2006

Susceptibility of Primary Eosinophils to Infection with HIV-1 Strain HTLV-IIIB

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Susceptibility of Primary Eosinophils to Infection with HIV-1 Strain HTLV-IIIB

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By

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M.B.B.S. Nagpur University, 2001

2006

Wright State University

WRIGHT STATE UNIVERSITY

SCHOOL OF GRADUATE STUDIES

September 11, 2006

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Jai G. Marathe ENTITLED Susceptibility of Primary Eosinophils to Infection with HIV-1 Strain HTLV-IIIB BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Marathe Jai G. M.S., Program in Microbiology and Immunology, Wright State University, 2006. Susceptibility of Primary Eosinophils to Infection with HIV-1 Strain HTLV-IIIB.

Over the past two decades, much research has been done in the field of human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS). However, many of the aspects of pathogenesis of HIV infection and its persistence in the body, despite treatment, remain a mystery. Recent evidence suggests that HIV positive patients develop eosinophilia, especially in the later stages of infection and AIDS. Eosinophils are CD4 positive cells that have the potential to be infected by HIV. Studies have shown that an eosinophilic cell line, AML14.3D10, can be productively infected with a T-cell tropic, CXCR4-using (X4) strain of HIV-1. In this study, primary human eosinophils from four healthy volunteers were shown to be susceptible to infection with a T-cell tropic, CXCR4-using (X4) strain of HIV-1, HTLV-IIIB. This data was supported by results from quantitative polymerase chain reaction (Q-PCR), which detected high HIV copy numbers in

infected eosinophil samples. In two out of four donors, these copy numbers were comparable to those obtained from infected AML14.3D10, used as a positive control. In all four donors, the number of viral copies detected in infected eosinophils were significantly ($p<0.05$) higher than those detected in infected peripheral blood mononuclear cells (PBMCs). Donor variability was observed in viral loads detected. No correlation was observed between the viral load and the production of p24. However, infected eosinophils showed higher amounts of p24 production, as compared to infected PBMCs with or without IL-2, in three out of four donors suggesting productive infection. Therefore, it is concluded that primary human eosinophils are susceptible to productive infection by X4 HIV-1.

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AKNOWLEDGEMENTS

I would like to thank my committee members Dr N. Bigley and Dr. L. Arlian for their support and valuable advice during my project. I would also like to thank Dr. D. Wooley for her constant encouragement, guidance and patience. Above all, I want to thank her for teaching me analytical and critical thinking.

I want to thank all members of Wooley laboratory: Melissa Leaver, Jude Atem and Darshini Trivedi for their friendship and for making the laboratory a great place to work in. I would also like to acknowledge Dr. P. Lauf for his help with translation of a reference written in German.

Above all I would like to thank my parents, my in-laws, Ameet and all loved ones for their love, constant encouragement and support, without whom I could never have successfully completed my project.

I. INTRODUCTION

1. Human immunodeficiency virus (HIV):

1.1. History

A new virus was first isolated and associated with acquired immunodeficiency syndrome (AIDS) in 1983 ((1). Initially called by various names like human T-cell leukemia virus IIIB (HTLV-IIIB) and AIDS-associated retrovirus (ARV), it was subsequently classified as a lentivirus belonging to the family Retroviridae and named human immunodeficiency virus (HIV) (22). Shortly thereafter, another retrovirus isolated from West African patients was recovered which was genetically different from the original HIV-1 and was named HIV-2. Within a few years of the discovery of HIV-1, two different strains were observed. One strain was macrophage-tropic and showed no syncytia formation in the infected culture, these were called as non-syncytia inducing (NSI) strain. The other strain was T-cell tropic and called as syncytia inducing (SI) strain because infected cultures showed multi-

nucleated giant cell formation(5, 14, 40). It was much later that the reason for this became apparent when further research showed that SI viruses used chemokine receptor (CCR5) and NSI viruses used chemokine receptor (CXCR4) to gain entry into the cells(3). Accordingly, these strains are now called as R5 and X4 strains.

HIV1-IIIB is an X4 lab-adapted strain. Culture fluids isolated from bone marrow and peripheral blood of AIDS patients were used to establish a permanent and productive infection in H9 cells. The virus isolated and characterized from this infection was originally termed as HTLV-IIIB. This virus is capable of infecting PBMCs and a number of T-cell lines like CEM, H9, HeLa CD4+ etc. Recently it was shown to infect an eosinophilic cell-line AML14.3D10 (39, 48).

1.2. Lifecycle:

It is an enveloped virus with a diploid single-stranded RNA (ssRNA) genome. The envelope bears viral gp120 glycoprotein, which is antigenic. Interaction between the gp120 and the CD4 receptor molecule (26), along with specific co-receptors such as CXCR4 and CCR5 on the cell surface allow the fusion of envelope with the host cell's membrane (3). This is the mechanism of viral entry into a

susceptible host cell. Once the ssRNA is injected into the host cell, it is converted to double-stranded DNA copy (ds DNA) by the enzyme reverse transcriptase (RT) (24, 51). This dsDNA is integrated into the host genome and is now called provirus. Using the host cell machinery viral genes are transcribed and translated. Transcription of provirus yields both viral mRNA as well as viral genomic RNA. Translation of viral mRNA yields immature proteins which are cleaved by viral proteases to yield mature proteins. Assembly of these proteins around diploid ssRNA strands results in the formation of viral particles which then bud out of the cell acquiring an envelope in this process.

1.3. Human cells infected with HIV:

Earliest studies described HIV infection of peripheral blood mononuclear cells (PBMCs)(1). Knowledge acquired over the past two decades shows that HIV infection is not limited to PBMCs alone and has been shown to occur in a large variety of cells from different organs(22). HIV has been shown to infect cells from various systems like:-

- Hematopoietic system- T-lymphocytes, B-lymphocytes, Macrophages, Stem cells, Dendritic cells
- Nervous system- Astrocytes, Oligodendrocytes, Choroid plexus ganglia cells, Microglia

- Gastro-intestinal system- Columnar and goblet cells, Enterochromaffin cells, Hepatocytes, Kupffer cells
- Genito-urinary system- Renal tubular cells, Cervix derived epithelial cells, Cervix, Prostate, Testes
- Skin and musculo-skeletal system- Langerhans cells, Fibroblasts, Synovial membrane, Osteosarcoma cells
- Other- Myocardium, Retinal cells, Trophoblasts, Fetal chorionic villi, Fetal adrenal cells
- However, eosinophil, an important cell of the immune system has been largely neglected.

2. Eosinophils:

Eosinophils are specialized antigen presenting cells of the immune system. They are responsible for the production of certain immunomodulatory cytokines and for activation of CD4+ T-cells. They have an important pathogenic role in various disorders like allergies, vasculitic granulomatous diseases etc (17, 45). Their key protective role is in the innate response against parasitic infections.

Figure 1: Schematic representation of an HIV virion:

The viral diploid ssRNA genome is enclosed within the core. ssRNA is shown to carry the enzyme reverse transcriptase. Envelope forms the outermost covering of the virus particle and bears important viral proteins like gp41, a transmembrane protein, and gp120, non-covalently bound to gp41.

Figure 1: Schematic representation of an HIV virion

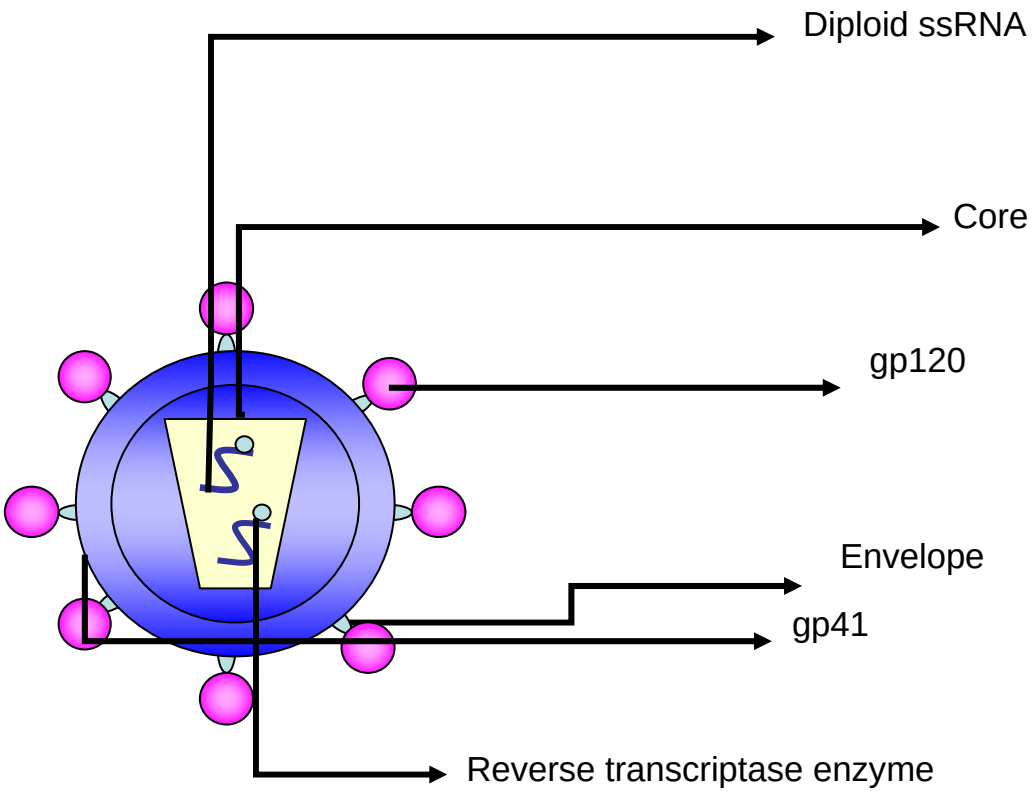
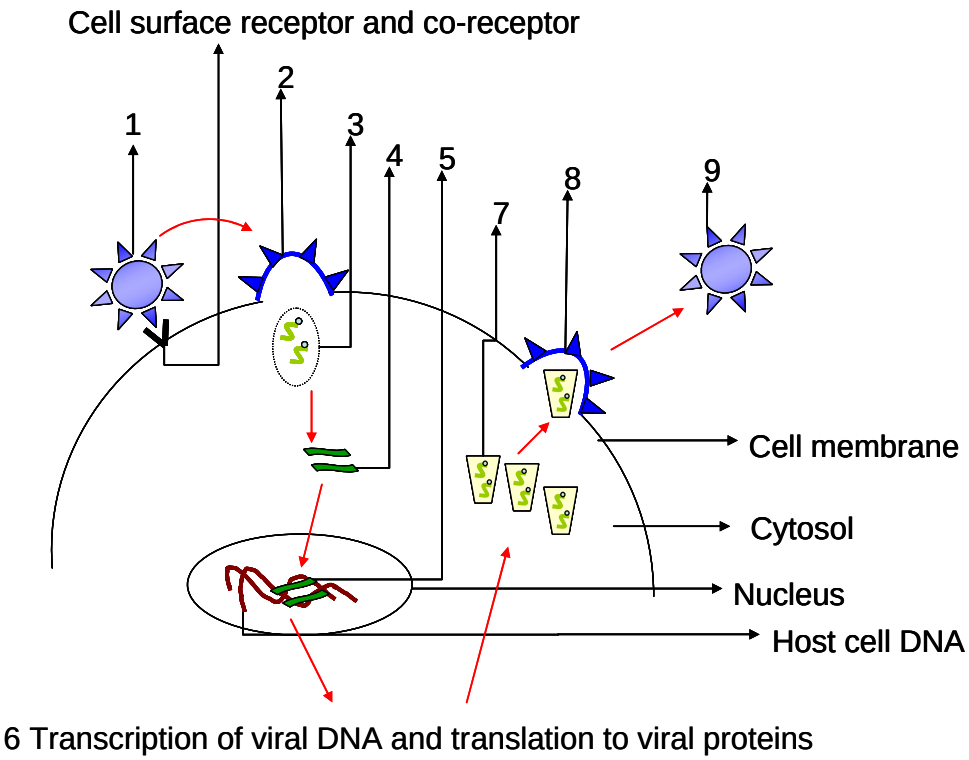


Figure 2: Diagrammatic representation of lifecycle of HIV:

Each step in the lifecycle of HIV is numbered in the sequence it occurs.

1- Binding of HIV to suitable receptors
2- Fusion of viral envelope with cell-surface (Viral entry)
3- Pre-integration complex in the cell cytosol (reverse transcription occurs)
4- Formation of double stranded DNA (ds DNA) which migrates into the nucleus
5- Integration of ds DNA into the host cell genome (viral DNA called as provirus)
6- Transcription of viral DNA and translation to viral proteins
7- Assembly of virus particle
8- Budding of virus from cell-membrane
9- Mature virus

Figure 2: Diagrammatic representation of lifecycle of HIV:



2.1. Importance of eosinophils in HIV infection:

Eosinophils are CD4+ cells and as early as 1989 it was shown that they can bind HIV protein gp120 (25). In developing countries like Africa, there is a high incidence of eosinophilia due to parasitic infections. Also, various studies have shown that in the later stages of AIDS, HIV positive patients develop eosinophilia (9, 12, 32, 34, 37, 42). These high numbers of circulating eosinophils represent potential hosts for HIV infection and its establishment and spread throughout the body. Thus, in depth study of HIV infection of eosinophils has a special significance.

2.2. Previous studies

In spite of this obvious importance, only three isolated studies done in the last decade have focused on this (10, 16, 47). Out of these, one study (10) demonstrated the presence of HIV DNA in primary eosinophils isolated from 2 out of 18 HIV sero-positive patients thus providing evidence that eosinophils are targeted by HIV ***in vivo***. These studies, however, have certain shortcomings. The most important short coming was that none of these studies were quantitative. Two of the studies (16, 47)did

not look at normal eosinophils. Freedman and his group used eosinophil precursors which were then processed to form mature eosinophils. In the peripheral blood, only mature eosinophils are found and hence the results of this study could not be extrapolated to the ***in vivo*** conditions. Also, this study stated that HTLV-IIIB strain could not infect eosinophils. Later studies provided evidence that eosinophils could be infected with HTLV-IIIB. Weller and his group of researchers performed an elaborate study to study HIV-1 infection of eosinophils. However, they isolated eosinophils from peripheral blood of patients with eosinophilia. These patients have phenotypically altered eosinophils (31) and hence they are probably not a good study model. They only group that used primary normal eosinophils was Conway et al. (10). The importance of this study was that it was done using primary eosinophil isolates from blood and it showed that not only could eosinophils be transiently infected ***in vitro*** but also that the eosinophils isolated from HIV positive patients are infected ***in vivo***, but the researchers had not specified the stage of HIV infection in the HIV-positive patients or their drug treatment status. The eosinophil count of HIV positive patients was not mentioned. They also had not characterized the HIV strain that had been identified in

the eosinophils isolated from two of the HIV positive patients.

In an effort to further the research in this field, our lab has been focusing on HIV infection of eosinophils. The most recent studies published were done in our laboratory (39, 48) and both studies were done using an eosinophilic cell line AML14.3D10. These conclusively proved that an eosinophilic cell line can be successfully infected with HIV. However, infection is strain dependent i.e. X4 HIV-1 strain, HTLV-IIIB, could infect the eosinophilic cell line but a macrophage-tropic; CCR5-using (R5) strain could not. In view of these results being obtained from a cell-line, it is now important to conduct a study using primary eosinophils.

3. AML14.3D10 cell line:

AML14.3D10 cells are a sub-clone of a human myeloid cell line AML14 (2). AML14.3D10 cells exhibit eosinophilic phenotype and also produce large amounts of eosinophil granule proteins like major basic protein (MBP). The cells can proliferate in the absence of cytokine stimulation and their doubling time is about 48 hours. The cells are CD4+ and also CXCR4+. This is currently the best cell line model

for study of eosinophils and was used as a positive control.

4. Peripheral blood mononuclear cells (PBMCs):

PBMCs comprise of lymphocytes and monocytes. These were the first cells shown to be infected with HIV. Within a few years of the discovery of HIV-1, two different strains were observed. One strain was macrophage-tropic and showed no syncytia formation in the infected culture, these were called as non-syncytia inducing (NSI) strain. The other strain was T-cell tropic and called as syncytia inducing (SI) strain because infected cultures showed multi-nucleated giant cell formation(5, 14, 40). It was much later that the reason for this became apparent when further research showed that SI viruses used chemokine receptor (CCR5) and NSI viruses used chemokine receptor (CXCR4) to gain entry into the cells(3). Accordingly, these strains are now called as R5 and X4 strains. The present study was done using an X4 strain to infect PBMCs either treated with or without interleukin-2. The major focus of this description is on T-cells for two reasons: firstly, because the X4 strain used in the study is not expected to infect monocytes; and secondly because culturing PBMCs overnight in a flask before infection would cause most of

the monocytes to be removed due to adherence of these cells to the flask.

4.1. Susceptibility of T-cells to HTLV-IIIB infection:

It has been demonstrated that HIV can enter quiescent cells but does not integrate into the host genome (38, 50, 51). However, certain cytokines like IL-2, IL-4, IL-6, IL-7 or IL-15 make resting T-cells permissive to HIV-1 infection (44).

4.2. Effect of interleukin-2 (IL-2) on lymphocytes and their infection with HIV-1 X4 strains:

IL-2 is a cytokine. It is a chemotactic agent and has also been known to enhance the viability of lymphocytes and to cause clonal expansion of stimulated T-cells. Circulating T-cells are in resting stage. They do not express IL-2 receptors nor does IL-2 stimulate them (4). However, recently, IL-2 treatment was administered to HIV positive patients (49). The results of this study suggest that IL-2 increased the CD4+ cell counts without increasing viral loads. This observation is in contradiction to the study (44), mentioned earlier, which studied ex vivo T lymphocytes and concluded that cytokine stimulation with

IL-2 can render resting T-cells permissive to HIV-1 infection.

In addition to their use as a positive primary cell control for infection, this study also compares infection of unstimulated isolated peripheral PBMCs with that of IL-2 stimulated PBMCs.

5. 8E5/LAV cell-line:

8E5/LAV cell-line was developed from A3.01 parent T-cell line. A3.01 cells were infected with lymphadenopathy associated (LAV) virus and then exposed to 5-iodo-2'-deoxyuridine (IUdR) (15). From the clones surviving a series of three exposures to IUdR a single clone 8E5 was isolated. This clone had a single HIV proviral copy, secreted high levels of p24 but had no reverse transcriptase activity. On propagation, these cells produced defective virus particles and have no un-integrated viral DNA. DNA obtained from 8E5/LAV cells was used to generate the standard curve for real-time polymerase chain reaction (Q-PCR) experiments.

II. Materials

Primary eosinophils and peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors between the ages of 21-65 years. Primary neutrophils, monocytes and lymphocytes were isolated from the same donors. AML14.3D10 cell line was maintained in our laboratory. Granulocyte-monocyte colony stimulating factor (GM-CSF) was purchased from R&D systems. Native Interleukin-2 (IL-2) was purchased from Hemagen Diagnostics, Inc., Waltham, MA. Eosinophil isolation kit, CD16 Microbeads, CD14 Microbeads, CD3 APC antibodies, CD14 APC antibodies, IgG control for APC antibodies were purchased from Miltenyi Biotec. CD16 PerCp-Cy5.5 antibodies and IgG control for PerCp-Cy5.5 were purchased from BD biosciences. p24 antigen enzyme-linked immunosorbent assay (ELISA) was done using Retro-Tek HIV-1 p24 antigen ELISA kit from Zeptometrix Corporation. Primers and probes used for quantitative polymerase chain reaction (Q-PCR) were purchased from Integrated DNA Technologies (IDT). Hema3 stain used for hematological staining was purchased from Fisher Scientific. The various

virus strains used were human T-cell lymphotropic IIIB virus (HTLV IIIB) strain and 8E5/LAV strains maintained in our laboratory.

III. Methods

Cell culture:

AML14.3D10, an eosinophilic cell line was cultured in RPMI 1640 (90%) supplemented with 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 20% heat-inactivated fetal calf serum, 5 X 10^{-5} M β-Mercaptoethanol, and 1mM sodium pyruvate. Cells were maintained at sub-confluency and passaged every three/four days. Cells were also passaged one day prior to infection with HTLV-IIIB.

8E5/LAV cell-line was cultured in 90% RPMI 1640 and 10% fetal calf serum supplemented with 2mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were maintained at sub-confluency and passaged every three/four days. The cells were cultured in a biosafety level 3 (BSL3) laboratory and all BSL3 practices were followed while handling the cells.

Isolation of primary leukocytes from peripheral blood:

Processing of whole blood

60 ml of peripheral blood was collected from healthy donors between the ages of 21 and 65 years in a syringe containing heparin. Blood was diluted 1:4 with PBS,containing 2mM EDTA. 20 ml of diluted blood was layered on 20 ml of Lymphoprep, a sucrose gradient with density of 1.077 ± 0.001 g/mL (Greiner Bio-One, North Carolina USA). Peripheral blood mononuclear cells (PBMCs) were separated from the granulocyte and erythrocyte cell pellet by centrifugation at 20^0C and 1800 rpm for 30 min. The PBMC layer was removed by pipeting and placed into a separate tube. The remaining Lymphoprep was aspirated and discarded.

Blood smears were also prepared from undiluted whole blood.

Isolation of eosinophils:

Eosinophil isolation kit (human) was used for obtaining primary eosinophils. The erythrocyte-granulocyte cell pellet obtained in the above step was processed and then magnetic labeling was done as per manufacturer's protocol.

Isolation of eosinophils was done on CS columns (Miltenyi Biotec, Auburn CA USA) as per manufacturer's protocol by negative selection. Effluent from the column was centrifuged at 20^0C 1100 rpm for 10 min to obtain an eosinophil cell pellet. Eosinophils were resuspended in 10 ml supplemented RPMI 1640 medium (RPMI 1640 medium, 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin, 20% heat-inactivated fetal calf serum). Cells were incubated overnight at 37^0C with 5% CO_2.

Isolation of neutrophils

Neutrophils were used as a positive CD16 cell control for flow-cytometry experiments. For this neutrophils were isolated using CD16 microbeads (Miltenyi Biotec.) from the granulocyte pellet following the manufacturer's protocol. Neutrophils were not cultured but were stained the same day for flow-cytometry.

Processing of peripheral blood mononuclear cells (PBMCs):

PBMCs were removed from the sucrose gradient as described above. They were washed two times with PBS containing 2mM EDTA and counted with trypan blue. After an additional wash with the same buffer, they were incubated overnight in 10 ml of the same media as described for

eosinophils. For two of four experiments, PBMCs were split into two 10 ml samples; one received 10% IL-2 and the other was cultured without IL-2.

Monocytes were used as a positive CD14 cell control for flow-cytometry experiments and were isolated using CD14 microbeads (Miltenyi Biotec.) following the manufacturer's protocol. Monocytes were not cultured but were stained the same day for flow-cytometry. Effluent obtained from the LS column used for positive isolation of monocytes was rich in lymphocytes. This was used as the positive CD3 cell control for flow-cytometry.

Culture conditions for eosinophils:

Determination of GM-CSF effect on viability of eosinophils:

Eosinophils were cultured in six-well plates in media (RPMI 1640 medium, 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin, and 8% heat-inactivated fetal calf serum). Each well was treated differently; either the well received no GM-CSF, received 36pM GM-CSF everyday or 36pM GM-CSF every third day. Cells were fed every three days. Viability of eosinophils was determined by cell counting using trypan blue.

Determination of effect of different sera on viability of eosinophils:

Isolated eosinophils were cultured in different media with 50pM GM-CSF. Stock medium containing RPMI 1640 with 2mM L-glutamine, 100U/ml penicillin, and 100µg/ml streptomycin was prepared. Different types of sera used were fetal calf serum (FCS), homologous human serum (HSS), autologous human serum (AHS). Each serum was either heat inactivated at 56^0C for 30 min (HI) or was used in the un-inactivated form (UI). From the stock medium aliquots were removed for preparation of different media. Media were prepared by adding one of the following sera, 20% FCS-HI or 20% FCS-UI, 10% FCS-HI + 10% HHS-HI, 10% FCS-UI + 10% HHS-UI, 10% FCS-HI + 10% AHS-HI, 10% FCS-UI + 10% AHS-UI.

In order to obtain autologous human serum, 5 ml of donor's whole blood was centrifuged at 1100 rpm for 15 min. This separated the white and red blood cell pellet from the serum. Serum was removed, placed into a separate tube, and then used for preparation of culture media.

Infection with HTLV-IIIB:

AML14.3D10 cells were split 1:2 on the day before infection. After overnight incubation as described previously, all the cells (AML14.3D10, eosinophils and

PBMCs) were counted with trypan blue. One and a half million viable cells of each cell type were transferred into each of two separate tubes. One tube was used for infection with HTLV-IIIB and the other was a negative control, treated with conditioned medium. Infections were performed in a biosafety level-3 (BSL-3) facility. Cell suspensions were centrifuged and supernatants were removed. Cell suspensions were incubated in 1 ml of HTLV-IIIB stock or mock-infected in conditioned medium. Virus stock that was heat inactivated at 56^0C was also used as a control for infection. The cell suspensions were incubated for three hours at 37^0C and 5% CO_2, then washed three times in PBS and cultured in appropriate media as described above. Five picomolar GM-CSF was added to the eosinophil cultures. For two of four donors, PBMCs were cultured without IL-2. For the remaining two donors, the samples that had been stimulated with 10% IL-2 on the day of isolation, received 10% IL-2 post-infection. Four hundred microliters of supernatant was frozen at -80^0C immediately after infection for p24 ELISA.

For donor 4, eosinophil cell pellets on the day of infection (D0), were frozen for DNA processing after collection of samples for p24 ELISA.

Cells were cultured for 72 hours post-infection. The samples were then screened by phase contrast microscopy for syntitia formation. Cells were counted with trypan blue and centrifuged. Six hundred microliters of the supernatant was removed for p24 ELISA. The remaining supernatant was removed. One wash was done with PBS and the cell pellets were processed for obtaining DNA.

Table 1: Infection conditions for different cells from individual donors

X= Samples processed from each donor

Donor / Sample I.D.	1	2	3	4
AML 3D10 + Mock	X	X	X	X
AML 3D10 + Heat Inactivated	X	X		X
AML 3D10 + HTLV IIIB	X	X	X	X
Eosinophils + Mock D0				X
Eosinophils + Heat Inactivated D0				X
Eosinophils + HTLV IIIB D0				X
Eosinophils + Mock D3	X	X	X	X
Eosinophils + Heat Inactivated D3			X	X
Eosinophils + HTLV IIIB D3	X	X	X	X
PBMCs -IL-2 + Mock	X	X	X	X
PBMCs -IL-2 + Heat Inactivated				X
PBMCs -IL-2 + HTLV IIIB	X	X	X	X
PBMCs +IL-2 + Mock			X	X
PBMCs +IL-2 + HTLV IIIB			X	X

DNA isolation and quantification:

The DNeasy kit from Qiagen was used for isolation of total cellular DNA using the manufacturer's protocol. DNA was quantified using a Nano-drop spectrophotomer. DNA was diluted in 0.5X Tris-EDTA (TE) buffer to a concentration of 1.25 ng/µl.

p24 antigen capture ELISA:

Retrotek HIV-1 p24 Antigen ELISA kit from Zeptometrix Corp. was used and the manufacturer's protocol was followed.

Preparation of cytopreps:

During the isolation of primary blood cells, cell samples were kept aside for preparation of cytopreps. Cell suspensions were prepared at a density of 150,000-175,000 cells per 100µl. FCS buffer containing 1% FCS in phosphate buffered saline (PBS) was prepared. Cytospin specimen holders; labeled slides, cyto-cups and clamps were assembled, as per instructions (Shandon Lipshaw Inc.). Seventy five microliters of FCS buffer with 100µl cell suspension was applied to the bottom of the cyto-funnel and the samples were centrifuged at 640 rpm for 5 min. After unclipping the clamps and separating the cytofunnels from

the slides, the slides were air-dried for 10 min inside the biological safety hood.

Hematological staining:

Cytoslides and blood smears were stained with Hema3 Protocol stain (Fisher Sci.). Slides were fixed with the fixative for 5X 10sec. Staining in solution I was done for 5X 10 sec and in solution II for 3X 10 sec. The slides were then washed with tap water till the water ran clear and then air-dried.

Light Microscopy and fluorescence microscopy:

Manual differential leukocyte counts (DLCs) were performed on the blood smears using light microscopy. Stained blood smears were also analyzed to determine purity.

Fluorescence microscopy was also performed on the cytopreps to detect auto-fluorescence of cells. Filters used were:

Blue = excitation wavelength 420-495 nm used for green fluorescence

Green = excitation wavelength 510-560 nm used for red fluorescence

Staining of samples for flow cytometry:

Appropriate numbers of isolated leukocytes (maximum number $1X10^6$) were centrifuged at 1500 rpm for 5 min at 25^0C. The cell pellet was resuspended in 50 µl staining buffer (0.1% NaN_3 and 1%FCS in PBS). Antibodies were appropriately diluted (as determined empirically by performing similar experiments with serial dilutions of antibodies and their control immunoglobulins). Fifty microliters of cells and fifty microliters of diluted antibody were incubated on ice on a linear shaker for 30 min. The cells were washed with 2 ml PBS and centrifuged at 1500 rpm for 5 min at 25^0C. Supernatant was decanted and the cell pellets were re-suspended in 400 µl of 3% paraformaldehyde (PFA) and 300 µl PBS. The antibodies used were CD16 PerCp-Cy5.5, CD 16 PerCp-Cy5.5 immunoglobulin (Ig) isotype control (Ms IgG_1, κ), CD3 APC, CD14 APC, CD3 and CD14 APC Ig isotype control (Mouse IgG2a).

Flow cytometry was done on the FACSCalibur machine from BD biosciences and analysis of data was performed using the FACSComp software.

Quantitative analysis of HIV-1 infection:

Quantitative real-time PCR (Q-PCR) was performed using the high-throughput ABI PRISM® 7900HT Sequence Detection

System. HIV-1 primers were designed for the relatively conserved polymerase sequences between Pol 2116-2137. The forward and reverse primers consisted of 22 bases each whereas the probe was 24 bases. Dye used for the probe was 6-FAM (fluorescein) with an emission wavelength of 520nm. The quencher was TAMRA with an emission wavelength of 583nm. FAM and TAMRA form a classic fluorescent resonance energy transfer pair (FRET). The principal of FRET pairs is that the two dyes, when initially tethered together, will show transfer of energy from one (from FAM) to the other molecule (to TAMRA) so that emission occurs at a higher wavelength of 580nm instead of at 520nm. Once the molecules separate, this transfer no longer occurs and hence fluorescence of FAM can be detected.

HIV-1:

Forward primer: 5’-GGA AGG CCA GGG AAT TTT CTT C-3’

Reverse primer: 5’-CTT CTG AGA GGG AGT TGT TGT C-3’

Probe: 5’-6-FAM-CAG AAC AGA GCC AAC AGC CCC ACC-36-TAMSp-3’

β-globin primers were used as an endogenous control in the multiplex reaction. HIV proviral copy number was normalized to β-globin copy number during analysis of Q-PCR results. The primers for β-globin were 20 bases and the probe was 25 bases. The dye and quencher for the probe were selected such that they did not interfere with the dye and

quencher for HIV primers. Hence JOE-NHS ester (JOEN) with wavelength of 555nm and BHQ-1 (black hole quencher-1) were used. Black hole quencher is a fluorescent acceptor molecule like TAMRA but instead of fluorescing at a higher wavelength, BHQ converts the energy to heat.

Forward primer: 5′-ACA CAA CTG TGT TCA CTA GC-3′

Reverse primer: 5′-CAA CTT CAT CCA CGT TCA CC-3′

Probe: 5′/56-JOEN/-TGC ACC TGA CTC CTG AGG AGA AGT C-/3BHQ-1/3′

The primers were diluted in Tris-EDTA (TE) buffer to give a 20X mix contain 18µM concentration of the forward and reverse primers and 5µM concentration of the probe. Multiplexing was done. Reactions were setup in a 96-well plate. One microliter of each primer was added to 10 µl of 2X Taqman Universal Master Mix (Applied biosystems) along with 8 µl (10 ng) sample DNA to make up a total volume of 20 µl. A standard curve was generated using serial two-fold dilutions of 8E5LAV DNA from 0.3125 ng to 10 ng. The standard samples were run in triplicates while the controls and test samples were run in duplicates. Standard curves for HIV-1 and β-globin were accepted when the efficiency of amplification was 85-100% i.e. the slopes were between -3.74 to -3.32 (11). Efficiency (E) of amplification was calculated from the slope of the standard curve as -

$$\%E = [(10^{(-1/slope)}) - 1] \times 100$$

The viral DNA copy number obtained was normalized with the copy number obtained for β-globin and then expressed as the number of HIV copies per 1000 cells. Thus the expression is:

Number of HIV copies per 1000 cells = (HIV copy number/ β-globin copy number) X 2 X 1000 (11).

Determination of multiplicity of infection (MOI) for HTLV-IIIB stock virus:

One milliliter of stock virus was diluted 1:5 with media prepared for Aml14.3D10 cell-line. Two hundred microliters of this dilution was plated in the first column of a 96-well micro-titer plate. From this, serial 5 fold dilutions were made through column 7 by transferring 40 µl of virus dilution from previous well into 160 µl of AML14.3D10 media. The last two columns received only media. In a second 96-well plate, 50 µl AML14.3D10 cell-suspension containing 20,000 cells was plated in the rows A to F. The last two rows received only 50 µl media. Fifty microliters of the virus dilution was now transferred to this plate thus giving an additional two-fold dilution. The final plate set-up is shown in figure 3. This plate was incubated at 37^0C and 5% CO2, in a moist chamber, for 3 days. On the

third day, 50 µl of media was added to each well. On day 7, 50 µl (20,000 cells) of uninfected Aml14.3D10 cells were added to a fresh 96-well plate and 50 µl of the infected samples were transferred to corresponding wells of this plate. Fifty microliters of fresh media was added on day 10 and 13 post-infection. On day 14, syntitia (giant cells) were counted under phase contrast microscope. Wells having 3 or more obvious syntitia were scored as positive. Supernatant from each well was removed and used for p24 ELISA. P24 ELISA was done as per the manufacturer's protocol.

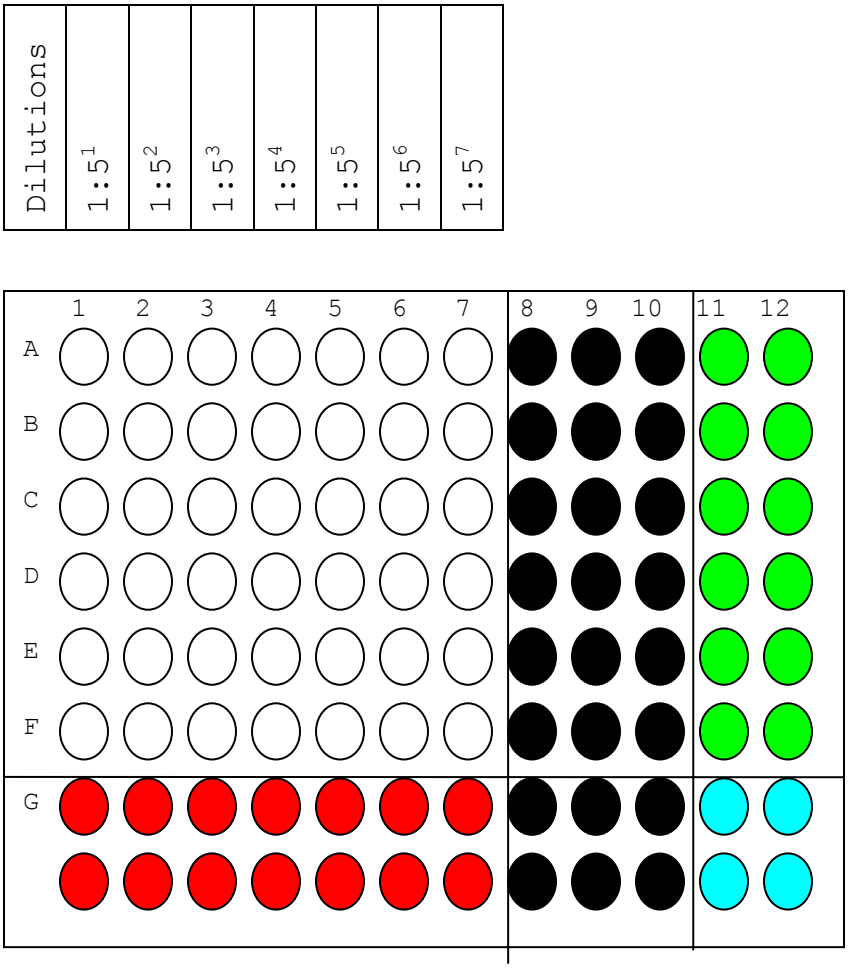
Analysis of data:

Data analysis was done using Student's T-test. The variates chosen were the two sample sets to be compared (e.g. eosinophils infected and PBMCs infected), two-tailed test was applied and limit was set at 0.05%. Anything less than 0.05% ($p<0.05\%$) was considered significant.

Figure 3: $TCID_{50}$ plate set-up

The following picture shows the initial set-up of a 96-well plate for calculating $TCID_{50}$. The final virus dilutions are written on top of the plate and legend is given at the bottom of the plate.

Figure 3: $TCID_{50}$ plate set-up



○	Aml14.3D10 cells with serial dilutions of HTLV-IIIB virus
●	Cells only in media
●	HTLV-IIIB virus dilutions in media
●	Media only
●	Columns left blank

IV. Results

Eosinophils were isolated from healthy donors with no history of eosinophilia. Donors had no risk factors suggestive of exposure to HIV. The number of eosinophils isolated from donors varied individually. Manual and automated differential leukocyte counts (DLC) were performed to ensure that the eosinophil counts were within the normal range 0-7% of the total white blood cells (Table 2). This range has been used by CompuNet Clinical Laboratories who performed the automated DLC counts. Eosinophil counts varied from 3 X 10^6 to 47 X 10^6 cells per 60ml of peripheral blood i.e. 2.97% to 8.41% by manual DLC.

Stained cytopreps were screened to check for purity of eosinophils. When stained with modified Wright stain (Hema3), eosinophilic granules appear red or red/orange, the nucleus, usually bi-lobed, and the cytoplasm are blue in color. This characteristic stain of the eosinophils is very different from the other white blood cells (WBCs) (Figure 5). Neutrophils have lilac colored granules and a dark-blue multi-lobed nucleus whereas lymphocytes and

monocytes which are non-granular cells have a single large dark violet nucleus (Figure 4). Lymphocytes are smaller in size as compared to the monocytes. The purity of isolated eosinophils was determined by screening 500 cells in several fields from stained cytopreps at 1000X magnification. The purity of eosinophil isolates for every donor was determined to be greater than 99%. This was also confirmed by doing flow-cytometry. As shown in figures 6-9, overlay of histograms of eosinophils stained with CD3, CD14 or CD16 antibodies showed no positive shift when compared to eosinophils stained with isotype (negative) control. Hence a detailed quadrant analysis was performed which helped in identification of contaminating cell populations. Among the contaminating cells, neutrophils are CD4 negative and hence are not expected to be infected with HTLV-IIIB. Similarly, monocytes also cannot be infected with X4 strains. Contaminating lymphocytes are <0.5%.

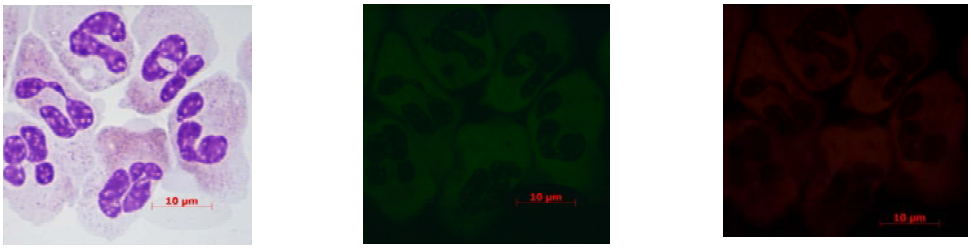
Figure 4: Isolated neutrophils and PBMCs at 1000X

Panel i shows cells stained with modified Wright stain, panel ii contains images showing green auto-fluorescence and panel iii images show orange/red auto-fluorescence of the cells. Horizontal panel a. shows neutrophils and panel b. shows PBMCs

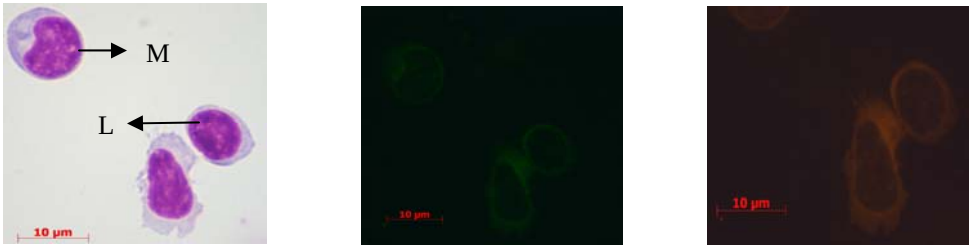
Figure 4: Isolated neutrophils and PBMCs at 1000X

i ii iii

a. Neutrophils



b. PBMCs



M = Monocyte

L = Lymphocyte

Figure 5: Isolated eosinophils at 1000X

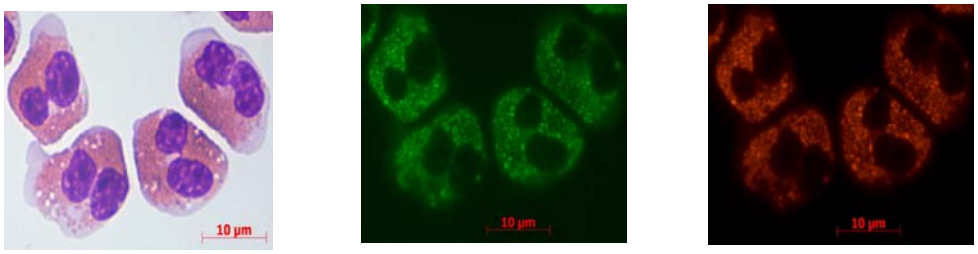
Panel i shows cells stained with modified Wright stain, panel ii contains images showing green auto-fluorescence and panel iii images show orange/red auto-fluorescence of the cells. Horizontal panels a-d show eosinophils from donors 1-4 respectively.

Figure 5: Isolated eosinophils at 1000X

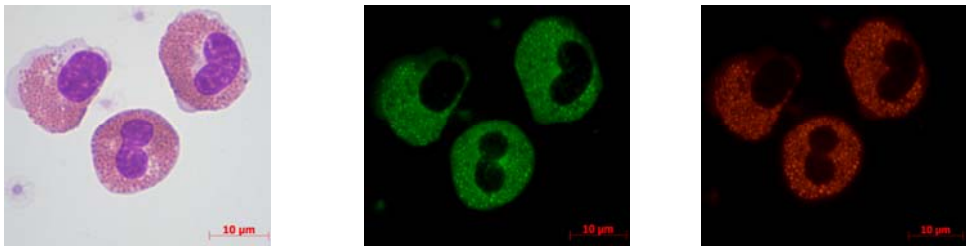
i ii iii

Eosinophils

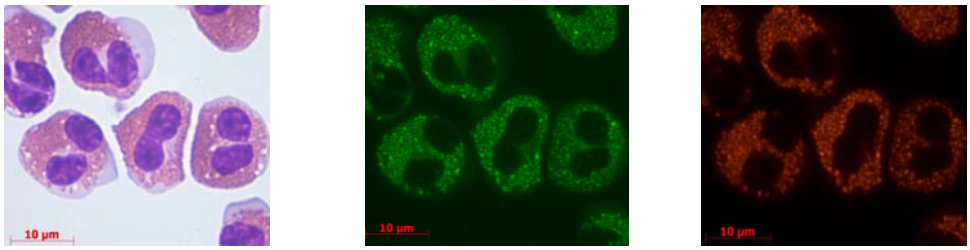
a.



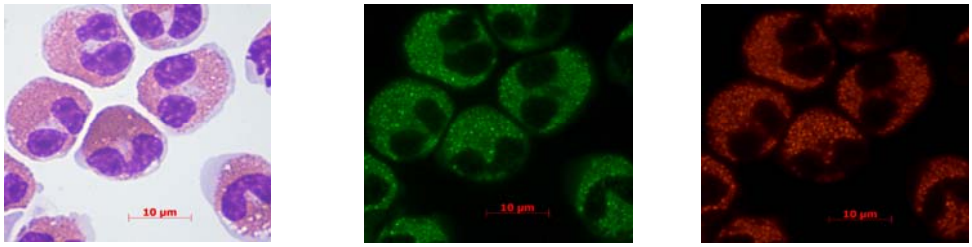
b.



c.



d.



We also observed that eosinophils exhibit an intense green fluorescence owing to the eosinophilic granules (Figure 5) as reported by previous groups (27, 46). Though other WBCs exhibit some auto-fluorescence, it is not as intense as eosinophils (Figure 4). Eosinophils have an excitation maxima at 370 nm and 450 nm with emission at 520 nm (27, 46). Therefore, while performing flow-cytometry, we used eosinophils as FL1 (green channel) positive cells and then used FL3 (PerCP-Cy5.5) and FL4 (APC) labels to identify contaminating cell populations. The excitation wavelength for PerCP-Cy5.5 conjugate is 490, 675 nm and emission is 695 nm while the excitation wavelength for APC is 650 nm and emission is at 660 nm.

Figure 6: Flow-cytometry analysis for donor 1:

Panel A shows the overlay of histograms for eosinophils stained with different antibodies CD3-APC (A1), CD14-APC (A2), CD16-PerCP-Cy5.5 (A3). Panel B shows the overlay of histograms for CD3+ lymphocytes (B1), CD14+ monocytes (B2), CD16+ neutrophils (B3). Grey areas represent cells stained with mouse immunoglobulin isotype control. Quadrant analysis to identify contaminating cells in isolated eosinophils is shown in panels C-E. C1 (lymphocytes), D1 (monocytes), and E1 (neutrophils) were labeled with Ig isotype control. C2 (lymphocytes), D2 (monocytes), and E2 (neutrophils) were labeled with CD3-APC, CD14-APC and CD16-PerCP-Cy5.5 antibodies respectively and the positive cell populations were gated. C3 shows CD3+ cells (R4 gate) in isolated eosinophil sample stained with CD3-APC antibody. Similarly, D3 and E3 show CD14+ (R5 gate) and CD16+ (R6 gate) cells in isolated eosinophil sample.

R4 = 0.42%

R5 = 0.05%

R6 = 0.14%

Figure 6: Flow-cytometry data analysis for donor 1:

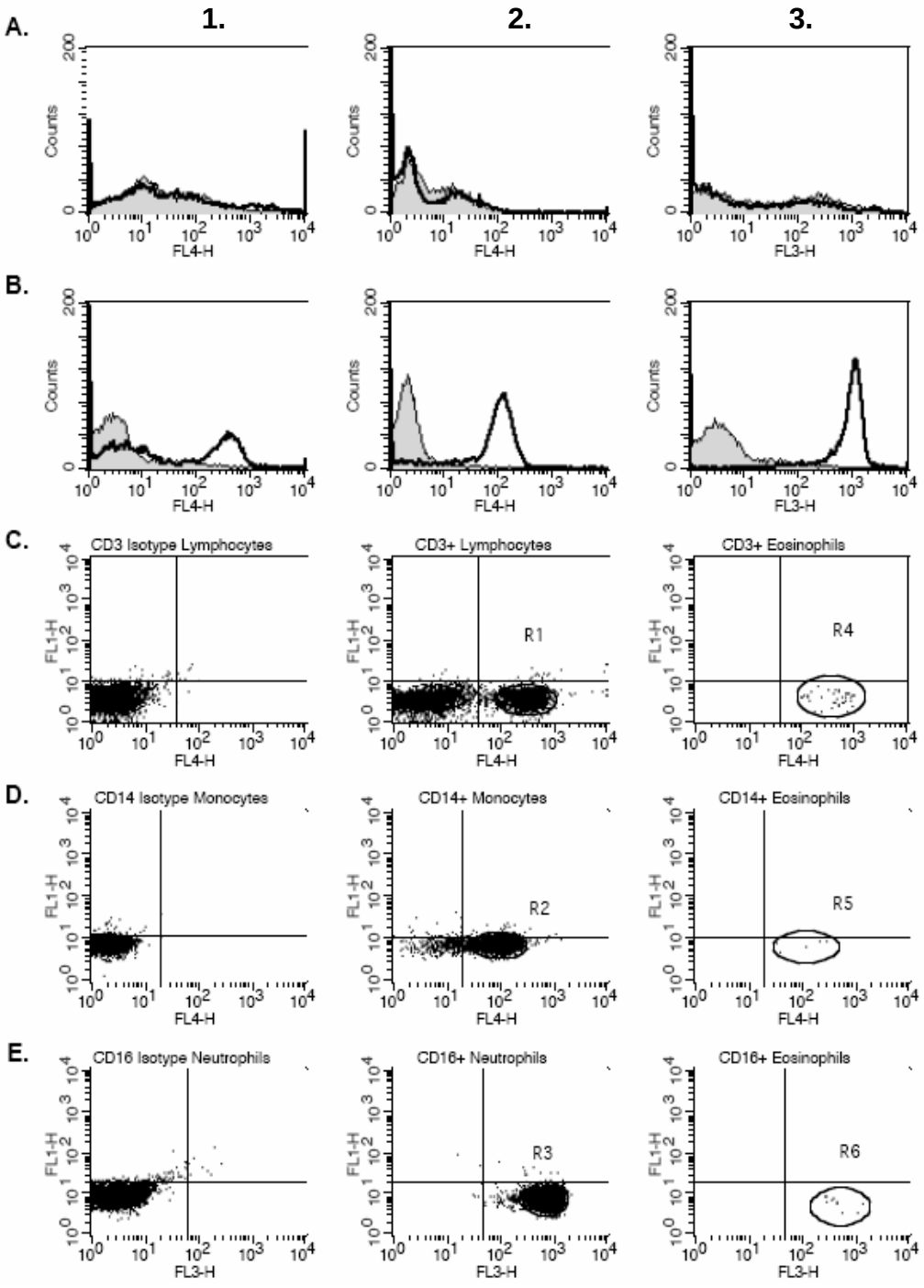


Figure 7: Flow-cytometry data analysis for donor 2:

Panel A shows the overlay of histograms for eosinophils stained with different antibodies CD3-APC (A1), CD14-APC (A2), CD16-PerCP-Cy5.5 (A3). Panel B shows the overlay of histograms for CD3+ lumphocytes (B1), CD14+ monocytes (B2), CD16+ neutrophils (B3). Grey areas represent cells stained with mouse immunoglobulin isotype control. Quadrant analysis to identify contaminating cells in isolated eosinophils is shown in panels C-E. C1 (lymphocytes), D1 (monocytes), and E1 (neutrophils) were labeled with Ig isotype control. C2 (lymphocytes), D2 (monocytes), and E2 (neutrophils) were labeled with CD3-APC, CD14-APC and CD16-PerCP-Cy5.5 antibodies respectively and the positive cell populations were gated. C3 shows CD3+ cells (R4 gate) in isolated eosinophil sample stained with CD3-APC antibody. Similarly, D3 and E3 show CD14+ (R5 gate) and CD16+ (R6 gate) cells in isolated eosinophil sample.

R4 = 0.18%

R5 = 0.06%

R6 = 1.68%

Figure 7: Flow-cytometry data analysis for donor 2:

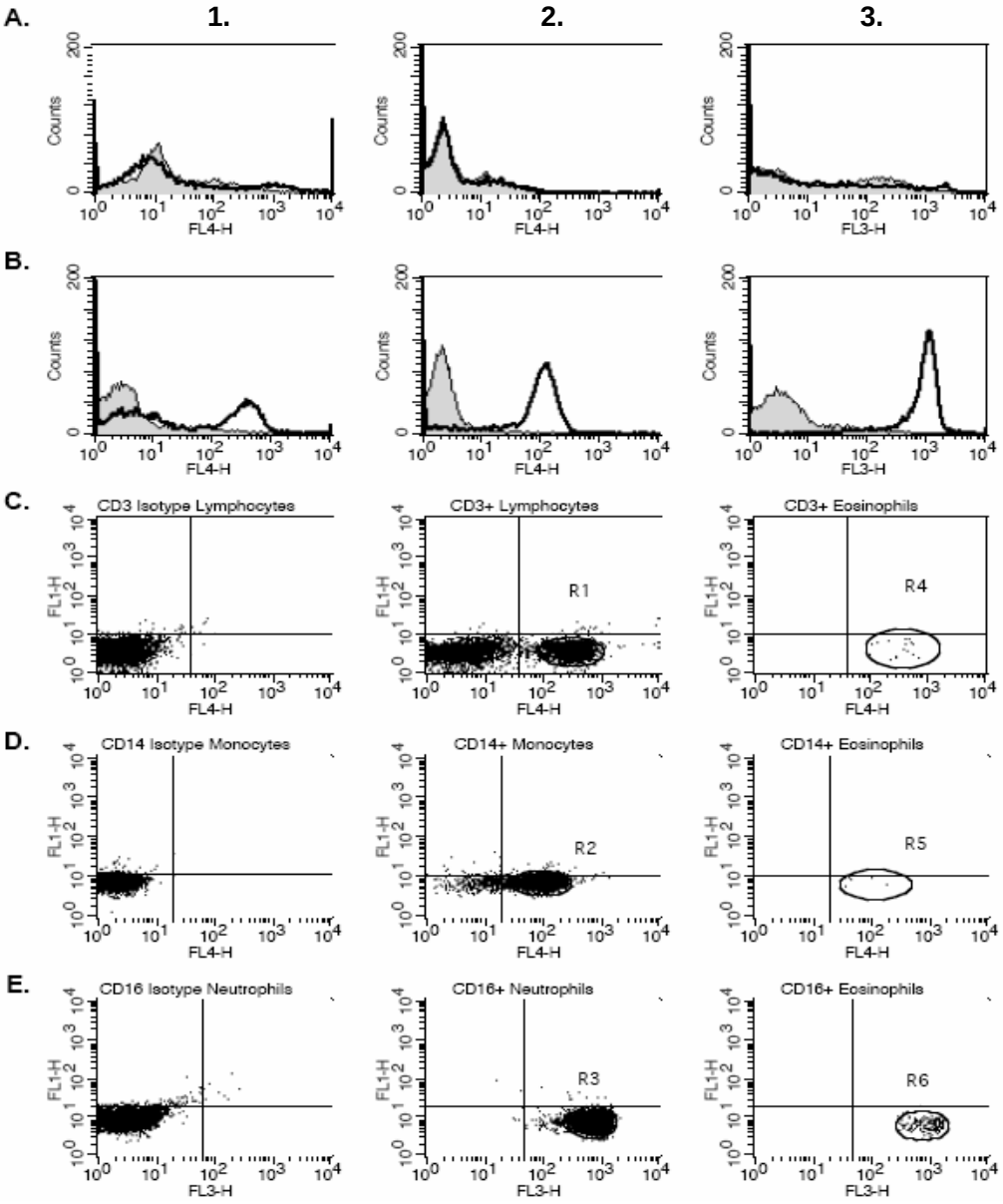


Figure 8: Flow-cytometry data analysis for donor 3:

Panel A shows the overlay of histograms for eosinophils stained with different antibodies CD3-APC (A1), CD14-APC (A2), CD16-PerCP-Cy5.5 (A3). Panel B shows the overlay of histograms for CD3+ lumphocytes (B1), CD14+ monocytes (B2), CD16+ neutrophils (B3). Grey areas represent cells stained with mouse immunoglobulin isotype control. Quadrant analysis to identify contaminating cells in isolated eosinophils is shown in panels C-E. C1 (lymphocytes), D1 (monocytes), and E1 (neutrophils) were labeled with Ig isotype control. C2 (lymphocytes), D2 (monocytes), and E2 (neutrophils) were labeled with CD3-APC, CD14-APC and CD16-PerCP-Cy5.5 antibodies respectively and the positive cell populations were gated. C3 shows CD3+ cells (R4 gate) in isolated eosinophil sample stained with CD3-APC antibody. Similarly, D3 and E3 show CD14+ (R5 gate) and CD16+ (R6 gate) cells in isolated eosinophil sample.

R4 = 0.16%

R5 = 0.00%

R6 = 0.23%

Figure 8: Flow-cytometry data analysis for donor 3:

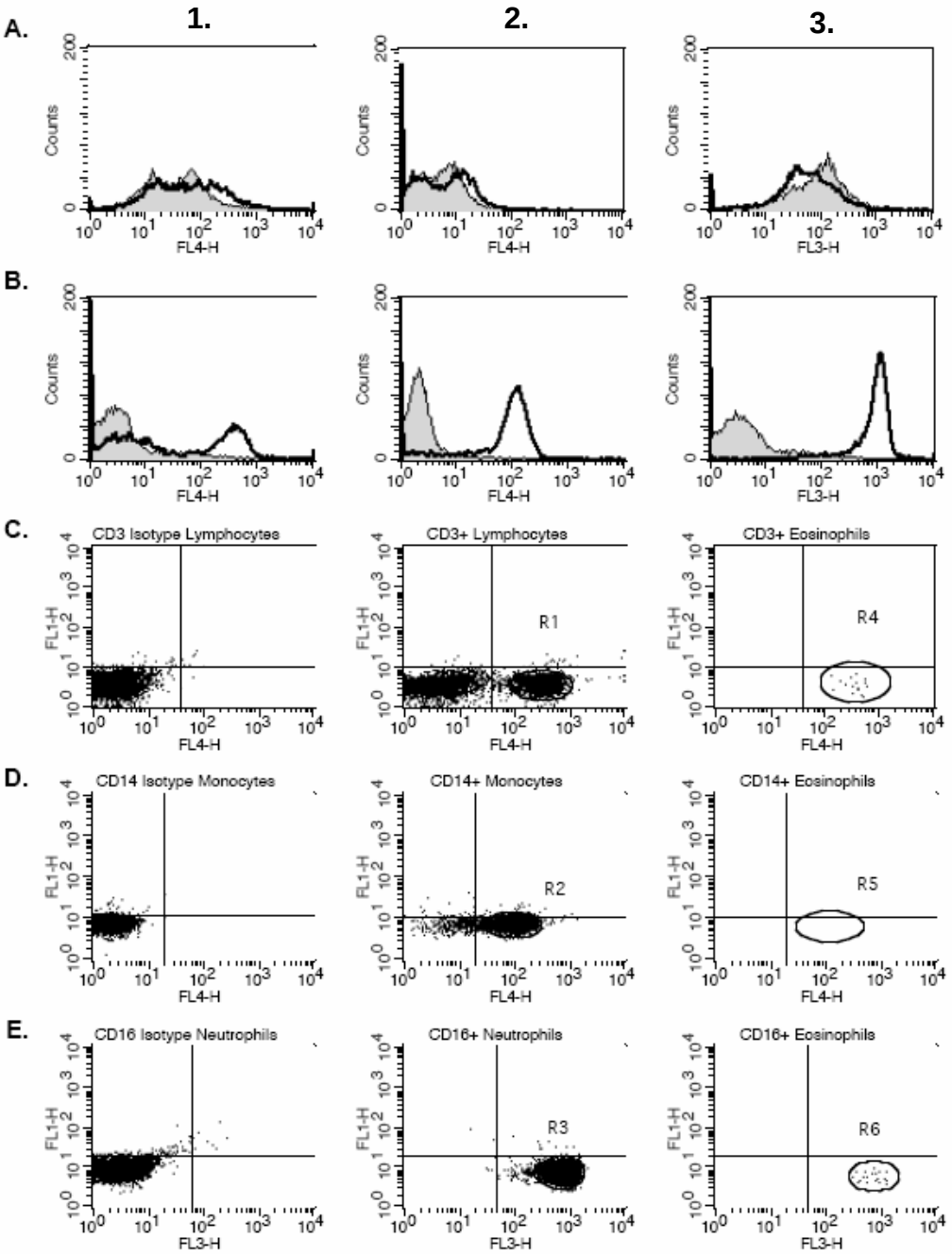


Figure 9: Flow-cytometry data analysis for donor 4:

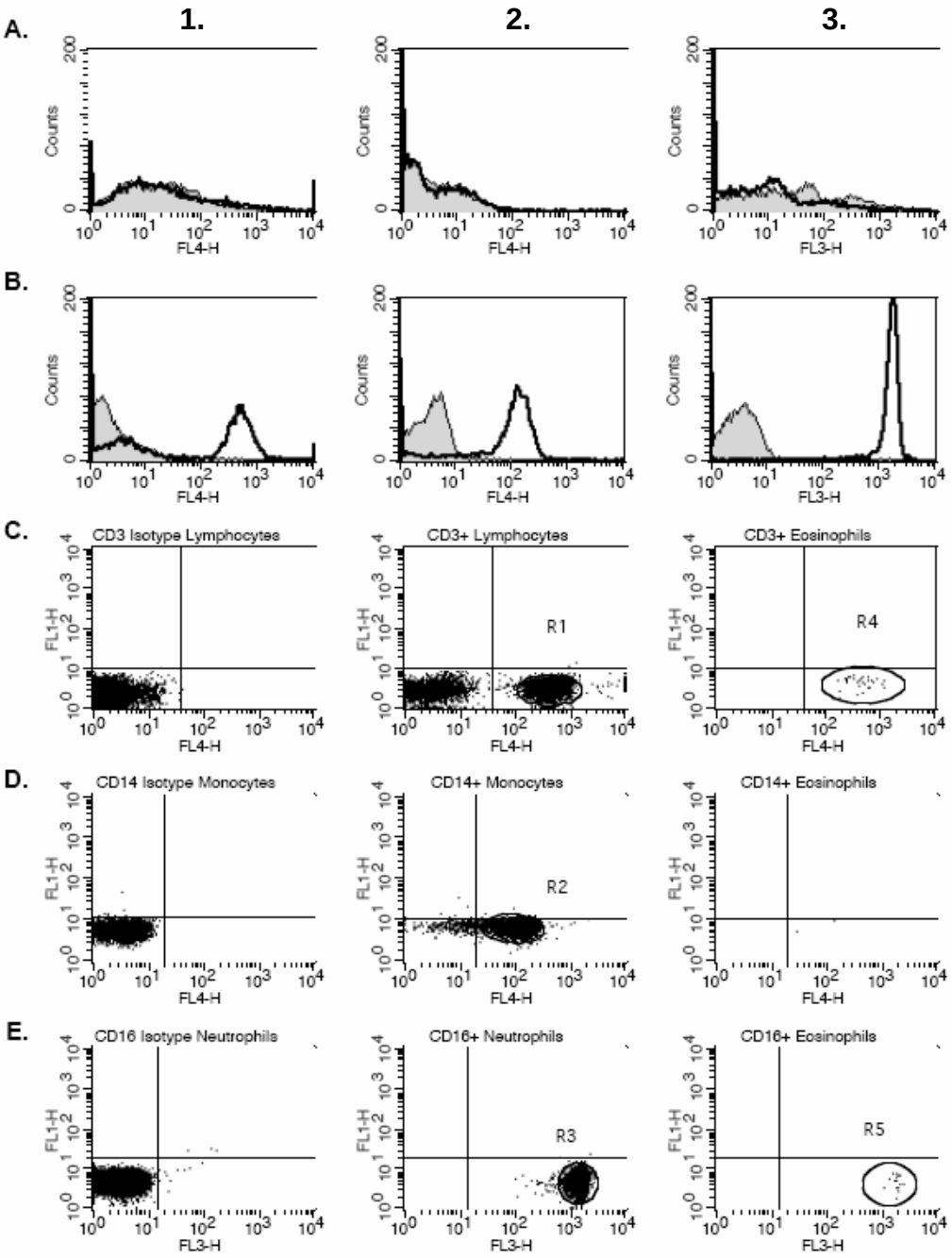
Panel A shows the overlay of histograms for eosinophils stained with different antibodies CD3-APC (A1), CD14-APC (A2), CD16-PerCP-Cy5.5 (A3). Panel B shows the overlay of histograms for CD3+ lymphocytes (B1), CD14+ monocytes (B2), CD16+ neutrophils (B3). Grey areas represent cells stained with mouse immunoglobulin isotype control. Quadrant analysis to identify contaminating cells in isolated eosinophils is shown in panels C-E. C1 (lymphocytes), D1 (monocytes), and E1 (neutrophils) were labeled with Ig isotype control. C2 (lymphocytes), D2 (monocytes), and E2 (neutrophils) were labeled with CD3-APC, CD14-APC and CD16-PerCP-Cy5.5 antibodies respectively and the positive cell populations were gated. C3 shows CD3+ cells (R4 gate) in isolated eosinophil sample stained with CD3-APC antibody. Similarly, D3 and E3 show CD14+ (R5 gate) and CD16+ (R6 gate) cells in isolated eosinophil sample.

R4 = 0.31%

R5 = 0.02%

R6 = 0.17%

Figure 9: Flow-cytometry data analysis for donor 4:



The lifespan of eosinophils is less than 48 hours. Hence, to determine optimal growth conditions for eosinophils with or without GM-CSF supplementation to culture media were tested as described. Eosinophils cultured in FCS H medium without GM-CSF supplementation had lower viability as compared to those with GM-CSF supplementation as shown in figure 10. There was significant difference between viability of cells which received GM-CSF everyday and those that received no GM-CSF.

The next question naturally was whether different sera affected the viability of eosinophils. We observed that eosinophils cultured in RPMI medium supplemented with 20% heat-inactivated (H) fetal calf serum (FCS) had an average viability of 94.15% and 80.60% on days 3 and 6 post-isolation. The corresponding viability for heat-inactivated human autologous serum (HAS) was 75.61% and 59.91% whereas for heat-inactivated human homologous serum (HHS) it was 70.61% and 50.93%. Thus there was an average of 25.18% higher viability of eosinophils when cultured in heat-inactivated FCS as opposed to using heat-inactivated human sera. There was a trend towards increased survival of eosinophils cultured in medium containing 20% fetal calf serum which was heat inactivated at 56^0C for 30min (FCS H) (Figure 11).

Figure 10: Effect of GM-CSF on eosinophil viability

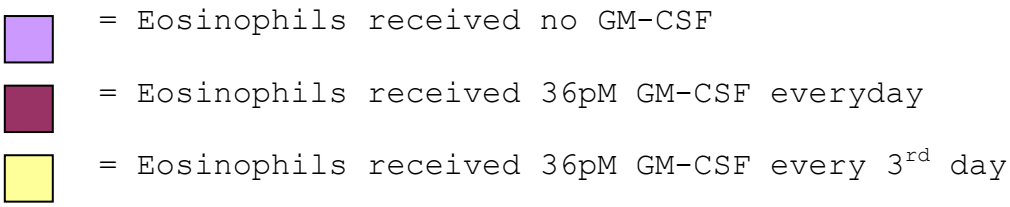


Figure 10: Effect of GM-CSF on viability of eosinophils

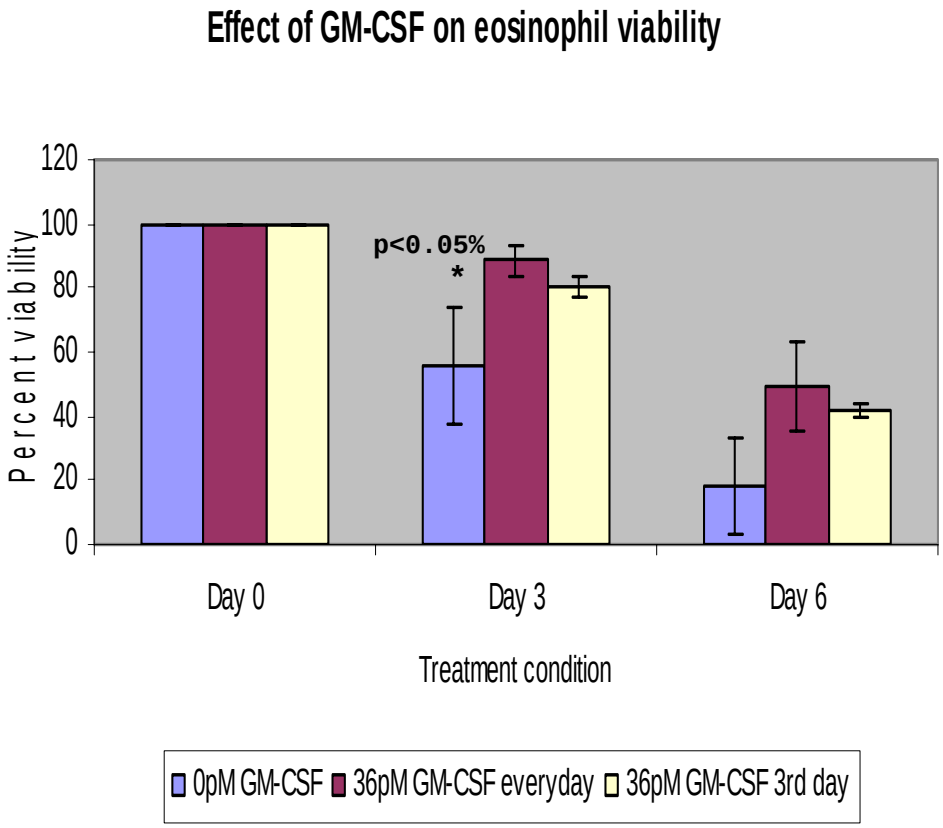
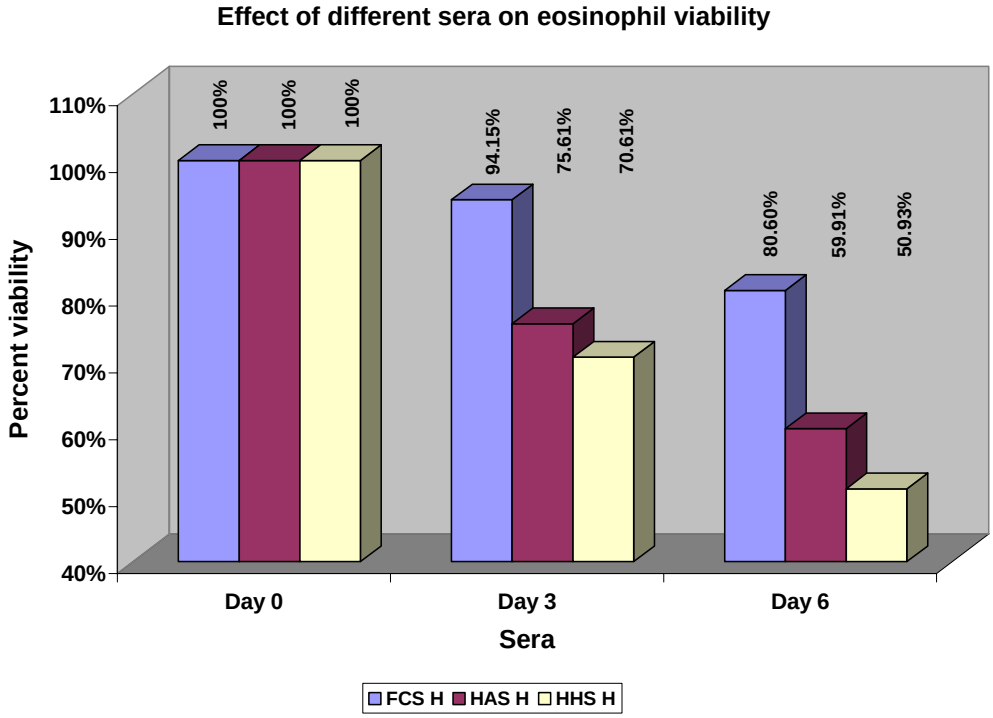


Figure 11: Effect of different types of sera on eosinophil viability

- FCS H = Medium containing 20% fetal calf serum which was heat-inactivated at 56^0C for 30min
- HAS H = Medium containing 10% human autologous serum and 10% FCS both of which were heat-inactivated at 56^0C for 30min
- HHS H = Medium containing 10% human homologous serum and 10% FCS both of which were heat-inactivated at 56^0C for 30min

Figure 11: Effect of different types of sera on eosinophil viability



During analysis of Q-PCR data, we observed that primary human eosinophils can be infected with HTLV-IIIB. Donor variability exists in the amount of infection as shown in figures 12-17. However, we primarily observed that infection of eosinophils was not significantly different from infection observed in AML14.3D10 cells in two out of four donors. All samples of primary PBMCs without IL-2 stimulation and in primary PBMCs with IL-2 stimulation showed low amounts of infection with HTLV-IIIB. There was no significant difference ($p<0.05\%$) between infection of primary PBMCs with or without IL-2 stimulation but there is a significant difference between detection of proviral copies per 1000 cells for infected eosinophils and infected PBMCs with or without IL-2 stimulation ($p>0.05\%$) when analysed with Student's T-test. Table 2 summarizes the HIV-1 proviral copy number per 1000 cells for experiments done on donors 1 and 2 while table 3 summarizes data for donors 3 and 4.

Table 2: HIV-1 copy number per 1000 cells

	Mean HIV copies per 1000 cells
Donor 1	
AML Mock infected	0
AML + Heat-inactivated virus	0
AML Infected	6426.35
Eos Mock infected	0
Eos Infected	1763.4
PBMCs Mock infected	0
PBMCs Infected	27.96
Donor 2	
AML Mock infected	0
AML + Heat-inactivated virus	0
AML Infected	5398.58
Eos Mock infected	0
Eos Infected	2722.22
PBMCs Mock infected	0
PBMCs Infected	98.01

Table 3: HIV-1 copy number per 1000 cells for Donor 3 and Donor 4

	Mean HIV copies per 1000 cells
Donor 3	
AML Mock infected	0
AML Infected	4032.31
Eos Mock infected	0
Eos + Heat-inactivated virus	4966.57
Eos Infected	3842.57
PBMCs Mock infected	0
PBMCs Infected	30.02
PBMCs + IL-2 Mock infected	0
PBMCs + IL-2 Infected	19.76
Donor 4	
AML Mock infected	0
AML + Heat-inactivated virus	0
AML Infected	16598.02
Eos Mock infected Day 0	0
Eos + Heat-inactivated virus Day 0	1295.01
Eos Infected Day 0	433.49
Eos Mock infected Day 3	0
Eos + Heat-inactivated virus Day 3	206.01
Eos Infected Day 3	489.98
PBMCs Mock infected	0
PBMCs + Heat-inactivated virus	0
PBMCs Infected	155.72
PBMCs + IL-2 Mock infected	0
PBMCs + IL-2 Infected	143.49

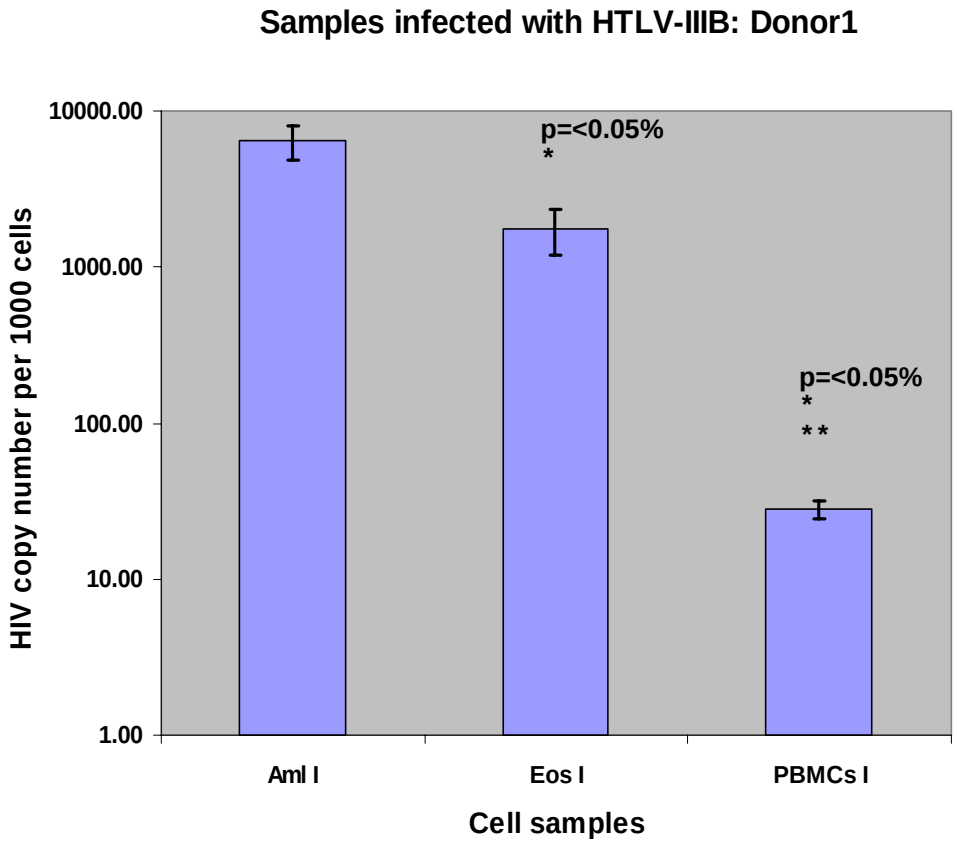
Figure 12: Comparison between infection of AML14.3D10 cells, eosinophils and PBMCs without IL-2 stimulation for donor 1.

AML I = AML14.3D10 cells infected with HTLV-IIIB

Eos I = Eosinophils infected with HTLV-IIIB

PBMCs I = PBMCs infected with HTLV-IIIB without IL-2 stimulation

Figure 12: Comparison between infection of AML14.3D10 cells, eosinophils and PBMCs without IL-2 stimulation for donor 1.



***** = significant difference between AML I and Eos I

****** = significant difference between AML I and PBMCs I, and Eos I and PBMCs I

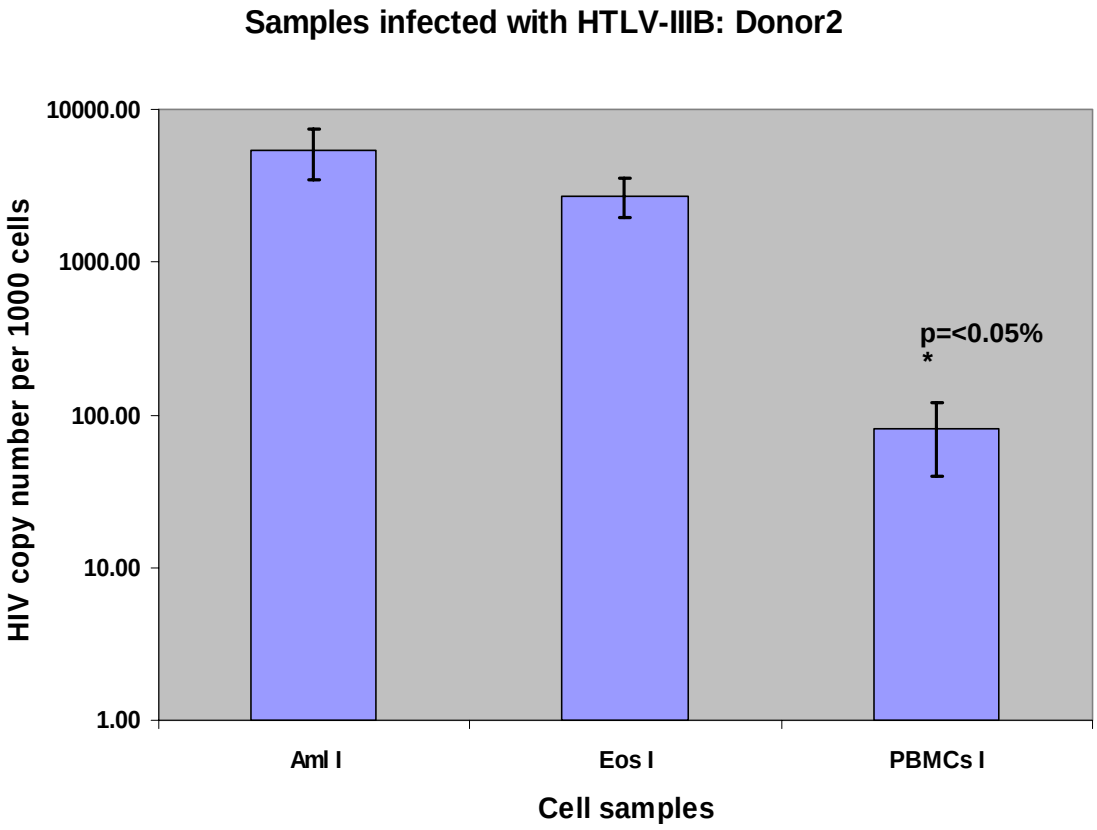
<u>Figure 13:</u> Comparison between infection of AML14.3D10 cells, eosinophils, and PBMCs without IL-2 stimulation for donor 2.

AML I = AML14.3D10 cells infected with HTLV-IIIB

Eos I = Eosinophils infected with HTLV-IIIB

PBMCs I = PBMCs infected with HTLV-IIIB without IL-2 stimulation

Figure 13: Comparison between infection of AML14.3D10 cells, eosinophils and PBMCs without IL-2 stimulation for donor 2.



***** = significant difference between AML I and PBMCs I, and Eos I and PBMCs I

<u>Figure 14:</u> Comparison between infection of AML14.3D10 cells, eosinophils and PBMCs without IL-2 stimulation for donor 3.

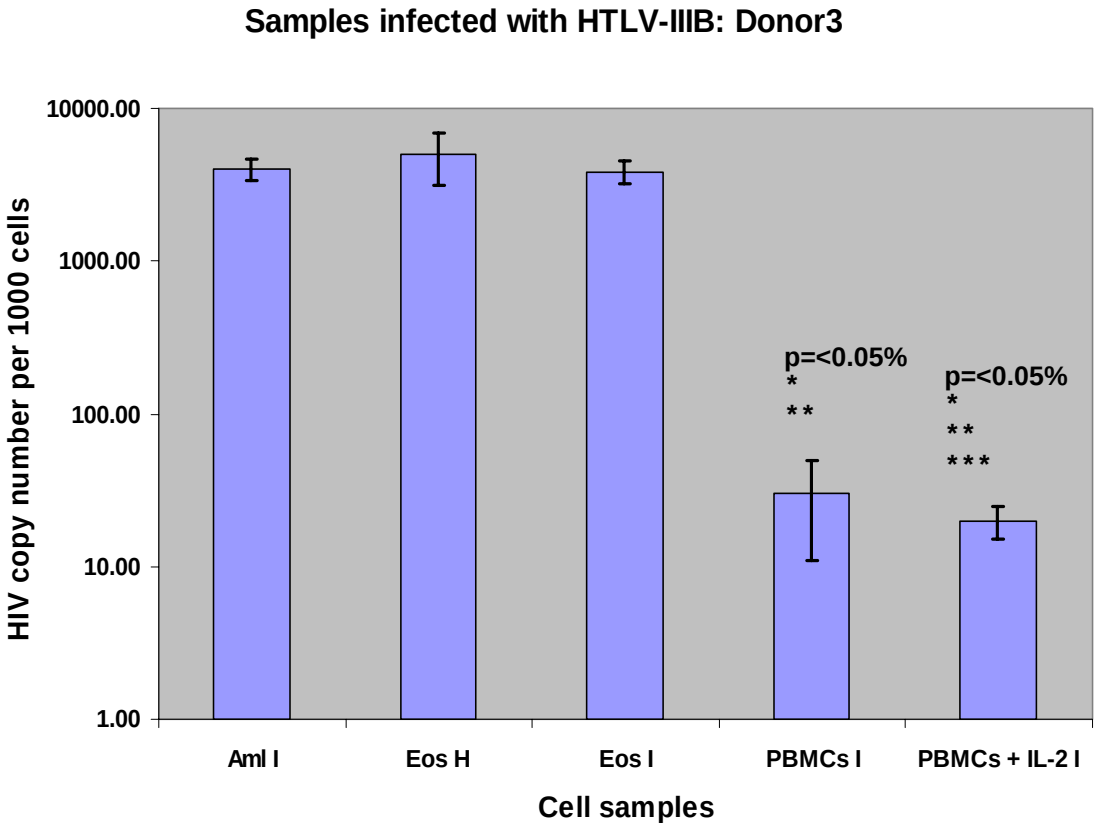
AML I = AML14.3D10 cells infected with HTLV-IIIB

Eos I = Eosinophils infected with HTLV-IIIB

PBMCs I = PBMCs infected with HTLV-IIIB without IL-2 stimulation

PBMCs + IL-2 I = PBMCs infected with HTLV-IIIB with IL-2 stimulation

Figure 14: Comparison between infection of AML14.3D10 cells, eosinophils and PBMCs without IL-2 stimulation for donor 3.



***** = significant difference between AML I and PBMCs I, and Eos I and PBMCs I

****** = significant difference between AML I and PBMCs + IL-2 I, and Eos I and PBMCs + IL-2 I

******* = significant difference between PBMCs I and PBMCs + IL-2 I

Figure 15: Comparison between infection of AML14.3D10 cells, eosinophils and PBMCs without IL-2 stimulation for donor 4.

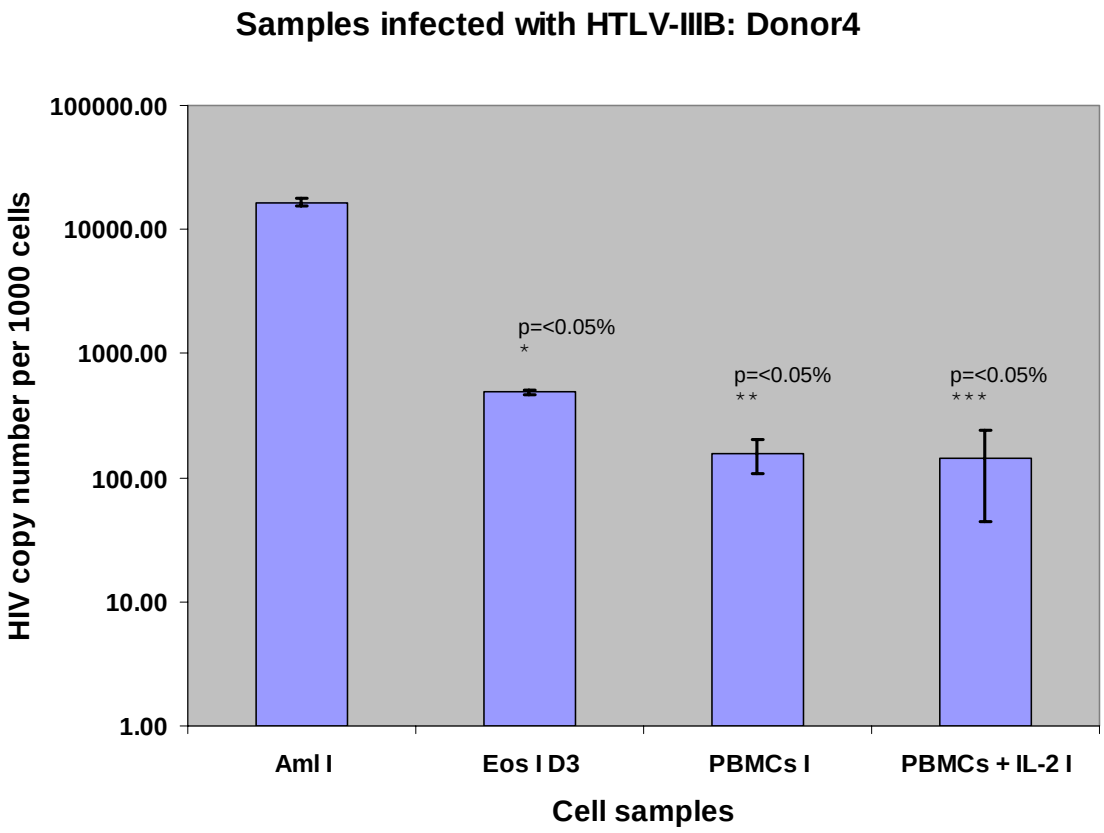
AML I = AML14.3D10 cells infected with HTLV-IIIB

Eos I D3 = Eosinophils infected with HTLV-IIIB with DNA harvested three days post-infection

PBMCs I = PBMCs infected with HTLV-IIIB without IL-2 stimulation

PBMCs + IL-2 I = PBMCs infected with HTLV-IIIB with IL-2 stimulation

Figure 15: Comparison between infection of AML14.3D10 cells, eosinophils and PBMCs without IL-2 stimulation for donor 4.



***** = significant difference between AML I and Eos I D3

****** = significant difference between AML I or Eos I D3 and PBMCs I

*******= significant difference between AML I or Eos I D3 and PBMCs + IL-2 I

<u>Figure 16:</u> Comparison between infection of eosinophils infected with heat-inactivated HTLV-IIIB and eosinophils infected with HTLV-IIIB on day 0 and day 3 for donor 4.

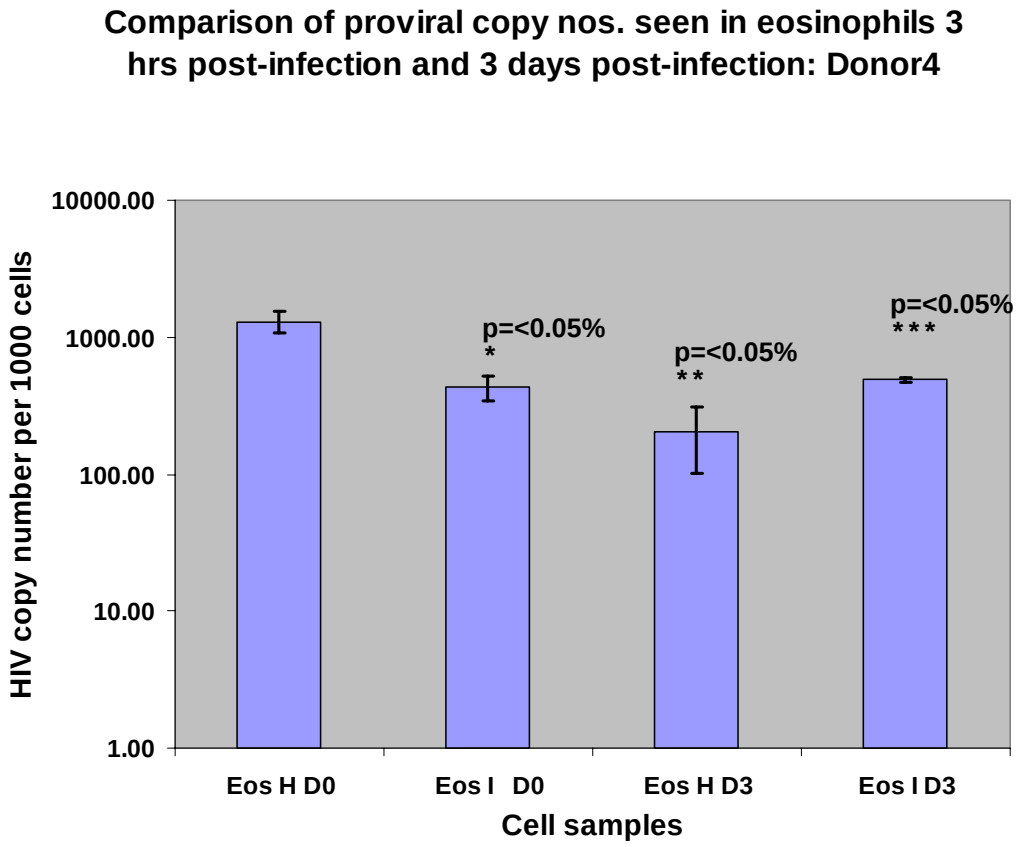
Eos H D0 = Eosinophils infected with heat-inactivated HTLV-IIIB with DNA harvested three hours post-infection on day 0

Eos I D0 = Eosinophils infected with HTLV-IIIB with DNA harvested three hours post-infection on day 0

Eos H D3 = Eosinophils infected with heat-inactivated HTLV-IIIB with DNA harvested three days post-infection

Eos I D3 = Eosinophils infected with HTLV-IIIB with DNA harvested three days post-infection

Figure 16: Comparison between infection of eosinophils infected with heat-inactivated HTLV-IIIB and eosinophils infected with HTLV-IIIB on day 0 and day 3 for donor 4.



***** = significant difference between Eos H D0 and Eos I D0

****** = significant difference between Eos H D0 and Eos H D3

*******= significant difference between Eos H D3 and Eos I D3

Figure 17: Comparison between infection of AML14.3D10 cells, eosinophils and PBMCs without IL-2 stimulation with heat-inactivated HTLV-IIIB for donor 4.

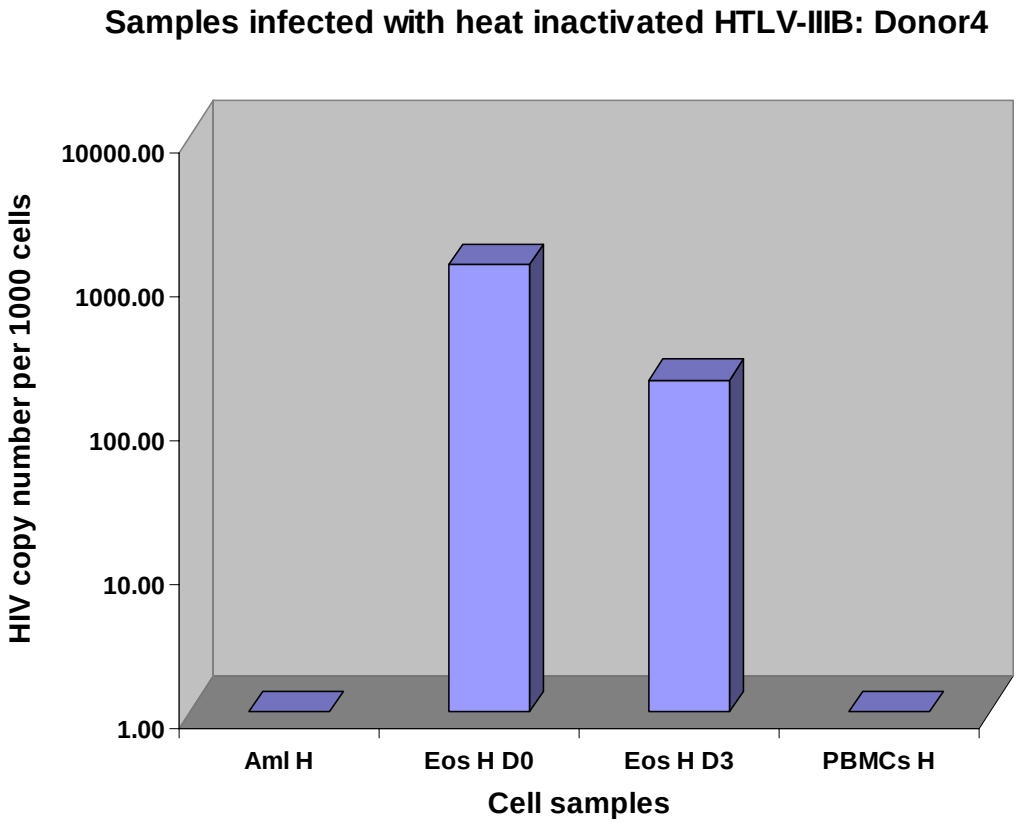
AML H = AML14.3D10 cells infected with heat-inactivated HTLV-IIIB DNA harvested three days post-infection

Eos I D0 = Eosinophils infected with HTLV-IIIB with DNA harvested three hours post-infection on day 0

Eos H D3 = Eosinophils infected with heat-inactivated HTLV-IIIB with DNA harvested three days post-infection

PBMCs H = PBMCs infected with heat-inactivated HTLV-IIIB DNA harvested three days post-infection

Figure 17: Comparison between infection of AML14.3D10 cells, eosinophils and PBMCs without IL-2 stimulation with heat-inactivated HTLV-IIIB for donor 4.



Assessment of productive HIV infection was done by screening samples for p24 production by using ELISA. However, p24 production did not correlate with the proviral copy numbers detected by Q-PCR (figure 18). HIV-1-infected AML14.3D10 cells produced significantly higher amounts of p24 (figure 18 v). Quiescent PBMCs had the least amount of p24 production. A lot of donor variability was also observed. No positive p24 result was obtained for any cell sample infected with heat-inactivated HTLV-IIIB virus.

Figure 18: p24 production for individual donors

Figure 18 i. = donor 1

Figure 18 ii. = donor 2

Figure 18 iii. = donor 3

Figure 18 iv. = donor 4

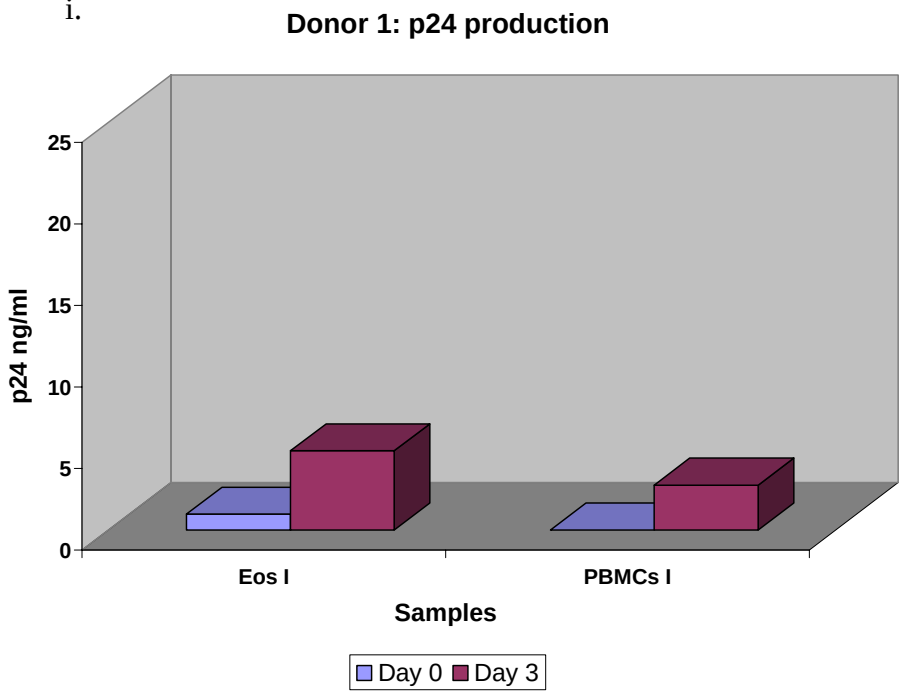
Figure 18 v. = p24 production by Aml14.3D10 cells used as positive control in each experiment

Day 0 samples were collected 3 hours post-infection to determine residual virus

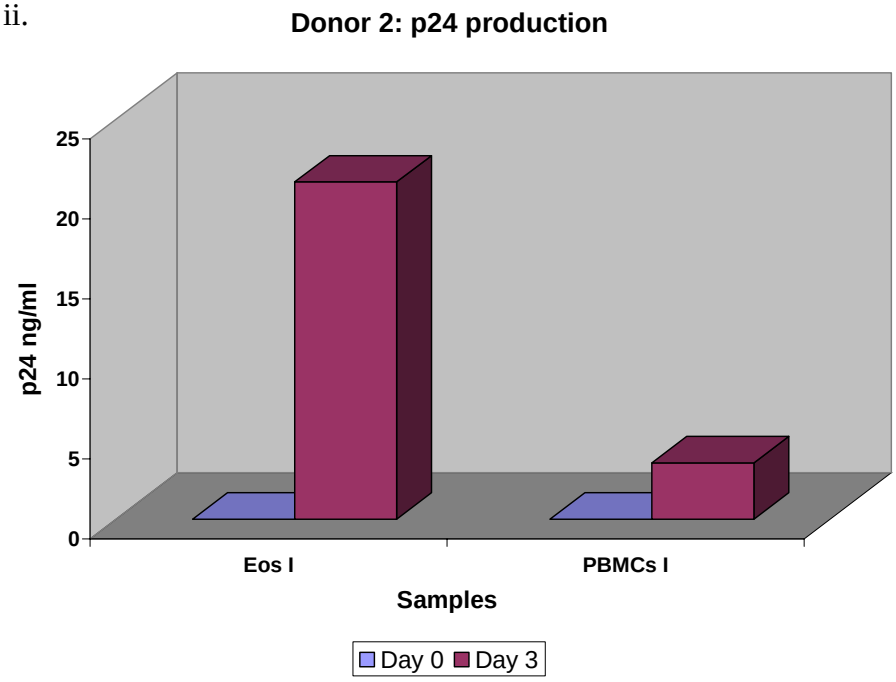
Day 3 samples were collected 60 hours post-infection to determine p24 production indicating productive infection

Figure 18: p24 production

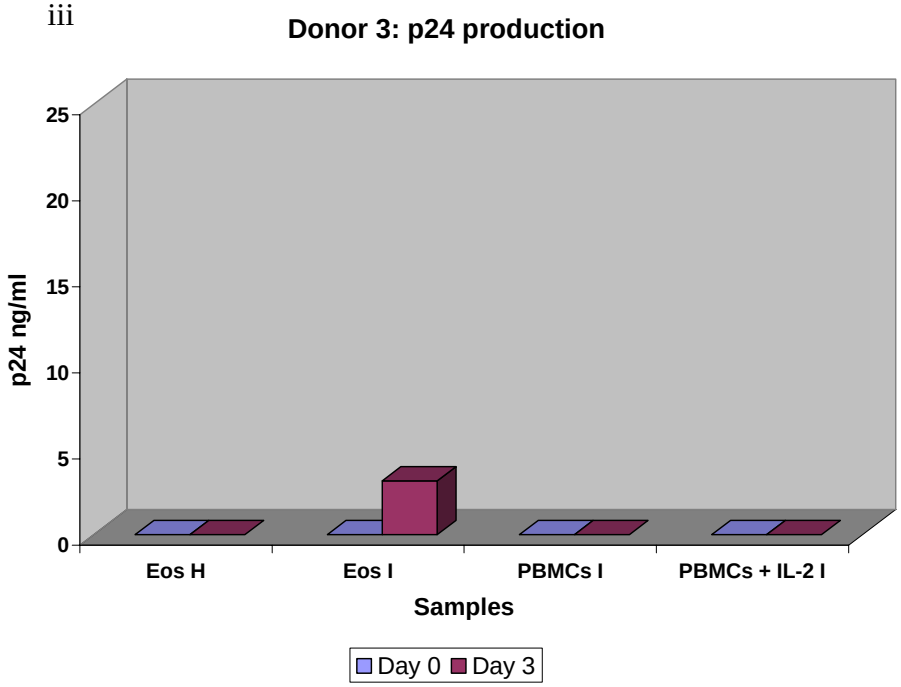
i.



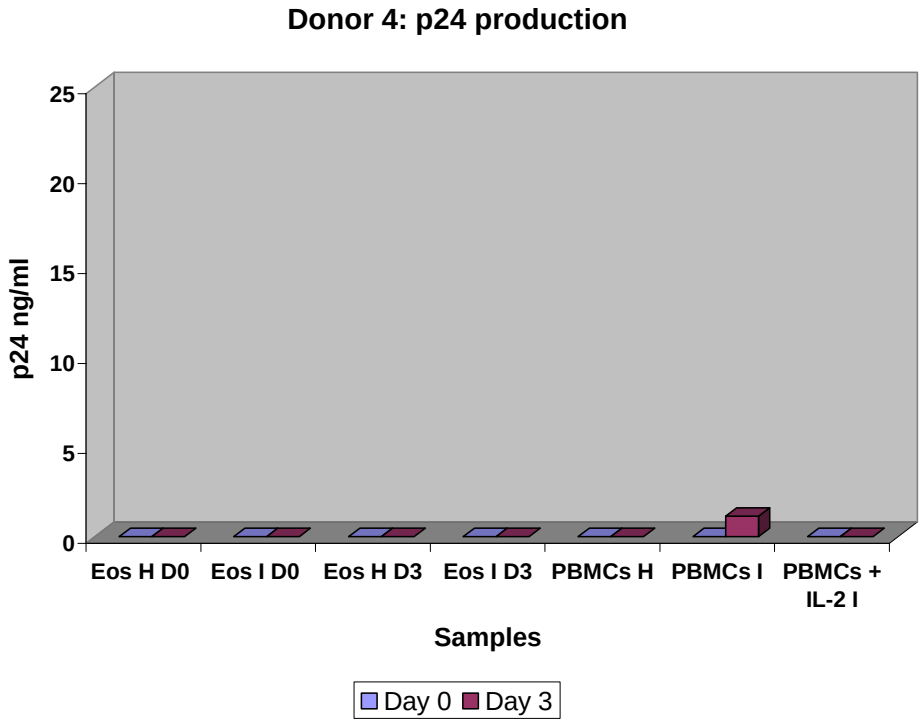
ii.



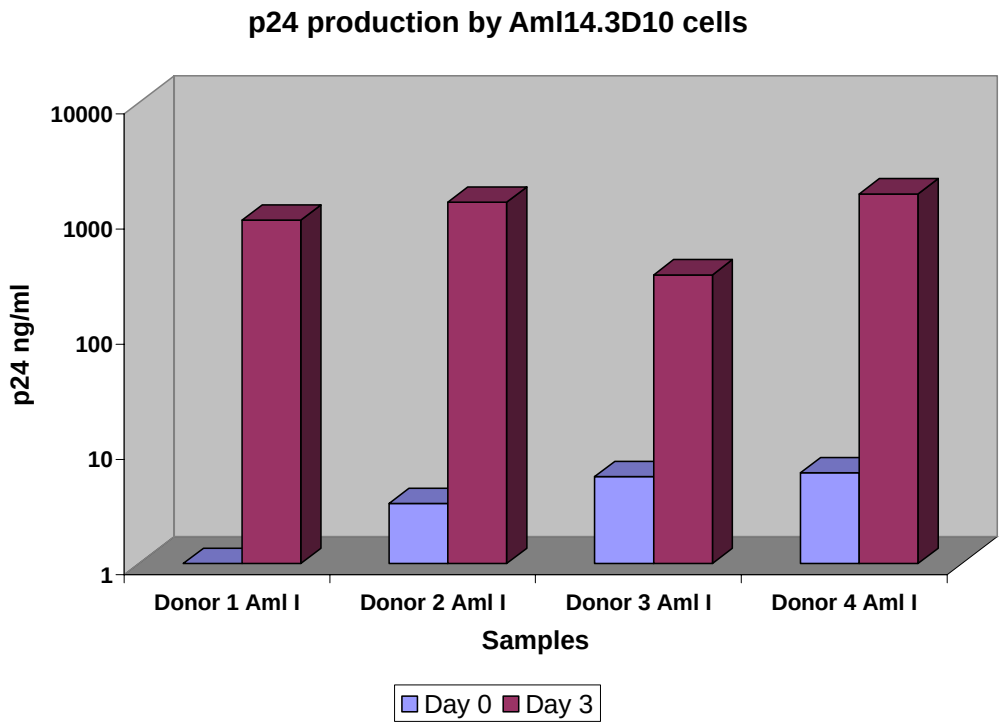
iii


Donor 3: p24 production
25
20
15
10
5
0
p24 ng/ml
Eos H
Eos I
PBMCs I
PBMCs + IL-2 I
Samples
Day 0
Day 3

iv.


Donor 4: p24 production
25
20
15
10
5
0
p24 ng/ml
Eos H D0
Eos I D0
Eos H D3
Eos I D3
PBMCs H
PBMCs I
PBMCs + IL-2 I
Samples
Day 0
Day 3

v.


p24 production by Aml14.3D10 cells
10000
1000
100
10
1
p24 ng/ml
Donor 1 Aml I
Donor 2 Aml I
Donor 3 Aml I
Donor 4 Aml I
Samples
Day 0
Day 3

Determination of multiplicity of infection was done on day 14 post-infection. Wells were scored positive or negative for p24 production. Table 4 shows the results for detection of p24 production in the plate for $TCID_{50}$ determination.

Calculation of $TCID_{50}$ was done using the Karber formula (19)

The equation used is:

$$M = xk + d\,[0.5 - (1/n)\,(r)]$$

$M = TCID_{50}$

xk = index of the highest dilution with all positive wells

r = sum of the number of negative responses

d = spacing between dilutions

n = wells per dilution

Calculation done was as follows:

$M = 7 + 1[0.5 - (1/6) \times (12)]$

$M = 5^{3.833}$

$M = 10^{2.68}$

Correcting for initial dilution,

$TCID_{50}/ml = 5 \times 10^{2.68}$

$TCID_{50}/ml = 2.51 \times 10^{3}$

Since 1ml of virus stock was used to infect 1.5×10^{6} cells, the multiplicity of infection is 0.002

Table 4: Wells scored positive or negative depending on the p24 production for replicates

Dilutions	5	5^{-2}	5^{-3}	5^{-4}	5^{-5}	5^{-6}	5^{-7}
A	+	+	+	+	–	–	–
B	+	+	+	–	–	–	–
C	+	+	+	–	–	–	–
D	+	+	+	–	–	–	–
E	+	+	+	+	–	–	–
F	+	+	+	–	–	–	–

V. DISCUSSION

HIV-1 infection has been widely studied in T-cells, macrophages and more recently, in dendritic cells. However, the possibility of infection in eosinophils has been neglected. In this study we have shown that eosinophils are highly susceptibility to infection with HIV-1 strain, HTLV-IIIB.

Eosinophil population forms a very small subset of the total leukocyte cells. Moreover, they have no specific cell surface marker. Hence, it was a challenge to isolate eosinophils from peripheral blood. By using microbead magnetic labeling followed by negative selection, isolation of relatively high numbers of eosinophils was possible (Appendix 1). However, individual donor variability was present and the number of eosinophils isolated varied from donor to donor.

As suggested by flow cytometry data analysis, the purity of isolated eosinophils was >99%. This was also confirmed by screening of stained cytopreps. Nagase et al. have reported that (29) expression of CXCR4 was

decreased on addition of GM-CSF but a 24 hour incubation at 37^0C up-regulated the expression of CXCR4. However, our own results confirm the previously reported findings (23, 30) that the viability of eosinophils was increased in the presence of GM-CSF. Thus, it was necessary to strike a balance between the down-regulation of CXCR4 co-receptor, which could affect the ability of HIV-1 to infect eosinophils, and addition of GM-CSF, which affects the survival of eosinophils. This was achieved by culturing eosinophils overnight, prior to infection, at 37^0C without the addition of GM-CSF. Post-infection, 5pM GM-CSF was added to the eosinophil culture. It has been documented that the eosinophil viability starts increasing with the addition of as little as 1pM GM-CSF and plateaus at 10pM (30) and addition of 5pM GM-CSF during infection with HIV-1 has been used previously (47). Using this method, a reasonable number of eosinophils survived till post-infection day 3 (Appendix B).

Q-PCR detection of HIV DNA copies in eosinophil cellular DNA supports the hypothesis that eosinophils are susceptible to HIV-1 infection. Further analysis reveals that HTLV-IIIB infected eosinophils show significantly higher HIV copy number per 1000 cells as compared to PBMCs with or without IL-2 stimulation. However, in two out of 4

donors, no significant difference was observed between amount of infection in AML14.3D10 cells and eosinophils. Even in donors with significant difference between the amount of infection between AML14.3D10 cells and eosinophils, eosinophils still show a very high HIV copy number. This suggests that the rate of infection in eosinophils, which are terminally differentiated, might approach that of a dividing cell-line. This is an interesting phenomenon which needs further in-depth investigation regarding the interaction between eosinophils and HIV and the dynamics of infection.

The observation of a high HIV copy number in eosinophils infected with HTLV-IIIB which was heat inactivated at 56^0C for 30 minutes was also unusual and unexpected. However, there was a significant decrease in the number of proviral copies detected 3 hours post-infection and those detected 3 days post-infection (figure 16). It has been demonstrated that thermal treatment of HIV at 56^0C for 30 minutes results in >5 log decrease in the proportion of original infectious $dose_{50}$ (ID_{50})(28). Other studies also confirm this finding (18, 33, 41). Following thermal treatment, even though there was a decrease in infectivity, the structure and function of viral envelope proteins was preserved (35) and there was enhanced binding

of gp120 to CD4 receptor (18). Also, eosinophils are antigen-presenting cells (36). Thus, various possibilities could be considered. It is possible that increased gp120 binding to CD4 could mediate viral entry; and it has been demonstrated that some part of reverse transcription and minus strand formation is detected in virions prior to infection and entry into cells (43, 52). Since the primers used are in the pol region, the detection of HIV copies could be this partially transcribed DNA. However, there was no detection of proviral copies in AML14.3D10 cells and PBMCs infected with similarly heat-inactivated virus. The second possibility was that primary eosinophils might phagocytose the entire virion as documented for dendritic cells (13, 21) and there is detection of the partially reverse transcribed genome by Q-PCR. The absence of viral copies in this scenario could be explained by the fact that lymphocytes are not phagocytic cells and phagocytosis in AML14.3D10 cells has not been documented. However, there is no documented evidence that eosinophils have either the DC-SIGN or DC-SIGNR receptors which are known to be responsible for HIV trans-infection of cells like dendritic cells (20). The last possibility to consider is ineffective inactivation of virus following thermal inactivation at 56^0C for 30 minutes. This could be safely disregarded because in

this scenario, AML14.3D10 cells and PBMCs should also show some amount of infection. To conduct a further study of this finding it would be necessary to allow the infected cells to remain in culture for a longer period of 7-10 days. It is important to note if there is a time-dependent decay in viral load in eosinophils infected with heat-inactivated virus. Experiments can also be done to study productive infection in these infected eosinophils. Also an assessment of eosinophils for expression of DC-SIGN or its related family members is warranted. In-depth study of trans-infection in eosinophils can also be done. It is also necessary to see if the HIV DNA detected in this study is integrated into the host cell genome. This can be done by using the previously described *Alu*-LTR PCR method (6-8).

Donor variability was observed in the p24 amounts and there was no co-relation between the number of proviral copies detected and the observed p24 production. The low levels of p24 production observed PBMCs could be a result of infection of quiescent cells, in whom HIV integration does not occur (38, 50, 51). Eosinophils infected with HTLV-IIIB showed p24 production in three out of four donors indicating a productive infection. The amount of p24 produced was significantly less than that produced by AML14.3D10 cells. However, it must be borne in mind that

AML14.3D10 cells divide continuously thus amplifying the initial infection whereas eosinophils are terminally differentiated cells. When compared to infected PBMCs, infected eosinophils not only had a higher proviral copy number consistently, but also showed higher amounts of p24 production. Thus study of eosinophil infection with HTLV-IIIB could provide another significant clue in the pathogenesis of HIV infection and progression to AIDS.

Summary of findings

A. *Reasonable numbers of eosinophils can be isolated from 60 milliliters of peripheral human blood.*
B. *Isolated eosinophils are greater than 99% pure.*
C. *Eosinophils show a green and red–orange auto-fluorescence.*
D. *Addition of GM-CSF to culture media increases eosinophil viability significantly by day 3.*
E. *Eosinophils can be infected with HTLV-IIIB.*
F. *The HIV copy numbers detected in infected eosinophils are significantly higher than those observed in quiescent PBMCs with or without IL-2 stimulation.*
G. *Eosinophils infected with heat inactivated HTLV-IIIB virus show high HIV copy numbers.*
H. *Infected eosinophils produce p24 indicating a productive infection but there is no p24 production detected for eosinophils infected with heat inactivated HTLV-IIIB virus.*
I. *Amount of p24 produced is more in infected eosinophils as compared to PBMCs with or without IL-2 stimulation*
J. *There is no correlation between the detection of HIV copy numbers and the amount of p24 produced.*

VI. APPENDIX

Appendix A: Number of eosinophils isolated from 60ml peripheral blood from each donor:

Donor id	Granulocytes per 60 ml	Eosinophils per 60ml	% eosinophils from total granulocytes
110-05	199440000.00	17460000.00	8.75
111-05	302250000.00	6240000.00	2.06
112-05	105315000.00	4200000.00	3.99
113-05	135000000.00	2500000.00	1.85
117-05	210000000.00	4650000.00	2.21
118-05	480000000.00	7650000.00	1.59
119-05	500000000.00	10890000.00	2.18
121-05	270000000.00	7875000.00	2.92
122-06	202500000.00	20550000.00	10.15
125-06	315750000.00	3200000.00	1.01
127-06	282500000.00	4225000.00	1.50
129-06	537500000.00	40200000.00	7.48
130-06	425000000.00	16500000.00	3.88

Appendix B: Post-infection 60 hour viable cell counts

Sample id	Cell counts	% viability
Donor 1		
AML M	7380000	492.00
AML H	7995000	533.00
AML I	6970000	464.67
Eos M	328000	21.87
Eos I	369000	24.60
PBMCs M	307500	20.50
PBMCs I	553500	36.90
Donor 2		
AML M	7933500	528.90
AML H	8118000	541.20
AML I	6211500	414.10
Eos M	410000	27.33
Eos I	676500	45.10
PBMCs M	574000	38.27
PBMCs I	553500	36.90
Donor 3		
AML M	1850000	123.33
AML I	3733333	248.89
Eos M	121000	8.07
Eos H	231000	15.40

Eos I	57750	3.85
PBMCs M	381500	25.43
PBMCs I	395900	26.39
PBMCs + IL-2 M	440000	29.33
PBMCs + IL-2 I	397850	26.52
Donor 4		
AML M	5227500	348.50
AML H	4350000	290.00
AML I	2900000	193.33
Eos M	60000	4.00
Eos H	160000	10.67
Eos I	185000	12.33
PBMCs M	380000	25.33
PBMCs H	285000	19.00
PBMCs I	400000	26.67
PBMCs + IL-2 M	405000	27.00
PBMCs + IL-2 I	64350	4.29

Appendix C: List of Abbreviations

AIDS	Acquired immunodeficiency virus
AML H	AML14.3D10 cells infected with heat-inactivated virus
AML I	AML14.3D10 cells infected with HTLV-IIIB virus
AML M	AML14.3D10 cells mock infected
APC	Allophycocyanin
ARV	AIDS related virus
BHQ	Black hole quencher
CCR5	Chemokine receptor 5
CXCR4	Chemokine receptor 4
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
Eos H	Eosinophils infected with heat-inactivated virus
Eos I	Eosinophils infected with HTLV-IIIB virus
Eos M	Eosinophils mock infected
FAM	Fluorescein
FCS H	Fetal calf serum heat-inactivated at 56^0C for 30 min
FRET	Fluorescence Resosnance Energy Transfer

GM-CSF	Granulocyte-monocyte colony stimulating factor
HAS	Human autologous serum
HHS	Human homologous serum
HIV	Human immunodeficiency virus
Hr	Hours
HTLV-IIIB	Human T-cell leukemia virus-IIIB
Ig	Immunoglobulin
IL-2	Interleukin-2
JOEN	6-carboxy-4', 5'-dichloro-2', 7'-dimethoxyfluorescein NHS ester
MOI	Multiplicity of infection
Min	Minutes
µl	Microliter
ng	Nanogram
NSI	Non-syntitia inducing
PBMCs	Peripheral blood mononuclear cells
PBMCs H	PBMCs infected with heat-inactivated virus
PBMCs I	PBMCs infected with HTLV-IIIB virus
PBMCs M	PBMCs mock infected
PBMCs+IL-2	PBMCs stimulated with IL-2
PBMCs+IL-2 I	PBMCs+IL-2 infected with HTLV-IIIB virus
PBMCs+IL-2 M	PBMCs+IL-2 mock infected

PFA	Paraformaldehyde
RNA	Ribonucleic acid
RT	Reverse transcriptase
SI	Syntitia inducing
ssRNA	Single-stranded RNA
$TCID_{50}$	Tissue-culture infectious dose 50
TAMRA	Carboxytetramethylrhodamine

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