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Human Immunodeficiency Virus-1 Productively Infects Mature Terminally Differentiated Eosinophils in HIV/AIDS Patients

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Human Immunodeficiency Virus-1 Productively Infects Mature Terminally Differentiated Eosinophils in HIV/AIDS Patients

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

By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Jude N. Atem ENTITLED Human Immunodeficiency Virus I Productively Infects Mature Terminally Differentiated Eosinophils in HIV/AIDS Patients BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Atem, Jude N. M.S., Program in Microbiology and Immunology, Wright State University, 2008. Human Immunodeficiency Virus I Productively Infects Mature Terminally Differentiated Eosinophils in HIV/AIDS Patients.

Eosinophils express membrane CD4 protein and can bind HIV-1 glycoprotein (gp) 120. Therefore, eosinophils could serve as host cells for HIV-1 infection in vivo, especially in the late phase of the infection. In culture, HIV-1 infects eosinophil precursors and primary eosinophils. Additionally HIV-1 proviral DNA sequences have been detected in the eosinophils of some HIV-1 positive patients. Since elevated levels of eosinophils occur during HIV-1 infection and parasitic infections, it implies that co-infection of parasites and HIV-1 could cause a much greater increase in the level of potential HIV-1 susceptible eosinophils. Therefore HIV-1 infection of eosinophils could partly explain the rapid spread of HIV-1 and the exacerbation of the disease especially in developing countries where HIV-1 and parasitic infections run concurrently. This study is a critical analysis of the current knowledge on HIV-1 infection of eosinophils focused on highlighting evidence that HIV-1 can productively infect mature human eosinophils in HIV/AIDS patients.

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INTRODUCTION

The degree or extent of HIV-1 infection depends on the efficiency of the virus/receptor interaction and the intracellular milieu of the host cell. Since eosinophils express membrane CD4 and CXCR4 molecules and can bind to HIV-1 gp 120 (1, 2) it is logical that they can serve as potential host cells for HIV-1 productive infection in vivo. Therefore eosinophilia that occurs alongside skin infections reported in HIV/AIDS patients could play a significant role in HIV-1 infection (3, 4).

In HIV/AIDS individuals, co-infection with parasites also leads to eosinophilia. Therefore the level of HIV-1 susceptible eosinophils is cumulatively elevated as a result of the co-infection. Concomitant immune activation due to parasitic infections makes host cells more permissive to HIV-1 infection favors rapid HIV-1 replication. Heightened levels of eosinophils and their infection by HIV-1 could ultimately lead to alterations in eosinophil function and cause immune dysregulation (5, 6).

Therefore co-infection of eosinophils by HIV-1 and parasites could account for the exacerbation of HIV/AIDS and the decline of the health status of patients especially in developing countries where the two run concurrently (7, 8).

Like monocytes, eosinophils could be latent viral reservoirs that enhance the replication, dissemination and trafficking of HIV-1 especially in the gut associated lymphoid tissues (GALT) or the mucosal associated lymphoid tissues (MALT) where eosinophils predominate(9, 10). Therefore HIV-1 infection of eosinophils could pose an enormous challenge to treatment and vaccine development and could be linked to some extent to the failure reported with the use of the Highly Active Antiretroviral Treatment (HAART) (11, 12).

It is therefore important that studies are conducted not only to ascertain and quantitate HIV-1 infection of eosinophils but also to demonstrate the effect of HIV-1 infection on the normal function of eosinophils and find out new treatment protocols that can target HIV-1 reservoirs. I intend to provide a succinct analysis of the current knowledge available on the infection of mature terminally differentiated human eosinophils by HIV-1, starting with a review of the general biology of HIV-1 and eosinophils.

HUMAN IMMUNODEFICIENCY VIRUS-1

The virus implicated as the cause of the acquired immune deficiency syndrome (AIDS) was first isolated in 1983 from a patient with multiple lymphadenopathies (13). In 1986 human immunodeficiency virus-1 (HIV-1) replaced the multiplicity of names for the virus (14).

Structure and Genome

HIV-1 is a lentivirus, a member of the retrovirus family. It is roughly spherical with a genome made of two single stranded RNA molecules. The genome is comprised of nine genes, grouped into accessory, regulatory and structural genes that are flanked by end terminal repeats. The gag-pol-env structural genes encode the antigenic p24 and p17 matrix protein, viral enzymes and the envelope glycoproteins, gp120/41, respectively. A core protein or capsid, made of p24 structural units, encases the single stranded RNA molecules that are closely bound to viral enzymes. Surrounding the capsid is the phospholipid envelope with an underlying protein shell, the p17 matrix protein(15). The phospholipid envelope bears surface and transmembrane gp120/41 proteins alongside host proteins acquired during the budding process.

Cellular Tropism

Human immunonodeficiency virus 1 infects a wide range of susceptible cells throughout the organ systems of the body. HIV-1 utilizes CD4 protein (16), and the co-receptors CXCR4 and/or CCR5, chemokine receptors that are members of the G-protein coupled 7 transmemberane receptor family to initiate entry and infection of host cells(17-21). Dendritic cells (DCs) utilize lectin like receptors, DC-SIGN, to trap, without being infected, and transfer HIV-1 to T cells in the mucosa(22).

HIV-1 infects cells in organ sytems like the gastrointestinal system, the genito-urinary system, the, the musculo-skeletal system, the nervous system and hematopoeitic sytem comprised of monocytes/macrophages, T cells, B cells, DCs and stem cells. Peripheral blood mononuclear cells (PBMC)/macrophages and T helper lymphocytes (CD4 T cells) are the most susceptible human host cells with infection of CD4+ T cells resulting in the highest HIV-1 titers (23).

Co-receptor molecules determine HIV-1 cellular tropism. The R5 HIV-1 strains that infect monocytes and macrophages utilize CCR5 co-receptors and do not induce syncytium formation are termed M-Tropic or non syncytium inducing (NSI) HIV-1 (24). T-tropic (T4) or syncytium

inducing (SI) strains infect T lymphocytes and utilize CXCR4 co-receptors and cause the formation of giant syncytial cells (25). Only the T4 strains infect eosinophils since they express only the CXCR4 and CCR3 co-receptors on their cell membrane.

Life Cycle of HIV-1

HIV enters host cells in a pH independent manner by the attachment of the lipid envelope borne gp120/41 to CD4 protein (16, 17, 26). Attachment causes conformational changes in the surface gp120 followed by the binding of the transmembrane gp41 molecule with CXCR4 or CCR5 (18, 21). This leads to the fusion of the virus with host cell membrane. As a result, the viral core or the nucleocapsid that contains viral (v) RNA and enzymes is released into the host cytoplasm where vRNA is transcribed by reverse transcriptase (RT) into a double stranded copy DNA (cDNA). The cDNA is then transported to the host nucleus as part of a pre integration complex and it is integrated into the host genome by the action of the viral enzyme, integrase (IN). The provirus, that is, the integrated cDNA is transcribed by host enzymes into viral mRNA and genomic RNA. Subsequently, full length mRNA is translated into the gag-pol polyprotein that is cleaved by the viral protease into gag and pol proteins. Other spliced or subgenomic mRNA

molecules are also translated into other viral proteins. Assembly of the viral proteins and genomic RNA into mature viral particles occurs at host cell membrane where the virus buds off and acquires the HIV-1 lipid envelope of HIV-1 (23). The sequence of events from virus-host fusion through integration of viral DNA into host genome to the manufacture of virion particles constitutes HIV productive infection

HIV-1 Disease Progression

Infection by HIV-1, like other lentiviruses, leads to slow degenerative disease. The progress of the infection can be divided into three phases, an acute viremic, a latent asymptomatic and a chronic symptomatic phase. Within weeks of the acute phase or primary HIV-1 infection (PHI) an initial peak of plasma viremia is noted accompanied by a rapid depletion of CD4+ T cells. One to 6 months following PHI, seroconversion occurs. This implies high levels of anti HIV-1 antibodies are produced while HIV-1 specific CD8+ cytotoxic T cells (CTLs) are activated resulting in a substantial fall in plasma viremia (27). The PHI progresses to the asymptomatic or latent phase of infection.

At the asymptomatic stage, in addition to a fall in plasma viral load there is a transient recovery of the CD4+ T cells to a steady state or set point that predicts the

prognosis of disease (28). The transient recovery is followed by a steady decline in CD4 T cell levels as a result of the direct killing of infected cells, up regulation of specific CTLs, low production of CD4+ T cells or apoptosis.

Once CD4+ T cells fall below 200 cells /µl, further decline becomes acute and the infection enters the chronic symptomatic phase at which point the patient's immune system becomes overwhelmed by opportunistic infections (OIs). Therefore, the acquired immunodeficiency syndrome (AIDS) is an end stage disease marked by the depletion CD4+ T cells (<200/µl blood) and a totally compromised immune system (23).

EOSINOPHILS

Ultrastructure

Eosinophils are terminally differentiated tissue dwelling granulocytes that are found mostly in sub mucosa tissues. They have a characteristic bi-lobed nucleus with no nucleoli connected by a visible filament that has no chromatin. Mature eosinophils are filled with secretory granules that can be grouped into four types, namely, primary, secondary or specific, the small dense secretory granules and vesiculotubular microgranules (29-31).

Functions of Eosinophils

Eosinophils are pleiotropic phagocytic granulocytes that function as antigen presenting cells in the immune system(32). They are involved in numerous disease processes including parasitic infections, allergies, exfoliative dermatitis, connective tissue diseases, vasculitis granulomatous diseases, and neoplasias. During parasitic infections, upon activation, eosinophils migrate to tissue sites and degranulate. They release release toxic proteins and lipid mediators that kill especially the larval form of helminthic parasites. Thus eosinophils play a major defensive role against helminthic infections. On the hand,

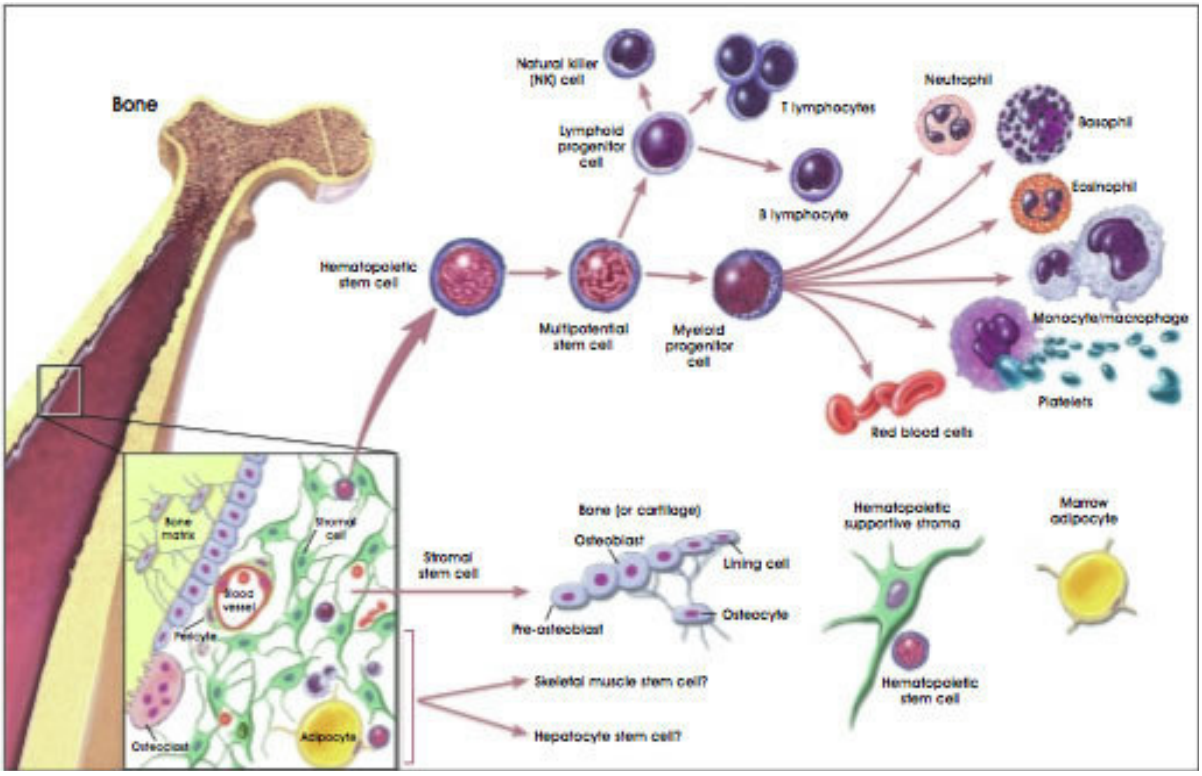
their toxic proteins cause tissue damage in allergic disorders(33).

Eosinophils also release growth factors and chemokines that include GM-CSF, IL-3, IL-5, IL-16, MIP-1α, RANTES and eotaxin. They are also a source of proinflammatory cytokines including IL-1, IL-6, IL-8, TNFα and immunoregulatory cytokines like INF-γ, IL-2, IL-4 and IL-10(29, 30, 34). Therefore, eosinophils play both a protective and pathological role, and regulate immune responses as well.

Development of Eosinophils

The self renewing pluripotent hematopoeitic stem cells (HSCs) in the bone marrow are the precursors of all blood cells. HSCs initially give rise to two cell lineages, the myeloid progenitors and the lymphoid progenitors. The lymphoid progenitors in turn give rise to the natural killer cells, the B and T lymphocytes, so called because they mature in the bone marrow and thymus gland respectively. B cells further differentiate into antibody secreting plasma cells upon antigenic activation; and T cells differentiate into two classes of effector cells, the cytotoxic T cells (CTL), whose main function is to kill cells infected with virus, and T helper cells that activate other cells like macrophages and B cells (35).

Figure 1. Development of blood cells from bone marrow



Adapted from http://stemcells.nih.gov/info/scireport/chapter5.asp

Myeloid progenitors further differentiate to basophils, eosinophils, neutrophils, erythrocytes, mast cells, platelet-generating megakaryocytes, blood monocytes that mature to tissue macrophages and dendritic cells (DCs)that also generated by lymphoid progenitors. Neutrophils, basophils and eosinophils are generally called polymorphonuclear leukocytes or granulocytes since their cytoplasm is packed with granules and their nuclei are irregularly shaped and segmented (36). The development of

eosinophils from the immature stage to the mature terminally differentiated cell is a continuum of unique morphological changes and proceeds in this order (36, 37),

Myeloblast→Promyelocyte → myelocyte → metamyelocyte →band cell→ mature eosinophil

Development from the myeloblast to myelocyte occurs by mitosis and from the myelocytes to mature eosinophils it is non mitotic. The promyelocytes are large cells with a single large euchromatic nucleus and a well developed nucleolus. The primary, non crystalloid core or azurophilic granules are synthesized at this stage. These granules contain the Charcot Leyden Crystal proteins. Promyelocytes divide and differentiate into myelocytes (36, 37).

The smaller less dense secondary or specific granules with a crystalloid core are synthesized at the myelocyte stage. These specific granules contain eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), eosinophil derived neurotoxin (EDN), and the major basic protein (MBP) that gives eosinophils the characteristic red stain with eosin. Myelocytes are only considered to be in the eosinophil lineage if their cytoplasm contains these secondary or specific granules (38).

Further division and differentiation of the myelocytes forms the metamyelocytes. Here total cell and nucleus size

reduce and indentation of the nucleus begins accompanied by the appearance of noticeable cytoplasmic organelles. Further condensation of the nuclei chromatin and the formation of more specific granules leads to the development of the band cell with its unique band shaped nucleus that has no visible filament. The band cell finally matures to the end stage non differentiating mature eosinophil (35, 38)

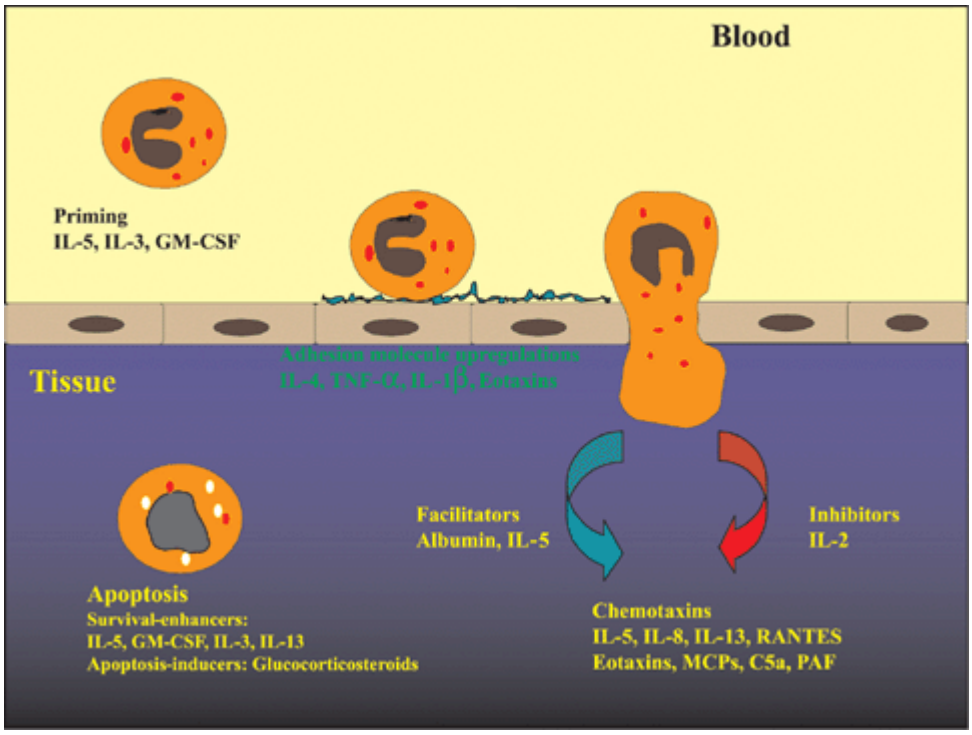
Specification of the eosinophilic lineage in the bone marrow is under the control of the transcription factors; PU.1 C/EBP and GATA-1 (39-41). Eosinophil expansion, differentiation and maturation are regulated by the eosinophilopoietins, IL-3, IL-5 and GM-CSF. Eotaxin and IL-5 control recruitmentment into circulation and tissues.

Eosinophil Trafficking

Once released from bone marrow, eosinophils remain in circulation for about 12 – 16 hours and then transmigrate into inflammatory sites or the mucosa tissues, especially the gastrointestinal tract (GIT) where they are prevalent (42). Tissue trafficking is initiated by priming of eosinophils with the cytokines IL-3, IL-5 and GM-CSF that are released by tissue macrophages and eosinophils themselves. Once primed eosinophil become more sensitive to chemoattractants and are induced to express L-selectins on

their cell membrane, while endothelial cells express P- and E-selectins. Expression of selectins enhances eosinophil adhesion to the vascular epithelial cell (43)

Figure 2. Transendothelial Migration of Eosinophils



Adapted from Lampinen, M et al 2004

L-selectins bind to Mucosal Addressin Cellular Adhesion Molecule (MAdCAM) -1 on endothelial cells while the endothelial P- and E-selectins bind to the carbohydrate molecules P-selectin Glycoprotein Ligand (PSGL) – 1 (CD162) and Sialyl Lewis X respectively. The reversible binding of selectins to their ligands is responsible for the slow rolling motion of eosinophils seen in blood vessels (35, 38).

Table 1.Eosinophil adhesion molecules and their ligands

	Eosinophil receptor	Ligand	Ligand upregulated by
Selectins	L-selectin (CD62L)	MAdCAM-1, CD34	
Integrins	VLA-4 (CD49d/CD29)	VCAM-1, fibronectin	IL-1, TNF-α, IL-4, IL-13
	VLA-6 (CD49f/CD29)	Laminin	
	CD11a/CD18	ICAM-1,-2,-3	IL-1, TNF-α, IFN-γ, eotaxin, IL-5
	CD11b/CD18	ICAM-1, fibrinogen, C3bi	
	CD11c/CD18	Fibrinogen, C3bi	
	α4β7	MAdCAM-1, VCAM-1, fibronectin	IL-1, TNF-α, IL-4
	αdβ2	VCAM-1	
Ig-like	ICAM-1/-3, PECAM-1 (CD31)	PECAM-1, αvβ3	
Carbohydrate	PSGL-1 (CD162)	P-selectin	Histamine, leukotrienes
	Sialyl Lewis X	E-selectin	Histamine, leukotrienes
Others	CD44	Hyaluronate	

Adapted from Lampinen, M., et al 2004

The rolling motion activates integrin receptors on eosinophils, leading to a much firmer adhesion between integrins and their ligands. The rolling cells are transiently arrested, setting the stage for extravasation.

Some of the integrins involved in eosinophil migration include CD11a/CD18 [leukocyte functional antigen (LFA) -1], CD11b/CD18 [Mac-1 or complement receptor 3 (CR3)], CD11c/CD18 [complement receptor 4 (CR4), p150, 95] that bind to the intercellular adhesion molecule (ICAM) -1 on endothelial cells and VLA-4 which bind to the vascular cell adhesion molecule (VCAM) -1 (44-46) .

Extravasation is mediated by the interaction between platelet endothelial cell adhesion molecule (PECAM)-1 and PECAM-1 or CD31 molecules expressed on both eosinophils and endothelial cell junctions. Eosinophils migrate to tissues along a chemotactic gradient created by the secretion of chemotactic factors like eotaxin-1, platelet activating factor (PAF), C5a, IL4, IL5, IL13 and RANTES secreted. Movement along the endothelial cell junction is characterized by adhesion/deadhesion and is mediated by cytokine priming, actin polymerization/depolarization systemand signal transduction mechanisms (35, 38).

Eosinophils then secrete enzymes that break the basement membrane of the endothelial cells and migrate in respond to chemotactic factors to mucosa tissue or inflammatory sites. IL5 and eotaxin-1 are the most important chemotactic factors involved in the circulation of eosinophils to tissue site (44).

RELEVANCE OF HIV-I INFECTION OF EOSINOPHILS

Eosinophilia, independent of allergies, parasitic infections, malignancies and other causes occurs in many HIV/AIDS patients. It is detected in association with skin diseases during the later stage of infection or chronic symptomatic stage (4, 7, 47-49), when the switch from the Th1 to Th2 cytokine profile occurs (50). HIV-1 infection of eosinophils could therefore make the pathology of the skin diseases or the general health of the patients worse.

During helminthic infections, the prevailing T_H2 cytokine profile perpetuates an abnormal increase in eosinophils aimed at mounting a defense against the infection (6). The resultant eosinophilia implies an increase in the level of HIV-1 sussceptible host cells. Since parasites are also exogenous stimuli, they activate immune cells and favor HIV-1 replication (5, 51). Therefore co-infection of parasites and HIV-1 would be very significant in the pathogenesis of AIDS, especially in developing countries where the two thrive concurrently.

In a cell culture model, it was demonstrated that only HIV-1 X4 strains, the predominant strain during the later phase of HIV-1 infection, can infect eosinophils (52-54). Since CD4 T cells are also infected by the HIV-1 X4; infection of eosinophils in the mucosal lining and in the

lamina propria in particular must play an important role in the activation of CD4 T cells, their infection by HIV-1 and possibly depletion of memory T cells in this region.

HIV-1 infection of eosinophils may cause dysregulation of eosinophil gene expression leading to abnormal cytokine profiles, alterations in normal cellular functions including abnormal immune regulation, poor anti helminthic activity, erratic degranulation and the release of proinflammatory cytokines and toxic proteins causing tissue damage. The dysregulation of eosinophils due to HIV-1 infection may also explain the compromised immunity reported in the GALT or the inadequate recovery of gut immunity despite the prolong use of HAART (11). The inefficiecy of HAART may therefore be explained by eosinophils also acting as HIV-1 reservoirs which makes it difficult for anti retroviral drugs to reach the integrated virus (55).

Since the productive infection of eosinophils by HIV-1 and concurrent parasitic infections could be the basis of the exacerbation of HIV/AIDS and the cause of a rapid collapse in patients' health especially in developing countries, and can also be linked to some extent to the failure of treatment protocol is one that the scientific community must address rigorously.

EVIDENCE OF HIV-1 INFECTION OF EOSINOPHILS

In the 15 years since eosinophils were shown to be CD4+ cells that bind HIV-1 gp120, (1), only three independent research groups (56-58) and our lab(52, 53) have been involved in studies to elucidate HIV-1 infection of eosinophils. In 1991, after failing to demonstrate HIV-1 infection of primary eosinophils, Freedman et al focused on HIV-1 infection of differentiating eosinophil precursors in bone marrow cultures (56).

They obtained bone marrow aspirates from 5 donors and purified them by centrifugation over ficoll gradients. The purified aspirates were cultured for 56 days on Iscove's Modified Dulbecco's Medium (IMDM) supplemented with human recombinant IL-5 to promote eosinophil maturation and differentiation. They infected cell cultures with three strains of HIV-1, namely, RF, RUT and HTLV-IIIB between day 7 and 21 and assessed the levels of CD4 proteins, HIV p24 antigen and gp160/41 by immunostaining with their own monoclonal antibodies (mAbs). One hundred cells from the control-uninfected culture were examined to determine the percentage of eosinophils that expressed CD4 molecules over time. They quantitated CD4 expression on each cell based on the intensity of the immunostaining with anti-CD4 mAb and scored them on a scale from 0 to 3. Enzyme-Linked

Immunosorbent Assay (ELISA) was used to determine p24 levels in culture supernatants. In situ hybridization with a 35S labeled DNA probe was used to determine vRNA in cell cultures. They measured RT activity in infected eosinophil cultures alone, and in co-cultures with Jurkat T-cells to ascertain the infectivity of the infected eosinophils (56).

Microscopic examination established that greater than 95% of cultured cells observed on day 7 as promyelocytes developed to eosinophils precursors after day 21 and matured to terminally differentiated eosinophils on day 56. The characteristic bi-lobed nucleus of eosinophils was also observed in cultures between day 49 and 63. CD4 expression was highest by day 28 for the uninfected eosinophil control culture and dropped steadily to insignificant levels by day 56. Therefore, to achieve maximum infection, cultures were infected between day 7 and 21 when CD4 expression was highest(56).

They first observed infection on the 14-day-old cultures with the cells expressing p24 and gp120/41 antigen on their cell membrane as detected by immunostaining. They detected infection in the two cell cultures inoculated with HIV-1 RF strain, in one of two inoculated with the RUT strain but no infection was observed in the two cell cultures inoculated with the IIIB strain. They detected

vRNA using in situ hybridization in 4% of infected cells and no vRNA was detected in contaminants that made up less than 5% of cell culture. Viral RNA, p24, nor gp160/41 was detected in control-uninfected cultures. Following the infection of eosinophil cultures RT activity increased from day 7 through day 49 indicative of active HIV-1 replication. A rise in RT activity, marked by an increase in p24 levels at day 42 after infection, was also observed in Jurkat T cells co-cultured with infected eosinophils. Based on the RT activity and p24 release, these results provide credible evidence for HIV-1 infection of eosinophils in vitro(56).

In the next study, Conway et al. demonstrated that eosinophils are potential host cells of HIV-1 in HIV/AIDS patients (57). They obtained peripheral blood from eighteen HIV-1 positive patients and three normal subjects. Eosinophils were isolated by Ficoll-Hypaque centrifugation and sedimentation in 4.5% high molecular weight dextran. They stained the cells with Diff-Quick reagents and obtained eosinophils that were greater than 95% pure. After decanting contaminating mononuclear cells and some erythrocytes, further purification was achieved by fractionation over six layer metrizamide discontinuous gradients. They washed the eosinophil fraction and treated

it with 0.2% NaCl erythrocyte lysis solution to eliminate any contaminating erythrocytes. They cultured eosinophils from the seronegative subjects with GM-CSF in RPMI-supplemented medium. Control eosinophils and peripheral blood mononuclear cells (PBMC) isolated from the seronegative individuals were then incubated with HTLV-IIIB. Using the DuPont ELISA Kit they detected p24 release from culture supernatants of the infected control eosinophils and PBMCs. After harvesting DNA from eosinophils isolated from HIV/AIDS patients and from control eosinophils infected in vitro with HIV-IIIB, PCR was performed. Chemiluminescent probes were then used to detect and visualize HIV-1 gag genes in the amplicons (57).

Conway et al observed transient in vitro infection of the control eosinophils with peak production of p24 antigen on day 4, while the peak p24 release in infected PBMCs was observed on day 7. HIV-1 proviral sequences were recovered in the eosinophils of 2 of 18 HIV-1 positive patients and in the PBMCs of all eighteen HIV-1 positive patients. In one of the seropositive subjects, they observed stronger proviral sequence signals in the infected eosinophils than in the PBMCs and One month after their infection, proviral sequences were still recovered in the eosinophils of the patients. In control eosinophils alone or culture media

alone and control cells incubated with heat inactivated virus no infection was observed. Therefore based on the purity of the eosinophils as detected by staining with Diff quick reagents and microscopic exam, p24 release and detection of proviral DNA was due to infection by HIV-1. This study provided the first and only in vivo evidence of HIV-1 infection of eosinophils (57).

In 1995, Weller et al. proposed that HIV-1 could infect mature terminally differentiated human eosinophils and induce apoptosis and necrosis (58). They isolated peripheral blood eosinophils from individuals with hypereosinophilic syndrome (HES) and helminthic infections by centrifugation over ficoll paque and isotonic percoll gradients. They ruled out the presence of any contaminating lymphocytes and monocytes using flow cytometry with anti-CD5, Leu-1 or anti-CD3, leu-4, and anti-CD14, Leu-M3 mAb respectively. Weller et al. used a gene-probe-mycoplasma assay to rule out contamination of their lab maintained viral stock and/or used freshly obtained viral stocks that were known to be mycoplasma free. Mycoplasmas are very common laboratory contaminants that cause significant changes in cellular metabolism and nucleic acid in cell cultures that can skew research data leading to erroneous results (59). Upon isolation of eosinophils, cytological

staining and microscopic examination revealed neutrophils as the only contaminants. The purified eosinophils were cultured in supplemented medium with or without a 3T3 fibroblast feeder layer with 5pM to 50pM granulocyte colony stimulating factor (GM-CSF) for three weeks. The cells were then infected with the T-tropic HIV-1 strains, MN and HTLV-IIIB, and the viability of infected and uninfected eosinophils was assessed by trypan blue exclusions. Since viable cells have intact cell membranes unlike dead cells when mixed with Trypan blue and visualized under a microscope, viable cells remain clear because their cell membranes do not absorb the dye while the dead cells appear blue since they absorb the trypan blue (60). They used light and electron microscopy to assess HIV-1 induced apoptosis and necrosis. They performed ELISA and PCR, respectively, to detect p24 release and the gag viral DNA in cultures of infected eosinophils. Flow cytometry of propidium iodide stained nuclei, and electrophoresis were also used to evaluate the cytolytic effects of HIV-1 infection.

The viability of eosinophils declined significantly after infection by HIV-1. Such a decline was not observed in uninfected eosinophil cultures or cultures incubated with inactivated HIV-1. A progressive release of p24 was

observed in cell cultures of infected eosinophils, in infected control mononuclear leukocytes, and uninfected H9 cells co-cultured with infected eosinophils. They detected no p24 from purified eosinophils alone, eosinophils cultured with uninfected H9 cells, eosinophils incubated with inactivated HIV-1, or H9 cells alone. (58). The addition of phorbol esters promoted a greater p24 release in infected eosinophil cultures, while high GM-CSF concentration enhanced HIV-1 replication and caused HIV-1 induced apoptosis and necrosis. They also recovered ^{35}S-methionine labeled HIV-1 proteins from H9 cells co-cultured with infected eosinophils, demonstrating that eosinophils can be infected by HIV-1. Gag DNA was detected by PCR in MN infected eosinophils and control mononuclear cells, but not in media or buffer alone, which further confirmed HIV-1 replication in eosinophils. Electrophoresis and flow cytometry with propidium iodide confirmed apoptosis and necrosis in infected cell cultures only. No syncytium formation or viral budding were observed in this study. This study once more affirmed the possibility of productive infection of eosinophils and went ahead to establish cytolytic effects of the infection on eosinophils (58).

For close to 10 years, our lab has tried to establish evidence for HIV-1 productive infection of eosinophils in

vivo. In the first study, the eosinophilic cell line AML14.3D10 that stains positive for eosinophil granules and expresses mRNAs for eosinophil cationic protein was incubated with HTLV-III. The cell lines bear CXCR4, CCR5 molecules on their membrane and are also CD4+ as determined by flow cytometry. They are therefore similar to primary eosinophils. Wooley et al. (52, 60) demonstrated for the first time the infection of an eosinophil cell line by HIV-1. The AML14.3D10 cell lines were incubated with T tropic and M Tropic HIV-1 strains for 1 hour at 37 degrees and 7% CO_2, washed with culture medium, resuspended and split twice per week and observed with an inverted phase contrast microscope. After p24 assay using the RETRO TEK antigen capture ELISA kit, since p24 antigen was detected only in cell line cultures infected with the T Tropic HTLV-IIIB and not M Tropic strains, it was determined that HIV-1 infects eosinophils in a strain dependent manner despite the expression of the CCR5 co-receptor by AML14.3D10(52).

In continuation of the above study, Taylor et al. (52, 53, 60) created the cell line model AML14.3D10.CCR3 model, by engineering the AML14.3D10 cell line to express CCR3, and exposed them to R5 and X4 HIV-1 strains. No infection of cells incubated with the R5 strain was reported. On the other hand increasing levels of p24 antigens, indicative of

viral entry and productive infection, were detected in the cell line exposed to the X4 strain. To decipher which co receptors are used for entry by the X4 strain, they incubated the cell line with the CXCR4 co-receptor antagonist, AMD3100, and then infected the cell line with HTLV-IIIB. AMD3100 completely blocked infection by X4, demonstrating that the X4 strain uses the CXCR4 co-receptor exclusively for infection (53). This eosinophilic cell line model created in our lab is unique with respect to providing evidence for HIV-1 tropism to eosinophils.

In a 10 year study carried out amongst 200 Kenyan car washers exposed to the parasite Schistosoma mansoni, researchers found out that 6 weeks following successful treatment, 133 seronegative car washers resisted re infection by the parasite but the HIV/AIDS subjects became reinfected(61). HIV-1 infection lives eosinophils in an activated state and compromises their resistance to the parasite, a clear indication of dysfunction or alteration of the eosinophil antihelminthic functions due to HIV-1 infection.

Even though these studies provide solid basis for HIV-1 infection of eosinophils, there is much controversy whether productive infection takes place or eosinophils barely phagocytose viral material. It is against this

background that I shall look at the shortcomings of these studies and provide proposals for an experimental model for the achievement of more convincing evidence of HIV-1 infection of HIV/AIDS patients.

CRITIQUE OF PREVIOUS STUDIES

However ground breaking the results of the previous studies on HIV-1 infection of eosinophils are, they are not definitive since most of the experimental models were based on highly controlled in vitro techniques or cell line models that do not directly capture physiological conditions. Therefore evidence of in vivo infection cannot be inferred from most of them.

Due to their unsuccessful attempt to infect mature eosinophils, Freedman et al. used eosinophil precursors in bone marrow tissue (56). However the immature forms of eosinophils and monocytes, the promyeolocytes and myelocytes, share common characteristics, therefore it is possible that the results observed could have been skewed by HIV-1 infection of monocyte precursors. Since the internal milieu of a cell and its cytokine profile are critical in the efficiency of viral replication, HIV-1 infection of eosinophil precursors might not necessarily provide any sound evidence for infection of the mature cell if differences exist. Since the internal milieu and cytokine profile are related to the expression level of receptor molecules like CD4 protein on eosinophils, it could explain why more immature than mature eosinophils were susceptible in culture. In addition to the use of

eosinophil precursor cells, they also used three laboratory strains of HIV-1, RF, RUT and IIIB but did not achieve infection with the IIIB strain. The extent of infection is not known. Therefore more critical and convincing facts are needed to make a solid case that HIV-1 can productively infect primary eosinophils in vivo.

The detection of HIV proviral DNA in mature human eosinophils of 2 out of 18 HIV/AIDS patients using traditional polymerase chain reaction (PCR) (57) is the most compelling in vivo evidence of productive infection of eosinophils till date. Unfortunately inadequate information is given about the health status of the patients, especially the stage of their infection, type of treatment if any, duration of infection and co-infections if any. In this study (57), the type of receptors used by HIV-1 for infection and the strain of the virus are not determined. Also they do not indicate whether or not the HIV-1 infected eosinophils can cause infection upon incubation with uninfected cells. Even though proviral DNA was detected using the traditional PCR, the technique has a low sensitivity, a short dynamic range, is laborious and requires gel analysis of amplicons. As such there is the possibility of contamination of the eosinophils with HIV-1 infected lymphocytes or PBMCs that could erroneously be

seen as positive result. Therefore eosinophil isolation and processing in this project did not guarantee absolute purity. Greater than 99% pure eosinophils can be achieved by centrifugation and negative isolation. Coupled to this the extent of infection could be quantitated using quantitative real time (Q) PCR (62), which did not exist at the time. In addition unlike traditional PCR, with Q PCR there is no processing of cells for gel electrophoresis. Therefore the possibility of eosinophils being contaminated by HIV-1 infected lymphocytes or monocytes diminishes or is eliminated. In the in vitro experiments only transient infection is determined and the infection is not also quantitated. These shortcomings necessitate further studies that would address them.

The Weller group achieved resounding success in demonstrating HIV-1 infection of eosinophils, and HIV-1 induced apoptosis and necrosis in vitro. However, like the previous study, the infection was not quantitated, and only the T-tropic laboratory strains of HIV-1, MN and HTLV-IIIB, were used for infection. In addition these results were based on in vitro conditions that do not adequately replicate physiological conditions in the human body (58).

Cell lines are immortalized cells; even though they support some cellular functions similar to the primary

cells they are physiologically very distinct from cells in in vivo (63). As such while clues on HIV-1 strain dependent infection of eosinophil and receptor use can be drawn from previous work in our lab, such findings are not a paradigm for the infection of primary eosinophils. Therefore HIV-1 infection of cell line cultures as demonstrated in our lab cannot be used by itself as the evidence of HIV-1 infection of primary eosinophils in patients (52, 53, 63).

Based on the shortcomings inherent in the previous studies conducted, it is clear that a combination of the methodologies these studies will provide the experimental model that can be used to demonstrate HIV-1 productive infection of eosinophils in HIV/AIDS patients. This shall involve a rigorous and sensitive method of isolating greater than 99.9% pure eosinophils, the detection, amplification and quantitation of proviral DNA using quantitative real time (Q) PCR. Unlike traditional PCR in which the amplicons are detected only at the end of the experiment and then processed for resolution with gel electrophoresis, Q PCR detects amplicons in real time as the experiment progresses. It is an automated computer program which is very precise, robust and sensitive. It detects a two fold change and has a long dynamic range. It

does not involve any post product processing therefore a lower risk of contamination (62, 64).

EXPERIMENTAL DESIGN FOR FUTURE STUDIES

The goal is to isolate primary eosinophils that are greater than 99% using negative selection negative selection, to demonstrate and quantitate the degree of HIV-1 productive infection of the eosinophils from HIV/AIDS patients using quantitative real time PCR.

Isolation of eosinophils

Peripheral blood (PB) will be collected from healthy seronegative individuals and HIV/AIDS patients after their informed consent. Important data about patients' infection including the stage of infection, assessed by their viral load and CD4 T Cell count will be collected. Their history of antiretroviral therapy if any will also be recorded. Blood will be collected in EDTA, centrifuged over Ficoll-Paque density gradients and the granulocyte layer collected. Eosinophils will be isolated from the granulocytes by using the eosinophil isolation kit (65, 66). Cell isolates will be magnetically labeled with a cocktail of biotinylated antibodies, including CD2, CD14, CD16, CD19, CD56, CD123 and CD235a, and antibiotin microbeads and passed through the magnetic field of the magnetic cell sorter, Vario MACS. Purified eosinophils will therefore be obtained by negative selection as CD16- cells. Negative selection guarantees the integrity of eosinophils

isolated since the cells will not be activated as result of labeling with their monoclonal antibodies. In addition the use of negative selection is advantageous since eosinophils do not have exclusive markers that differentiate them from other granulocytes. Cytological staining with modified Wright stain will be performed on cytopreps to ensure that isolated eosinophils are free of contaminating monocytes and lymphocytes. Further confirmation of purity would be ascertained by light microscopy and flow cytometry. Eosinophils will be enumerated by trypan blue exclusion using the hemacytometer. It is expected that over 99% pure eosinophils will be obtained by this technique (65, 66).

It must be noted that processing of blood shall be done on the same day blood is collected from donors because eosinophils are very short lived outside the body. Also since there is diurnal relationship to eosinophil levels, that is, the levels are highest in the morning and evening period and lower in the afternoon (67), blood collection must be carried out at the same time each day.

Assessment and quantitation of Infection

Once purified eosinophils are obtained some will be cultured and incubated in CO_2 and the rest used for cellular DNA preparation. The DNeasy kit, by Qiagen, will be used to isolate cellular DNA while primers and probes will be

designed that are specific for conserved regions of the HIV-1 gag-pol-env. Absolute Q PCR will then be used to detect proviral DNA and quantitate the extent of HIV-1 infection. The quantitation of the proviral DNA will be achieved by comparing the real-time PCR product in isolates with a standard 8E5/LAV cell line that has one proviral DNA copy (68) the number of proviral DNA copies in eosinophils will be extrapolated from the standard curve generated from the cell line. This will give a succinct indication of the quantitative infection of eosinophils. Beta globin gene, used as an endogenous control, will be amplified in a multiplex reaction for normalizing target cDNA. The value of the reference gene is also extrapolated from a standard curve. To achieve the normalization, the cDNA value is divided by the value of the endogenous gene (62).

This proposed study will also incorporate the determination of the type and expression level of receptors and co receptor used in the in vivo infection hence confirm the strain of HIV-1 that infects the cell, whether T Tropic, M Tropic or dual tropic. The membrane CD4, CXCR4 and CCR5 proteins of eosinophils from healthy individuals will be blocked with their specific antagonists pyridoxal 5'phosphate, TN14003 and TAK-779 respectively (69-71). The cells will then be infected with different HIV-1 strains.

The study should also demonstrate that the infected eosinophils can cause infection of another permissive cell type. This will be achieved by culturing infected eosinophils with H9 cells which T cell lines and the pro-monocyte line, U937. This will be valuable in verifying whether eosinophils can serve as HIV-1 reservoirs or whether the the infected eosinophil can cause cell-cell infection.

Schematic representation of experimental model.

1. Measure viral load and CD$ T cell count
2. Isolate PB eosinophils from healthy and HIV/AIDS donors by centrifugation and negative selection
3. Prepare cytopreps and perform cytological staining
4. Enumerate eosinophils and ascertain viability using a hemacytometer and trypan blue exclusion
5. Microscopic and flow cytometric analysis of eosinophils
6. Incubate control eosinophils with HIV-1 H9 T lines and U937 pro-monocyte. Block co receptors with specific antagonists
7. Measure p24 release from control eosinophils incubated with HIV-1 and eosinophils alone using ELISA
8. Isolate DNA from patient eosinophil using DNeasy kit. Use real time PCR to amplify conserved region to

detect proviral DNA and quantite extent of HIV-1 infection.

Limitations of the Proposed Model

The above approach successfully addresses some of the pitfalls in the previous studies. Vital statistics about the patient's health status are collected, isolation of eosinophils yields pure cells and the extent of infection is quantitated. However this model also has some inherent pitfalls.

Eosinophils survive about 12-16 hours in peripheral blood but predominate in the gastrointestinal tract. Therefore HIV-1 infection of tissue eosinophils should be addressed. The use synthetic CXCR4, CCR5 and CD4 antagonists to verify HIV-1 tropism to eosinophils could provide solid evidence but the results will not be definitive since the antagonists used are non physiologic and it is not known what receptors are expressed in the tissues. This model does not address the possibility that eosinophils phagocytose viral material that can be falsely detected as productive infection.

CONCLUSION

Even though Conway et al. recovered HIV-1 proviral sequences in mature primary eosinophils from two of eighteen HIV-1 positive patients and provided compelling evidence for the infection of eosinophils many questions remain (57). Given their low levels in PB, concerns about the isolation of pure eosinophils or eosinophils free of cellular contaminants have been raised. Also, the argument that eosinophils phagocytose HIV-1 particles and thus present as infected cells is also a genuine concern. However in our lab we have successfully isolated greater than 99% pure primary human eosinophils and used quantitative real time pcr (Q-PCR), a more robust and sensitive DNA amplification protocol, unlike traditional PCR used in the Conway study, to detect and quantitate proviral DNA in eosinophils infected with HTLV-IIIB.

In the most recent in vitro study productive infection was determined but no correlation was found between viral load or p24 release and proviral DNA detection. However the lack of such a correlation does not devalue the fact there was productive infection. It is important to note that eosinophils are mainly tissue dwelling and do not recirculate from tissues to PB. Therefore the level of p24 release from PB eosinophils relative to PBMC and

circulating lymphocytes may not be significant but critical in the mucosal or gut tissues.

As researchers galvanize their efforts towards discovering more efficient treatments and vaccines for HIV-1, prevention remains the key in curbing the spread of this pandemic. In developing countries where HIV-1 incidence and mortality are alarming there must be a concerted effort between the public and private sectors to stem up campaigns to increase awareness about HIV/AIDS, its socio economic impact and preventive measures. The high incidence of helminthic infection in these countries, coupled to the high incidence and prevalence of HIV-1 makes a bad scenario worse as a result of co infection. As such deworming as proposed by others (72) is one step in the right direction towards maintaining good health for many HIV/AIDS patients. The worst deworming can do is derail funding from other research projects but it would have left millions of people healthier even if helminthic infections were found to have no role in HIV-1 infection.

Given the above evidence for the in vivo infection of eosinophils by HIV-1, it is necessary for researchers to engage in studies that will elucidate the mechanism of infection and the significance of the infection especially populations hard hit by parasitic infections and HIV-1.

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