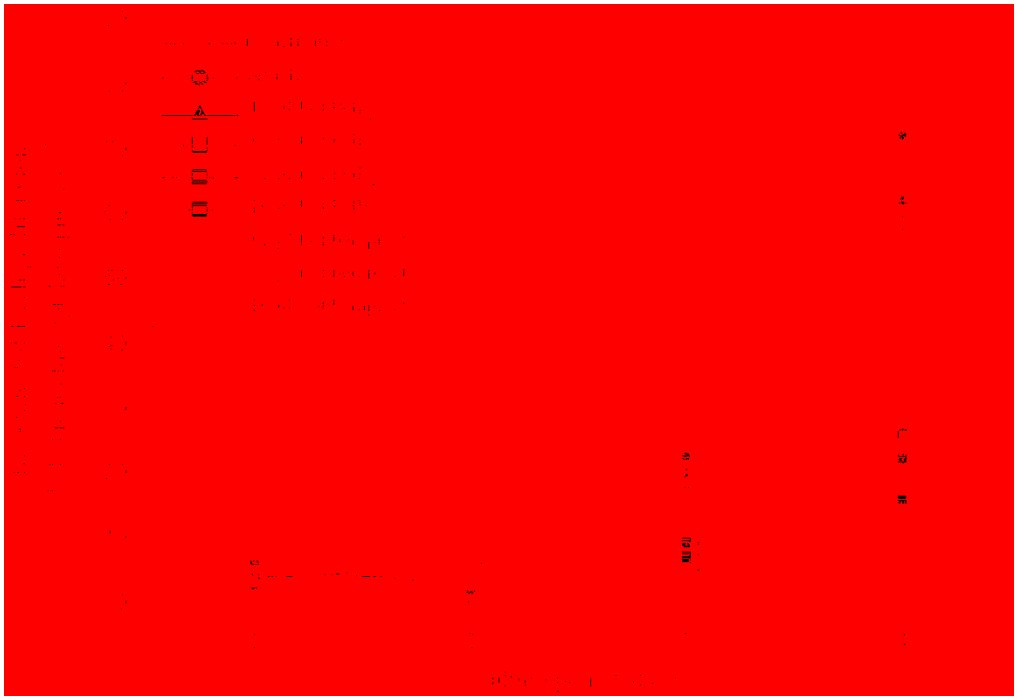

Figure 4 PGE_2 and Beraprost inhibit proliferation of HIV infected T cells. Tonsillar $CD4^+$ $CD69^+$ T cells were infected with HIV_{IIIB} for 2hrs. Then 1 X 10^5 cells were stimulated with 5µg/ml of PHA in the presence or absence of PGE_2 or Beraprost up to 4 days. 1µCi of [^{3}H] thymidine were added during the last 12 h culture period. Dose and time response experiments are shown. Error bars represent mean ± SEM of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared to the control (vehicle).

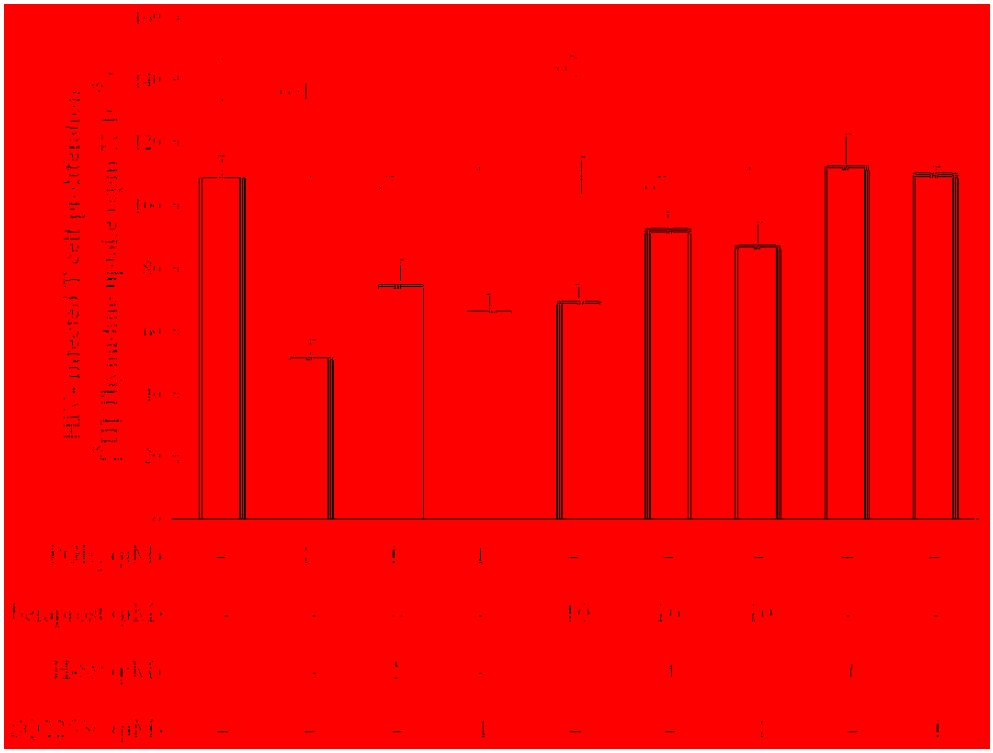

Figure 5 PGE_2 and Beraprost repress proliferation of HIV infected T cells. Tonsillar $CD4^+$ $CD69^+$ T cells were infected with HIV_{IIIB} for 2hrs. After preincubation with H-89 or SQ22536 for 30 min, the HIV-infected tonsillar T cells were treated with the indicated amount of PGE_2 or Beraprost for 4 days. 1μCi of [^{3}H] thymidine were added during the last 12 hrs culture period. Error bars represent mean ± SED for each group (n =3); *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Error bars represent mean ± SEM of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

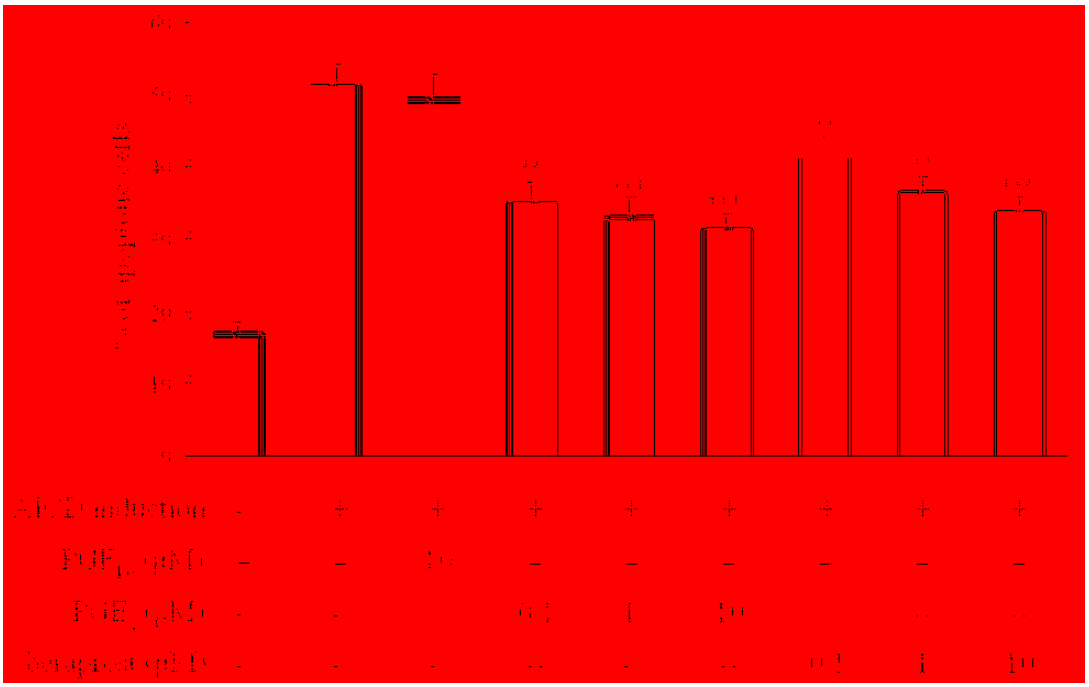

Figure 6 PGE_2 and Beraprost partially suppress the AICD of T cells. Purified tonsillar T cells (1 X 10^7 cells) were cultured in a 60-mm dish with immobilized anti-CD3/anti-CD28 Ab in the presence of IL-2 (20U/mL) for 3 days. Viable cells were purified by Ficoll density-gradient centrifugation, infected by HIV-1 for 2h and incubated with IL-2 alone for additional 48 h, followed by a 12 hrs incubation with immobilized anti-CD3 and anti-CD28 Ab in the presence or absence of indicated PGs (0.1-10 μM). Viability of T cells was measured by a flow cytometer after staining with annexin V and PI. Error bars represent mean ± SEM of three independent experiments. **, $p < 0.01$; ***, $p < 0.001$ compared to the control (AICD induction).

Figure 7 PGE_2 and Beraprost decrease activation-induced phenotypes of HIV-infected T cells. Tonsilar $CD4^+$ $CD69^+$ T cells were infected with HIV_{IIIB} for 2h. Then 4 X 10^5 cells were stimulated with 5μg/ml of PHA in the presence or absence of PGE_2 or Beraprost for 3 days. The expression of HLA-DR (A), CD25 (B), CD45RO (C) and CD95 (D) were measured by flow cytometer. Error bars represent mean ± SEM of three independent experiments; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared to the control (vehicle).

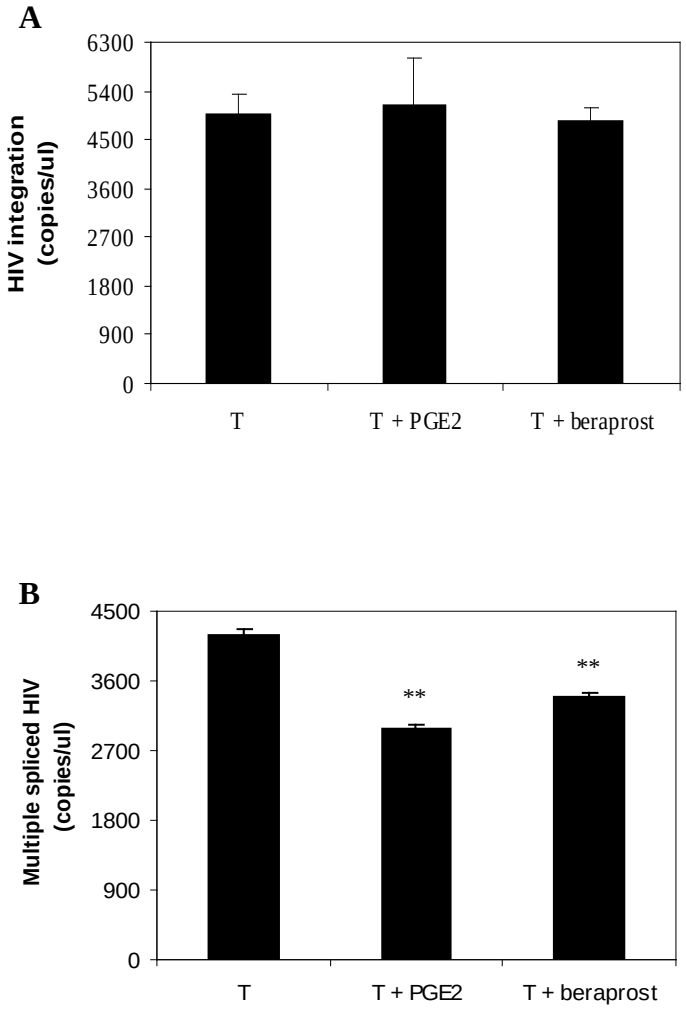

Figure 8 PGE_2 and Beraprost have no effect on HIV integration, but inhibit transcription of HIV-infected T cells. Tonsillar $CD4^+CD69^+$ T cells were infected with HIV-1 for 2hrs. After extensive washing, 4 X 10^5 cells were cultured with prostaglandin E_2 or Beraprost (0.1 μM) in 48-well plate for 2 days. (A) Alu-PCR was performed to determine the HIV integration. (B) Multiple spliced HIV was determined using real time PCR. **, $p < 0.01$ compared to the control (T).

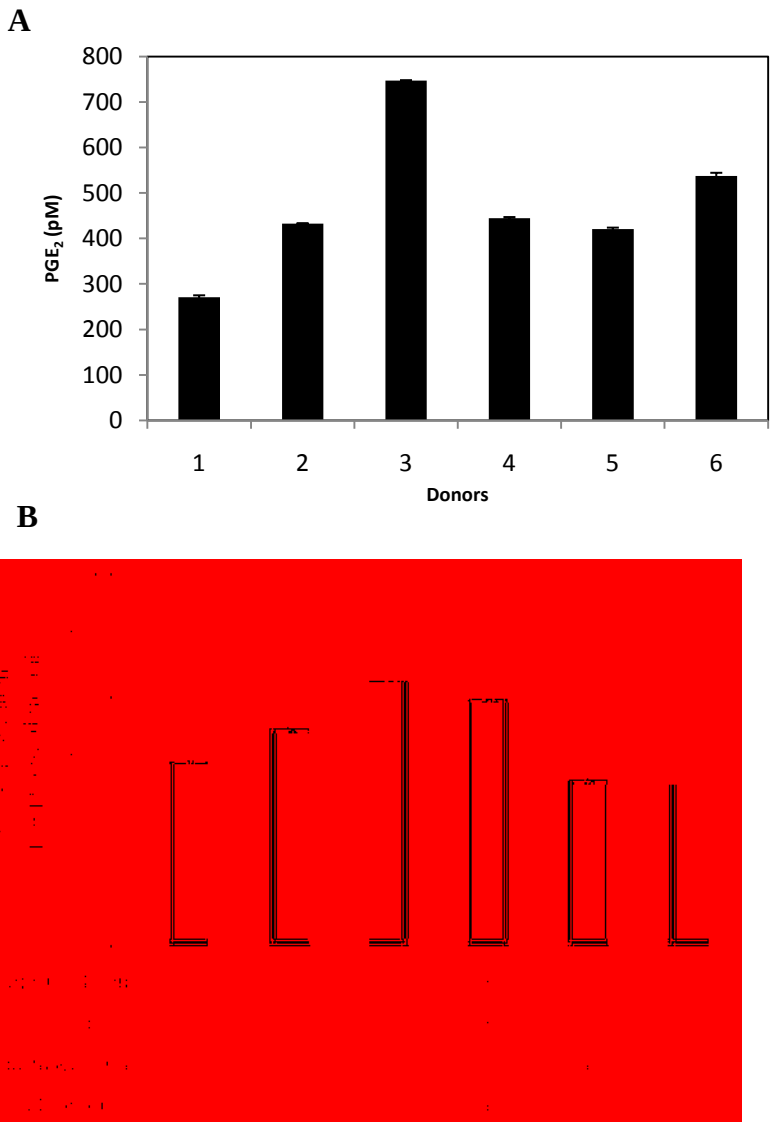

Figure 9 Prostaglandin produced by freshly isolated FDC inhibits HIV production. (A) FDCs produce PGE_2. FACS-sorted FDCs (1x 10^6/mL) from six donors were cultured for 6 days, after which the culture supernatant fluid was examined for PGE_2 production using ELISA. Data are expressed in picograms PGE_2 per milliliter of culture fluid and represent the means ± SEM for duplicate wells. (B) Tonsillar $CD4^+$ $CD69^+$ T cells were infected with HIV for 2hrs, stimulated with 5μg/ml of PHA in the presence of IL-2 (20U/mL). FDCs (4×10^4 cells/well) were incubated with 1μM indomethacin or CAY10404 for 30 min, followed by a further incubation with T cells (4×10^5 cells) in the presence of indomethacin or CAY10404 for 9 days. The concentration of p24 was measured by ELISA. Error bars represent mean ± SEM of three independent experiments; *, $p < 0.05$.

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ABBREVIATIONS

Ab, antibody; **AAT**, alpha-1-antitrypsin; **AC,** adenylate cyclase; **Ags**, antigens; **APC**, antigen presenting cells; **AICD,** activation-induced cell death; **CO**X, cyclooxygenase; **CM**, complete medium; **FBS**, fetal bovine serum; **CXCR4**, CXC chemokine receptor 4; **FDCs**, follicular dendritic cells; **GC**, germinal center; **GFP**, green fluorescence protein; **GPCR**, G-protein coupled receptor; **HAART**, highly active antiretroviral therapy; **HIV**, human immunodeficiency virus; **Jaks**, Janus kinases; **hTNFR-Ig**, human TNFR-immunoglobulin; **ICs**, immune complexes; **MAP**, mitogen activated protein; **NFAT**: Nuclear Factor of Activated T cells; **PKC**, protein kinase C; **PKA**, protein kinase A; **PHA**, phytohaemagglutinin; **PBMC**, peripheral blood mononuclear cells; **PMA**: phorbol myristate acetate; **PG**, prostaglandin; **RGS**, regulator of G-protein signaling; **SDF-1**, stromal-cell derived factor 1 or CXCL12; **STAT**, signal transducers and activators of transcription; **TCR**, T cell receptor; **^{3}H-TdR**: tritiated thymidine incorporation; **Tx**, thromboxane; **TNF-α**, tumor necrosis factor alpha.