HETEROGENEITY OF MONOCYTES IN HIV PATIENTS

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M.Sc

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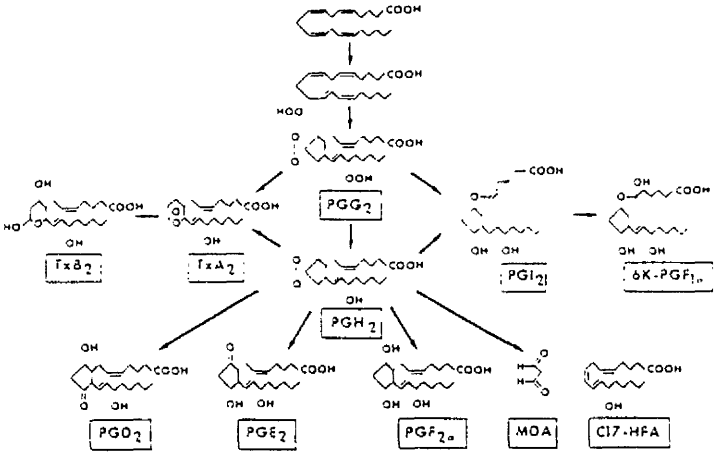

Scheme of products formed via the cyclooxygenase using arachidonic acid

ABSTRACT

Monocytes from human immunodeficiency virus type 1 (HIV-1) infected patients have an increased heterogeneity of phenotype and function. The phenotype of peripheral blood monocytes was characterized by flow cytometry using monoclonal antibody markers against various surface antigens. The functional aspect of monocytes in vivo was investigated by measuring plasma interleukin-6 (IL-6) in HIV-infected patients to see if this cytokine correlated with any other parameters in the blood. This was followed by a study of the cytokines gene expression in peripheral blood monocytes. The accessory cell function of monocyte, was looked into by considering the effect of monocyte-derived prostaglandin E_2 (PGE_2) on lymphocyte proliferation, and monocyte interleukin-1 alpha (IL-1α) and tumour necrosis factor alpha (TNF-α) production, in anti-CD3 stimulated whole blood cultures of HIV-1-infected patients.

Light microscopy study of peripheral blood of HIV patients showed large mononuclear cells whose identity was difficult to distinguish morphologically. In contrast, flow cytometry study using monoclonal antibodies to CD14 antigens, revealed these cells to be monocytes.

Monocyte numbers in the peripheral blood of HIV patients, based on anti-CD14 staining, showed no significant difference with disease progression. However, because of a progressive reduction in total white cell count, there was a significant increase in the proportion of these monocytes with disease progression ($p < 0.05$). Patients showed no significant difference in monocyte numbers compared to controls. However, the proportion of monocytes in patients ((median and interquartile range; 14.0 (8.8-18.5%)) showed a significant increased compared to controls (8.0 (7.0-12.3)%), ($p < 0.05$).

Monocyte membrane CD4 expression was significantly decreased with disease progression ($p < 0.01$), in parallel with T-cell CD4 expression. Flow cytometry showed that a proportion of monocytes could be heterogeneous, showing an extra discrete subset of cells which were larger, and expressed phenotypic evidence for activation or maturation. These large monocytes had increased expression of the markers CD11b, HLA-DR, CD45 and CD16, some of which suggested cell activation. The mean size of the larger monocyte population was 56 % (SD = 12 %) greater than normal. The proportion of activated monocytes with the larger phenotype correlated with disease progression ($\chi^2 = 32.1$, $p < 0.001$), and this was more prominent in the CDC4 disease category (AIDS). However, monocytes in the extra population never exceed 50%.

The functional aspect of monocytes was investigated by monitoring cytokine and prostaglandin production in whole blood cultures, in order to evaluate as closely as possible the in vivo situation. HIV patients' peripheral blood monocytes, purified by adherence, showed increased expression of the genes for TNF-α compared to TNF-β, whereas monocytes from controls showed a relatively increased expression of TNF-β over TNF-α. Some HIV patients had high plasma IL-6 levels which correlated significantly with the number of monocytes in the activated population ($r = 0.25$, $p = 0.05$), during disease progression. HIV patients' monocytes produced high constitutive PGE_2 levels in vivo (617 (479-755) μg/ml) which could not be increased by LPS stimulation, in whole blood cultures.

Monocyte accessory cell function was assessed by supporting lymphocyte proliferation in anti-CD3 stimulated whole blood cultures of HIV-infected patients. Lymphocyte proliferation was quantified by lymphocyte IL-2 receptor (CD25) expression in response to anti-CD3. Since IL-2 receptor expression showed a positive correlation with tritiated (^3H)-Thymidine incorporation (r =

0.77, p = 0.01), this marker was used to quantify lymphocyte proliferation. The approach devised in this thesis to examine proliferation by CD25 expression allowed the simultaneous measurement of cytokines and PGE_2 in culture, which would not have been possible using the harvesting techniques of traditional tritiated thymidine incorporation. The patients' responses to anti-CD3 were divided into three groups: those with either negative, poor or good responses. Monocyte PGE_2 levels in cultures were found to correlate inversely with the lymphocyte responses in HIV- infected patients. Patients with negative responses had high levels of PGE_2, while patients with good responses had low PGE_2 levels. This suggested the hypothesis that high constitutive PGE_2 synthesis by monocytes in HIV patients contributed to the reduced lymphocyte proliferation which is seen in disease progression. IL-1α and TNF-α in some culture supernatants were found to increase with the lymphocyte proliferative response. Both cytokines showed high post-stimulation levels, however some patients showed a low spontaneous TNF-α production in vivo.

These observations suggested that there exists a more dynamic situation of recruitment, activation and maturation of peripheral blood monocytes driven by HIV infection, which results in a broader phenotypic profile. Peripheral blood monocyte in HIV patients produced high levels of PGE_2 and this could inhibit T-cell proliferative responses. PGE_2 production in monocytes, driven by HIV infection in vivo, might in part account for the HIV-related T-cell anergy in these patients and raises the possibility that this may be ameliorated with indomethacin treatment.

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AUTHOR'S DECLARATION.

These studies represent original work made by the author, and have not been submitted in any form to any other University. Where use has been made of material provided by others, acknowledgement has been made to the person concerned. Part of this work has been published (1).

P.M. ABEL

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ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome.
c.p.m	count per minutes.
C5 des Arg	C5 des Arginine.
Con A	Concanavalin A
DNA	Deoxyribonucleic acid.
CDC	Centre for disease control, Atlanta, USA.
EDTA	Ethylene diamine tetra-acetic acid.
FITC	Fluorescein isothiocyanate.
HIV	Human immunodeficiency virus.
Ig	Immunoglobulin
IL	Interleukin.
LPS	Lipopolysaccharide, endotoxin
PBS	Phosphate buffered saline solution.
PE	Phycoerythrin.
PGE_2	Prostaglandin E_2.
PGL	Generalised lymphadenopathy.
PHA	Phytohaemagglutinin
PI	Propidium Iodide.
RNA	Ribonucleic acid.
r-IL-2	Recombinant interleukin-2.
mRNA	messenger RNA.
NF-kB	Nuclear factor kappa B.
S.E.M	Standard error on the mean.
TNF	Tumour necrosis factor.
WHO	World health organisation.

DEFINITIONS

Accessory cells A non-lymphocytic cell which functions as a modulator of immune responses, lymphocyte function and development. They are usually Ia positive, showing MHC restriction when interacting with T-helper cells in the initiation of an immune response. Examples of accessory cells are mononuclear phagocytes, dendritic cells and langerhans cells.

AIDS Acquired immunodeficiency syndrome is a severe immunodeficiency occuring in adults as a result of a deficiency of helper T-lymphocytes and other cells important to the immune system eg. monocytes. It usually follows HIV infection and the disease is characterized by repeated severe infections and a rare skin tumour called Kaposi's sarcoma.

Antibody An immunoglobulin capable of specific combination with antigens.

Antigen A molecule that initiates an immune response when introduced into tissues.

B-lymphocytes These are subset of lymphocytes which are CD19 positive and have the ability to synthesize immunoglobulins.

Cell surface markers These are proteins expressed on the cell surface, which are characteristic of certain phenomena occurring in the cell.eg. cellular activation, cellular differentiation and cell proliferation. These proteins have either specific names or designated with specific numbers eg. CD16, CD11b and CD25.

Cytokines This is a group of soluble proteins released from cells including these cells performing immunological functions. Some of these proteins are termed monokines since they are produced from monocytes. Others are termed lymphokines since they are mainly produced by lymphocytes.

Extra monocyte population A subset of the monocyte population which appeared in the peripheral blood of some HIV-infected patients, especially those with AIDS. These monocytes are 1.5 times larger than normal, and expressed activation markers.

Granulocytes A polymorphonuclear leucocyte of the blood, of which three types can be differentiated morphologically and by the staining properties of their cytoplasmic granules. These are neutrophil, eosinophil and basophil granulocytes.

Heterogeneity a different kind of nature, diverse in character and varied in content. Monocytes in HIV patients were heterogeneous in that they present subsets of populations, which contain cells that were different in size and expressions of cell surface markers.

Human immunodeficiency virus (HIV) This is a member of the lentiviridae subfamily of retrovirus affecting humans. It is the infective agent that caused the acquired immunodeficiency syndrome (AIDS).

Immunoglobulins (Ig) THese are members of a family of proteins each made up of light chains and heavy chains linked together most commonly by sulphide bonds. The members are divided into immunoglobulin classes and subclasses determined by the amino acid sequence of their heavy chains. Humans have five Ig classes: IgM, IgG, IgA, IgD and IgE.

Lymphocytes A spherical cell from 7-12 μm in diameter, with large round nucleus and little cytoplasm, present in the blood. Two types of lymphocyte exist and play an important role in the immune system: T and B lymphocytes.

Monocytes are one of the cell populations which made up the mononuclear phagocyte system. They are generated by myeloid differentiation in the bone marrow. At the final stage of maturation, they expressed a myeloid differentiation antigen termed CD14 and this molecule is monocyte specific. They then circulate in the blood as mature monocytes. Once these cells acquire an adherent phenotype, they settle into tissues where they mature and become resident macrophages.

Mononuclear phagocytes The phagocytic cells of which the mature functioning form is the macrophages. All cells of the mononuclear phagocyte lineage originate from the bone marrow. It is the new term which replaces the old term of 'reticulo-endothelial cells'.

T-cells Lymphocytes that are derived from the thymus,and play a major role as antigen reactive cells and effector cells in cell mediated immunity. It cooporates with B-cells in antibody production against thymus dependent antigens.

Chapter 1

Introduction

Chapter 1

1 Introduction.

1.1 The human immunodeficiency virus (HIV).

1.1.1 Definition of human immunodeficiency virus (HIV).

The human immunodeficiency virus (HIV) was found to be the infective agent that caused the acquired immunodeficiency syndrome (AIDS), (2). It was classed as a member of the lentiviridae subfamily of retrovirus affecting humans. Two strains of the virus have been identified to date. Human immunodeficiency virus type one (HIV-1) is concentrated in America, Central Africa, Europe and other parts of the world. Human immunodeficiency virus type two (HIV-2) is associated mainly with West Africa. HIV-1 is the virus type related to the present thesis.

1.1.2 Structure of human immunodeficiency virus (HIV-1).

High resolution electron microscopy showed the HIV-1 virion as an icosahedral structure in a lipid bilayered envelope containing 72 spikes (3) (Appendix 1.1.2). These spikes correspond to two major viral-envelope glycoproteins, gp120 and gp41. Also studded in the envelope were various host proteins acquired during virion budding, including Class I and Class II histocompatibility antigens. The core of HIV-1 contained four nucleocapsid proteins, p24, p17, p9 and p7. The myristylated p17 protein was associated with the inner surface of the viral lipid bilayer, whereas the phosphorylated p24 polypeptide formed the major component of the inner shelf of the nucleocapsid. The p7 protein bound to the genomic RNA via zinc-finger structures and together with p9 formed the nucleoid core. In the viral core, there existed two copies of the single-stranded HIV-1 genomic RNA in

association with virus enzymes, namely the reverse transcriptase, integrase and protease.

1.1.3 The genomic structure of HIV-1

The HIV-1 genome shared the basic structure of all known retroviruses, including nucleotide sequences called gag encoding core structural proteins, evn encoding envelope glycoproteins and pol encoding reverse transcriptase, endonuclease and viral protease enzymes required for viral replication. HIV-1 contained in its 9-kilobase RNA genome not only the three essential genes but six other genes, including vpr, vif, tat, rev, nef and vpu genes, whose products regulated viral replication in various ways (Appendix 1.1.3).

The Tat gene potently transactivates the expression of all viral genes. The Nef gene function is not clear, but it points to the inhibition of HIV-1 replication. Vpr on the other hand appeared capable of moderate stimulation of the HIV-1 long terminal repeat (LTR) as well as a wide array of cellular and viral promoters. Vif and Vpu, appeared to have important functional roles during the late stages of virion morphogenesis. Vpu protein promotes the efficient release of the budding virions from the surface of the cell. The product of the Vif gene was necessary for full infectivity of the released HIV-1 virions, since mutations in this gene impairs the cell-free transmission of the resultant viruses. The product of the Rev gene acted at a post-transcriptional level by activating the cytoplasmic expression of the unspliced and singly spliced forms of HIV-1 RNA that encode the products of the gag, pol, and env genes.

1.2 Clinical aspects of human immunodeficiency virus type one (HIV-1) infection.

1.2.1 Disease progression in HIV-1 infection.

Throughout HIV-1 infection, patients suffer from a variety of symptoms which can be followed clinically as the disease progresses. Most people show no symptoms when initially infected by HIV-1. Yet some individuals develop a disorder resembling mononucleosis with symptoms ranging from fever, swollen glands and rash. This resolves quickly. The first sign that the immune system is affected becomes apparent with the development of chronically swollen lymph nodes (i.e lymphadenopathy), followed by a persistent drop in CD4 positive T-cell count to less than 400 cells/ml. The impairment in cell-mediated immunity becomes evident as the patient fails to respond to skin tests measuring delayed hypersensitivity (i.e. the individual's ability to mount a cellular immune response against specific proteins injected under the skin).

As the CD4 positive T-cell count drops below 200 cells/ml, overt disease may set in. The patient may develop thrush, a fungal infection of the mucous membranes of the tongue and oral cavity. This may be accompanied by persistent viral (Herpes simplex) and fungal (Candida albicans) infections of the skin and mucous areas, resulting in painful sores surrounding the mouth, anal and genital areas.

The development of disseminated opportunistic infections at sites beyond the skin and mucous membranes reflects a severe decline in the immune functions and is the hallmark of the acquired immunodeficiency syndrome (AIDS). The infections include

Pneumocystis carinii pneumonia (PCP) in the lungs, the parasitic infectious toxoplasmosis infecting the brain leading to seizure and coma, and chronic cryptosporidiosis causing chronic diarrhoea. These may be lethal to the patients. AIDS patients also suffer from a variety of symptoms which physicians call AIDS-related complex (ARC). These symptoms comprise unexplained fevers, persistent night sweats, chronic diarrhoea and wasting (cachexia).

Several groups of workers have attempted to classify the clinical symptoms of HIV-1 infection to improve management of patients and to have a better understanding of disease progression. The Walter Reed Army Medical Center in Washington DC developed a classification system in 1984 (4). A new system was followed from the Center for Disease Control (CDC) in Atlanta, USA, in 1986 (5). The World Health Organisation (WHO) updated the CDC classification system in 1990 (Table 1.2.1), (6), but both groups reconciled their systems in 1991 in a joint WHO/CDC classification system for HIV infection and disease.

1.2.2 Effects of human immunodeficiency virus type one (HIV-1) infection on the immune system.

HIV-1 infection is accompanied by depletion of CD4 positive T-helper lymphocytes (7), a cell type vital for the functioning of the immune system. This progressive depletion of T-helper cells leads to a general decline of the immune functions and hence may be the primary factor determining the clinical course in patients.

The eventual development of lymphadenopathy i.e. chronically swollen lymph nodes, is an early sign of the impairment of the immune system. HIV-1 was found to overstimulate B lymphocytes

which are abundant in lymph nodes, and keep them in a state of chronic activation.

The loss of cell-mediated immunity in patients offered direct evidence of an immune disfunction. The development of anergy (8) i.e. total absence of delayed hypersensitivity, put patients at risk of chronic infection. This led to severe decline in immune functions reflected by the emergence of chronic or disseminated opportunistic infections

1.2.3 Effect of human immunodeficiency virus type one (HIV-1) on the nervous system.

Human immunodeficiency virus type one (HIV-I) invades the central nervous system in the course of viral infection. The patient suffers from impaired memory, altered concentration and psychomotor retardation (9). Possible mechanisms for central nervous system invasion by HIV-1 include virus carried to the brain via latently infected blood monocytes or lymphocytes or as cell-free virus infecting the brain capillary endothelial cells (10). Southern blot analysis has shown HIV-specific DNA in the brain tissue of AIDS patients with dementia (11) and the predominant infected cells in the brain are macrophages and microglial cells (12,13).

Dissemination of virus infection from brain tissues to neighbouring neurons or glial cells resulted in spinal cord disease (14). This became evident when spinal cords of AIDS patients who had suffered from vacuolar myelopathy were examined. They revealed vacuolation of the white matter with infiltration by macrophages within which HIV-1 expressions was observed (15).

1.3 Consequences of human immunodeficiency virus type one (HIV-1) infection.

The human immunodeficiency virus gp120 glycoprotein bound with high affinity to CD4 molecules which acted as receptors on cell membranes. The receptor-bound HIV-1 virion penetrated the cells via a pH-independent membrane fusion event (16), involving either classic-mediated endocytosis (17) or virus-mediated membrane fusion (16). This entry was directed by the virion bipeptide envelope glycoprotein, gp120, non-covalently linked via amino terminal interactions to a transmembrane protein, gp41, embedded in the lipid membrane that surrounds the virion (18).

Later evidence supported membrane fusion as the dominant mechanism for virion entry (19). However, recently, alternative methods of uptake of HIV into cells which lack detectable surface CD4 or CD4 transcripts have been shown to exist. HIV infection of cells (e.g. monocytes) was seen to happen through complement (20) or Fc-receptor (21) antibody-dependent mechanisms. Data indicated that complement receptor (CR3) and FcgRIII on cells acted as a port of entry for complement (CR3)-coated and IgG-coated HIV-1.

1.3.1 Latency during HIV-1 infections.

Once the HIV-1 virion entered a CD4 positive cell, its RNA genome was reverse-transcribed to a double stranded DNA genome which integrated into the host cell genome as the HIV-1 provirus. The provirus had the ability to establish a latent or persistent form of infection by staying in the provirus state for months or years. Quantifying HIV protein or nucleic acid in blood leucocytes of seropositive subjects revealed a low frequency of productive infection (22). DNA and RNA amplification

based on polymerase chain reaction, showed that 1 in 1000 peripheral-blood CD4 positive T-cells from patients with AIDS expressed HIV-1 RNA (23). In contrast , this was a marked underestimate of the true viral burden. Approximately 1 in every 100 of the CD4 positive T-cells contained detectable HIV-1 DNA. Thus, for every T-cell actively producing virus, nine other T-cells contained latent virus.

Both T cells and monocytes harboured proviral DNA and produced progeny virions in vivo, but the relative proportions of infection in these cells was unclear. Recently, it was found that a fraction (<1%) of both T cells and monocytes in blood carried a latent infection in all stages of HIV-1 disease and could serve as reservoir throughout AZT therapy (23).

Infected cells e.g. lymphocytes (24) and monocytes, acted as the reservoir of persistent HIV infection (25). When the infected cells encountered mitogens, antigens or cytokines, these could reactivate the provirus and caused initiation of productive infection. HIV replication was able to induce some cellular genes, including those coding for cytokines, which in turn could amplified both HIV replication and cellular activation. In vitro work involving cytokine stimulation and HIV infection of normal monocytes in culture, revealed that IL-4 was the most potent enhancer of HIV-1 replication (26).

1.3.2 Cytopathic effects of HIV-1 infections.

The ability of HIV-1 to kill the CD4 positive subset of human T lymphocytes had profound implications for the immunodeficiency induced by the virus. HIV-1 replication and cell death increased when infected T-cells became activated. The immune response that should

have protected against the HIV-1, had the effect of increasing the proliferation of the virus. However, the precise mechanisms of the HIV-1 cytopathic effect remained unclear.

One mechanism of HIV-1-induced killing in vitro involved cell fusion and the formation of syncytia mediated by the gp41 env protein following gp120 env protein interaction with CD4 (27,28). This fusion process required the presence of HIV-1 env proteins on the surface of infected cells and involved uninfected CD4-expressing cells that came in contact with virally infected cells expressing HIV-1 env proteins. The fusion of these cells led to the formation of multinucleated cells, also called syncytia, that were shortlived. Syncytial formation may involve the fusion of several CD4 positive lymphocytes, thus offering one mechanism for depletion of this cell type. In other cases, it involved the fusion of CD4 positive T-lymphocytes to CD4 positive mononuclear cells such as monocytes (29).

1.3.3 Cellular activation in HIV-1 infection.

The strategy of HIV replication was to use transcriptional mechanisms very similar to those used by cellular genes involved in the activation of immunocompetent cells (lymphocytes and monocytes). Its target was the inducible cellular genes, such as nuclear factor (NF-kB) and ras proto-oncogene which participated in the cascade of events linking membrane activation and HIV reactivation.

1.3.3.1 T-lymphocyte activation and HIV genome transcription in HIV-1 infection.

Most circulating T-cells in the blood are in the resting (G_0) state (30). Their activation induced a set of cellular genes, including

protooncogenes (31) whose functions rendered T-cells fully immunocompetent. HIV-1-infected CD4-positive T-lymphocytes, could be activated by antigens, mitogens, cytokines e.g. tumour necrosis factor (TNF), interleukin-2 (IL-2) and interleukin-1 (IL-1), or gene products of different viruses (e.g. human T-lymphotropic virus type one [HTLV-1] and Herpes simplex), creating a cellular environment that promoted a high level of HIV-1 provirus expression (32) (Appendix 1.3.3.1). Specific antigen recognition occured through the binding of antigen to the T-cell receptor (TCR) complex (TCR-CD3-CD4). Soluble cytokines such as tumour necrosis factor (TNF) acted through its specific receptor (TNF-R), whose expression was upregulated by mitogen (phytohaemagglutinin; PHA).

The binding of these ligands to their respective receptors on the cell membrane, created a cascade of events from the cell membrane, through the cytoplasm to the nucleus. This resulted in the induction of host factor expression, notably the nuclear factor (NF-kB) transcription factors in the cytoplasm. NF-kB was then liberated from the inactive complex which it formed with an inhibitor (I-kb), followed by its activation by Protein Kinases (33). The free NF-kB travelled to the nucleus where it interacted with its DNA recognition sites to mediate gene transcription.

Once in the nucleoplasm, these NF-kB proteins, which normally regulate the expression of various T-cell genes involved in growth, including IL-2 (34) and the alpha subunit of IL-2 receptor (35), bound to and activated the NF-kB enhancer elements present in the U3 region of the HIV-1 proviral long terminal repeat (LTR) (36).

The induction of the HIV-1 enhancer element resulted in the transcription of HIV-1 genes followed by low translation of HIV-1 mRNA. The early low level HIV-1 transcription allowed early synthesis of the HIV-1 regulatory genes, including the HIV-1 tat proteins. Tat, acting through the TAR (transactivation response) element (37), caused amplification of viral gene expression, leading to a high level of expression of all genes sequences linked to the HIV-1 long terminal repeat (HIV-1-LTR).

1.3.3.2 Monocyte activation in HIV-1 infection.

HIV-1-infected monocytes could be activated by a number of activating agents, including lipopolysaccharide (LPS), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-1 (IL-1), tumour necrosis factor alpha (TNF-alpha) and gene product of viruses e.g Herpes simplex (38). These ligands caused membrane activation followed by a cascade of events involving the induction and release of NF-kB from its inhibitor (I-kB) (39). The free form of NF-kB then migrated to the nucleus where it bound to the 10 or 11 bp NF-kB enhancer-motifs present in a variety of promotors, including HIV-1, Cytomegalovirus, TNF-alpha and IL-2 promoters (40). The binding of NF-kB to the HIV-1 enhancer in the HIV-1 long terminal repeat (HIV-1-LTR) element, led to the transcription of HIV-1 genes followed by translation of HIV-1 mRNA.

Some work focusing on in vitro HIV reactivation, provided evidence for the mechanism of HIV expression in activated monocytes. In vitro studies showed that LPS stimulated the translation of HIV-1 long terminal repeat (HIV-1-LTR) constructs transfected into the monocyte / macrophage-like cell line (U937) (41). This effect was found to be

mediated by induction of nuclear factors NF-kB. Evidence involving cytokines emerged when the TNF-alpha promoter was found to possess Kappa B-like regions (42). Since TNF-alpha could cause activation of HIV-1-CAT construct transcription, it was evident that its binding at membrane level could itself induced HIV-1-LTR expression via NF-kB.

1.4 Infection of the cells of the immune system by HIV-1.

1.4.1 Definition of monocytes.

Monocytes, blood phagocytes which belong to the mononuclear phagocyte system, are generated by myeloid differentiation in the bone marrow (43). At the final stage of maturation, they express a myeloid differentiation antigen termed CD14, which is a 53 kDa glycoprotein anchored in the plasma membrane via phosphatidylinositol linkage (44,45). This molecule is monocyte specific. Although this monocyte surface antigen is lacking on the surface of premyeloid cells, its expression could be induced by reagents known to drive premyeloid cell differentiation along the monocytic pathway (46). Eventually, monocytes enter the peripheral blood circulation as adherent 12-20 um diameter cells and display bean-shaped nuclei with finely granular cytoplasm containing lysosomes, phagocytic vacuoles and cytoskeletal filaments.

Once peripheral blood monocytes acquire an adherent phenotype, they settle into tissues where they mature and become resident macrophages. Macrophages activated by a variety of stimulation, assume different forms; becoming tissue macrophages, epithelioid cells or other macrophages. These cells are found in a wide variety of tissues, including alveolar macrophages in the lungs, the

Kupffer cells of the liver, follicular dendritic cells of the lymphoreticullar system (47) and the macrophages and microglia of the brain.

1.4.2 Cell specificity of HIV-1 infection.

HIV-1 had the capacity to infect and incapacitate the cells of the immune system (48). The virus showed a selective tropism for certain cells of the immune and central nervous systems, expressing the CD4 molecules. CD4, the cellular receptor for HIV-1 (49), bound with high affinity to the viral envelope glycoprotein gp120 (50). The major CD4 positive cells of the immune system were T-helper lymphocytes and cells of the mononuclear phagocyte lineage, namely monocytes and macrophages.

Fresh isolates of HIV-1 showed dual tropisms for CD4+-T-lymphocytes and monocyte derived macrophages, and this tropism was not lost through infection of any one cell type (51). However, monocytotropic HIV-1 isolates were present in the peripheral blood throughout the course of HIV-1 infection and the majority of these isolates had a non-syncytium-inducing (NSI), non-T-cell line-tropic phenotype (52).

All cells expressing CD4 in the immune system were potential targets for HIV infection. Consequently, the cellular/tissue distribution of HIV-1 infection was determined by cells expressing the CD4 molecules on their surfaces. These included lymphocytes (53,54), monocytes (55,56), lung fibroblasts (57), skin and oral mucosa Langerhans cells (55,58), alveolar macrophages (59,60), astroglia and microglia of the brain (61,62) and of spinal cord (15) and eosinophils

(63). Follicular dendritic cells of lymph nodes were a target for HIV-1 infection in vivo (64,65).

Several lines of evidence indicated that cells of the mononuclear phagocyte lineage harboured HIV-1. Electron microscopy study revealed HIV-1 particles associated or budding from the processes of follicular dendritic cells (66) and in lymph nodes of patients with persistent generalysed lymphadenopathy and AIDS (67).

In other work involving isolation techniques, virus was recovered from the adherent monocyte-enriched fraction of blood from seropositive subjects, only after cocultivation with mitogen-stimulated lymphoblasts from seronegative donors (68,69). Recovery of virus from non-adherent T-cell enriched fraction was unsuccessful (69). However, virus has been isolated from T-cells as well as monocytes in some studies (24,70).

1.5 Monocyte phenotype in HIV-1 infections.

1.5.1 Monocyte marker expression in peripheral blood.

1.5.1.1 Monocyte marker expression in normal peripheral blood.

For monocytes to function normally, they express various receptors which are of great importance in immune function. These include CD4, MHC Class II antigens, adhesion molecules CD11a and CD11b, and receptors for the Fc domain of immunoglobulin (Ig), Transferrin receptor (CD71) and IL-2 receptor (CD25).

CD14 is a myeloid differentiation antigen specific to monocytes, detectable with a variety of monoclonal antibodies (eg. Mo2, MY4, Leu M3) on the surface of peripheral blood monocytes (71,72,73).

Monocytes when activated in vitro, shed CD14 by phosphatidyl inositol-specific phospholipase C cleavage of this molecule (45). This CD14 shedding might have a key role in the regulation of CD14 expression during monocyte maturation to macrophages.

CD4 is another important molecule expressed on monocytes. This molecules binds with high affinity to MHC ClassII molecules and plays a role in antigen presentation. Anti-CD4 had the ability to stimulate monocyte by binding CD4 molecules (74).

The Fc receptors consist of a group of closely related membrane glycoproteins present on monocytes and other cells participating in host defence (75). Monocytes express three distinct types of Fc gamma receptors which show different affinities and selectivities for different IgG subtypes (Table 1.5.1). The higher-affinity receptors, Fc gamma receptor I and Fc gamma receptor II are most important in mediating uptake of complexes and opsonised particles (76,77). Fc gamma receptor III, expressed in a small percentage (10%) of monocytes (78), is a hallmark of activated mononuclear phagocytes. These receptors play an important role in the clearance of immune complexes and within inflammatory sites.

CD11a and CD11b are the respective alpha-chains of the heterodimeric proteins LFA-1 (the receptor for the intercellular adhesion molecule-1 (ICAM-1)) and CR3 molecules (the specific receptor for the C3bi complement fragment involved in phagocytosis). Both CD11a and CD11b are noncovalently linked to the beta-chain (CD18) in the molecule.

Monocytes also express major histocompatibility class II antigens (MHC II) including HLA-DR, HLA-DQ and HLA-DP. These played an essential role in monocyte-T-cell-antigen interaction which was MHC class II restricted.

Peripheral blood monocytes stimulated by lipopolysaccharide (LPS), phorbol myristate acetate (PMA) or muramyl dipeptide (MDP) in culture, express a plasma membrane antigen, Mo3e, identified by a murine monoclonal antibody and immunofluorescence flow cytometry (79). Mo3e may be an important activation marker.

1.5.1.2 Monocyte marker expression in peripheral blood of human HIV-1-infected persons.

Monocytes from HIV-infected patients were in a state of activation and therefore expressed several surface markers. A striking feature of these monocytes was a significant level of expression of Fc gamma receptor III (CD16), normally associated with activation and the maturation of monocytes to macrophages (80). Dual fluorescent staining using monoclonal antibodies specific for Fc gamma III (anti CD16) showed that 38.5 $\pm$ 3.2% of CD14 positive monocytes in HIV-1 infected patients were CD16 positive, as compared to 10.4+/-1.0% for normal individuals (n=29, $p<0.005$).

Other studies using dual staining of patients' peripheral blood by flow cytometry, revealed a CD14 positive monocyte population with 29.4 $\pm$ 5.2% of monocytes co-expressing IL-2 Receptor (CD25) whereas CD14 monocytes from normal subjects were essentially CD25 negative (2.0 $\pm$ 0.42%, $p<0.001$, n=15). These CD25 positive monocytes also expressed an increased level of HLA-DR which might favour monocyte

T-cell interactions and the transmision of HIV-1 (81). In a further study, there was no difference in HLA-DR, HLA-DQ or HLA-DP expression on isolated monocytes from HIV-1 infected patients and controls (82), although earlier conflicting studies reported significantly decreased HLA-DR, HLA-DQ and LFA-1 antigen expression on monocytes and macrophages of HIV-1 infected patients (83). Other early work regarding marker expression by monocytes in HIV-1 infected individuals showed no difference from controls with respect to expression of CD4, CD11, transferrin receptor (CD71), MHC antigens (84) and the Fc gamma receptors (FcgIII) and CR3 receptors (85). One other report revealed decreased expression of MHC class II antigens on AIDS patients' monocytes using dual colour flow cytometry (86).

Several studies concentrated on monocyte marker expression on infected-monocytes in culture. The high level of CD4 expression in monocytes changed with time in culture and the ability of the cell to bind HIV became CD4 independent (87). In vitro maturation of peripheral blood monocytes in culture was associated with a decrease in membrane CD4, while HLA-DR and Fc gamma receptor III expression increased. Cell at all stages of maturation were succeptible to HIV-1 infection, even mature macrophages without CD4 detected by immunofluorescent staining (88). The proportion of monocytes expressing the Fc gamma receptors and CR3 molecules before and after activation with LPS, appeared to be similar in both infected individuals and controls (85). When monocytes were infected by HIV or gp120 in culture, it was found that more than 25% of the monocytes became IL-2 receptor (CD25) positive and HLA-DR positive before any productive infection, suggesting that activation occured in vitro before productive infection (81).

Some work has been done to investigate marker expression on promonocytic cell lines infected with HIV. Immunofluorescence analysis using monoclonal anti-IL-2r alpha antibody, showed that HIV infection itself induced IL-2R alpha expression moderately in U937 and THP-1. IL-6 and IFN-gamma induced a high level of IL-2R alpha expression both in uninfected and infected THP-1 cell lines (89). Promonocytic cell line U937 as a monocyte model, have shown induction of differentiation and increased expression of mature monocyte surface markers (except for HLA-DR) after in vitro infection with HIV (85,90,29).

1.5.2 Monocyte number in HIV-1 infection.

Monocytes form between 1-9 percent of peripheral blood leucocytes. The mean monocyte count in normal adults is 400 per microliter. Men tend to have higher monocyte counts than woman (91). Monocytosis occured when the count exceeded 800 per microliter in adults. An increase in the number of peripheral blood monocytes was directly proportional to the increase in the total blood monocyte pool and the monocyte turnover rate (92). Adults infected with HIV-1 were found to have abnormalities in their monocyte counts.

Study of patients' monocytes by microscopy showed moderate to marked (93) reduction in counts compared to controls. However, other reports using morphology found no difference in monocyte count from normals (94,95,96).

Dual fluorescence flow cytometry study using anti-CD14 (Leu M3) as a monocyte specific marker, showed the total number of

monocytes to be reduced in AIDS patients compared to controls (95). Other flow cytometry studies revealed a decrease in CD4 positive monocytes in HIV-1 infected patients compared to controls, although the total number of circulating monocytes remained unchanged (97). The number of CD4+ monocytes was found to decrease in parallel with CD4+-T cell counts, but the CD4+ monocyte counts were observed to be significantly higher than control in persons with early stage of HIV disease (98). However other flow cytometry studies using Leu M3 in one study and in another case Leu M3 in conjunction with CD4 monoclonal antibody, showed significant increases in monocyte count and CD4 positive monocytes (84) in AIDS patients, compared to controls. The mean number of adherent monocytes in peripheral blood measured by the method of Currie and Hedley, was significantly decreased in HIV-1 infected patients compared to controls (29).

1.6 Monocyte function in human immunodeficiency virus type one (HIV-1) infection.

1.6.1 Monocyte chemotaxis in human immunodeficiency type one (HIV-1) infected patients.

Chemotaxis is the directional movement of cells in response to a chemical substance in the environment. The cells become orientated and move towards (positive chemotaxis) or away from (negative chemotaxis) the source via a concentration gradient of the substance. Monocyte chemotaxis involves the movement of monocytes towards infected sites in response to inflamatory stimuli and signals, including formyl peptides, C5a, leucotriene B4, cytokines, denatured proteins, products of cell damage and bacterial substances.

Monocytes were reported to exhibit decreased in vivo chemotaxis in HIV-1-infected patients compared to controls (8). With the development of a suitable method of screening for HIV-1, it was discovered that monocytes fron HIV-1-infected patients showed reduced chemotaxis compared to controls (99). HIV-1 related factors in the serum of patients were found to be responsible for the in vivo decreased monocyte chemotaxis (99,100). As a result, peripheral blood monocytes were unable to migrate to inflamatory sites, thus contributing to inadequate host responses to opportunistic organisms, characteristic of disease. Further evidence revealed that the circulating gp41, the HIV-1 transmembranous envelope protein, reduced monocyte chemotaxis in HIV-1-infected individuals (101).

In vitro studies revealed a marked reduction of monocyte chemotaxis in HIV-1-infected patients compared to controls (100,102,). One of these studies (100) reported monocytes to have reduced chemotaxis responses observed over a wide range of concentrations to three different stimuli, i.e. N-formyl methyl leucyl phenylalanine (fMLP), lymphocyte derived chemotactic factor and C5 des Arginine (C5 des Arg). The same group of workers also observed that monocytes from AIDS patients exhibited significantly reduced migration to optimal concentrations of Giardia lamblia in comparison to migration by monocytes from control subjects.

One group of worker (103), however, found normal monocyte chemotaxis in response to casein and zymosan-activated serum in AIDS patients.

1.6.2 Monocyte phagocytosis during HIV-1 infection.

Phagocytosis is the ingestion of cells, particles or microorganisms which then become enclosed in cytoplasmic phagosomes. Monocytes phagocytose target particles by either immune (receptor-specific) or nonimmune (non-specific) mechanisms.

Non-specific phagocytosis is a natural ability of monocytes to engulf any particles without involving receptor binding. In contrast, receptor specific phagocytosis involves monocyte attachment to microorganisms opsonised with antibody, via their cell surface receptors (Fc receptors) or, if the organism was opsonized with a fragment of the third complement component (C3b) through activation of the complement system, attachment was achieved through the cell surface receptor CR3 (i.e. the receptor for C3b). Once the binding occured, the phagocyte membrane became activated and formed pseudopodia which extended around the microorganism. The microorganism eventually became completely engulfed in a vesicle called a phagosome which was then internalised into the cytoplasm.

In vivo evaluation of mononuclear phagocytes C3 and Fc-receptor mediated clearance in HIV-1-infected patients, revealed a striking defect in C3-specific clearance (104,105). HIV-1-infected patients were found to release large number of C3-sensitised erythrocytes back into the circulation and efficient phagocytosis did not occur.

These in vivo reticuloendothelial system defects in HIV-1-infected patients led workers to investigate peripheral blood monocyte phagocytosis defects in vitro. Monocytes from AIDS patients were unable

to phagocytose avian erythrocytes in an in-vitro antibody-dependent cell-mediated cytotoxicity assay when compared to controls (106). Most studies indicated an impairment in the immune mechanism of monocyte phagocytosis in HIV-1-infected patients. Monocytes from patients, in vitro, had reduction in both Fc and C3 receptor-mediated phagocytosis of Candida guilliermundii when compared to controls (107).

In contrast, some investigators reported no anomalies in C3-dependent monocyte phagocytosis in HIV-1-infected patients. The mean number of Candida pseudotropicallis (108), and Candida albicans (103), and the phagocytic index (109) in HIV-1-infected patients showed no significant difference from controls.

Firm evidence suggested no anomalies in the non-immune mechanism of monocyte phagocytosis in HIV-1-infected patients. In vitro studies showed no significant difference in non-specific monocyte phagocytosis of fluorescent latex beads quantified by laser flow cytometry in patients compared to controls (99,107).

1.6.3 Monocyte killing of intracellular pathogens in human immunodeficiency virus infection.

Monocytes killed ingested microorganisms by an intracellular oxidative respiratory burst and generation of reactive metabolites mechanism activated during phagocytosis (110). The phagosomes containing ingested microorganisms fused with the enzyme-containing granules in the cytoplasm, forming vacuoles called phagolysosomes within which the killing process occured.

1.6.3.1 Monocyte killing of intracellular microorganisms in normal individuals.

Different pathogens, eg. Candida (yeast) strains, were lysed by different oxidative mechanisms in monocytes. Moreover, the Candida strains differ in their capacity to ferment carbohydrates and in their antigenic characteristics (111). The fact that different strain of Candida are lysed through different pathways of oxidative mechanisms, was obvious when it was demonstrated that Candida pseudotropicales but not Candida albicans, can be killed even in the absence of myeloperoxidase (112). Candida albicans was killed only by myeloperoxidase mechanism (112), while Toxoplasma gondii was lysed by several different pathways (113). Another mechanisms by which monocytes killed targets was antibody-dependent cell cytotoxicity (ADCC). This process involved the killing of certain cell-types by effector cells carrying, bound to Fc receptors, antibody specific for the target cells. Monocytes were important effectors of damage to antibody-sensitized cells (106). The attachment of the Fc-receptor positive monocyte to the target was folllowed by phagocytosis and killing of the target.

Collaboration between monocytes and lymphocytes was essential for the elimination of these intracellular pathogens. Lymphocytes secreted cytokines which activated monocytes to regulate the respiratory burst reactions. Normal monocytes in culture released low amount of reactive oxygen product and showed little pathogenicity. However, stimulation of the monocytes by IFN-gamma, produced enhance superoxide levels (114-119). IL-2 was found to augment the cytotoxicity of normal monocytes (120).

1.6.3.2 Monocyte killng of microorganisms in HIV-1-infected patients.

Monocytes were found to show reduced killing of intracellular microorganisms in HIV-1-infected patients. The evidence for deficient microbicidal functions of monocytes in HIV-1 infection was contradictory. The discrepancies between the results were probably due to the different method employed to evaluate lysis of the microorganisms by the monocytes. Emphasis should have been placed on the type of microorganisms used and the method by which they were killed.

Some investigators found defective microbicidal activity in monocytes from patients with HIV-1 infection. In contrast with normals, HIV-1-infected patients presented decreased monocyte candidacidal activity for Candida pseudotropicallis that gradually detoriorated as the clinical symptoms progressed towards AIDS (108). Monocytes from HIV-1-infected subjects also showed decreased ability to kill Toxoplasma gondii after activation with rIFN-gamma in vitro (121). This defect was most striking in patients with persistent generalised lymphadenopathy (PGL). In both AIDS and assymptomatic patients with HIV-1 infection, monocyte oxidative burst responses were reduced compared with healthy individuals (110). There was a clear reduction in antibody-dependent cell-mediated cytotoxicity (ADCC) activity by monocytes from HIV-1-infected patients to chicken red blood cells in vitro, compared to controls (106).

Other investigators reported no anomalies of intracellular killing by monocytes in HIV-1-infected patients. Peripheral blood monocytes showed no significant difference in the percentage killing of Crytococcus neoformans and spores of Aspergillus fumigatus, and

Thermoascus crustaceus in HIV-1-infected patients and controls (109). This was consistent with previous finding that killing of Toxoplasma gondii by cultured AIDS monocytes was normal (122). During the course of antibody dependent cellular cytotoxicity (ADCC), Fc gamma receptor (FcγR+) positive monocytes acting as effector cells, recognize and bind to IgG-sensitized target cells. This process induces the lytic potential of the monocytes, and leads to the death of the target cells. There was no significant difference either in monocyte antibody-dependent cell-mediated cytotoxicity (ADCC) activities between HIV-1-infected patients and controls (123).

1.6.4 Monocyte accessory cell functions in human immunodeficiency virus infection.

Accessory cell functions involve cells acting as modulators of immune responses, lymphocyte function and development. Monocyte accessory cell function can involve processing and presenting antigens to T-lymphocytes, resulting in the induction of immune responses. Such induction showed MHC class I or II restriction and activated the secretion of cytokines by and clonal proliferation of T-cells. Monocytes also had the ability to secrete cytokines which induced the proliferation of both B and T lymphocytes. These cytokines included IL-1, IL-6 and TNF.

It was observed that monocytes showed abnormal accessory cell function in HIV infection. Peripheral blood monocytes from HIV-infected patients ex-vivo exhibited alterations in accessory cell functions (124) so that normal lymphocytes showed reduced response to tetanus toxoid and herpes antigens, as compared to normal monocytes cocultured with autologous lymphocytes.

In other work done in vitro, coculture of HIV-infected monocytes with autologous uninfected T-lymphocytes, significantly decreased T-cell proliferative responses and secretion of IL-2 (125).

Other investigators working on cell lines, found decreased accessory cell function in the U937 cell line infected with HIV in culture (126). This decrease was found to correlate with production of large amount of virus detectable by reverse transcriptase activity. The accessory cell defect was partly overcame by the addition of r-IL-2 or IL-1 to HIV infected U937 cells and T-cells cocultivated in anti-CD3 stimulated cultures.

However, IFN- gamma production by infected monocytes remained unaffected (125). The same group of workers also found that monocyte IL-1 production triggered by LPS was not impaired by HIV infection.

1.6.5 Monocyte cytokine production in human immunodeficiency virus type one (HIV-1) infection.

Cytokine is the collective name for a group of soluble proteins mediating the interactions between cells involved in both natural and specific immunity. These include monokines, interferons, growth factors and colony stimulating factors. Monokines are soluble proteins secreted by mononuclear phagocytes with regulatory effects on lymphocyte functions e.g. Interleukin-1 (IL-1). It mediates the effector function of mononuclear phagocytes and acts as a costimulator of lymphocyte activation. Lymphokines are non-antibody proteins released by primed T- lymphocytes. Both mononuclear phagocytes and

T-lymphocytes produce other cytokines called colony stimulating factors (CSF) which regulate the proliferation of immature leucocytes in the bone marrow.

Monocytes secrete a variety of cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), tumour necrosis factors (TNF) and interferons (IFN).

Conflicting results have been reported regarding monocyte cytokine secretion in HIV-infected patients. Some workers found impairment in cytokine prodution by monocytes in HIV-infected patients while other workers reported no abnormalities.

1.6.5.1 Abnormal cytokine production in HIV-1-infected patients.

Abnormal cytokine production has been detected in vivo in HIV-1-infected patients. Spontaneous IL-1 released from monocytes in HIV-infected patients was observed to be significantly greater than in controls (127). A subset of these patients with high levels of serum immunoglobulin had elevated IL-1 production (2.9 times higher than controls).

Peripheral blood monocytes was also found to produce IL-6 (128). Serum IL-6 mean level measured by the B9 cell growth assay, in serum samples of 40 HIV-infected patients and 10 controls was found to be raised in 15 patients with stage 1 and 2 disease (25.2 $\pm$ 1.8 U/ml) and stage 3 and 4 disease (46 $\pm$ 1.7 U/ml) as compared to normals. Patients with late stage disease (5 and 6) had mean serum IL-6 value not different from controls ($p=1.0$). In one report, a significant increase in plasma IL-6 levels was also detected in patients as compared to controls (129).

HIV-infected patients had levels of TGF-alpha at optimum levels to induce CD16 expression (80) on monocytes.

1.6.5.2 Abnormal cytokine production by normal monocytes infected by HIV-1 in culture.

It was observed that infection of normal monocytes by HIV-1 in culture directly induced the production of IL-1 and TNF-alpha (130). The event happened via CD4 receptor interaction with HIV and this was not an active infection since the effect was mimicked by anti-CD4 (OKT4A). Anti-CD4 stimulation of monocytes resulted in the production of increased levels of IL-1, nanogram amounts of IL-1-beta and TNF (74,130). Monocytes infected by HIV-1, in culture, only produced 20% of the amount of IL-1 activity of the uninfected controls in response to stimulation with either latex or LPS (131). However, purified gp120 bound to monocytes in culture, induced an increased production of IL-1 compared to controls (74).

IL-1 inhibitory activity released by long term cultures of monocytes infected in vitro, was found to inhibit lymphocyte proliferation (132). HIV-1 induced normal monocytes infected in culture to secrete significantly increased levels of TNF-alpha, TNF-beta and IFN-alpha compared to controls (133). These cytokines were suggested to potentiate viral replication by autocrine or paracrine routes.

1.6.5.3 Abnormal cytokine prodution by monocytic cell lines infected with HIV-1 in culture.

Monocyte cell lines THP-1 and U937, when infected with HIV-1, did not produce IL-1-beta. LPS (10 ug/ml) and TPA (100uM) stimulation induced the infected cells to produce IL-1 levels 8 to 15 folds

more than non-infected controls. Monocyte-colony -stimulating factor (M-CSF) and TNF-alpha were elevated less than two fold in HIV-infected cells as compared with uninfected cells (134). However, IFN-gamma suppressed HIV production in monocytic cell line THP-1), while IL-2 and IL-6 augmented virus production (89). IL-6 on the other hand induced HIV expression in infected primary monocyte-derived macrophages (MDM) and in synergy with TNF-alpha (135).

One report revealed that infection of the promonocytic cell line U937 with HIV-1 did not increase the expression of IFN-alpha 1, IFN-alpha 2, IFN-beta, IL-1-alpha, IL-1-beta, IL-6 and TNF-alpha (136). Infection of U9-IIIB cells resulted in low constitutive level of TNF and IL-1 but not IFN. However, coinfection with Sendai virus or stimulation with LPS, resulted in 10-20 fold higher levels of IFN-beta, IL-6 and TNF-alpha.

1.6.5.4 Abnormal cytokine production in cultures of monocytes from HIV-infected patients.

TNF-alpha production was found to be strongly enhanced in monocytes from HIV-1-infected patients after stimulation with LPS compared to TNF-alpha production of activated monocytes from healthy donors (137).

1.6.5.5 Normal cytokine production by HIV-1-infected peripheral blood monocytes in vitro.

Some workers reported no abnormalities in cytokine responses. Studies done using Northern blot and bioassay techniques, showed that HIV infection did not cause TNF gene translation in activated monocytes cultures infected with a macrophage tropic HIV strain. There was no detectable effect of HIV on the normal monocyte-

derived macrophage TNF production induced by LPS (138). Another study showed that in vitro infection of monocytes with HIV or r env did not cause release of TNF-alpha, IL-1-beta, IL-6 or GM-CSF. LPS stimulated monocytes showed increased expression similar to controls (139). In one case, normal monocytes infected with HIV or recombinant (r) gp120 inthe absence of LPS showed no production of either form (i.e. protein or mRNA) of IL-1-beta, IL-6 and TNF-alpha. When LPS was added, as low as 0.5 ng/ml, expression of cytokines was induced (140).

1.6.6 Monocyte production of prostaglandin E_2 (PGE_2)

PGE_2 could be biosynthesised from arachidonic acid or the PG endoperoxides (PGH_2 or PGG_2), (141,143). (see p.2).

1.6.6.1 PGE_2 production by normal monocytes in vitro.

Normal peripheral blood adherent monocytes in culture, were found to inhibit T-lymphocyte proliferation induced by mitogens, antigens, or allogenic cells (144,145) and Pokeweed mitogen-driven polyclonal plasma cell generation (146). Monocyte inhibitory activity was largely PGE_2-dependent for both T-lymphocyte proliferation (147,148) and for B-lymphocyte maturation into plasma cells. The mechanism by which PGE_2 suppressed the in vitro immune response was unclear. However, the multiple effects of IL-1 on monocytes and monocyte-derived macrophages included the induction of the synthesis of PGE_2 (149).

1.6.6.2 PGE_2 production by monocytes in human immunodeficiency virus infection.

Several studies have been done on PGE_2 production in HIV-infection. Results showed abnormal PGE_2 production by HIV-infected monocytes from HIV-infected patients. PGE_2 levels determined by high-performance liquid chromatography and radiography in supernatants isolated from monocyte culture, showed that in 3 out of 8 patients, PGE_2 levels were markedly higher (736,419 and 208 pg/ml), than the mean values from 7 controls(73 ± 51 pg/ml) (150). Anhanced level of PGE_2 was detected in monocytes from HIV-positive drug users with or without zymosan stimulattion. Leukotriene B4 increases were not detected. This pointed to an altered cyclo-oxygenase arachidonic acid metabolism in monocytes from HIV-infected drug users, and that this was associated with severe cellular immune-dysfunction characteristic of AIDS (151).

Signal transduction also played a major role in PGE_2 release by monocytes. One report showed that purified gp120 (50-400 ng/ml) stimulated the release of PGE_2 and IL-1 from human monocytes in culture (74). An increase in PGE_2 was initially detected with 50 ng of gp120 per ml. After a 12 fold increase, PGE_2 levels reached a plateau at 200 ng of gp120 per ml. (The amount of PGE_2 produced in response to gp120 was comparable to that obtained with LPS or Con A.). The production of IL-1 by monocytes in response to increasing amount of gp120 paralleled that of PGE_2. Involvement of CD4 signal transduction and PGE_2 production was demonstrated by the ability of OKT4 and OKT4A monoclonal antibodies to increase monocyte PGE_2 (152).

HIV infection was associated with spontaneous proliferation of T-lymphocytes which was CD4 dependent, and this increased with

disease progression (153). However, PGE_2 seemed to have an inhibitory role on lymphocyte proliferation in HIV infection and might be one of the factors involved in the severe immune disfunction associated with AIDS. Ultrafiltrates from HIV-infected monocyte cultures showed an increase of PGE_2 levels and this had an inhibitory effect on autologous immune T-cell proliferation (154). This activity was significantly reduced in similar ultrafiltrates prepared from indomethacin-treated cultures. Further evidence showed that indomethacin between 1-10 μM enhanced PHA-induced lymphocyte proliferative responses in vitro, with cells from patients with AIDS and AIDS-related complex, and this may have therapeutic potential in some patients with AIDS (155).

However, one group of investigators (156) reported that monocytes from HIV-infected patients produced spontaneously a factor which inhibited IL-2Ra (Tac) expression, and IL-2 production by normal stimulated lymphocytes in culture. This factor was a 29Kd cationic protein molecule and was not PGE_2.

1.7 Scope of the present thesis.

Observation of peripheral blood leucocytes of HIV-1-infected patients under the microscope, revealed the presence of abnormal cells which resembled both lymphocytes and monocytes. This thesis presents results of phenotyping studies on these cells. Earlier work reported monocytes to be devided into two heterogenous subpopulations as a consequence of the continuous spectrum of physical and functional properties related to monocyte maturation and activation (157). Preliminary flow cytometry analysis done in this laboratory, revealed an extra leucocyte population in HIV-1-infected blood. The first

task of this thesis was to identify and phenotype this extra leucocyte population.

1.7.1 Phenotyping of Mononuclear Phagocytes in HIV Infection

In the present thesis, monocyte phenotype was investigated to see if the activated phenotype has any influence on functions in vivo. Monocyte phenotype was studied using monoclonal antibody labelling of peripheral blood leucocytes by flow cytometry. The phenotype studied included monocyte numbers based on CD14 and CD4 expression in disease progression, and monocyte activation markers expression in peripheral blood. Emphasis was placed on the extra monocyte population, to see if the activated phenotype of these cells influenced their functions in vivo.

Monocytes were characterized by expression of CD14. The etiologic agent of AIDS, HIV, displayed a selective tropism for the cell-surface CD4 glycoprotein (49), also expressed on monocytes (55,56). There was a need to follow the expression, in vivo, of both monocyte surface markers with disease progression in HIV infection. Since various investigators had seen a heterogeneity in function of monocytes in vitro, it was necessary to see if any of the monocyte subsets in vivo showed evidence of activation and if this had any association with function.

1.7.2 Functional of Mononuclear Phagocytes in HIV Infection

To understand monocyte function in vivo, two steps were undertaken. Firstly, IL-6 and PGE_2 secretion, and IL-1 and TNF gene expression were investigated in peripheral blood monocytes of HIV-infected patients. Secondly, the accessory cell functions of monocytes were investigated to assess the effect of monocyte TNF-a, IL-1a and PGE_2

production on lymphocyte proliferation in anti-CD3-stimulated whole blood cultures of patients.

The cytokines' levels were measured by ELISA, while lymphocyte proliferation was quantified as the proportion of lymphocyte IL-2 receptor (CD25) expression. The PGE_2 level was quantified by a PGE_2 radioimmunoassay.

Monocyte IL-6 was studied because increased level of this cytokine was considered to be a remarkable characteristic found in the serum of HIV patients (129), and this was accompanied by increased number of B-cells and elevated IgG levels. HIV gp120 had been observed to bind monocyte CD4 (150) in vivo, and induced the production of PGE_2 (74). PGE_2 in contrast, had been found to inhibit lymphocyte proliferation (154). It was important to assess monocyte accessory cell functions by considering monocyte PGE_2 inhibitory effect on T-cell proliferation, in parallel with monocyte IL-1α stimulatory effect and monocyte TNF-α effect on T-lymphocyte proliferation.

Stage 1

Asymptomatic
Acute retroviral infection
Persistent generalised lymphadenopathy (PGL)

Stage 2

Weight loss, < 10% of body weight.
Mucocutaneous manifestations (seborrhoeic dermatitis, prurigo, fungal nail infections, recurrent oral ulcerations, angular cheilitis).
Herpes Zoster.
Recurrent upper respiratory tract infections (eg. bacterial sinusitis).

Stage 3

Weight loss, > 10% of body weight (A).
Unexplained chronic diarrhoea, > 1 month (B).
Unexplained prolonged fever (intermittent or constant), > 1 month (C).
Candidiasis, oral.
Candidiasis, vulvovaginal, > 1 month.
Oral hairy leukoplakia.
Pulmonary tuberculosis.

Stage 4

Bed-ridden, > 50% of the day during the last month.
Candidiasis of the oesophagus, trachea, bronchi or lungs.
Cryptomegalovirus (CMV) disease of an organ other than liver, spleen or lymph nodes.
Herpes simplex infection, mucocutaneous (> 1 month), or visceral (any duration).
HIV dementia (encephalopathy).
Isosporiasis with diarrhoea, > 1 month.
Kaposis sarcoma (KS).
Lymphoma.
Mycobacterium tuberculosis (extrapulmonary).
Mycobacteriosis / atypical, disseminated.
Mycosis, disseminated/ endemic (eg. histoplasmosis, coccidioidomycosis).
Pneumocystis carinii pneumonia (PCP).
Progressive multifocal leukoencephalopathy (PML).
Salmonella septicaemia, non-typhoid.
Toxoplasmosis of the brain.
Wasting syndrome due to HIV (A with B or C, see stage 3).

Table 1.2.1 WHO clinical staging system for HIV infection and disease.

Common Nomenclature	CD Designation	Size	Affinity
FcRI	64	75kD	IgG1>IgG3>IgG4>IgG2
FcRII	32	40kD	IgG1>IgG3=IgG4>IgG2
FcRIII	16	50-70kD	IgG1=IgG3 (not igG2, IgG4)

Table 1.5.1 shows the common nomenclature, CD designation, sizes and affinities of the leucocyte Fc receptors.

Chapter 2

Materials and Methods

Chapter 2

2 Materials and Methods

Flow cytometry techniques, involving the labelling of cells by fluorescent monoclonal antibodies against various cell surface antigens, was used to phenotype peripheral blood monocytes.

Two facets of monocyte function were considered. Firstly, in vivo plasma interleukin-6 (IL-6) and prostaglandin E_2 (PGE_2) levels, plus cytokine gene expression in peripheral blood monocytes were studied. The ELISA technique was used to monitor plasma IL-6 levels in peripheral blood of patients, and radioimmunoassay was used to assess PGE_2 levels in lipopolysaccharide (LPS) stimulated whole blood cultures. Monocyte cytokine gene expression was done by polymerase chain reaction (PCR) techniques. Secondly, monocyte accessory cell function was assessed by using ELISA techniques and radioimmunoassay to measure cytokines and PGE_2, respectively, in anti-CD3 stimulated whole blood cultures. Both cytokine (IL-1α and TNF-α) levels were compared to lymphocyte response assessed by the proportion of lymphocyte IL-2 receptor (CD25) expression in response to anti-CD3 stimulation.

2.1 The Patients.

A sample of 153 HIV-1-infected patients from Ruchill and Falkirk hospitals in Glasgow, were involved in the experiments which comprised this thesis. Samples of peripheral blood from these patients, were sent to the Department of Immunology, Glasgow University, to be monotored for diagnostic cell surface markers. A group of 109 patients were subdivided into groups of disease categories; including 40 patients in CDC2, 27 in CDC3 and 38 in CDC4. The other 44 patients were taken into

consideration irrespective of their disease categories. A total of 28 healthy laboratory personnel were used as controls. The study of monocyte surface marker expression involved 51 of the patients, including the two patients on whose cells the effect of paraformaldehyde on monocyte heterogeneity was investigated. The measurement of plasma interleukin-6 (IL-6) levels was performed on a group of 63 patients and the same patients were used for assessment of other parameters in peripheral blood, including the total white cell count and monocyte and lymphocyte subsets. Prostaglandin E_2 (PGE_2) levels in LPS stimulated cultures was quantified in 14 patients, while cytokine gene expression was assessed in a small group of 4 patients. The effect of PGE_2 on lymphocyte proliferation, and cytokine production in anti-CD3 stimulated whole blood cultures, involved 19 patients.

These patients were HIV-infected subjects whose blood samples were sent on a regular basis to be monitored for surface markers at the Department of Immunology. They formed part of the group of Glasgow HIV-infected patients. The cumulative figure for HIV-infected patients in Glasgow was 462 HIV positive individuals among which 60 had AIDS. In Scotland, the cumulative figure was 1943 HIV positive patients, among which 294 were AIDS patients.

Controls utilised in the experiments reported in this thesis, were healthy laboratory personnel with median age of 33 years. All personnel were recruited from the Department of Immunology.

2.2 Classification of disease progression in HIV infection.

Data about disease and symptoms occuring throughout the course of the disease progression were collected in HIV-infected

patients. Several groups of medical scientists and practitioners attempted to classify the diseases and symptoms occuring throughout the course of the disease, in order to improve the management of patients and to better understand the disease progression. Four staging system had been developed up to date. These are the Walter Reed, the CDC, the WHO, and the CDC/WHO staging systems of disease progression in HIV infection.

2.2.1 The Walter Reed classification of disease progression in HIV infection.

In 1984, the Walter Reed Army Medical Center developed a classification system which classified disease progression in six stages (4). Stage zero described the exposure to HIV through any of the known transmission routes. Stage 1 was the period of acute infection. Stage 2 covered the period when patients developed chronic lymphadenopathy. Subclinical immune dysfunction described patients in stages 3 and 4. Patients who developed skin and membrane immune defects were classified as stage 5. Stage 6 patients had systemic immune deficiency (AIDS).

2.2.2 The CDC classification system of disease progression in HIV infection.

In 1986, the Center for Disease Control (CDC), Atlanta, USA, published a system for classifiying conditions in adolescents and adults within the spectrum of clinical findings attributed to HIV infections (5). It was classed in groups called CDC. Acute infection was CDC1, asymptomatic but HIV infected was CDC2, progressive symptoms was CDC3, and acquired immunodeficiency syndrome (AIDS) was CDC4.

2.2.3 WHO staging system for disease progression in HIV infection.

In 1990, the World Health Organisation (WHO), published an interim proposal for a staging system for HIV infection and disease (Table 1.2.1) which incorporated some measures of immune function which had come into common usage since 1986 (6). This interim staging system consisting of four stages (Stages 1,2,3 and 4) developed on consensus reached by expert consultants, was based on clinical criteria, and included laboratory information involving the use of CD4 positive lymphocytes and / or total lymphocytes determination. Stage 1 was the asymptomatic and the persistent generalised lymphadenopathy (PGL) patients. Stage 2 were patients with early (mild) disease. Stage 3 were patients with intermediate (moderate) disease. Stage 4 were patients with late (severe) disease (equivalent to AIDS).

The laboratory information subdivided each clinical category into 3 strata (A, B, C), depending on the number of CD4 lymphocytes per mm3 (>500, 200-500, <200). If the CD4 counts were not available, the total lymphocytes was used instead as an alternative laboratory marker and also subdivided in 3 different strata (>2000, 1000-2000, <1000).

2.2.4 The CDC/WHO classification system for HIV infection and disease in adolescents and adults.

The CDC/WHO classification system classified adolescents and adults diagnosed as HIV seropositive. It was represented by categories arranged along two axes. One axis covered clinical categories of conditions related to HIV infection. These were categories A, B, and C. The second axis comprised of laboratory categories defining cell-

mediated immune status measured by CD4+ lymphocyte counts/mm3 and /or total lymphocytes.

2.3 The Cells used in the experiments.

The cells used in the experiments were leucocytes from peripheral blood of HIV-infected patients and normal individuals.

2.4 Phenotyping the monocytes.

2.4.1 Assessing cell counts.

2.4.1.1 Total white cell count in peripheral blood.

Peripheral blood (40µl) was added to 20ml buffer solution, followed by 3 drops of saponin which lysed the red blood cells. The total white cell count was assessed on a Coulter counter (Coulter Electronics, England) as the number of cells per ml.

Mononuclear cell counts were performed by light microscopy, after staining the cells in a blood film. This staining was performed by making a film of blood which was air dried. The slide was then fixed for 10 minutes in 100% methyl alcohol, then put into May-Grunwald (50 v/v with water) for 10 minutes. The slide was then rinsed and drained, then mounted with DPX and a coverslip.

2.4.1.2 The differential white cell count.

The differential white cell count in peripheral blood was assessed by flow cytometry. Peripheral blood leucocytes were labelled with fluorescent anti-CD14 and anti-CD45 monoclonal antibodies, and fixed in paraformaldehyde by the normal staining method (refer to section 2.4.2: Labelling cells with conjugates). The data from the stained

cells was aquired by a Becton/Dickinson FACScan flow cytometer, registered on computer disks and analysed on a Hewlett-Packard computer by the Simulset computer programme. The Simulset program displayed on computer screen, the different leucocyte populations on the basis of PE and FITC fluorescence, and granularity and size parameters. The statistics showing the numbers and percentages of cells in the each leucocyte population were also displayed simultaneously.

2.4.2 Labelling cells with conjugates.

2.4.2.1 The conjugates.

Monoclonal antibodies were obtained as directed by Becton-Dickinson Immunocytometry Systems. For simultaneous two-colour immunofluorescence, antibodies directly conjugated with fluorescein isothiocyanate (FITC) and phycoerythrin (PE) were used to label the leucocytes. Dual staining with anti-CD14 as monocyte specific marker in combination with another antibody which specified the mononuclear cell phenotypes were used. The right combination of CD14 (i.e Leu M3 PE or Mo2 FITC) was used, depending on whether the other antibody specific for a phenotype was conjugated to FITC or PE.

The conjugates utilised were as prescribed by Becton and Dickinson. The control conjugate was a nonspecific mouse monoclonal antibody (MsIgG1-RD1/MsIgG1-FITC). CD14 conjugated to PE was an anti-human Leu-M3 (DAKO: Cat. No 7497). CD14 conjugated to FITC (Mo2) was from Coulter (Lot. 2909 K023) and both CD14 conjugates were monocyte specific. Anti-CD4 (T4) conjugated to FITC was from DAKO (Lot: 2400D063), and binds specifically to CD4, the receptor for HIV.

The antibodies used to investigate monocyte activation were as follows; CD11b purchased from DAKO (Lot NR: P0206), was anti-Leu 15 and was conjugated to PE. It binds specifically to the receptor (CR3) for the complement C3. RD-PE (I3-RD1) purchased from Coulter (Lot No: 1529D054), binds the human histocompatibility antigen HLA-DR. CD16 (DAKO) bound Fc gamma receptor III and CD71 (DAKO) labelled transferrin receptors on monocytes.

Other antibodies were against the interleukin-2 receptor (CD25) (DAKO) to characterize lymphocyte proliferation, and CD8 (DAKO) to specify T-suppressor cells.

2.4.2.2 The reagent solution used in phenotyping.

FACS Lyse Fluid.

The original stock of FACS Lysing solution (Becton and Dickinson, Belgium) was diluted 1 in 10 in distiled water.

Phosphate Buffering Solution (PBS azide).

The original stock of PBS contained 0.2% sodium azide and had a concentration of X25 (diluted 1 in 25 in distilled water for use).

2.4.2.3 Labelling whole peripheral blood with conjugates by the normal staining method.

5µl of each conjugate was added to 100ml of peripheral blood collected in EDTA and incubated at room temperature, in the dark, for 10 minutes. 2ml FACS lysing fluid (Becton and Dickinson) dilution 1 in 10 in distilled water, was added to the blood to lyse the red blood cells, incubated for 10 minutes in the dark to prevent quenching of the

fluorochrome, and centrifuged at 1000 r.p.m for 5 minutes. The supernatant was removed, 2ml PBS Azide, diluted 1 in 25 in distilled water, added and the lot recentrifuged at 1000 r.p.m for another 5 minutes. The supernatant was removed, and 0.5ml of 1% paraformaldehyde pH 7.4 was added to the leucocytes and the data acquired on a Becton/Dickinson FACS Scan flow cytometer. (Since HIV seropositive peripheral blood samples were hazardous, the procedure was conducted in isolation, in a hood in the category three containment laboratory).

2.4.3 Acquisition of data from labelled cells.

The Becton/Dickinson flow cytometer.

The Becton/Dickinson flow cytometer was a machine capable of collecting and measuring the intensity of fluorescent and scattered light from fluorescent labelled cells (Appendix 2.4.3). The collected light was transformed into electrical signals and digital information which could be registered and analysed by computer. The machine was composed of various units assembled together. These were an argon laser, light collecting units, light detection units, a preamplifier, a pulse processing and amplifier unit and an analogue to digital converter unit connected to a Hewlett-Packard computer.

The light collecting units.

Laser light (488nm) reflected from laser stearing mirrors passed through the sample sucked up into a flow chamber. Light transmitted and scattered from the flow chamber was collected by collection lenses. The forward collection lens was of small numerical

aperture and gathered a cone of light from 1-20 degrees off the laser beam axis. This angle of light minimized the effect of changes on the forward scatter measurements, thus minimizing the dependence on the particle size. The right-angle collection lens was of a high numerical aperture to collect right angled scattered light. This allowed for the particle density to be measured. The mixture of right-angled scattered and fluorescent light collected from the stained cells were separated by an optical filtration system which allowed specific independent and correlated measurement to be made.

The different wavelengths were separated by dichroic mirrors, interference and absorption filters. Dichroic mirrors were oriented 45 degrees to the incident beam while the absorption and interference filters were orthogonal. A mirror of 500nm shortpass dichroic filter reflected wavelengths shorter than 500nm (the 488nm scattered light) towards the right-angle scatter detector. Longer wavelengths traversed this mirror to a second mirror of 560nm shortpass dichroic filter allowing wavelengths greater than 560nm to reflect through a 578nm filter with a 28nm half-peak bandpass and onto the PE fluorescence detector. Shorter wavelengths (500-560nm) incident at the second mirror pass on through a 530nm filter with a 30nm half-peak bandpass and onto the FITC fluorescence detector.

Although the bandpass filters selected separate wavebands, the emissions from the two fluorochromes overlaped such that some fluorescence from one fluorochrome was able to pass to the detector intended to measure the fluorescence from the other and vise versa. This spectral overlap was corrected during signal processing.

The light detecting unit.

Two kinds of diodes were used as detectors. PIN diodes, a solid detector of low sensitivity and of wide characteristics, detected forward scatter and light absorption. Photomultiplier tubes (PMT) with low spectral response and good signal characteristics detected FITC and PE fluorescence.

Signal processing.

Light falling on the detectors generated a current which fed into the preamplifier which generated a smoothed voltage pulse output (0-10V) with amplitude proportional to the number of photons reaching the detectors. The shape of the pulse was determined by the size and speed of the particles, the width of the illuminating beam and the distribution of the fluorochrome on or within the particles. Small particles with narrow illuminating beams produced pulses with fast rise times and larger particles produced slower pulses.

The pulse processing unit.

The pulse processing unit retained fast output pulses from the preamplifier, providing an analogue voltage memory of the signal which was held long enough for further processing to take place. The sustained output voltage from the pulse processing unit was proportional to the height, the area and the width of the pulse from the preamplifier. Logarithmic amplification amplified weak signals and compressed large signal, so that both weak and strong signals were displayed on the same scale.

An analogue to digital converter (ADC) translated each continuous voltage analogue range into a scale represented by a binary number. The result was a stream of numbers which was held into computer memory or on computer discs.

Analysis of the acquired data.

The computer used programmed software to read the stored data and displayed them as frequency histograms and double parameters correlated plots known as cytograms or dot plots. The dot plot was a two-dimentional extension of the frequency histogram. The locations in the memory corresponded to a two-dimensional array of an ADC correlated against the channels of another ADC. The memory was then read onto the screen to produce a square plot where each cell was represented as the coordinates appropriate to the measured values.

Statistical analysis of the displays were done by the computer. Markers were set at specific channels of a histogram and percentage total and mean value generated for selected events. Cytograms were analysed by setting quadrants around areas of interest and specific statistics were generated. Gates were set around specific areas on the cytograms allowing more complex analysis to be performed.

2.5 Functional aspects of monocyte experiments.

2.5.1 The growth medium.

Hepes Saline/RPMI growth medium.

Stock RPMI 1640 (10x, Gibco) diluted in 0.01M Hepes-bicabonate buffer pH 7.2 (Gibco) was supplemented with 2 mM L-Glutamine (Gibco BRL, Paisley) and 100 Units/ml penicillin / 200 µg/ml streptomycin (Gibco). The medium was neutralized with 1m NaOH and used for incubating peripheral blood mononuclear cells. This was termed wash medium.

The wash medium was supplemented with 10% feotal calf serum (Gibco) and was used for peripheral blood mononuclear cells in microexudate coated flasks to purify monocytes.

Iscoves growth medium.

Iscoves growth medium was prepared by adding 1 ml streptomycin to 180 ml Iscoves.

2.5.2 The cell cultures.

2.5.2.1 LPS stimulated whole blood cultures.

The culture medium content was 180ml 0.01M Herpes Saline (Gibco), 20ml RPMI (10x, Gibco) and 2 ml L-Glutamate (Gibco). 100 units/ml Penicillin/Streptomycin (Gibco) was added to the content. 50µl whole blood preserved in 10U/ml heparin (Leolabs) and 200µl medium was placed in the wells of a 96U-shaped Nunclon culture plate. 2.5µg of LPS was added to the content of the wells to stimulate the mononuclear cells. The cultures were incubated for 24 hrs at 37°C in 5% CO_2 in an air humidified incubator (Amersham), then centrifuged at 300 x g for 5 minutes before harvest of supernatants.

2.5.2.2 Anti-CD3 stimulated whole blood cultures.

The growth medium was prepared by adding 1ml streptomycin to 180ml Iscoves. Whole blood (1.5ml) was diluted in 13.5 ml growth medium to make up a 1/10 whole blood dilution. A volume of 100 µl whole blood (1/10), to which was added 25µl 1/100 diluted 0.1mg/ml anti-CD3 monoclonal antibody (UCH T-1) (Serotec) of the IgG1 subclass, was cultured in wells of a 96 U-shaped Nunclon culture plate. The cultures were incubated at 37°C in a CO_2 incubator for 3 days. After incubation, the cultures were centrifuged at 1400 r.p.m for 5 minutes, followed by harvests of supernatants and blood.

2.5.2.3 ^{3}H-Thymidine incorporation in anti-CD3 stimulated whole blood cultures.

The 3 day old anti-CD3 stimulated whole blood culture wells were pulsed with 25µl 1µCi ^{3}H-Thymidine (Amersham), and the whole culture incubated for 4 hours at 37°C in 5% CO_2 in an air-humidified incubator. After incubation, the cultured plate was placed in a Multi-cell harvester (Titertek Cell Harvester, Skatron, Norway) which produced an aerosol mixture and harvested the cell bound DNA on paper. The harvested cell bound DNA on paper, was sealed in a transparent Titertek non-toxic plastic film (Flow Labs, Irvine) containing EcoScint A scintilation fluid (Optiphase 'Hisafe' 3; LKB). A β-plate rack was used to hold the paper in position, then transfered to a β-counter (LKB 1215 Rack beta) which counted the radioactivity from the cells in count per minutes (cpm).

2.5.3 Measuring PGE_2 levels produced from mononuclear cells.

2.5.3.1 Radioimmunoassay for prostaglandin E_2 (PGE_2).

Prostaglandin E_2 (PGE_2) was measured by a radioimmunoassay using competitive adsorption to dextran coated

charcoal (7). PGE_2 (Sigma) was dissolved at 1mg/ml in ethanol and stored at -20°C. Standards for the assay were prepared by diluting this stock solution in assay buffer. Tritiated PGE_2 ([5,6,8,11,12,14,15(n)-^{3}H]PGE_2) at radioactive concentration of 0.1mCi/ml (3.7 MBq/ml) was obtained from Amersham International plc. Rabbit anti-serum to PGE_2 was donated by Dr. Alison Severn (Dept. of Immunology, Glasgow University). The assay buffer constituted of 50mmol Tris.HCl at pH7.4 with 0.1% gelatin. Dextran coated charcoal was prepared by suspending 0.5g activated charcoal (Sigma) and 0.1g Dextran 70 (Sigma) in 25ml assay buffer without gelatin.

A standard PGE_2 assay was performed to determine the standard curve for PGE_2 each time the assay was done. 40µl standard PGE_2 at concentrations (1000, 500, 200, 100, 50, 20, 10 and 0pg/40µl), 40µl ^{3}H-PGE_2 (diluted 1:500 in buffer), 40µl of anti-serum (diluted 1:500 in buffer) and 40µl of medium were incubated at 4°C in a 96-well round-bottomed tissue culture plate (Nunclon). Wells without anti-serum were included to determine the non-specific counts and the total radioactive counts. These contained 40µl ^{3}H-PGE_2 and 120µl buffer in the non-specific count wells and 40µl ^{3}H-PGE_2 plus 180µl buffer in the total radioactive count wells.

40µl of sample, 40µl ^{3}H-PGE_2 (diluted 1:500 in buffer), and 40µl anti-serum (diluted 1:500 in buffer) were incubated together at 4°C in a 96-well round-bottomed tissue culture plate (Nunclon). 40µl buffer was also included.

After a four hour incubation at 37°C, 60µl dextran-coated charcoal was added to each well, and the plate incubated on ice for 10

minutes. The plate was centrifuged at 300 x g for 3 minutes on a Sorvall RT6000b centrifuge, and 160µl supernatant removed from each well. Scintillation fluid (Optiphase 'Hisafe' 3;LKB) was added, and the samples counted in a beta-counter (LKB 1215 Rackbeta). Percentage inhibition of binding (of ^{3}H-labelled PGE_2 to antibody, by PGE_2 in sample or standard) was calculated as follows;

$$\text{percentage (\%) inhibition of binding} = 10 - \frac{(1\text{-cpm-NSC})}{(0\text{ cpm-NSC})}$$

where cpm= counts per minute for wells containing no PGE_2 and NSC= non-specific counts; this represented counts due to ^{3}H-PGE_2 unbound to antibody and not adsorbed by dextran-coated charcoal.

The standard curve was determined by plotting percentage (%) inhibition of binding against pg PGE_2/40µl on a semi-log graph. The amount of PGE_2 in each sample was extrapolated from the standard curve by reading its percentage (%) inhibition of binding to its corresponded weight in pg/40µl. The sensitivity of the assay was 250-500 pg/ml.

2.5.4 Detection of gene expression in mononuclear cells.

2.5.4.1 RNA extractions from peripheral blood monocytes, and lymphocytes.

a) Harvesting the monocytes and lymphocytes.

5ml of peripheral blood was added to 5ml Lymphoprep (Nycomed Pharma, AS Oslo, Norway) or Ficoll Hypaque (S.G = 1.077) (Flow Lab) and centrifuged at 1400 r.p.m for 30 minutes. After centrifugation, three layers were observed; a top layer containing plasma, a middle

layer or interface containing the monocytes and lymphocytes and a bottom layer containing granulocytes and red cells in the separation medium. Mononuclear cells were harvested from the interface between the separation medium and the serum, then added to 10 ml RPMI 1640 wash medium, and centrifuged at 1400 r.p.m for 10 minutes. The supernatant was removed and the pellets (cells) suspended in culture medium i.e wash medium plus 10% feotal calf serum (Gibco). The cells was incubated in a microexudate coated flask (plastic growth bottle) at 37°C for 45 minutes. The monocytes adhered to the plastic bottle and the lymphocytes stayed in suspension. After incubation, the medium containing the lymphocytes was removed, and the monocytes left in the plastic bottle for RNA extraction.

b) Homogenisation.

RNAzol: Guanidine Isothiocyanate (0.2ml RNAzol/10million cells) from BRL Bathesda, USA, was added to the cells. The RNA was solubilized by agitating the lysate a few times through a plastic pipette.

c) RNA extraction.

0.2ml chloroform was added to 2ml of homogenate. The samples were tightly covered, shaken vigourously for 15 seconds and placed on ice (or at 4°C) for 15 minutes. The suspension was centrifuged at 12000g for 15 minutes. After the addition of chloroform and centrifugation, the homogenate formed two phases; the lower Phenol-chloroform phase and the upper aqueous phase. RNA remained exclusively in aqueous phase whereas DNA and proteins remained in the interphase and organic phase.

d) RNA precipitation.

The aqueous layer was transfered to fresh tubes, equal volumes of isopropanol were added and the samples were stored for 45 minutes at 20°C. The samples were centrifuged at 12000 r.p.m for 15 minutes at 4°C. RNA precipitated (often invisible before centrifugation) formed a white translucent pellet at the bottom of the tube.

e) RNA wash.

The supernatant was removed and the RNA pellet washed twice with 75% ethanol by vortexing and subsequent centrifugation at 12000 r.p.m for 8 minutes at 4°C. 0.8ml of ethanol per 50-100µl RNA was used. The pellets were dried for 10-15 minutes. It was important not to dry the pellets completely, as it would have greatly reduced its solubility.

f) Reconstitution of RNA.

The pellets were disolved in 0.5% SDS or in 1ml EDTA, pH7 solution by vortexing or by passing few times through a plastic pipette tip.

2.5.4.2 Detection of the genes.

The quality and quantity of this RNA was confirmed by spectrophotometry and gel electrophoresis. This RNA was reversed transcribed and amplified in a 30-cycle PCR using suitable primers for TNF-α (33 base pairs) and TNF-β (21 base pairs) which were specific for sequences in the first intron of each gene. These were synthesised to order (Genosis, Cambridge,UK).

2.5.5 Cytokine measurement in peripheral blood and in culture supernatants.

Soluble interleukin-1 alpha (IL-1α), interleukin-2 (IL-2), and tumour necrosis factor-α (TNF-α) were measured by ELISA technique. IL-2, IL-1α and TNF-α kits were purchased from Amersham. For all three cytokines' ELISAs, monoclonal anti-TNF-α or anti-IL-2 and anti-IL-1α antibody was diluted in coating buffer and 100 µl added to each of 96 wells. After overnight incubation at 37 °C, the plate was washed three times with washing buffer. Test samples, appropriate negative control and TNF-α and IL-2 and IL-1α standard dilutions were added in duplicate wells and incubated overnight at 37 °C. After four washings, polyclonal rabbit anti-TNF-α or IL-2 and IL-1α conjugated to horseradish peroxidase was added to each of 96 wells utilized (1 hour,37°C). Alkaline-phosphatase- conjugated goat anti-rabbit Ig was dispensed into each well and incubation proceeded for 1 hour at 37°C. After four washes, 200 µl substrate reagent was added per well and after incubation at room temperature, the plate was read on a standard ELISA reader at 405 nm (at six 15-min intervals for 90 min). The duplicate readings were averaged and the zero standard potical density was subtracted from the sample optical density. The optical density for the standards were plotted versus the concentration of standard. The cytokine concentration was then interpolated from the curve.

2.6 Statistical methods.

The variables not normally distributed were expressed in medians and ranges, and were compared by Mann-Whitney U-test. Data expressed as mean ± s.e.m are normally distributed. Comparison of two sets of normally distributed data was made using student's t-test. P values less than 0.05 were considered significant.

Chapter 3

RESULTS

Monocyte phenotype and function in HIV-1-infected patients.

Chapter 3

3 RESULTS

Monocyte phenotype and function in HIV-1-infected patients.

3.1 Peripheral blood monocyte phenotype in HIV-1 infection.

A survey of monocyte phenotypes involving assessment of monocyte numbers and expression of different cell surface markers, in peripheral blood, was conducted in 10 HIV-infected patients who had double monocyte populations. The proportion of monocytes in the extra population was compared to the disease categories in 63 HIV-infected patients, followed by a comparison of proportion of CD4 positive monocytes in the extra monocyte population to disease progression in 41 HIV-infected patients. A survey of activation markers on peripheral blood monocytes was also conducted on 10 patients.

3.1.1 Identification of monocyte heterogeneity in HIV infection.

3.1.1.1 Identification of normal peripheral blood leucocyte populations by flow cytometry.

Peripheral blood from a normal person was labelled with anti-CD14 and anti-CD45 monoclonal antibody markers by flow cytometry techniques. Figure 3.1.1.1 showed the distribution of peripheral blood leucocytes based on granularity and size parameters. The distribution showed four cell populations; population 1,2,3 and 4. Population 1 had cells of smaller size with very low granularity. Population 2 contained large cells with low granularity. Population 3 was seen to contain cells with high granularity and large size.

Population 4 contained cells of small size and very low granularity and these were cell debris and dead cells.

3.1.1.2 Analysis of normal blood leucocytes based on anti-CD14 and anti-CD45 fluorescent monoclonal antibody markers.

The antibody fluorescent markers used was anti-CD14-PE in conjunction with anti-CD45-FITC. These labelled the cell populations on the basis of expression of CD14 and CD45, but to varying degree. Anti-CD14 was monocyte specific and anti-CD45 labelled all leucocytes.

The pattern for normal peripheral blood leucocyte staining based on CD14 and CD45 was shown in Figure.3.1.1.2a. The distribution showed four cell populations. Population 1 had negative PE with high positive FITC staining, indicating no expression of CD14 antigen by this cell population. Population 2 had high positive PE with moderate FITC stainings, indicating that this cell population expressed high levels of CD14 antigen. Population 3 had low positive PE with moderate positive FITC stainings, indicating low expression of CD14 by this cell population. However, population 4 had negative PE and negative FITC stainings, showing no expression of CD14 or CD45 by this cell population.

Each cell population shown in Figure.3.1.1.2a was gated and further analysed for their distribution based on granularity and size parameters. Figure 3.1.1.2b,c,d,e revealed the identity of the populations in relation to granularity and size. Gate R1 (population 1) was the lymphocytes, figure 3.1.1.2c. Gate R2 (Population 2) was found to be monocytes, figure 3.1.1.2b. Gate R3 (Population 3) was granulocytes, figure 3.1.1.2d. Gate R4 (Population 4) was considered as background

fluorescence due to cell debris, dead cells and autofluorescence due to cells like eosinophills, figure 3.1.1.2e.

3.1.1.3 Identification of peripheral blood leucocytes of an HIV patient.

The distribution of an HIV-infected patient's peripheral blood leucocytes based on granularity and size parameters was shown in figure.3.1.1.3. To identify the leucocyte populations, the same procedure utilized in identifying leucocyte populations in normal blood was used. Unlike the distribution of normal peripheral blood leucocytes which contained four cell populations (populations 1, 2, 3 and 4), the distribution of peripheral blood from an HIV-infected patient showed an extra cell population. The leucocyte populations were as follows; 1, 2a, 2b, 3 and 4. Population 2a contained cells of average size and were moderately granular. This cell population was considered to be monocytes. Population 2b had cells which were less granular than that of population 2a, but were very large. Further analysis was needed to clarify the identity of this cell population, since it was unsure whether this cell population was monocytic or lymphocytic. Population 1 had cells of low granularity and small sizes. These were considered to be lymphocytes. Population 3 contained cells of high granularity and large size. These cells were considered to be granulocytes. Population 4 contained cells which were small and had low granularity. These were considered to be dead cells and cell debris.

3.1.1.4 Analysis of HIV positive peripheral blood monocytes using anti-CD14 and anti-CD45 fluorescent antibody markers.

The antibody fluorescent markers used was anti-CD14 in conjunction with anti-CD45-FITC. These labelled the cells on the basis of PE and FITC fluorescence but to varying degree. CD14-PE stained

monocytes and CD45-FITC stained leucocytes. The pattern for HIV positive peripheral blood leucocyte staining was shown in figure.3.1.1.4a with PE and FITC stainings represented, respectively. The distribution showed four cell populations: Population 2 had high positive PE with moderately positive FITC staining, indicating high expression of CD14 by this cell population. Population 2 was therefore considered as the monocyte population. Population 1 had negative PE with high positive FITC stainings. Population 3 showed low positive PE with moderately positive FITC stainings. Population 4 had negative PE and negative FITC stainings.

The cell population corresponding to the monocyte fluorescence was gated (Gate R1, Figure.3.1.1.4b) and its distribution based on granularity and size parameters shown in Figure.3.1.1.4c. Unlike normal peripheral blood, the monocyte population in peripheral blood from an HIV-infected patient was found to be heterogenous: it contained an extra monocyte population, Figure.3.1.1.4c. The expected monocyte population (the first monocyte population): i.e. population 2a, had granularity and size comparable to that of normal peripheral blood monocyte population (population 2b). The extra monocyte population, had lower granularity but were very large in size.

3.1.1.5 Analysis of HIV positive peripheral blood leucocytes using anti-CD14 and anti-CD45 fluorescent antibody markers.

Apart from the monocytes, the other cell populations based on fluorescence distribution, were gated and further analysed for their distribution based on granularity and size (Figure.3.1.1.5a). The identity of each cell population in relation to granularity and size was shown in Figure.3.1.1.5b,c,d,e. Gate R1 was found to be lymphocytes,

Figure.3.1.1.5c; Gate R3 granulocytes, Figure.3.1.1.5d and Gate R4 was staining due to background fluorescence, Figure.3.1.1.5e.

3.1.1.6 Quantifying the monocyte populations in HIV patients.

The size and granularity of the two monocyte populations were analysed on histograms. Figure.3.1.1.6a showed the distribution of the monocyte populations based on sizes. Markers were set on the distribution to obtain the statistics. The larger monocytes in the extra population, were approximately 30% greater in diameter, based on the relative size scale of the forward angle light scatter, and less granular than the normal monocyte population. Figure.3.1.1.6b showed the distribution of the two monocyte population based on granularity. The granularity was approximately 30% less which was consistent with the granule contents being diluted in an increased volume. Two-dimensional statistics quadrants were set on the granularity-size graph showing the two monocyte populations, Figure.3.1.1.6c. This allowed a further overall assessment of the two populations. The mean size of the larger monocyte population in 10 patients was 56% (SD = 12%) greater than normal.

3.1.1.7 Testing the effect of paraformaldehyde fixation on monocyte heterogeneity in periperal blood of HIV-patients.

The monocyte counts in peripheral blood was measured to test if paraformaldehyde fixation did not affect the monocyte count in the two monocyte populations in peripheral blood of HIV-infected patients. The monocyte counts were assessed by flow cytometry based on anti-CD14 and anti-CD45 labelling, at different times following paraformaldehyde fixation. The study was conducted on two HIV-

infected patients who had two monocyte populations present in their blood.

Table 3.1.1.7 showed the monocyte counts expressed as numbers and percentages in the two monocyte populations in patient 1 and patient 2 at times zero (0), 24 and 48 hours after fixation. Both Patients had no significant difference of monocyte counts, nor in the proportions of cells in both monocyte populations at any given time. The total cell number remained constant at any given time.

The results indicated that paraformaldehyde fixation of peripheral blood leucocytes did not affect monocyte counts in any of the monocyte populations. Paraformaldehyde therefore had no effect on the heterogeneity of the monocyte populations.

3.1.1.8 To test whether heterogeneity in monocyte population was due to storage time dependence.

The monocyte numbers present in peripheral blood of a normal person, was measured at different times after storage in a plastic EDTA bottle. The monocyte counts was assessed by flow cytometry using anti-CD14 and anti-CD45 labelling. The counts were expressed as cell numbers with the CD14 mean fluorescent intensity (Table 3.1.1.8).

Firstly, blood was sampled in the evening at time zero (0_e) on day 1, and incubated. Table 3.1.1.8 showed the monocyte counts and the fluorescent intensities in the evening at time zero (0_e) on day 1 and at time 17 , 20 , 22 and 24 hours after storage on day 2. There was no significant difference in the monocyte counts nor in the mean fluorescent intensities at any time.

Table 3.1.1.8 also showed the monocyte counts and the fluorescent intensities in another blood sample from the same person taken on the morning of day 2. The monocyte counts in this sample was considered at time zero (0_m) in the morning, 3, 5 and 7 hours on day 2. The mean fluorescent intensities was found to be constant throughout the 7 hours. There was no significant difference in the monocyte counts nor in the mean fluorescent intensities at any given time.

The results showed that in each case the monocyte number and CD14 mean fluorescent intensity remained constant. No other extra monocyte population appeared throughout the time of storage. The effect of heterogeneity of monocyte population was not due to storage time dependence nor to a diurnal effect.

3.1.2 Leucocyte count in peripheral blood.

3.1.2.1 Monocyte counts in disease progression.

The monocyte count based on CD14 expression and flow cytometry, obtained from 41 HIV patients at different stages of disease progression, was observed to decreased non-significantly with disease progression (Table 3.1.2.1). The biggest difference was between CDC2, 444 (381-571), and CDC4, 319 (239-478), ($p = 0.08$). However, the proportion of monocytes was found to increase slightly as disease progressed, but this increase was not significant. Flow cytometry also revealed an extra monocyte population containing large cells in the peripheral blood of HIV-infected patients. There was a significant increase in the number of large monocytes in this extra population with disease progression ($\chi^2 = 32.1$, $p < 0.001$).

3.1.2.2 Monocyte counts in patients and controls.

A study done on 10 patients of median age 32 years, showed no significant difference in monocyte count compared to 10 controls of median age 34 years (Table 3.1.2.2). However, patients had a significantly increased proportion of monocytes ($p < 0.05$). The proportion in patients was 14 (8.8-18.5) % cells/ml compared to 8 (7.0-12.3) % cells/ml in controls.

3.1.2.3 The lymphocyte subsets.

Patients lymphocyte counts and proportion were found to decrease significantly with disease progression (Table 3.1.2.1). In particular, a significant decrease in lymphocyte count existed between disease category CDC2 and CDC4 ($p < 0.001$). When considering the lymphocyte subsets, the $CD3^+$ lymphocyte (T-cell) count decreased with disease progression. Significant differences in T-cell count existed between patients of CDC2 and CDC3 ($p < 0.05$) and patients of CDC2 and CDC4 ($p < 0.001$). However, when patients were compared to controls (Table 3.1.1.2), no significant differences were found to exist in their proportion of T-cells.

Both T-cell subset counts i.e. $CD4^+$ T-cells and $CD8^+$ T-cells, decreased with disease progression (Table 3.1.2.1). Significant differences existed in $CD4^+$ T-cell counts between each disease category. However, the largest significant difference in $CD8^+$ T-cell count existed between CDC2 and CDC4 patients. When comparing patients to controls (Table 3.1.2.2), there was a significant decrease in their proportion of $CD4^+$ T-cells ($p < 0.01$). However, a significant increase existed in the proportion of their $CD8^+$ T-cells ($p < 0.01$).

3.1.3 Monocyte surface marker expression and disease progression in HIV-infected patients.

3.1.3.1 Monocyte CD4 expression and disease progression.

A comparison of CD4 positive monocytes in 41 HIV patients with disease progression, measured by flow cytometry, showed the CD4 + monocytes to correlate with disease progression ($r = 0.36$, $p = 0.01$). The parameters were displayed on Table 3.1.3.1 and illustrated in figure.3.1.3.1. The study was performed on 41 HIV-infected patients of whom 14 patients were at disease progression stage CDC2, 12 at stage CDC3 and 15 at stage CDC4.

Table 3.1.3.1 showed the median proportion of both CD4 positive monocytes and CD4 positive lymphocytes in peripheral blood of patients in each disease categories and for the whole group of patients. In addition, it was observed that the CD4+ lymphocytes also correlated with disease progression ($r = 0.4$, $p = 0.01$). The proportion of CD4+ monocytes significantly decreased with disease progression ($p < 0.01$) and this was in parallel with the significant decrease in the proportion of CD4+ T-lymphocytes.

When comparing the patients' CD4+ monocytes in each disease category, there was a significant decrease in the proportion of CD4+ monocytes in patients of CDC3 category, compared to those in CDC2 ($p < 0.05$). The proportion of CD4+ monocytes in patients of CDC3 category showed no significant difference to those of patients in CDC4. However, there was a significant decrease in the proportion of CD4+ monocytes observed in patients of CDC4 to those in CDC2 disease category ($p < 0.01$).

The illustration (Figure.3.1.3.1) showed the CDC disease categories displayed against the proportion of CD4 positive monocytes and the CD4 positive lymphocytes. The proportion of CD4 positive monocytes was found to decrease with disease progression whilst their heterogeneity increased (Figure 3.1.3.1). This heterogeneity was accentuated in the CDC4 disease category (AIDS), where patients had a wider range in the proportion of monocyte CD4 expression.

3.1.3.2 Monocyte heterogeneity in relation to disease status in HIV patients.

The proportion of monocytes in the extra monocyte population was compared to the disease categories in 63 HIV patients (Table 3.1.3.2). The extra monocyte population, based on CD14 staining, were arranged into groups: those having 0%, 1-5%, 6-20% and those having greater than 20% of the total monocytes. The disease categories indicated the severity of the disease. It was observed that the percentage of monocytes in the extra monocyte population increased with the severity of the disease (chi-square = 13.3, with 6 degrees of freedom, $p < 0.042$). In that case the null hypothesis was rejected, showing that this association of percentage of monocyte in the extra population with disease progression was significant.

3.1.4 Monocytes expression of surface markers in peripheral blood of HIV-patients.

Monocytes surface marker expression was measured in peripheral blood by labelling the cells with fluorescent monoclonal antibodies against different monocyte surface antigens. The positivity of the fluorescent intensities defining the expression of surface markers based on PE and FITC staining was measured on peripheral blood monocytes using flow cytometry.

The expression of 8 surface markers were considered, including CD14, CD4, CD11b, CD71, CD45, CD25, CD16 and HLA-DR. Table 3.1.4 showed a comparison of the various monocyte surface markers expressions between the first monocyte population in 10 patients and the monocyte population in 10 controls, and between the two monocyte populations in patients. The surface marker expressions were expressed as fluorescent intensity.

3.1.4.1 Monocyte surface marker expression on the monocyte population in patient and controls.

A comparison of fluorescent intensities of monocyte surface markers expressions between the first monocyte population in patients and the monocyte population in controls, showed patients' monocytes to express significantly greater mean fluorescent intensity of staining for CD11b, CD4 and CD25 (Table 3.1.4). All 3 surface markers showed significantly increased level of staining ($p < 0.01$ for CD11b and CD4, and $p \leq 0.05$ for CD25). The median fluorescent intensity of CD11b was 1513 (1084-1753) compared to 463 (394-522.5) in controls. That of CD4 expression was 92.5 (67.3-134.0) in patients compared to 35.2 (30.8-46.5) in controls, and CD25 expressions in patients was 30.0 (5.5-54.5) compared to 4.0 (2.0-9.5) in controls.

There was no significant difference in monocyte HLA-DR, CD14, CD45, CD71 and CD16 expressions between patients and controls, although HLA-DR and CD14 were expressed at high levels.

3.1.4.2 Monocyte surface marker expression on the two monocyte populations in patients.

A comparison of surface marker expression on the double monocyte populations in patients revealed the extra population with larger monocytes to express significantly greater mean fluorescent intensity of staining for CD45, CD16 and HLA-DR (Table 3.1.4). All 3 surface markers showed a significant increase level of staining: CD16 and HLA-DR ($p < 0.05$), and CD45 ($p < 0.01$). There was a greater expression of CD11b, CD14, CD4 and CD71, but these were non-significant.

The monocyte HLA-DR fluorescent intensity was 1273 (867-2350) in the extra monocyte population compared to 483 (47-1174) in the first monocyte population. The monocyte CD16 fluorescent intensity was 42 (12.75-68.75) in the extra monocyte population compared to 12 (10-38.25) in the first monocyte population. Monocyte CD45 fluorescent intensity was at 368 (273-489) in the extra monocyte population compared to 209.5 (152.5-283.5) in the first monocyte population.

Expressions of activation markers, including CD16, CD11b, CD71 and HLA-DR, plus the larger size of the cells in the extra monocyte population, would suggest that this was an activated phenotype.

3.2 Results of peripheral blood monocyte functions in HIV-1 infection.

Monocyte functions were assessed in vivo by making a comparison of plasma monocyte-derived IL-6 with other parameters, and by assessing mononuclear phagocyte gene expression. Monocyte accessory cell function was studied by investigating the effect of PGE_2

on lymphocyte response in patients, in parallel with monocyte IL-1α and TNF-α roles on lymphocyte proliferation.

3.2.1 Cytokine production by monocyte in HIV-1 patients.

3.2.1.1 In vivo plasma IL-6 levels in HIV-1-infected patients.

Plasma IL-6 measured in the peripheral blood of HIV-infected patients by commercial enzyme immunoassay, concurrent with monocyte phenotyping, had a statistically significant correlation with the proportion of monocytes of the activated phenotype ($r = 0.31$, $p = 0.02$). This correlation was stronger in the CDC4 AIDS group ($r = 0.44$, $p < 0.02$). A group of 63 HIV-infected patients was involved in the study, and their plasma IL-6 levels was measured at the same time as monocyte phenotyping. Plasma IL-6 level was measured with and compared to other parameters, including total white cell count (TWCC), monocyte number, lymphocyte subsets and IgG level, throughout disease progression (Table 3.2.1.1).

IL-6 level did not correlate with disease progression, and showed no correlation to the absolute monocyte numbers, total white cell count, B-cell counts, IgG level, nor any other lymphocyte subsets (CD4+ or CD8+ lymphocytes).

The plasma IL-6 levels were 10 (0-129.0) pg/ml in CDC2 patients, 58 (0-86.0) pg/ml in CDC3 patients and 13 (0-129.0) in CDC4 patients. No significant differences existed between the IL-6 levels in each disease category. The percentage of activated monocytes was 2 (1-7) % in CDC2 patients, 3 (0.3-7) % in CDC3 patients and 7 (4-26) % in CDC4 patients. There was a significant association between the number of

monocytes in the extra population and disease progression ($\chi 2 = 32.1$, $p < 0.001$).

3.2.1.2 Cytokine gene expression by peripheral blood monocytes assessed by polymerase chain reaction (PCR).

RNA was extracted from peripheral blood monocytes to identify the different cytokine genes expressed. This revealed that adherent monocytes from HIV patients expressed elevated level of TNF-α, rather than the -β form, while controls expressed elevated level of TNF-β rather than the -α form (Table 3.2.1.2). The study involved 4 HIV-infected patients, and 3 normal laboratory personnel as controls. The level of gene expression was compared semi-quantitatively with the signal from actin gene expression and assessed as follows. A negative expression was described by a minus (-) sign, a moderate level of expression, similar to actin, was awarded a plus (+) sign and a high level (at least twice the moderate level) of expression was awarded two plus (++) signs.

Semiquantitatively, there was greater constitutive expression of TNF-α in the HIV patients than controls, and greater TNF-β expression in controls than patients. Table 3.2.1.2 showed TNF-α and TNF-β genes displayed against their level of expressions in patients (1,2,3 and 4), and in controls (5,6,7).

Patients showed high levels of TNF-α expression while controls had a moderate level of expression. The reverse was seen for TNF-β expressions. The controls showed high level of TNF-β expressions, while patients 2,3 and 4 had moderate level, with the exception of patient 1 who expressed high levels. Very low level of IL-2 mRNA was present in

controls, and this suggest lymphocyte contamination, since monocytes do not express this cytokine. Actin gene expression was constant throughout. All stages of this experiment were done together so that each part served as a control for the other.

3.2.1.3 PGE_2 production by stimulated whole blood from HIV-infected patients.

In a study involving 14 HIV patients and 8 normal controls, the PGE_2 levels measured in the supernatants of LPS stimulated whole blood cultures showed no significant difference between the inducible and constitutive levels in patients. The constitutive level was high in patients and no further increase could be induced by LPS stimulation (Table 3.2.1.3). However, the controls showed a significant increase in the inducible PGE_2 level compared to the constitutive level ($p < 0.01$).

Table 3.2.1.3 showed the constitutive and inducible PGE_2 levels in HIV-infected patients and controls. There was a significantly higher constitutive PGE_2 level in patients compared to controls. The constitutive PGE_2 level in patients was 617.0 (479.0-754.5) pg/ml and 0.0 (0.00-0.82) pg/ml in controls ($p<0.001$). When comparing the inducible levels, a significantly higher inducible PGE_2 level existed in patients as compared to controls ($p < 0.001$).

3.2.2 Peripheral blood accessory cell function in HIV-1 infection.

3.2.2.1 Effect of PGE_2 on lymphocyte ^3H-thymidine incorporation and CD25 expression in anti-CD3 stimulated whole blood culture.

The effect of PGE_2 on lymphocyte proliferation was quantified by measuring lymphocyte CD25 expression and ^3H-thymidine

incorporation in anti-CD3 stimulated whole blood cultures, in 6 healthy laboratory personnel. CD25 expression was quantified by flow cytometry techniques and ^{3}H-thymidine incorporation by the β-emission from the cells.

PGE_2 was found to inhibit lymphocyte proliferation assessed by CD25 expression and ^{3}H-Thymidine incorporation, in anti-CD3 stimulated whole blood cultures. Table 3.2.2.1 displayed the CD25 expressions and ^{3}H-thymidine incorporation results against each concentration of PGE_2 and the control. The CD25 expression at each PGE_2 concentration showed a significant decrease of expression when compared to control which had a mean value of 48.67 $\pm$ 2.94 % ($p<0.001$) at all points. The ^{3}H-thymidine incorporation which had a mean value of 2412 $\pm$ 482 cpm at 1mg PGE_2 and 3576 $\pm$ 565 c.p.m at 100 pg PGE_2, showed a significant decrease in counts compared to control ($p < 0.05$). No significant difference in ^{3}H-thymidine incorporation existed at any other PGE_2 concentrations.

The lymphocyte CD25 expression was found to correlate with ^{3}H-thymidine incorporation in anti-CD3 stimulated whole blood cultures ($r=0.77$, $p=0.01$). PGE_2 was found to inhibit lymphocyte proliferation, since the proportion of CD25 expression and the mean ^{3}H-thymidine incorporation reduced with increasing amount of PGE_2. The result showed that CD25 expression could be used as a measure of lymphocyte proliferation at the same time as PGE_2 measurement in the supernatant.

3.2.2.2 Lymphocyte association with PGE_2 production.

The PGE_2 levels measured by radioimmunoassay in anti-CD3 stimulated whole blood cultures of 19 HIV-infected patients, was found to

show an inverse relationship to lymphocyte response (Table 3.2.2.2). There was a negative correlation between PGE_2 production and lymphocyte proliferation ($r=-0.37$, $p = 0.1$). The lymphocyte anti-CD3 response was assessed through flow cytometry by considering lymphocytes CD25 expression in each patients. Patients with 0-10 % CD25 expression were considered as negative responders, those with 10-40 % CD25 expression were poor responders and patients with greater than 40 % CD25 expression were good responders. There were 7 patients who were negative responders, 7 were poor responders and 5 were good responders.

Table 3.2.2.2 displayed the pre and post stimulated PGE_2 levels for patients with different degree of lymphocyte response to anti-CD3. Patients with negative response had elevated PGE_2 level, while those with good response had lower PGE_2 levels ($p < 0.05$).

There was no significant difference between the pre and post stimulated PGE_2 levels in any group of responders. No significant difference in the post-stimulated PGE_2 level was observed between any of the responder groups.

When the pre and post stimulated PGE_2 levels were compared in the whole group of patients (i.e 19 patients), no significant difference were observed. High levels of PGE_2 were being produced already in the pre-stimulated cultures, and further stimulation by anti-CD3 did not increase the PGE_2 levels.

3.2.2.3 Lymphocyte association with cytokine production.

TNF-α and IL-1α was measured and compared to lymphocyte response in pre and post anti-CD3 stimulated whole blood cultures. TNF-α and IL-1α were measured by ELISA, and lymphocyte response was quantified by flow cytometry using anti-CD25 monoclonal antibody labelling. The study was conducted on 19 HIV-infected patients. The parameters measured were TNF-α and IL-1α levels. These were expressed as pg/ml and lymphocyte response to anti-CD3 was expressed as the proportion of lymphocyte expressing CD25.

Table 3.2.2.3 displayed the pre and the post-stimulated TNF-α and IL-1α levels against the lymphocyte anti-CD3 response. Patients with 0-10 % lymphocyte CD25 expressions were termed as negative responders, those with 10-40 % lymphocyte CD25 expressions were poor responders and patients with greater than 40 % lymphocyte CD25 expressions were good responders. There were 19 patients of whom 7 were negative responders, 7 were poor responders and 5 were good responders.

a) TNF-α production in pre and post-stimulated anti-CD3 whole blood cultures of HIV patients.

The pre and the post stimulated TNF-α levels were compared to lymphocyte response in each group of responders. TNF-α levels were found to increase non-significantly with lymphocyte proliferation. The negative responders had low levels of TNF-α, while the good responders had elevated levels. The post-stimulated TNF-α levels were significantly increased in all three responder groups compared to the pre-stimulated levels ($p < 0.001$ for poor and good responders; $p < 0.05$ for negative responders).

Each group of responders was compared to each other to check for any significance in the TNF-α levels among the groups. There was a significant increase in the pre-stimulated TNF-α level in the poor responders compared to the other two groups of responders ($p < 0.05$). However, when the post-stimulated TNF-α levels were compared, a highly significant difference existed among the group of responders. The poor responders showed a highly significant increase in the post-stimulated TNF-α level compared to the negative responders ($p < 0.001$). There was also a highly significant increase in TNF-α level in the good responders compared to the poor and negative responders ($p < 0.001$).

b) IL-1α production in pre and post-stimulated anti-CD3 whole blood cultures of HIV patients.

The pre and post-stimulated IL-1α levels were compared to lymphocyte response in each group of responders (Table 3.2.2.3). There was a positive association between the IL-1α levels with lymphocyte response. This increase in IL-1α level was not significant ($r = 0.25$, $p =$ not significant). The negative responders showed low IL-1α level, while the good responders had elevated levels.

The comparison of the pre and post-stimulated IL-1α levels revealed a significant increase in IL-1α levels among the poor and good responders ($p < 0.01$ for poor responders; $p < 0.001$ for good responders).

A comparison of each the pre and post-stimulated IL-1α levels among the responder groups, revealed that the negative responders showed a significant decrease in the post-stimulated IL-1α level compared to the poor and good responders ($p < 0.05$ in each case).

No significant difference existed in the pre-stimulated IL-1α levels among the group of responders.

3.2.2.4 Lymphocyte stimulation in anti-CD3 stimulated whole blood cultures.

a) The distribution of peripheral blood leucocytes in unstimulated whole blood cultures.

Blood from unstimulated normal whole blood culture was labelled with anti-CD14 and anti-CD45 using flow cytometry techniques. The distribution of peripheral blood leucocytes in unstimulated normal whole blood cultures, based on granularity and size parameters, was shown in Figure.3.2.2.4a. The distribution showed two cell populations: a smaller cell population containing cell of small size and low granularity, and a large cell population containing cells of small size with a wide range of granularity.

b) Identification of the lymphocyte population in unstimulated normal whole blood cultures.

The labelling of peripheral blood leucocytes by anti-CD14-PE and anti-CD45-FITC, give a characteristic fluorescent distribution of leucocyte populations by flow cytometry. The fluorescence of unstimulated normal whole blood leucocytes was studied by labelling the cells with anti-CD14 conjugated to PE and anti-CD45 conjugated to FITC. These labelled the cell populations on the basis of their relative expression of CD14 and CD45. The fluorescence of the cell population corresponding to the lymphocytes (Figure.3.2.2.4b) was identified by the negative CD14 and the highly positive CD45 expressions. This lymphocyte fluorescence was gated (gate R1, Figure.3.2.2.4b), and its cell distribution displayed according to size and granularity (Figure.3.2.2.4c).

The cell population with small size and low granularity (Figure.3.2.2.4a) corresponded to the lymphocyte fluorescece (Figure.3.2.2.4b), and was identified as the unstimulated lymphocyte population (Figure.3.2.2.4c).

c) The distribution of peripheral blood leucocytes in anti-CD3 stimulated normal whole blood cultures.

The distribution of peripheral blood leucocytes in anti-CD3 stimulated normal whole blood culture was displayed according to granularity and size parameters (Figure.3.2.2.4d). The distribution showed two cell populations: a smaller cell population containing cells with a range of size and granularity, and a larger cell population containing small cells with a range of granularity.

d) Identification of the lymphocyte population in anti-CD3 stimulated whole blood cultures.

The fluorescence of anti-CD3 stimulated normal whole blood labelled with anti-CD14 and anti-CD45, was displayed on Figure.3.2.2.4e. The fluorescence of the cell population corresponding to the lymphocyte population was gated (gate R1, Figure 3.2.2.4e) and its distribution displayed according to size and granularity (Figure.3.2.2.4f). The smaller cell population containing a range of cells with increasing size and granularity (Figure 3.2.2.4d) was found to correspond to the anti-CD3 stimulated lymphocyte population (Figure.3.2.2.4f).

3.2.2.5 The distribution of lymphocyte population based on size parameters.

Both unstimulated and stimulated lymphocyte populations were displayed on histogram according to size parameters. Figure.3.2.2.5 showed the distribution of lymphocytes populations based on size. The stimulated lymphocyte population showed a skewed shaped distribution

of cell sizes, with a proportion of cells larger than that of the unstimulated population.

3.2.2.6 Lymphocyte proliferation in unstimulated whole blood culture.

Lymphocyte CD25 expression was measured in unstimulated normal whole blood, cultured for 3 days. The cultured cells were labelled with anti-CD2-FITC which was T-lymphocyte specific, and anti-CD25-PE which bind to IL-2 receptors. CD25 expression was used as a measure of lymphocyte proliferation. The distribution of lymphocyte CD25 expressions based on PE fluorescence in unstimulated whole blood, is shown in figure.3.2.2.6. To estimate the proportion of CD25 expression in the unstimulated lymphocyte population, the distribution of the control PE fluorescence was overlaid on that of CD25 expression. A marker was set at the point where both distribution intersected, so as to evaluate the excess CD25 expressed by the unstimulated lymphocyte population.

3.2.2.7 CD25 expressions in anti-CD3 stimulated whole blood cultures.

The lymphocyte population CD25 fluorescent distribution based on PE stainings in anti-CD3 stimulated whole blood cultures is shown in figure.3.2.2.7. The unstimulated lymphocyte population CD25 expression distribution, was overlaid on that of the stimulated lymphocyte population. A marker was set at the point where both CD25 distributions intersected, so as to evaluate the CD25 expression in the anti-CD3 stimulated lymphocyte population.

	Time (hours)		
	0	24	48
Patient 1			
Total cell number	1167	1136	1119
First monocyte population	1041 (89 %)	1018 (90 %)	991 (89 %)
Extra monocyte population	126 (11 %)	118 (10 %)	128 (11%)
Patient 2			
Total cell number	873	790	742
First monocyte population	805 (92 %)	689 (91 %)	674 (91 %)
Extra monocyte population	68 (8 %)	71 (9 %)	68 (9 %)

Table 3.1.1.7 Testing for formaldehyde effect on monocyte heterogeneity after formaldehyde fixation of peripheral blood leucocytes in HIV-infected patients. The number of cells remain constant throughout the experiment, showing that the heterogeneity of monocytes was not due to paraformaldehyde fixation. The number of cells are expressed as mean and the proportions of cells in each population are also given.

	DAY 1	DAY 2							
		Day 1 blood				Day 2 blood			
Time (hr)	0_e	17	20	22	24	0_m	3	5	7
Number of cells	521	436	426	407	367	567	475	421	461
Mean intensity	1172 (630)	1165 (611)	1167 (702)	1173 (656)	1176 (813)	1168 (597)	1172 (608)	1166 (629)	1169 (784)

O_e time zero in the evening.

0_m time zero in the morning.

Table 3.1.1.8 To test whether heterogeneity in monocyte population was due to storage time. The number of cells and fluorescent intensity of CD14 staining remained constant throughout the experiment, indicating that the heterogeneity of monocytes was not due to storage time. The number of cells is expressed as mean, and the fluorescent intensity as mean $\pm$ standard deviation.

Disease category	CDC2		CDC3		CDC4	
Number of patients	14		12		15	
Total White Cell Count	6344 (5067-7932)		5549 (4625-7338)		3982 (3027-5460)	
Monocyte count	444 (381-571) 7%		388 (278-444) 8%		319 (239-478) 9%	
Activated monocyte count	127 (63-444) 2%		167 (0-388) 3%		279 (159-1115) 7%	$p < 0.001$ $\chi^2 = 32.1$
Lymphocyte count	2079 (1429-2845) 33%		1614 (962-2191) 29%		1036 (717-1736) 26%	
T-cell count	1576 (114-224)	*	1283 (769-1645)	**	771 (523-1343)	***
CD4+ T-cell count	560 (355-730)	**	237 (180-419)	***	79 (29-132)	***
CD8+ T-cell count	1012 (726-1465)		887 (591-1468)		617 (402-967)	**
B-cell count .	213 (121-298)	***	88 (69-180)	**	163 (55-174)	
Granulocyte count	3814 (58%)		2903 (63%)		262 (69%)	

* $p = 0.05$

** $p = 0.01$

*** $p = 0.001$

Table 3.1.2.1 Peripheral blood leucocytes and mononuclear cell subpopulations evaluated using monoclonal antibody markers by flow cytometry techniques. Both the count and proportion of activated monocytes in the extra population, increased with disease progression. The cell counts are in medians and interquartile range. Where appropriate, the proportion of cells are given. The stars on the far right are the significance between CDC2 and CDC4.

	Controls		Patients
Number	10		10
Gender	male		male
Age	34 (33.8-38.0)	*	32 (27.5-34.0)
White cell count	6800 (6050-7200)	**	2964 (1992-4246)
Granulocytes (%)	59 (56.0-64.3)		59 (50.5-67.0)
Granulocyte count	4012 (3808-4372)	**	1749 (1497-1986)
Monocytes (%)	8 (7.0-12.3)	*	14.0 (8.8-18.5)
Monocyte count	554 (476-836)		415 (261-548)
Lymphocytes (%)	30 (26.8-35.3)		27.5 (20.5-36.8)
Lymphocyte count	2040 (1822-2400)	***	815 (608-1091)
(%) CD3+ lymphocytes	74.5 (68.0-77.5)		82.0 (69.0-86.3)
(%) CD4+ lymphocytes	41 (35-43)	**	11 (7.5-16.0)
(%) CD8+ lymphocytes	22.5 (19.8-30.8)	**	60 (51.3-68.0)

* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

Table 3.1.2.2 Comparison of the median values of variables between study groups of HIV-infected and control subjects. Patients show a significant increase in the proportion of monocytes compared to controls.

	Disease Categories					
	Whole group	CDC2		CDC3		CDC4	
Number of patients	41	14		12		15	
(%) CD4+ monocytes	76 (61-85)	84 (82-90.5)	*	73.5 (65.3-83.5)		68 (33-78)	* *
(%) CD4+ lymphocytes	18 (4-30)	34 (24.3-36.0)	* *	18.5 (12.5-28.5)	* * *	3 (2-9)	* * *

* $p < 0.05$
* * $p < 0.01$
* * * $p < 0.001$

$r=0.36$, $p=0.01$ for $CD4^+$ monocytes

$r=0.4$, $p=0.01$ for CD4+ lymphocytes

Table 3.1.3.1 showed the comparison between the CD4+ monocytes and the CD4+ lymphocytes with disease categories in peripheral blood of HIV-infected patients. The proportion of $CD4^+$ monocytes significantly decreases with disease progression, in parallel with the $CD4^+$ T-cells. The figures are in medians and interquartile range. The stars on the far right depict the significance between CDC2 and CDC4.

	Percentage of monocytes (%)			
	0	1-5	6-20	>20
Number of patients	14	24	14	11
Disease categories				
CDC2	6	8	1	2
CDC3	3	8	6	0
CDC4	5	8	7	9

n=63, χ^2=13.3 (6 degree of freedom), p=0.042

Table 3.1.3.2 A correlation between the proportion of monocytes in the extra monocyte populations and the disease categories in 63 HIV seropositive patients.

	Controls		Patients		
	Monocyte population		First monocyte population		Extra monocyte population
CD14	570 (462-746)		418 (173-927)		529 (198-1000)
CD4	35.5 (30.75-46.50)	**	92.5 (67.3-134.0)		140.5 (110-184.3)
CD11b	463.0 (394-522.5)	***	1513 (1084-1753)		1988 (1200-2359)
CD71	11.50 (5.75-15.5)		16.5 (9.5-32.75)		23.5 (17.25-32.0)
CD45	179.5 (142.2-211.2)		209.5 (152.5-283.5)	**	368 (273-489)
CD25	4.00 (2-9.5)	*	30 (5.5-54.5)		24.0 (7.5-59.0)
CD16	10.00 (7-11.5)		12 (10.00-38.25)	*	42.0 (12.75-68.75)
HLA-DR	694 (340-971)		483 (47-1174)	*	1273 (867-2350)

*P < 0.05 **p < 0.01 ***p < 0.001

Table 3.1.4 Comparison of the median fluorescent intensities of various monocyte surface markers between the monocyte populations in control and HIV-patients, and between the two monocyte populations in the patients. The large monocytes in the extra population show significant increase in the expression of CD45, CD16 and HLA-DR, and increase level of expression of CD11b and CD71. Expression of these activation markers and the large size of the monocytes, indicate that this extra population has an activated phenotype. The values are expressed in median with interquartile range.

		Disease Progression					
	Whole group	CDC2		CDC3		CDC4	
Number of patients	68	26		15		23	
IL-6 (pg/ml)	14.5 (0-99.8)	10 (0-129.0)		58 (0-86.0)		13 (0-129.0)	
Monocyte number	382 (265-510)	533 (305-564)		1614 (962-2191)		408.0 (234-466)	
Activated monocyte (%)	4 (1-10)	2 (1-7)		3 (0.3-7)		7 (4-26)	
Total White Cell Count	5270 (3666-6535)	6344 (5067-7932)		5549 (4625-7338)	*	3982 (3027-5460)	***
Lymphocyte number	1522 (996-2232)	2079 (1429-2845)		1614 (962-2191)		1036 (717-1736)	***
CD4+ T-cell number	234 (110-501)	560 (355-730)	**	237 (180-419)	***	79 (23-132)	***
IgG level	13.3 (11-18)	14 (11-20)		13 (11-16)		12 (9-17)	
B-cell number	162 (86-237)	213 (121-298)	**	88 (69-180)		163 (55-174)	**

*p < 0.05 **p < 0.01 ***p < 0.001

Table 3.2.1.1 Plasma IL-6 levels compared with cellular phenotype markers according to CDC categories in 64 HIV patients. Plasma IL-6 level correlated with the proportion of large activated monocytes in the extra population ($r = 0.31$, $p = 0.02$). No other parameters in the blood correlated to plasma IL-6 level. The stars on extreme right are the p values for the significance between CDC2 and CDC4. Medians and interquartile range are expressed.

	Patients				Controls		
	1	2	3	4	5	6	7
Actin	+	+	+	+	+	+	+
TNF-α	++	++	++	++	+	+	+
TNF-β	++	+	+	+	++	++	++

(-) = negative expression.
(+) = moderate expression.
(++) = high expression.

Table 3.2.1.2 Gene expression in peripheral blood monocytes of HIV-infected patients. A minus sign (-) means negative expression, a plus sign (+) moderate expression and two plus signs (++) as high expression. HIV-infected patients expressed high level of TNF-α and moderate TNF-β, while controls expressed high level of TNF-β and moderate expression of the - α form.

	PGE_2 Level (pg/ml)		
	Constitutive		Inducible
Controls n=8	0.0 (0-82.0)	**	204.0 (79.2-357.5)
	***		***
HIV-Patients n=14	617.0 (479.0-754.5)		638.5 (401.0-696.)

*p = 0.05 **p = 0.01 ***p = 0.001

Table 3.2.1.3 Median PGE_2 production in whole blood culture with LPS stimulation (10µg/ml for 24 hours). HIV-infected patients showed no significant difference between the constitutive and the inducible PGE_2 levels. HIV patients had already an elevated level of PGE_2, and no further stimulation by LPS could induce any further increase in the PGE_2 level.

PGE_2	CD25 expression (mean ± s.e.m) %		^3H-Thymidine incorporation (mean ± s.e.m) c.p.m	
1µg	32.5 ± 3.0	* * *	2412 ± 482	*
100 ng	35.8 ± 2.7	* * *		
10 ng	38.5 ± 2.7	* * *	4036 ± 1261	
1 ng	41.8 ± 3.2	* * *		
100 pg	42.3 ± 1.6	* * *	3576 ± 565	*
10 pg	45.5 ± 1.9	* * *		
1 pg	45.5 ± 1.8	* * *	5867 ± 1230	
Control (0 pg)	48.7 ± 2.9		9132 ± 2844	

The significance was done at each point and compared to control.

* $p < 0.05$

* * $p < 0.01$

* * * $p < 0.001$

Correlation of CD25 to ^3H-Thymidine

$r = 0.77$

$p = 0.01$

Table 3.2.2.1 The effect of PGE_2 on lymphocyte proliferation measured using CD25 expression and ^3H-thymidine incorporation by lymphocytes, in anti-CD3 stimulated whole blood cultures of normal subjects. PGE_2 was found to inhibit lymphocyte proliferation. CD25 expression correlated to ^3H-thymidine incorporation, showing that IL-2 (CD25) expression could be use as a measure of lymphocyte proliferation.

	Number of patients	PGE_2 Levels (pg/ml) at 0 and 24 hr	
		Pre-stimulation	Post-stimulation
Whole group	19	275.0 (234-339)	269.0 (224-309)
Response (% CD25 expression)			
Negative	7	309.0 (275.0-389.0)	269.0 (246-309)
Poor	7	246.0 (229-371)	270.0 (224-417)
Good	5	234 (229-291) *	224 (180-304.5)

*p < 0.05 **p < 0.01 ***p < 0.001

CD25 expression

0-10 % = negative responders

10-40 % = poor responders

>40 % = good responders

Correlation of PGE2 levels to lymphocyte response

r = - 0.37, p = 0.1

Table 3.2.2.2 Comparison of median PGE_2 levels to lymphocyte response in anti-CD3 stimulated whole blood culture of HIV-infected patients. PGE_2 showed a weak negative correlation to lymphocyte response. The star at the bottom is the p value of the significance between the good and negative responders.

		TNF-α			IL-1α		
	Number of patients	Pre-sti.		Post-sti.	Pre-sti.		Post-sti.
Whole group	19	2.0 (1.0-2.3)	***	26 (4-45.)	0 (0-0)	**	11.4 (7.3-19.3)
Negative responders	7	1.0 (1.0-2.0)	*	4.0 (2.0-4.0)	0 (0-0)		0 (0.0-15.6)
		*		***			*
Poor responders	7	4.0 (2.0-5.0)	***	30.0 (26-35)	0 (0-0)	**	12.0 (9.8-21.5)
		*		***			
Good responders	5	2.0 (1.0-2.5)	***	47.0 (45-63.5)	0 (0-3.5)	***	12.8 (10.7-20.4)
				***			*

CD25 expression

0-10 % = negative responders

10-40 % = poor responders

> 40 % = good responders

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

anti-CD3 stimulation.

pre-sti. = pre-stimulation

post-sti. = post-stimulation

Table 3.2.2.3 Comparison of median TNF-α and IL-1α levels to lymphocyte response in anti-CD3 stimulated whole blood of HIV-patients. Both TNF-α and IL-1α had a positive correlation with lymphocyte response. The stars at the bottom of the table, represent the significance between negative and good responders.

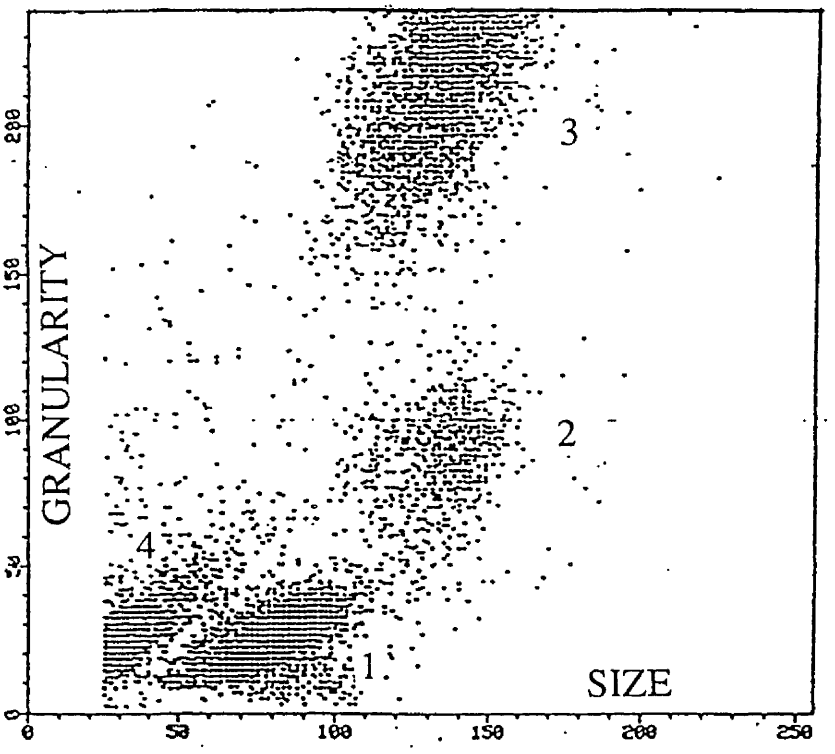


Figure 3.1.1.1 The distribution of normal peripheral blood leucocyte populations based on granularity and size parameters, using flow cytometry. This figure shows the characteristic distribution of one normal individual, indicating populations 1, 2, 3 and 4 (see text 3.1.1.1., p 80)

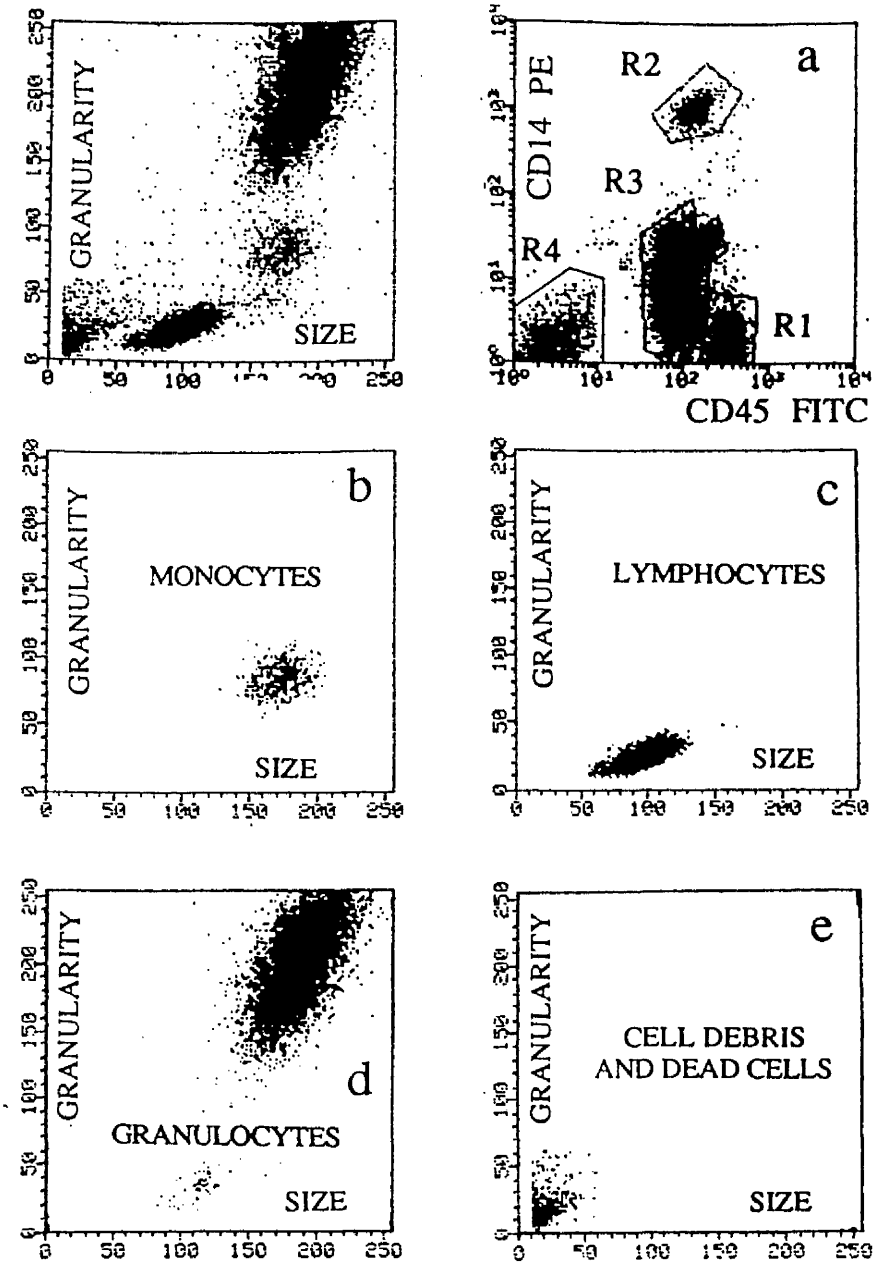


Figure 3.1.1.2 Identification of leucocyte populations in peripheral blood of a normal person by flow cytometry using anti-CD14 and anti-CD45 monoclonal antibody fluorescent markers.

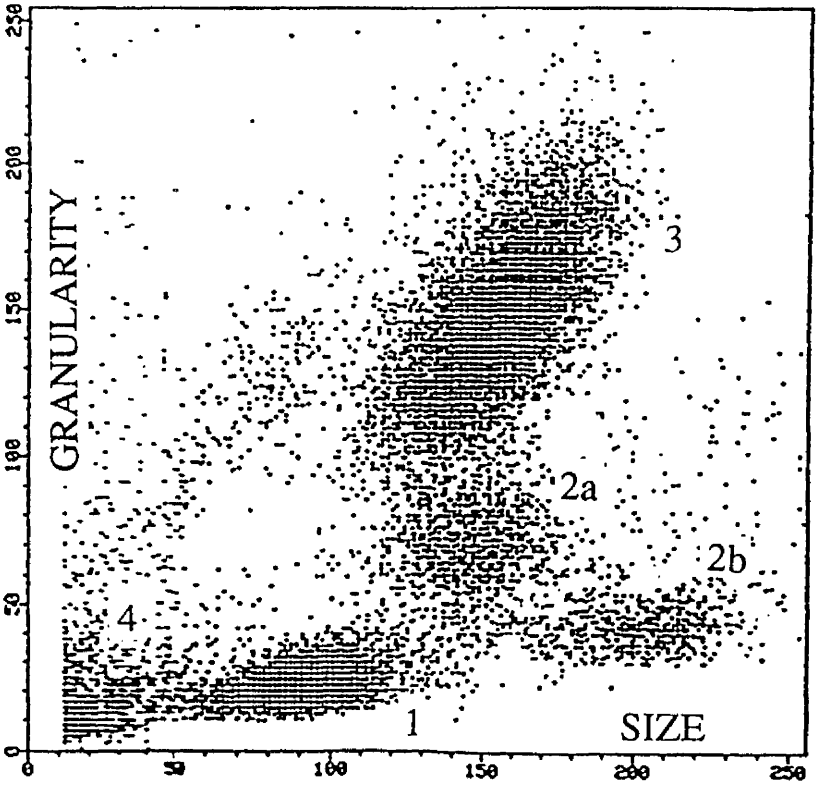


Figure 3.1.1.3 The distribution of peripheral blood leucocyte populations based on granularity and size parameters in peripheral blood of one HIV patient, by flow cytometry techniques.

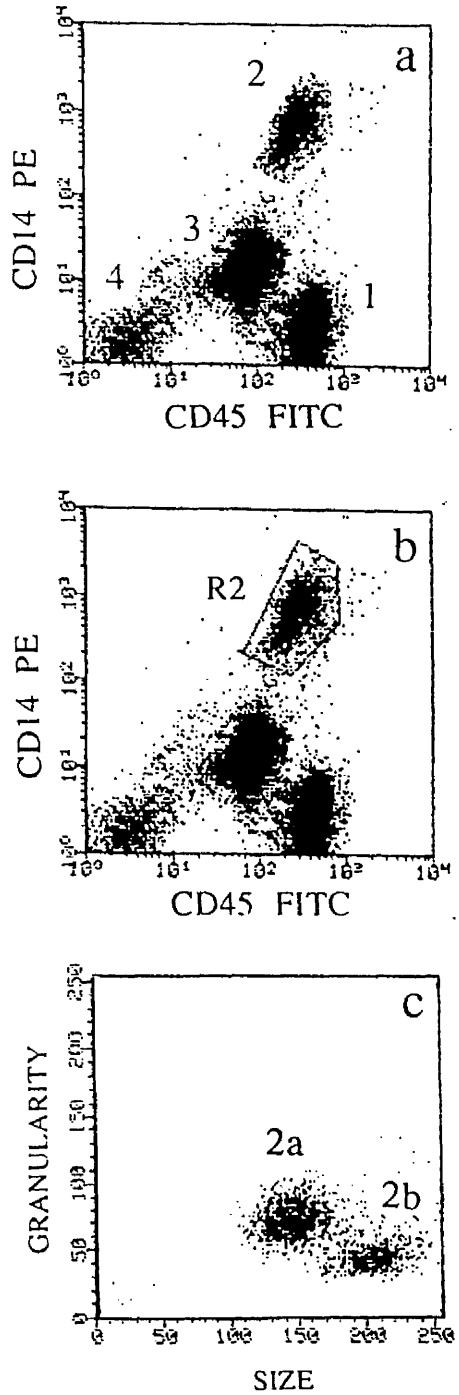


Figure 3.1.1.4 Identification of the extra monocyte population in peripheral blood of an HIV - infected patient by flow cytometry. Anti-CD14 and anti-CD45 fluorescent monoclonal antibody markers were used for labelling the cells. The extra monocyte population is depicted as population 2b (c).

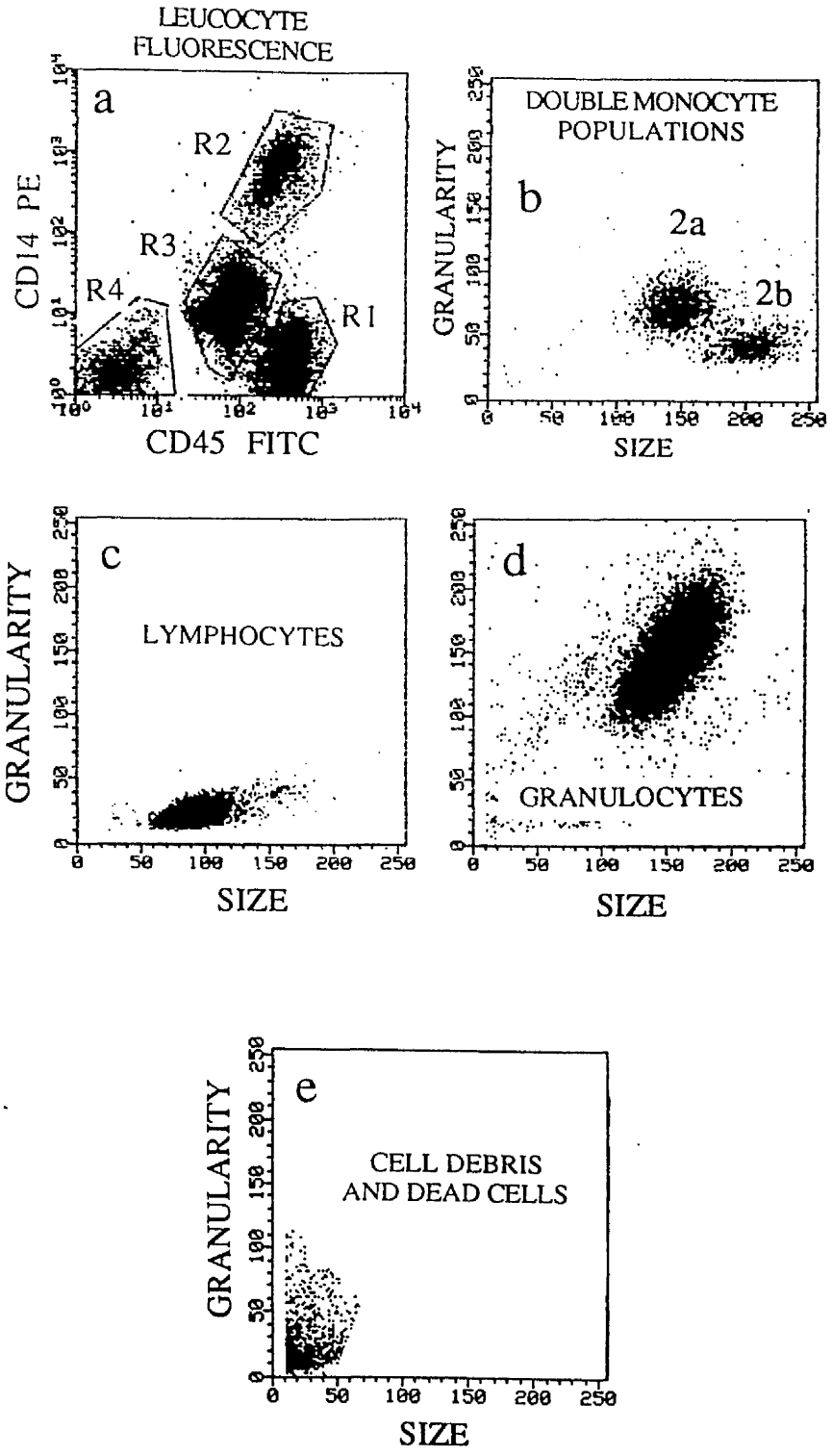


Figure 3.1.1.5 Analysis of peripheral blood leucocyte populations in an HIV-patient.

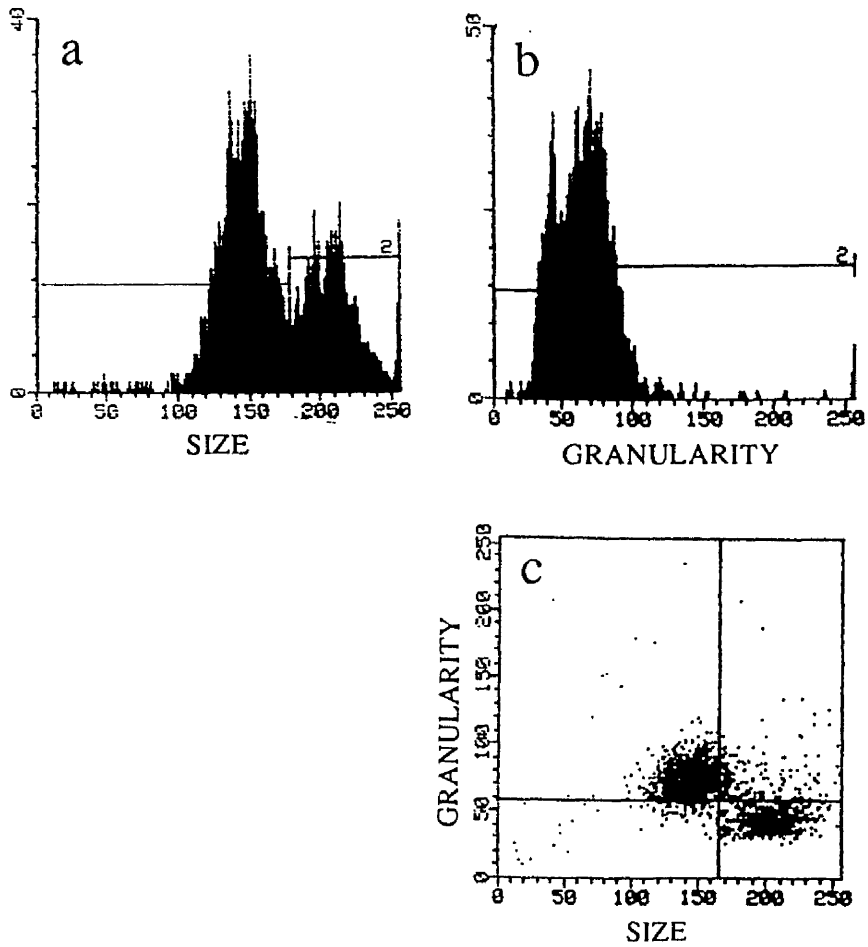


Figure 3.1.1.6 Quantifying the monocyte populations in an HIV-infected patient, according to size and granularity parameters. Markers and quadrants are set on the histogram and the dot plot, to quantify the monocytes in each population.

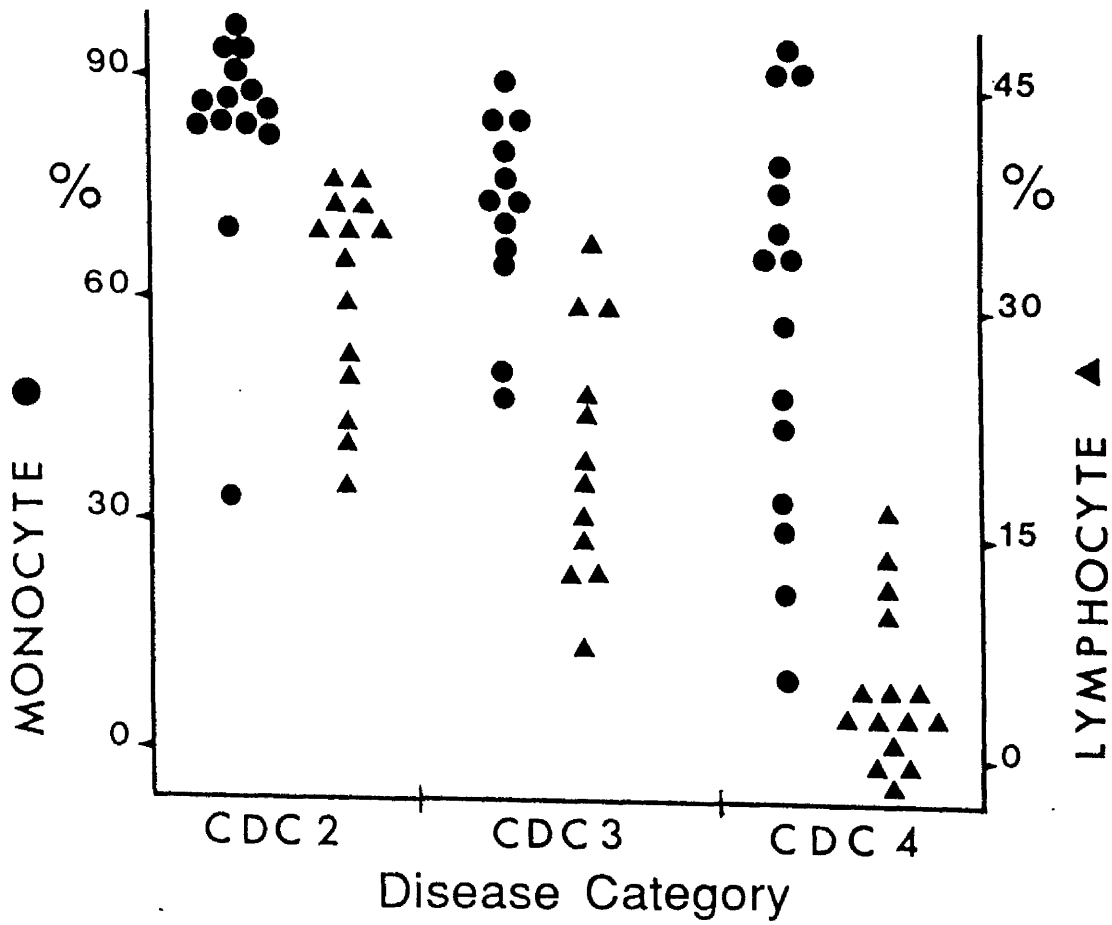


Figure 3.1.3.1 Comparison of the CD4+ monocytes and the CD4+ lymphocytes with disease progression in peripheral blood of HIV-infected patients. The disease categories are represented as CDC2, CDC3 and CDC4. Both the proportions of CD4+ monocytes and CD4+ lymphocytes decrease with disease progression. The CD4+ monocytes also had its heterogeneity increased with disease progression. this was more evident in disease category CDC4 where the monocytes show a wide range in the proportion of CD4 expression.

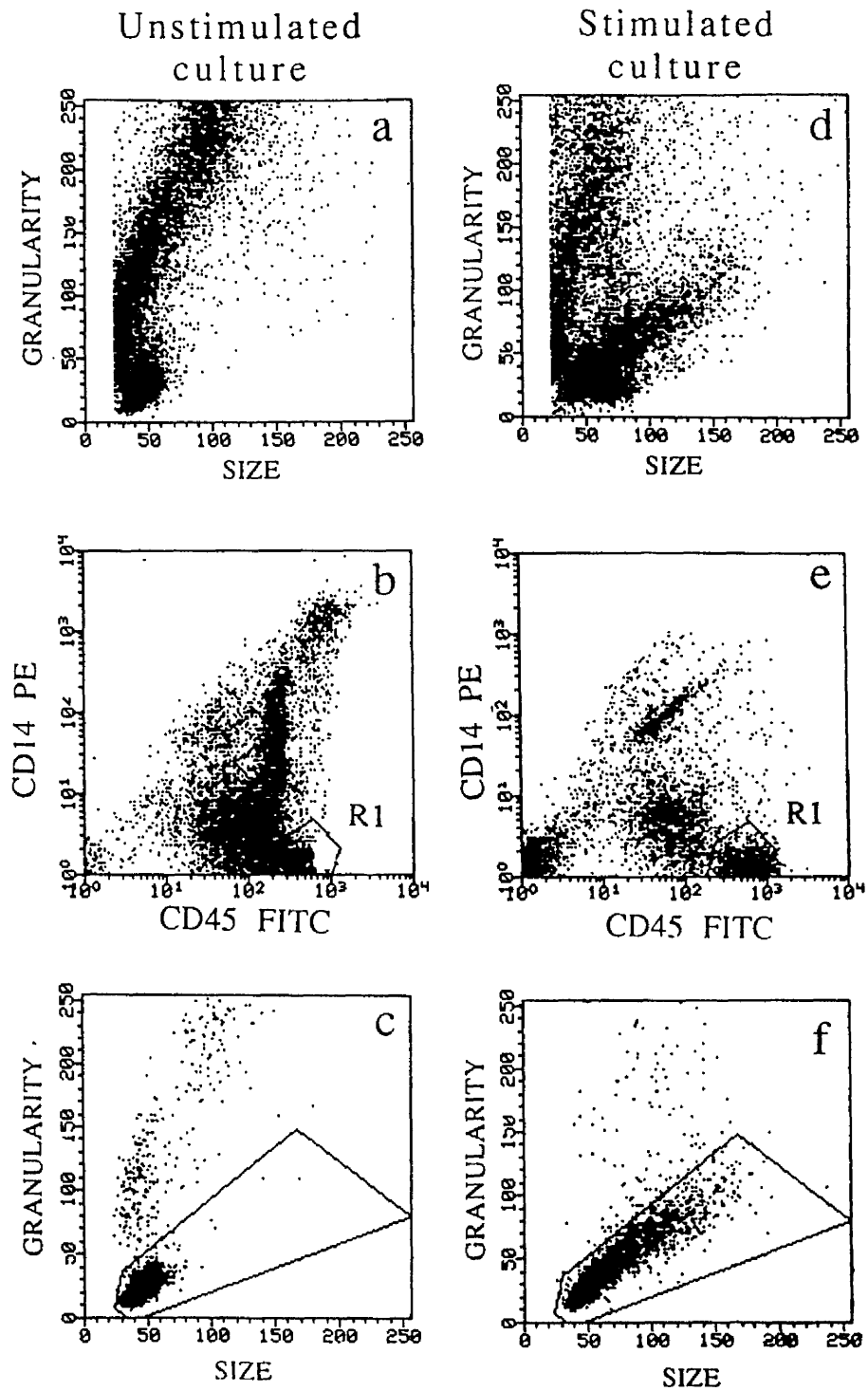


Figure 3.2.2.4 Identification of lymphocyte population in anti-CD3 stimulated whole blood culture. The lymphocyte fluorescence (b,e) in each respective culture are gated (RI), and its distribution based on granularity and size displayed (c,f).

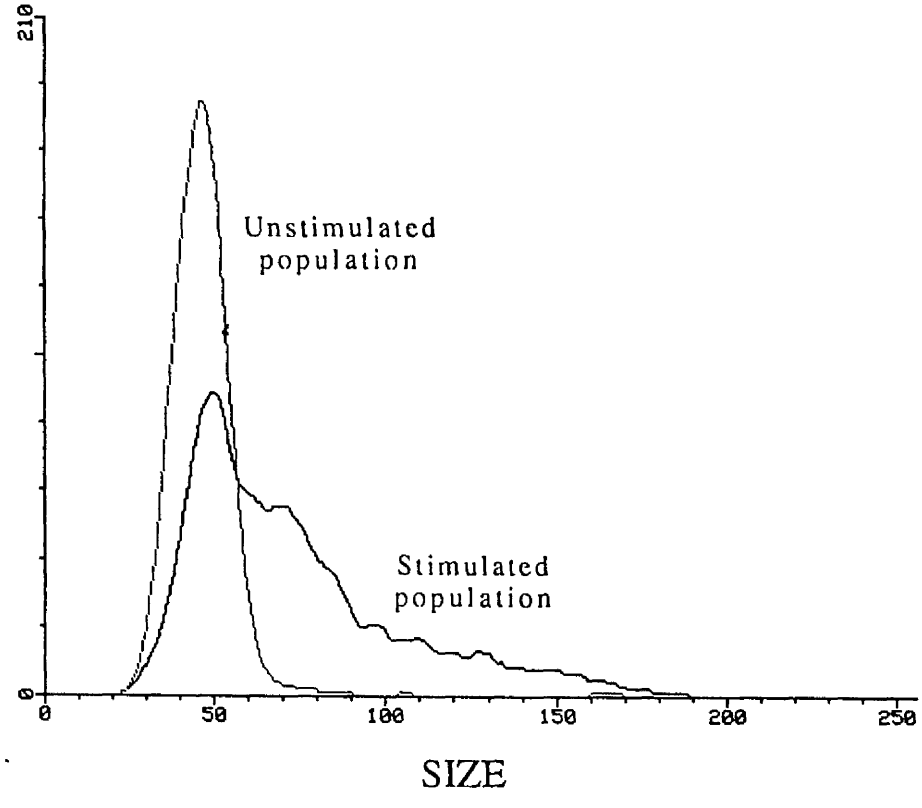


Figure 3.2.2.5 The distribution of lymphocyte populations based on size parameter, in whole blood cultures. The histogram shows both unstimulated and anti-CD3 stimulated lymphocyte population distribution of cell sizes.

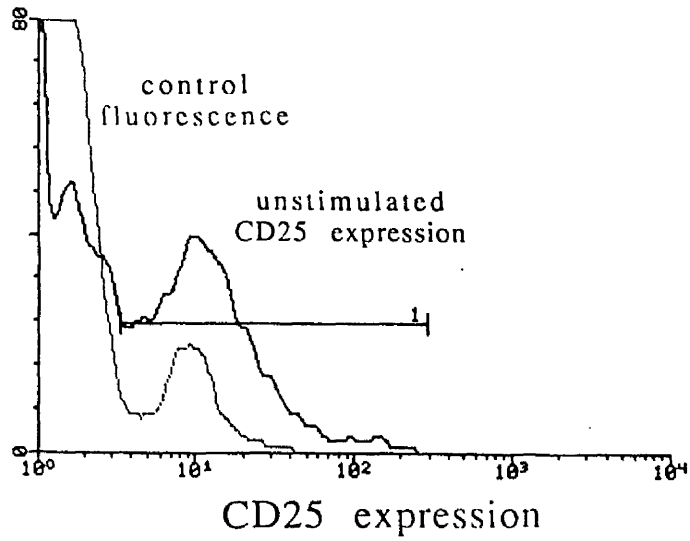


Figure 3.2.2.6 The distribution of lymphocyte interleukin-2 (CD25) expression in unstimulated whole blood cultures. The histogram compares the CD25 expression in the unstimulated lymphocyte population to the control fluorescence.

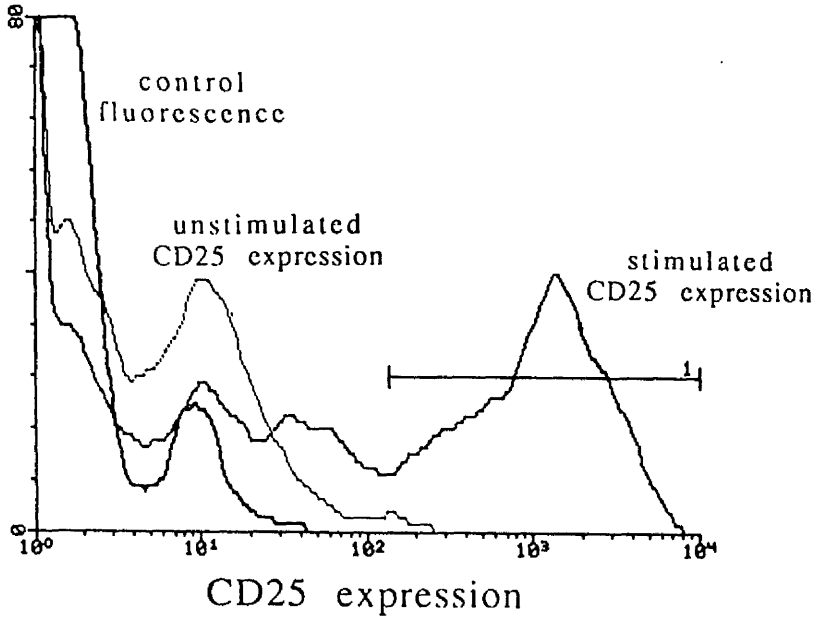


Figure 3.2.2.7 The distribution of lymphocyte interleukin-2 (CD25) expression in anti-CD3 stimulated whole blood cultures. The CD25 expression in the stimulated lymphocyte population, is compared to both the CD25 expression of the unstimulated lymphocyte population and the control fluorescence. The marker on the histogram delimits the CD25 expression due to the stimulated lymphocyte population.

Chapter 4

Discussion and conclusion.

Chapter 4

4 DISCUSSION AND CONCLUSION.

4.1 DISCUSSION.

Studies of monocytes in HIV infection have had a tendency to have been undertaken in vitro, and little had been done in vivo. The present thesis had investigated monocytes directly from peripheral blood to reflect as closely as possible the in vivo situation. The phenotype of the monocytes was examined to look for evidence of in vivo activation (157) and any influence of this on function. Phenotype (84) included monocyte numbers based on CD14 (95,84) and CD4 (98,84) expression during disease progression, and monocyte activation markers (80-86) expression in peripheral blood. Monocyte function (84,100,103) was studied firstly, by investigating associated plasma IL-6 (42) and PGE_2 (152) secretion in vitro, and gene expression of IL-1α and TNF-α (138) in cells taken directly from peripheral blood of patients. Secondly, the accessory cell functions (124-126) of monocytes were studied by comparing monocytes TNF (85,49), IL-1α (41,49) and PGE_2 Secondly, the accessory cell functions (124-126) of monocytes were studied by comparing monocytes TNF (85,49), IL-1α (41,49) and PGE_2 (152) production associated with T-cell proliferation in vitro (153,154).

4.1.1 Monocyte Phenotype in Peripheral blood of HIV Patients.

In order to elucidate the nature of the immune defect in AIDS and HIV infection (8,110,123), the number of peripheral blood

monocytes in infected patients was enumerated using flow cytometry. In view of monocytes being the reservoirs (24) for HIV and also effector cells in accessory cell functions (124), it was necessary to investigate the monocyte count in disease progression in patients. The myeloid differentiation antigen CD14 (44) detected on mature monocytes was monocyte specific, and was an appropriate marker for monocytes. Monocyte numbers based on CD14 staining and flow cytometry, was demonstrated to decrease non-significantly with disease progression, while the proportion of monocytes increased. This result matched a previous report (95) which found the total number of monocytes to be reduced in patients with AIDS-related complex and AIDS.

Observation of peripheral blood of HIV-infected patients by light microscopy (157), revealed large mononuclear cells resembling immature monocytes and activated lymphocytes. Light microscopy made it difficult to judge and estimate the monocyte and lymphocyte differential counts. Flow cytometry techniques using monoclonal antibodies against specific leucocyte surface markers, allowed some unusual characteristics of these cells to be observed. There was a double monocyte population in some HIV patients, predominantly those with AIDS. For the purpose of simplicity in this thesis, the usual monocyte population was termed as the first monocyte population, and it was accompanied by an extra monocyte population. Monocyte in the extra population were larger, less granular and had cell surface markers of activation. An earlier report (157) had demonstrated peripheral blood of HIV patients to show subsets of monocyte population. The proportion of monocytes in the extra activated population, reported in the present thesis, was negligible in control normal blood. However in HIV infection

the proportion was low at CDC category 2, at CDC3, and at CDC4 there were increasingly more activated monocytes. However, the proportion of cells in this extra population was rarely greater than 50%, and both its monocyte count and proportion increased significantly with disease progression.

The reduction in monocyte numbers with disease progression, accompanied by the significantly increased level of HLA-DR expression on activated monocytes in patients, suggest a potential defect in antigen-presenting cell populations which may have had a role in the emergence of the immune deficiency in HIV patients. HLA-DR is a major-histocompatibility antigen expressed on monocytes and is involved in antigen recognition during antigen presentation. Monocyte reduction in HIV infection was a possible consequence of the frequent exposure of monocytes to antigens (95).

The significance of the reported differences of total monocyte number between patients and control was still debatable. The results obtained by different investigators appeared to be conflicting. Some investigators studying AIDS patients' peripheral blood, had observed a moderated to marked reduction in monocyte numbers (93,99,100,123). Others, using similar methodology showed no significant change in monocyte numbers when compared to controls (94-96). However, the present thesis demonstrated no significant difference in total monocyte numbers in patients and controls, in agreement with a previous report (97). However, because the total white cell count fell with disease progression the proportion of monocytes was significantly

increased in patients, as found by an earlier group of investigators (158) who documented a significant increase in the proportion of patients monocytes. Likewise, these investigators were using the same technique as the present thesis to enumerate monocyte numbers.

Whatever the methodology or accuracy of the technique used to evaluate monocyte numbers, the results obtained seemed to be highly individual. Each HIV-infected patient had a tendency to show a characteristic of his own. There is a possibility that the mechanism underlying the immune deficiency might be responsible for the difference in cellularity (8) found throughout disease progression in each patient. If this is the case, the results of monocyte numbers could depend on each individual patient's characteristics.

Monocyte membrane CD4 expression was significantly decreased with disease progression, in parallel with T-cell CD4 expressions, as reported in the first cytofluorographic study which found similar results (98). The reason for this decrease in peripheral blood CD4 positive monocytes is uncertain. Potential explanations include the downregulation of monocyte membrane CD4, the CD4 positive monocyte taking residence in tissues, or the destruction of CD4 positive monocytes during progressive HIV-disease. The most plausible explanation for the decrease of monocyte CD4 expressions with disease progression would be the downmodulation of CD4 surface antigen after HIV infection. A previous study (159), demonstrated that in vitro HIV infection of peripheral blood monocytes down-modulated CD4 surface protein, although CD4 messenger RNA levels were not affected in

monocytic cell lines (160). This reduction in monocytes CD4 expression would have an important role in the pathogenesis of AIDS, as CD4 was the receptor for HIV attachment (49) and was important in monocyte T-cell interaction during the generation of an immune response. Each patient with AIDS showed a different level of activated monocyte CD4 expression. As a result, these patients, as a group with CDC4 disease category, had a wide range of monocytes CD4 expression. This abnormality may depend on the mechanism underlying the immune deficiency in each patient, and might be a direct consequence of the patients ability to respond to antigens.

Monocyte CD4 was critical in the uptake, processing and presentation of HIV gp120 antigen to human T-cells (161). However, this binding of monocytes CD4 by gp120 (49), activate monocytes to produce arachidonic acid metabolites via both the cyclooxygenase and 5-lipoxygenase pathways (17). These metabolites include PGE_2 (92) and leukotriene B4 which could both function as immunosuppressants. PGE_2 was a potential inhibitor of lymphocyte proliferation (154). Other consequence of the binding of HIV gp120 to monocyte CD4 (49) was the production of cytokines, including IL-α (40), TNF-α (130,74) and IL-6 (129). It was evident from those previous report that productive HIV-infections was not required to induce production of these cytokines, only the binding of the virus to the monocyte CD4 molecule was necessary.

One original observation made in this thesis is the description of two distinct monocyte populations which are likely to

exist in vivo. The two monocyte populations likely to exist in patients had a marked difference in the expression of surface markers. A significantly increased levels of surface markers were expressed on the larger monocytes of the extra population. The leucocyte specific marker CD45, together with two other activation markers, namely the class II histocompatibility antigen HLA-DR and the Fc gamma receptor III (CD16) which played a role in the clearance of immune complexes, were significantly increased in patients, in keeping with a previous report (80,81). As documented previously (84,85), the transferrin receptor (CD71), which is reported to be a marker for differentiation and activation for monocytes (162), and the complement receptor (CD11b), showed increased expression which was not significant. The monocyte specific surface marker CD14, with CD4, the receptor for HIV gp120 glycoprotein, had increased expression which was non-significant. When considering the larger size of the monocytes plus the significant increased expression of activation markers, this suggests that this was an activated phenotype. This in vivo observation was consistent with previous reports (163,164,95) which found increased expression of activation markers present on circulating monocyte.

HIV has the capacity to infect monocytes before infection of T-lymphocytes (165). Observations made in the present thesis offer a possible mechanism for enhancement of activated monocyte-T-cell interactions and the transfer of HIV from monocyte to CD4 positive lymphocytes. Increased HLA-DR expression on the activated monocytes would favour monocyte CD4-positive T-cell interactions, since helper T-cell recognised antigens in association with MHC class 11 determinants (166). As a result, this interaction favoured the release of cytokines,

including IL-1, by activated monocytes (167) which could then promote T-cell proliferation and consequently the replication of HIV in T-cells (165). Such was the case in the present thesis which demonstrated a positive correlation of IL-1 in culture with T-cell proliferation. This would be eventually followed by increased expression and release of virus (35-42) which could then productively infect (35-42) and activate other monocyte (38-42). This cycle of monocyte-T-lymphocyte interaction could persist with perpetual T-cell infection and depletion, supported by the data describing decreasing monocyte and T-cell count (95) in disease progression.

The main ligand for HIV-1 is the CD4 receptor (49,50) which is present on monocytes (55,56) macrophages (59,60), and T-lymphocytes (53,54). The virus entry into monocytes was directed by the viral envelope glypoprotein gp120 bound to CD4, and the viral transmembrane protein gp41, embedded in the lipid membrane that surrounds the virion (18). Recently other modes of virus entry into monocytes has been documented. Two other components (168) of human serum were found to enhance HIV infection and to mask HIV-1 neutralising antibody activity. HIV-I infection enhancing antibodies have been identified in the sera of seropositive patients (168) and Fc gamma Receptor III antibodies inhibited the enhancement of HIV-infection in vitro (169). On the other hand, HIV-I-infected cell lines were demonstrated to activate complement by both the classical and alternative pathways, leading to deposition of C3 fragments on the cell membrane during incubation with human serum without lysis of the cell (20). Yet HIV-infected cells, coated with complement formed rosettes by immune adherence with compliment receptor (CR3) bearing cells

(170). Although some results suggest that Fc gamma receptor III (21) and CR3 (20) on monocytes mediated antibody-dependent enhancement of HIV infectivity by a pathway different from the CD4 molecule, it is not clear whether antibody enhancement of HIV infection occured in vivo (171). The lack of Fc gamma receptor III and CR3 would likely hinder this route of infection on circulating monocytes in vivo. Incidentally, activated monocytes in vivo, had been found to express significant level of Fc gamma receptor III (80) and CDIIb (85-86) in keeping with data presented in this thesis.

The first monocyte population on the contrary, present an assymmetry in surface marker expression with respect to the activated population. The IL-2 receptor (CD25) a T-cell proliferation marker also expressed on monocytes, together with CD4 and CDIIb were significantly increased. This matched earlier reports which described patients' monocyte to show significantly increased expression of CD4 (98) and CD25 (81), while reduced (83) or no significant difference (84) in CDIIb expression.

The significant level of CD25 (IL-2 receptor) (81) in the first monocyte population may provide an insight in the immunopathogenesis of HIV-1 infection. The presence of IL-2 receptors on monocytes can bind to, absorb and deplete any soluble IL-2 circulating in the blood (172). This provided a plausible mechanism for any decrease in IL-2 levels and possibly contributed to the decreased T-cell proliferative capacity in AIDS patients (173), as observed in the present thesis. On the other hand any shedding of IL-2 receptor by

monocytes could contribute to the increased levels of soluble IL-2 receptor in AIDS patients (173,174). It is possible that monocytes in the first population become activated by HIV or cytokines (eg IL-2 or γ-IFN) and as a result become larger monocytes with activated phenotypes in the process of differentiation to macrophages. Since blood monocytes in HIV-infected patients have been shown to express phenotypic and functional changes characteristic of an activated or differentiated population (81,100,157) the possibility exist that the heterogeneity in phenotypes influenced functions in vivo. IL-2 receptor positive monocytes might be modulated by IL-2 and consequently express activation markers (80,81,82), which regulated the secretion of cytokines such as IL-1α (40), TNF-α (76), IL-6 (42), plus product as PGE_2 (92). The abnormalities in cytokines and PGE_2 levels would have a negative effect on accessory cell function (124,124,126), and favour increased expression of HIV (32-42). Such abnormalities of monocyte function is associated with AIDS (164). This supports the paradox of a cell population which was phenotypically differentiated, but functionally impaired (99,108,163,175,176).

4.2.1 Monocyte functions in peripheral blood of HIV patients.

The functional capacity of monocyte in vivo was assessed indirectly by measuring plasma IL-6 levels in the blood of patients. Interleukin-6 (IL-6), is a phosphoglycoprotein and a multifunctional cytokine induced by a variety of stimuli. Monocytes also produce IL-6 in vitro on stimulation with different signals, such as bacteria, viruses and other cytokines (128,177). Polyclonal B-cell activation and immunoglobulin (Ig) secretion are characteristics seen in HIV infection

(178,179). To study the role of B-cell stimulatory cytokines in this process, peripheral blood IL-6 level was examined in HIV-infection.

Peripheral blood mononuclear phagocytes isolated from healthy, non HIV-infected donors, primarily monocytes, can be induced in vitro by HIV to secrete IL-6 (180). Emphasis was now placed on whether monocyte IL-6 production was elevated in vivo in HIV-infected patients, to associate the possible role of overproduction of this cytokine in the B-cell activation associated with HIV infection. Also to see if there was any relation of IL-6 levels to any other parameters, such as monocyte numbers, total white cell count, and lymphocyte counts, or immunoglobulin level.

It has been reported that HIV-infected individuals have abnormally elevated serum immunoglobulin (Ig) levels (178), and that a proportion of HIV infected individual displayed abnormally elevated numbers of B-cell in the circulation (179). However, in the present report, IL-6 levels were shown to be elevated in HIV patients, in keeping with a previous report (128), but the IL-6 level did not correlate with disease progression, number of B-cells, total white cell count nor total serum IgG level (129). However, plasma IL-6 level did correlate with the number of larger monocytes with the activated phenotype. This is comparable to previous findings which associated phenotype with circulating cytokines levels in HIV patients where serum tumour growth factor beta (TGF-β) levels correlated with CD16 expression (33). Most dramatic was the 5-fold increase of plasma IL-6 level in patients of CDC3 disease category, compared to patients in CDC2 and CDC4. The

increase did not appear to be related to either disease status, the number of B-cell, nor IgG levels. Earlier report had showed that HIV-infected patients without any documented coinfections, had the same range of plasma IL-6 levels as bacterially coinfected HIV-infected donors (129,181,182). This suggests that HIV as well as bacteria, played a central role in inducing increased plasma IL-6 levels through peripheral blood mononuclear cell activation in vivo. Possibly, HIV is acting in these patients in a fashion similar to that seen in vitro, where it can directly stimulate monocytes to produce IL-6 (180).

The mechanisms of IL-6 induction by HIV are unclear. If the in vitro investigation of IL-6 in earlier reports are considered, an in vivo counterpart is expected to show elevated levels of this cytokine in HIV-infected patients. This elevated in vivo IL-6 level was demonstrated in the present thesis. It is possible that the monocytes secreting IL-6 in patients are also those productively infected with HIV. This idea could be supported by the report (128) which showed that patients infected with an HIV which was capable of replicating in monocyte cultures had significantly higher serum IL-6 levels than patients infected with HIV which was capable of replicating only in T-cell cultures. However, it has been reported that HIV could induce a transient increase of IL-6 gene expression in monocytes without infecting the cells, possibly by signal transduction via the cell surface IL-6 receptor protein (180). If that is the case, it seemed that activated monocytes were responsible for the elevated IL-6 level seen in vivo. This could give a plausible explanation for the existence of a correlation between plasma IL-6 levels and the monocyte population with the activated phenotype described in this thesis. The role IL-6 played in the pathogenesis of HIV disease is still

uncertain. IL-6 might affect the accessory cell function as a mediator of host responses during virus infection. The elevated level of this cytokine produced by activated monocytes could promote T-cell proliferation, as reported by in vitro experiments (183). It is also reported that activated T-cells replicate HIV in preference to resting T-cells (184). By inducing T-cell proliferation, IL-6 may contribute to the expansion of the pool of HIV-infected T-cells.

The finding that IL-6 upregulates the production of HIV in vitro, both directly and in synergy with TNF-α (135), provides a potential model of the physiological as well as pathogenic mechanisms that influence the degree of HIV expression in vivo. As suggested in a previous report (135), it is possible that IL-6 affects the expression of HIV by at least two mechanisms. In the presence of TNF-α, IL-6 increases the levels of viral transcription and of steady state RNA, resulting in a synergistic enhancement of HIV expression. In the absence of TNF-α, IL-6 acts post-transcriptionally, increasing the expression of HIV proteins without inducing new transcription of HIV-RNA. Understanding the role of TNF-α, IL-6 and other cytokines in HIV-infected cells may provide an insight into the mechanism associated with the viral burden and the deterioration of the immune functions in the disease progression to AIDS (23). This would emphasize the importance of the observation of monocyte heterogeneity reported in this thesis.

Apart from the in vivo IL-6 production, peripheral blood monocytes from HIV-infected patients were seen to express the genes

for TNF-α (138), which was detected by polymerase chain reaction (PCR) techniques. Despite that ability of TNF-α to mediate a variety of functions and to exert remarkably diverse effects on the immune system, surprisingly little is known about its relevance to the disregulation of the immune system in vivo in HIV disease. There are much data linking TNF-α to HIV, but the vast majority of these studies were performed in vitro, so their biological relevance is unclear. In the present thesis, an investigation of the expression of TNF by adherent monocytes ex vivo, demonstrated that there was greater expression of TNF-α in HIV patients than TNF-β, and greater expression of TNF-β than TNF-α in controls. In agreement with a previous report (140), only a little secretion of the alpha-form was detected constitutively or after stimulation in culture. The increased TNF-α expression with negligible secretion is supported by earlier investigation which found TNF-α to be absent in the serum of HIV patients in disease categories CDC3 and CDC4, while a minority of patients in CDC2 showed detectable levels of expression (185).

TNF-α and TNF-β (lymphotoxin) are partially homologous cytokines. They share 30% homology at the amino acid level. Although distinct biologically and immunologically, they share nearly identical biological activities (186). The two cytotoxins are encoded by distinct but closely linked genes in the major histocompatibility complex region of human chromosome 6 (187). TNF had been shown to be produced by monocytes, macrophages and lymphocytes subsets (188).

It is unclear why patients had elevated expression of TNF-α and low TNF-β, while controls have elevated TNF-β and low TNF -α

expression. The ability of normal peripheral blood mononuclear cells to produce TNF-α and TNF-β mRNA in response to IL-2 in the absence of other activation signals, had already been investigated in vitro (189). Previous reports (190,191) had demonstrated that TNF-α mRNA had a rapid appearance and disappearance rate in normal peripheral blood mononuclear cells, while TNF-β mRNA was expressed later and showed a sustained accumulation, suggesting independent expression of the mRNA coding for the two cytokines. From the results of the present thesis, it is apparent that HIV infection is predominantly regulating the production of TNF-α and β in HIV patients, while in controls both cytokines are modulated by IL-2 owing to lymphocyte contamination of the monocyte sample. Because of the rapid turnover rate of TNF-α in controls, this cytokine is apparently expressed at a lower level than TNF-β which showed sustained accumulation, and therefore seemed expressed at a higher level than the alpha form. In contrast, the burden of persistent HIV in patients upregulates TNF-α transcription in patients' mononuclear cells. Having a high turnover rate, TNF-α eventually accumulates rapidly and seems to be expressed at higher levels than TNF-β, in patients.

The mechanism(s) of constitutive TNF-β mRNA accumulation in control monocytes may operate on several levels. One possible explanation is that the promoter for TNF-β, which is repressed in normal T and B-cells (192), functions constitutively in monocytes. In addition, the present thesis revealed traces of IL-2 in controls due to lymphocyte contamination of the monocytes. However, no IL-2 was detected in HIV patients, owing to a purer monocyte sample. It is important to note that IL-2 is a cytokine produced by lymphocytes and not by monocytes. Thus,

TNF-β promoter in control monocytes, may also be functioning as a result of IL-2 activation, as found by earlier investigators (189). Constitutively, TNF-β promoter activity could be associated with the function of upstream positive regulatory elements that transcriptionally activate the promoter. In such situations, the positive regulatory elements would probably be recognized by DNA-binding proteins that are present or modified in cell-type specific manner in constitutive TNF-β mRNA expression, thereby activating element function. Post-transcriptional regulation of mRNA stability may also be contributing to this upregulation of TNF-β in these cells (193).

The main pathological findings in HIV infections are thought to be related to TNF-α (194), and TNF-α may also up-regulate HIV expression in vitro by activation of the nuclear factor kB (NF-kB), and HIV could therefore augment its own expression (40,41,42). It is possible that what is happening in vitro may have an in vivo counterpart. However, it has been established in vivo that HIV, which utilises a functionally important molecule (CD4) on the surface of immune competent target cells as its receptor for cell entry (48), has also evolved cellular mechanisms for the upregulation of its expression by certain cytokines operative in the normal homeostatic control of the immune system.

4.2.1.1 Accessory cell functions of monocytes.

The monocyte is an accessory cell in that it functions as a modulator of lymphocyte function in immune responses. Being DR-

positive, it shows MHC restriction in its interaction with helper T-cells when initiating an immune response. Both the monocyte and the T-cell require to be syngeneic for class II antigens. The ability of monocytes from HIV-infected patients to function as accessory cells for the induction of T-lymphocyte responses to soluble antigenic stimuli has been studied to a limited extent (124-126). Because T-lymphocyte activation is dependent on accessory cells (47), any defective accessory function of monocytes could compound the abnormalities in T-cell function observed in HIV patients.

The assay of IL-2 receptor (CD25) expression or lymphocytes stimulated with anti-CD3 allowed a parallel assessment of activation and cytokine release which would not have been possible using conventional proliferation assays with tritiated thymidine incorporation. With this system, a positive association between IL-1α secretion, TNF-α and T-cell proliferation was found. Also, an inverse association between PGE_2 production and proliferation was observed.

The classification of patients into groups based on their lymphocyte responses to anti-CD3 stimulation, revealed some characteristics of HIV-infection. The negative responders had defective monocyte accessory cell function, showing no IL-1α, low TNF-α, and elevated levels of PGE2 which could inhibit T-cell proliferation. A previous report (125) had demonstrated defects in accessory cell function of anti-CD3 triggered blood mononuclear cells which showed significantly decreased T-cell proliferative responses and low IL-1α levels. However, PGE_2 was the main factor in suppressing T-cell

proliferative responses (195). The good responders on the contrary had good monocyte accessory cell function, since both IL-1α and TNF-α levels were elevated, and low PGE2 levels favoured T-cell proliferation. The poor and good responders shared a significant difference in TNF-α levels suggesting that not only PGE2 but also TNF-α might be playing a role in inhibiting the T-cell proliferation. TNF-α has been reported to upregulate HIV expression (40,41,42) resulting in increased production of PGE2 (195) which inhibited T-cell proliferation. In addition TNF-α itself showed antiproliferative characteristics (196).

The findings concerning the lymphocyte responses to anti-CD3 in the present thesis was in agreement with the known roles of IL-1α, TNF-α and PGE2. IL-1 is one of the molecular mediators that participates in the activation of cells in the presence of antigen (197), and induces the appearance of IL-2 receptors in CD4 positive T-cells as well as the release of IL-2 by the same cells (198). In vivo, TNF acts in synergy with numerous other cytokines, particularly IL-I, with which it shared some properties (199) and also TFN-α (200). Some of the effects shown were beneficial components of cell-mediated immunity. TNF-α in synergy with IL-1α favoured lymphocyte activation (185). This was evident in a report (185) which showed a positive correlation between IL-1α and TNF-α levels in HIV-1 infected patients and this co-existed with an elevated level of soluble IL-2 receptor (sIL-2R) and IL-2. However under other circumstances, TNF was responsible for important immunopathology. It acts synergistically with TNF-α and causes enhanced antiproliferative effects (196). The multiple effects of TNF (201) which include lipoprotein lipase suppression (202), pyrogenic activity (203), and disturbed brain function (204) could account for

several of the pathological feature of AIDS, including wasting, fevers and dementia, respectively. Alternatively a deficient TNF response in AIDS might explained host susceptibility to several opportunistic microorganisms (205), particularly mycobacteria (206).

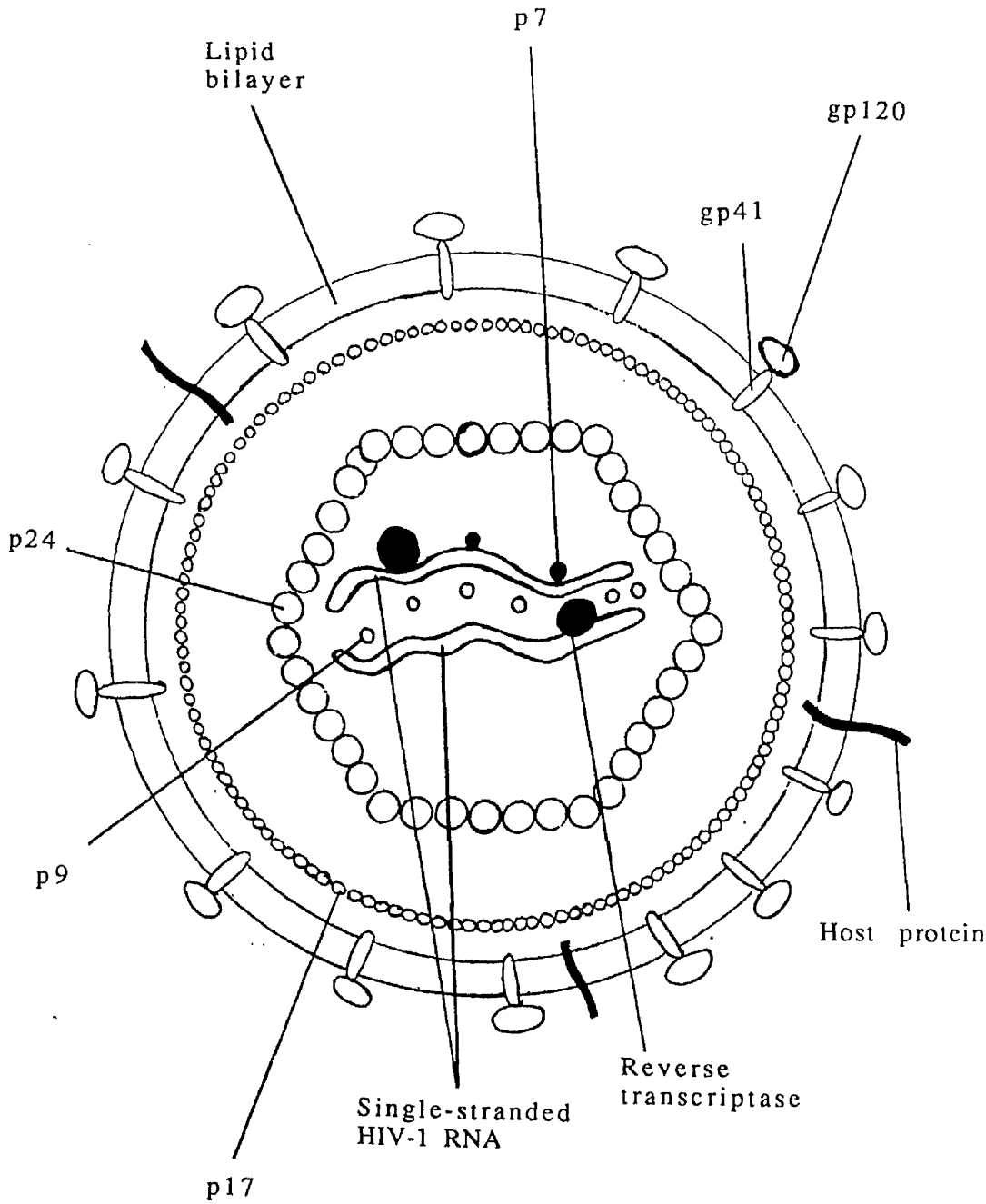
PGE_2 on the contrary is primarily a suppressor of immune function as demonstrated in vitro by its ability to inhibit lymphocyte proliferation and production of lymphokines (195). In addition, PGE_2 also functions as a feedback inhibitor of monokine production, monocyte-mediated tumor cell cytotoxicity, and phagocytosis (195). Thus, the initial exposure of monocytes to either soluble or intact virion gp 120 in vivo, may result in a PGE_2-mediated suppression of immune surveillance by lymphocytes (207). Furthermore, the ability to combat secondary opportunistic infections may be compromised because of the reduction in monocyte or lymphocyte function by PGE_2. The finding that monocytes from HIV patients constitutively produced PGE_2 is in keeping with the higher levels reported in a previous report (150) and the ability of gp120 to directly stimulate PGE_2 production by monocytes (74). The findings in the present thesis was in agreement with a previous report (154) that PGE_2 produced by HIV infected monocytes in vitro was a potent inhibitor of T-cell proliferative responses. There is a possibility that PGE_2 produced by monocytes infected with HIV in vivo might in part account for the HIV- related T-cell anergy in patients, and raise the possibility that this may be ameliorated with indomethacin (208) treatment.

4.3 CONCLUSION.

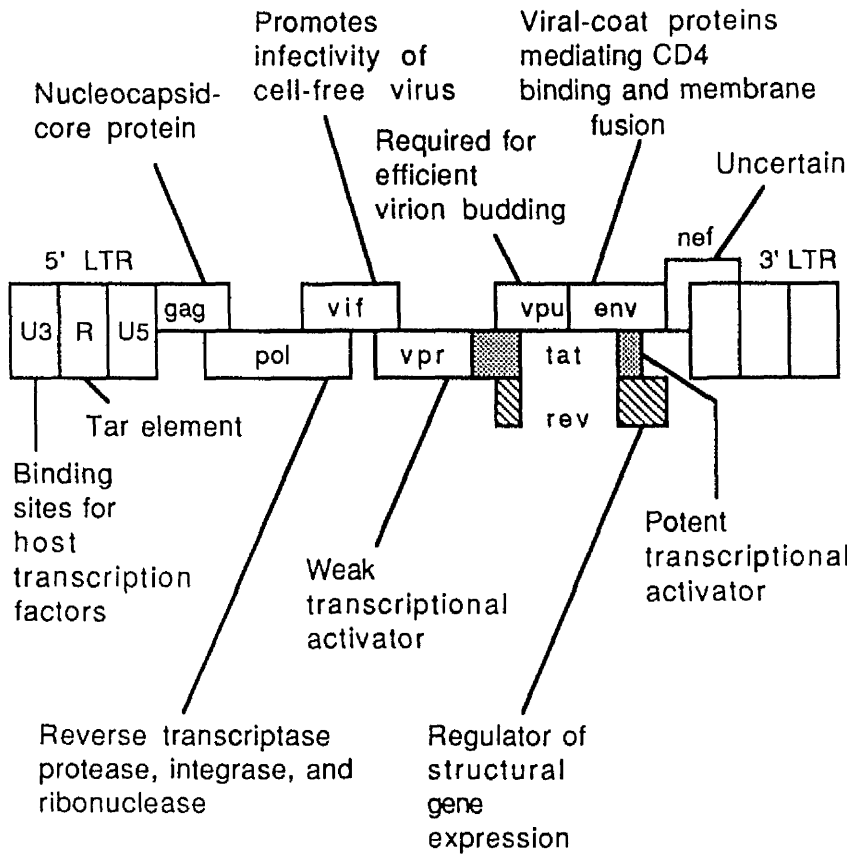
The number of monocytes in HIV infected patient was observed to decrease nonsignificantly with disease progression, while the proportion of monocyte increased. This was accompanied by a significant decrease in the number of CD4 positive monocytes with disease progression, in parallel with CD4 positive T-cells. HIV monocytes are different from normal controls, with increased intensity of CD11b and CD4 staining. Within HIV patients there is evidence for an in vivo activated monocyte phenotype which is larger, and has greater intensity of staining for CD11b, CD16, HLA-DR and CD71. This activated monocyte phenotype correlated with plasma IL-6 levels.

Monocytes from HIV patients have increased TNF-α and decreased TNF-β gene expression. This was evident with the increase in secreted TNF-α level in concert with an increase in IL-1α and both cytokines in synergy stimulated lymphocyte proliferation. HIV patients monocytes have high constitutive PGE_2 production which cannot be further induced with LPS, and this might have been a consequence of HIV modulation of monocytes via CD4 receptors, or monocyte activation by cytokines, including IL-1-α and TNF-α. This PGE_2 production was highest in mononuclear cell cultures which failed to respond to lymphocyte stimulation. This PGE_2 production may contribute to HIV-related immunosuppression.

APPENDICES

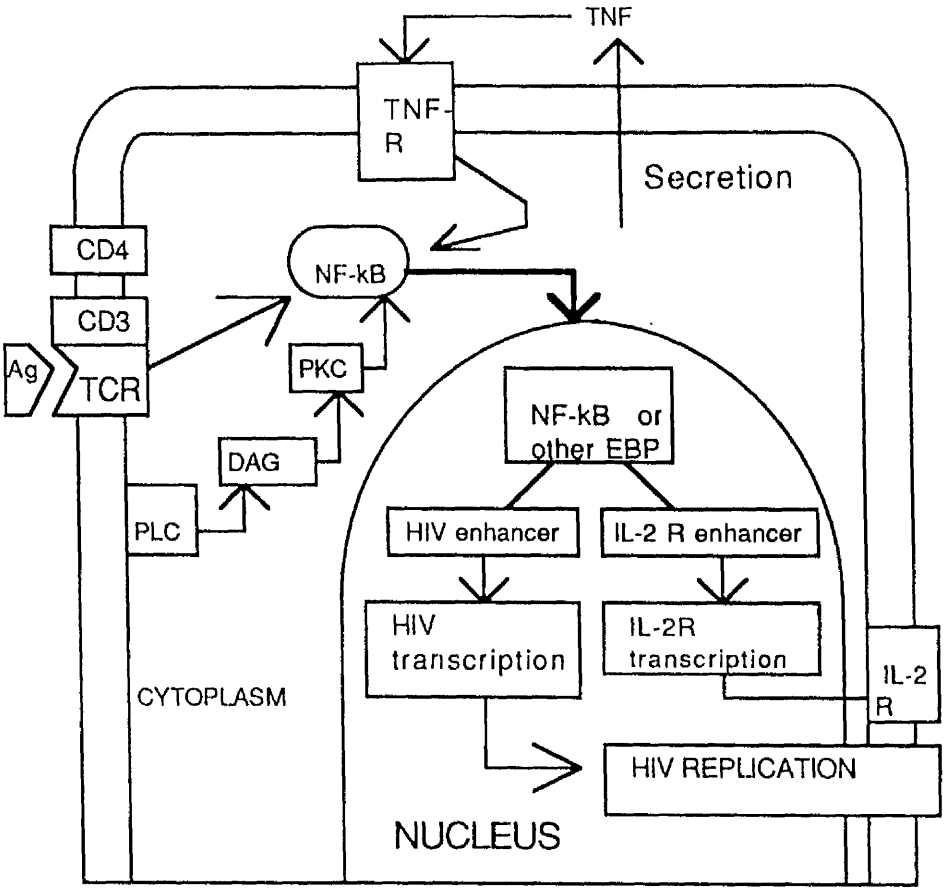


Appendix 1.1.2 A schematic diagram of HIV-1 virion (209). Each of the virion proteins making up the envelope (gp120 and gp41) and nucleocapsid (p24, p17, and p7) is identified. In addition, the diploid RNA genome is shown associated with reverse transcriptase, an RNA-dependent DNA polymerase.



APPENDIX 1.1.3

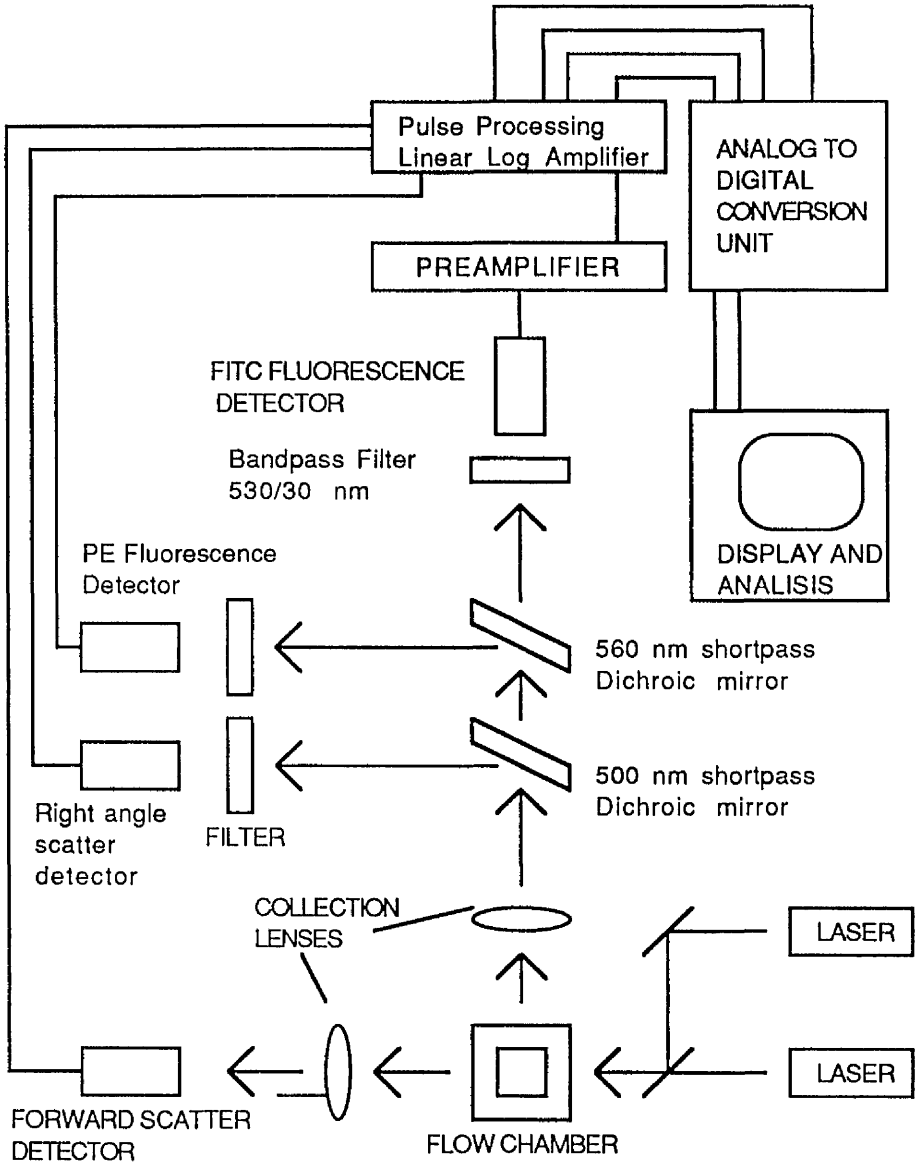
The genomic stucture of HIV-1 (209). Each of the known genes of HIV-1 are shown, and their primary functions are summarized. The 5' and 3' long terminal repeat (LTR) contained regulatory sequences recognized by various host transcription factors are depicted, and the Tat response elements (Tar) are indicated.



EBP Enhancer binding protein

APPENDIX 1.3.3.1

Diagram representing the signals which reactivate the HIV genome during activation of an infection (210). Soluble cytokines such as TNF acted through a specific receptor (TNF-R), whose expression was upregulated by mitogens. The T-cell receptor (TCR) complex (TCR-CD3-CD4) recognized specific antigens (Ag). Such transmembrane signals led to translocation of the NF-kB transcription factor from the cytoplasm to the nucleus, after activation of NF-kB by protein kinases. Protein Kinase C (PKC) activated by diacylglycerol (DAG), itself induced by Phospholipase C (PLC). The final step is the induction of HIV enhancer leading to HIV transcription.



APPENDIX 2.4.3

DIAGRAM SHOWING THE PRINCIPLE OF THE FLOW CYTOMETER.

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